Dual Drug-Loaded Electrospun Nanofibres for the Treatment of Corneal Diseases

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Declaration of Authorship

I, Essam Tawfik, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in this thesis.

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Date: _______________________________
Abstract

Corneal abrasion is a scratch on the surface of the eye, which can predispose a patient to corneal infection. Certain types of bacteria, such as *Pseudomonas aeruginosa* (*P. aeruginosa*) can lead to a more aggressive condition known as corneal ulcer. If this is left untreated, patients may suffer from vision loss. A potential approach to treat this clinical problem could be the administration of an anti-scarring agent and an antibiotic incorporated into one dosage form. This would accelerate the wound closure and treat any associated infection.

In this study we have demonstrated the performance of electrospun fibres loaded with an anti-scarring agent pirfenidone and a broad spectrum antibiotic moxifloxacin in separate compartments of multi-axial fibres. An optimisation of the preparation of these fibres has been performed. Pirfenidone was embedded in a water insoluble polymer, PLGA, to form the outer layer of these fibres, while moxifloxacin was dispersed in a water soluble polymer, PVA or PVP, in the inner layer. This led to extension of the release of pirfenidone, while providing a suitably rapid release of moxifloxacin from the fibres. An *in vitro* release study was performed using a range of approaches to study the release of each drug. The efficacy of the antibiotic moxifloxacin was tested against a Gram positive bacteria, *Staphylococcus aureus* (*S. aureus*), and the Gram negative *P. aeruginosa* by the zone of inhibition microbiological study. However, the efficacy of pirfenidone released from the fibres has been verified by a Western blot technique. More specifically, this was demonstrated by reducing the expression of alpha smooth muscle actin (α-SMA) protein that is generally upregulated in response to an injury. Finally, an *in vivo* study has been done to compare the performance of the fibres to conventional eye drops against an infected eye.

Overall, the findings suggest that the electrospun fibres are capable of extending the release of both drugs compared to the drugs in the solution form. Moreover, the antibiotic moxifloxacin has retained its effect against *S. aureus* and *P. aeruginosa*. Finally, the drug-loaded fibres downregulated α-SMA, suggesting the accelerated healing of the injured cornea.
Impact Statement

A current treatment of corneal abrasion includes the administration of an antibiotic, moxifloxacin hydrochloride, three times daily for two weeks, and can be less than satisfactory for many patients. The frequent dosing, as well as the amount of drug loss to the tear ducts that usually results from the administration of eye drop solutions can be the main limitations of this dosage form. Therefore, having a delivery system that contains the antibiotic moxifloxacin and the anti-scarring agent pirfenidone to be administered once daily could accelerate the healing of the abrasion or scar, prevent any infection and enhance the overall patient compliance.

All the aforementioned aspects have given incentives for finding a suitable dosage form which is likely to increase patient compliance by reducing the frequency of dosing and the overall duration of therapy. Electrospinning is a technique used for the production of micro- and nano-fibres. A high electric force is applied to overcome the surface tension of a viscous polymer solution, leading to solvent evaporation and thus the production of a fibrous mat. It is also possible to prepare single-layered (monoaxial) or multi-layered (coaxial or triaxial) fibres using a single- or multi-nozzle system, respectively. Moreover, this technique allows the loading of different drugs, and the ability to tailor their release rate by varying the polymers used. This delivery system may allow to control the release of the drugs along with allowing compartmentalised loading of the different active ingredients. Moreover, these fibres have been recognised for their application in wound healing and tissue regeneration fields. Their unique morphology plays an important role in accelerating the wound closure and helping the tissues to redevelop by acting as a scaffold.

The utilisation of these electrospun fibres has therefore been proposed as a mean of incorporating two drugs into separated compartments, by an electrospinning technique known as multi-layered electrospinning. In this study, an anti-scarring agent, pirfenidone, has been used to reduce the scar and to accelerate the rate of that wound healing. In addition, a broad spectrum antibiotic, moxifloxacin, has been added in order to prevent or even treat any
associated infection. Ultimately, one dose of a single dosage form can be administered to increase the patient’s compliance by eliminating the multiple dosage of one or more eye drop solutions and potentially reducing the duration of treatment.
Dedication

This thesis is dedicated to my father, mother, my wife, my brothers, my teachers, my friends and my colleagues, with a special dedication to my lovely daughter Banah and new son Nabil.
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# Table of Content

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Declaration of Authorship</td>
<td>2</td>
</tr>
<tr>
<td>Abstract</td>
<td>3</td>
</tr>
<tr>
<td>Impact Statement</td>
<td>4</td>
</tr>
<tr>
<td>Dedication</td>
<td>6</td>
</tr>
<tr>
<td>Acknowledgment</td>
<td>7</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>9</td>
</tr>
<tr>
<td>List of Figures</td>
<td>17</td>
</tr>
<tr>
<td>List of Tables</td>
<td>31</td>
</tr>
<tr>
<td>List of Abbreviation</td>
<td>35</td>
</tr>
<tr>
<td>Scientific Publications and Presentations</td>
<td>39</td>
</tr>
<tr>
<td>Chapter 1. Introduction</td>
<td>40</td>
</tr>
<tr>
<td>1.1. General Introduction</td>
<td>41</td>
</tr>
<tr>
<td>1.2. The Anatomy and Physiology of the Eye</td>
<td>42</td>
</tr>
<tr>
<td>1.3. Corneal Diseases</td>
<td>44</td>
</tr>
<tr>
<td>1.4. Pharmacological Management of Corneal Diseases</td>
<td>47</td>
</tr>
<tr>
<td>1.4.1. Corneal Infection Management</td>
<td>47</td>
</tr>
<tr>
<td>1.4.2. Corneal Fibrosis Management</td>
<td>48</td>
</tr>
<tr>
<td>1.4.3. Corneal Neovascularisation Management</td>
<td>49</td>
</tr>
<tr>
<td>1.5. Ophthalmic Dosage Forms</td>
<td>50</td>
</tr>
<tr>
<td>1.5.1. An Overview of Ocular Dosage Forms</td>
<td>50</td>
</tr>
<tr>
<td>1.5.2. Barriers to Ocular Absorption</td>
<td>56</td>
</tr>
<tr>
<td>1.6. Nanotechnology for the Treatment of Eye Disorders</td>
<td>59</td>
</tr>
<tr>
<td>1.6.1. Overview of Nano-carriers</td>
<td>60</td>
</tr>
<tr>
<td>1.6.2. Nano-carriers for Ocular Delivery</td>
<td>64</td>
</tr>
<tr>
<td>1.6.3. Overview of Nanofibres</td>
<td>73</td>
</tr>
<tr>
<td>1.6.4. Antimicrobial Application of the Nanofibres</td>
<td>74</td>
</tr>
<tr>
<td>1.6.5. Drug Delivery Application of Nanofibres</td>
<td>77</td>
</tr>
<tr>
<td>1.6.6. Wound Healing Application of the Nanofibres</td>
<td>79</td>
</tr>
<tr>
<td>1.7. Nanofibre Fabrication by Electrospinning</td>
<td>84</td>
</tr>
<tr>
<td>1.7.1. The Principle of Monoaxial Electrospinning</td>
<td>86</td>
</tr>
<tr>
<td>1.7.2. The Principle of Multi-layered Electrospinning</td>
<td>88</td>
</tr>
</tbody>
</table>
1.7.3. Factors Affecting Electrospinning 89
1.7.4. Alternative Nanofibre Production Methods 92
1.7.5. Electrospun Nanofibres for Ocular Drug Delivery 93
1.7.6. Electrospun Nanofibres as Ocular Scaffolds 95
1.8. Clinical Use of Moxifloxacin and Pirfenidone 97
   1.8.1. Clinical Use of Moxifloxacin 97
   1.8.2. Clinical Use of Pirfenidone 102
1.9. Thesis Aims 105
1.10. Thesis Overview 107

Chapter 2. Materials and Methods 109

2.1. Materials 110
   2.1.1. Moxifloxacin 110
   2.1.2. Pirfenidone 112
   2.1.3. Poly Vinyl Alcohol (PVA) 114
   2.1.4. Poly Vinyl Pyrroldione (PVP) 117
   2.1.5. Poly-Lactic-co-Glycolic Acid (PLGA) 118
   2.1.6. Solvents 120

2.2. Methods 121
   2.2.1. Electrospinning Techniques 121
   2.2.2. Characterisation Methods 122
      2.2.2.1. Light and Fluorescence Microscopy 122
      2.2.2.2. Confocal Microscopy 124
      2.2.2.3. Scanning Electron Microscopy (SEM) 126
      2.2.2.4. Transmission Electron Microscopy (TEM) 128
      2.2.2.5. Thermogravimetric Analysis (TGA) 130
      2.2.2.6. Differential Scanning Calorimetry (DSC) 131
      2.2.2.7. Fourier Transform Infrared Spectroscopy (FTIR) 134
      2.2.2.8. X-Ray Powder Diffraction (XRD) 136
      2.2.2.9. High Performance Liquid Chromatography (HPLC) 138
      2.2.2.10. Drug Release Studies 140
      2.2.2.11. Minimum Inhibitory Concentration (MIC) 144
      2.2.2.12. Zone of Inhibition 145
      2.2.2.13. Cellular Sub-Culturing 146
Chapter 3. Preparation of PLGA-pirfenidone/PVA-moxifloxacin coaxial fibres

3.1. Introduction
3.2. Materials and Methods
  3.2.1. Materials
  3.2.2. Methods
    3.2.2.1. Preparation of Monoaxial PLGA Fibres
    3.2.2.2. Preparation of Monoaxial PVA Fibres
    3.2.2.3. Preparation of Coaxial Fibres
    3.2.2.4. Size and Shape of Fibres
      3.2.2.4.1. Scanning Electron Microscopy
      3.2.2.4.2. Transmission Electron Microscopy
      3.2.2.4.3. Light and Fluorescence Microscopy
    3.2.2.5. Thermal Analysis
      3.2.2.5.1. Differential Scanning Calorimetry
      3.2.2.5.2. Thermogravimetric Analysis
    3.2.2.6. HPLC Assay Methods for Determination of Pirfenidone and Moxifloxacin in the Fibres
      3.2.2.6.1. Development of the HPLC Assay Using Water as the Dissolution Solvent
      3.2.2.6.2. Development of the HPLC Assay Using Acetonitrile as the Dissolution Solvent
      3.2.2.6.3. Development of the HPLC Assay Using PBS (pH 7.4) as the Dissolution Solvent
    3.2.2.7. Drug Loading [DL], Entrapment Efficiency [EE%] and Yield [Y%]
  3.3. Results and Discussion
    3.3.1. Morphological Characteristics of Fibres
      3.3.1.1. Blank Monoaxial PLGA Fibres
      3.3.1.2. Pirfenidone-loaded Monoaxial PLGA Fibres
      3.3.1.3. Blank Monoaxial PVA Fibres
3.3.1.4. Moxifloxacin-loaded Monoaxial PVA Fibres 176
3.3.1.5. Blank Coaxial PLGA/PVA Fibres 178
3.3.1.6. Dual Drug-loaded Coaxial PLGA/PVA Fibres 181
3.3.2. Fibre Size and Microscopic Analysis 181
3.3.3. Thermal Analysis 192
  3.3.3.1. Thermal Characterisation of the Drugs 192
  3.3.3.2. Thermal Characterisation of the Polymers 194
  3.3.3.3. Thermal Characterisation of the Polymer-drug Physical Mixtures 198
3.3.4. HPLC Analysis 199
  3.3.4.1. Aqueous Extraction Method 200
    3.3.4.1.1. Moxifloxacin alone 202
    3.3.4.1.2. Pirfenidone alone 204
    3.3.4.1.3. Moxifloxacin and pirfenidone in combination 206
  3.3.4.2. Organic Extraction Method 210
    3.3.4.2.1. Moxifloxacin alone 212
    3.3.4.2.2. Pirfenidone alone 214
    3.3.4.2.3. Moxifloxacin and pirfenidone in combination 216
  3.3.4.3. Phosphate Buffer Extraction Method 220
    3.3.4.3.1. Moxifloxacin and pirfenidone in combination 221
  3.3.4.4. Limit of Detection and Limit of Quantitation 223
3.3.5. Drug loading [DL], Encapsulation Efficiency [EE%] and Yield [Y%] 225
3.4. Conclusions 229

Chapter 4. Preparation of PLGA-pirfenidone/PVP-moxifloxacin multilayer fibres 230
4.1. Introduction 231
4.2. Materials and Methods 233
  4.2.1. Materials 233
  4.2.2. Methods 233
    4.2.2.1. Preparation of Monoaxial PLGA Fibres 233
    4.2.2.2. Preparation of Monoaxial PVP Fibres 235
    4.2.2.3. Preparation of Coaxial Fibres 236
4.2.2.4. Preparation of Triaxial Fibres 238
4.2.2.5. Conductivity and Viscosity Determination 240
4.2.2.6. Size and Shape of Fibres 240
  4.2.2.6.1. Scanning Electron Microscopy 240
  4.2.2.6.2. Transmission Electron Microscopy 241
  4.2.2.6.3. Light and Fluorescence Microscopy 241
  4.2.2.6.4. Confocal Microscopy 241
4.2.2.7. Thermal Analysis and Physical State Characterisation 241
  4.2.2.7.1. Thermogravimetric Analysis 242
  4.2.2.7.2. Differential Scanning Calorimetry 242
  4.2.2.7.3. Fourier-Transform Infrared Spectroscopy 242
  4.2.2.7.4. X-Ray Diffraction 243
4.2.2.8. Drug Loading [DL], Entrapment Efficiency [EE%] and Yield [Y%] 243
  4.2.2.9. Drug Release Profile 244
  4.2.2.10. Release Kinetic Modelling 244
  4.2.2.11. Swelling and Weight Loss Behavior 245
4.3. Results and Discussion 246
  4.3.1. Morphological Characteristics of Fibres 246
    4.3.1.1. Blank Monoaxial PLGA Fibres 246
    4.3.1.2. Pirfenidone-loaded Monoaxial PLGA Fibres 249
    4.3.1.3. Blank Monoaxial PVP Fibres 251
    4.3.1.4. Moxifloxacin-loaded Monoaxial PVP Fibres 253
    4.3.1.5. Blank Coaxial PLGA/PVP Fibres 255
    4.3.1.6. Drug-loaded Coaxial PLGA/PVP Fibres 258
    4.3.1.7. Blank and Drug-loaded Triaxial PVP/PLGA/PVP Fibres 259
4.3.2. Fibre Size and Microscopic Analysis 261
4.3.3. Thermal Analysis and Physical State Characterisation 281
  4.3.3.1. Thermogravimetric Analysis 282
  4.3.3.2. Differential Scanning Calorimetry 290
  4.3.3.3. Fourier-Transform Infrared Spectroscopy 298
4.3.3.4. X-Ray Diffraction

4.3.4. Drug Loading [DL], Entrapment Efficiency [EE%] and Yield [Y%]

4.3.5. Drug Release Profile

4.3.5.1. Monoaxial Fibres Release using Dialysis Bags

4.3.5.2. Coaxial Fibres Release using Dialysis Bags

4.3.5.3. Monoaxial Fibres Release using Custom-Made Cages

4.3.5.4. Coaxial Fibres Release using Custom-Made Cages

4.3.5.5. Triaxial Fibres Release using Custom-Made Cages

4.3.5.6. Drug Release Study Summary

4.3.5.7. Release Kinetic Modelling

4.3.7. Swelling and Weight Loss Behaviour

4.4. Conclusion

Chapter 5. In vitro Biological Efficacy Assessment of the Multi-Layered Electrospun Fibres

5.1. Introduction

5.2. Materials and Methods

5.2.1. Materials

5.2.2. Methods

5.2.2.1. Determination of the Antimicrobial Efficacy of the Multi-Layered Fibres

5.2.2.1.1. Minimum Inhibitory Concentration (MIC) Determination

5.2.2.1.2. Zone of Inhibition Determination

5.2.2.1.3. Sterility Testing

5.2.2.2. In Vitro Determination of the Anti-scarring potential of the Multi-Layered Fibres

5.2.2.2.1. Fibroblast Sub-Culturing

5.2.2.2.2. Inhibitory Concentration (IC) Determination

5.2.2.2.3. Cell Treatment with the Multi-Layered Fibres

5.2.2.2.4. Protein Quantification for Western Blot
5.2.2.2.5. Proteins Determination by the Western Blot Technique

5.3. Results and Discussion

5.3.1. Determination of the Antimicrobial Efficacy of the Multi-Layered Fibres

5.3.1.1. Minimum Inhibitory Concentration Determination
5.3.1.2. Zone of Inhibition Determination
5.3.1.3. Sterility Testing

5.3.2. In Vitro Determination of the Anti-scarring Efficacy of the Multi-Layered Fibres

5.3.2.1. Inhibitory Concentration Determination
5.3.2.2. Cell Treatment with the Multi-Layered Fibres
5.3.2.3. α-SMA Expression Determination using Western Blotting

5.4. Conclusions

Chapter 6. In Vivo Assessment of Drug-Loaded Coaxial Electrospun Fibres

6.1. Introduction

6.2. Materials and Methods

6.2.1. Materials
6.2.2. Animals
6.2.3. Methods

6.2.3.1. Pharmacokinetic Study

6.2.3.1.1. Calculation of the Administrated Drug Dose
6.2.3.1.2. UPLC Analysis
6.2.3.1.3. UPLC Method Validation
6.2.3.1.4. Ocular Pharmacokinetics Study

6.2.3.2. Infection Inhibition Study
6.2.3.3. Eye Irritation Study

6.3. Results and Discussion

6.3.1. UPLC Assay for Moxifloxacin and Pirfenidone Separation

6.3.1.1. Specificity
6.3.1.2. Linearity
6.3.1.3. Intra- and Inter-day Precision  398
6.3.1.4. Robustness  400
6.3.2. Ocular Pharmacokinetic Study  402
6.3.3. Infection Inhibition Study  405
6.3.4. Eye Irritation Study  409
6.4. Conclusion  417

**Chapter 7. Conclusion and Future Work**  418
7.1. Conclusions  419
7.2. Future Work  426
References  428
List of Figures

Chapter 1

Figure 1.1: The main constituents of the human eye. Adapted from (medicinenet.com, n.d.).

Figure 1.2: A schematic diagram of the anatomy of the cornea and pre-corneal tear film (James and Bron, 2011).

Figure 1.3: Corneal wound healing process. 1) Corneal basement membrane will be lost by an injury 2) Cytokines will be released into the stroma 3) Keratocytes will proliferate and migrate to form fibroblasts 4) Fibroblasts will trans-differentiate to myofibroblast by TGF 5) In normal conditions, myofibroblasts will undergo apoptosis to repair the cornea 6) In pathological conditions, myofibroblasts secrete irregular matrix 7) This will lead to hazy vision. Adapted from Chaurasia et al. (2015).

Figure 1.4: A schematic diagram of an intravitreal injection bypassing the anterior segment of the eye. Adapted from Ochakovski et al. (2017).

Figure 1.5: Different dosage forms for the anterior segment of the eye showing the barriers to the ocular drug absorption. Adapted from (Bachu et al., 2018).

Figure 1.6: Several types of NPs used in drug delivery. Adapted from Maximilien et al. (2015).

Figure 1.7: Scanning electron microscope image of randomly oriented PVA nanofibres network.

Figure 1.8: The application of fibres on an open wound to accelerate its closure with (a) showing the effect of bacteria on retarding the wound closure, (b) showing bacterium entry prevention by applying the fibrous membrane, (c) showing wound closure facilitated by the application of the fibrous membrane. Adapted from (Augustine et al., 2014).

Figure 1.9: Wound closure after treating with silver NPs loaded MADO fibres, blank MADO fibres and untreated control group at 0, 5, 10, and 15 days showing the accelerated wound closure of silver NPs loaded MADO fibres compared to the other groups. Adapted from (Nejad et al., 2015).

Figure 1.10: PVP fibrous network forming a mat after 2 hours of collection on aluminium foil.

Figure 1.11: A Taylor cone forming a stable jet after adjusting the electrospinning parameters (flow rate, tip-to-collector distance, applied voltage and polymer solution concentration).
Figure 1.12: A schematic diagram of the electrospinning process illustrating its main components and the Taylor cone. The use of monoaxial, coaxial or triaxial needle system can produce single-, double- or triple-layered fibres, respectively.

Chapter 2

Figure 2.1: Moxifloxacin hydrochloride chemical structure.

Figure 2.2: Pirfenidone chemical structure.

Figure 2.3: Partially and fully hydrolysed PVA structures in which the former one is the most commonly used type of PVA.

Figure 2.4: Polymerisation of PVP from N-vinyl pyrrolidone.

Figure 2.5: Hydrolysis of PLGA to D, L lactic acid and glycolic acid monomers

Figure 2.6: Electrospinning instrumentation showing its main components (Spraybase.com, n.d.).

Figure 2.7: Light microscopy principle showing the light pathway. Adapted from (clinicalgate.com, 2015).

Figure 2.8: Confocal microscopy principle showing the laser pathway. Adapted from (Fellers and Davidson, n.d.).

Figure 2.9: Scanning electron microscopy principle showing the electrons pathway. Adapted from (Bitesizebio.com, n.d.).

Figure 2.10: Transmission electron microscopy principle showing the electrons pathway. Adapted from (Bitesizebio.com, n.d.).

Figure 2.11: TGA instrumentation illustration showing the tested sample being hanged on a sample holder. Adapted from (Patel, n.d.).

Figure 2.12: DSC experimental setup showing the reference and the sample pans being placed on the DSC pans’ holders. Adapted from (evitherm.org, n.d.).

Figure 2.13: FTIR principle illustration showing the IR light pathway. Adapted from (instrumentationforum.com, n.d.).

Figure 2.14: A schematic diagram of the XRD goniometer. Adapted from (Husnain and Madhuku, 2017).

Figure 2.15: HPLC instrumentation illustration showing its main components. Adapted from (laboratoryinfo.com, 2019).

Figure 2.16: A schematic diagram of the rotating basket apparatus for dissolution test. Adapted from (Blaesi, 2014).
Figure 2.17: A schematic diagram of the dialysis bag (left image) and a custom-made cage in a glass container (right image).

Figure 2.18: The antibiotic MIC determination assay. The yellow colour indicates for the turbidity that occur due to the bacterial growth, while the white colour indicates the absence of bacterial growth.

Figure 2.19: The antibiotic disc diffusion assay. A, B and C are antibiotic discs with different bacterial susceptibility.

Figure 2.20: The haemocytometer gridded squares located in each corner (A-D). Blue stained cells that are located in these corners were visually counted. Adapted from (weberscientific.com, n.d).

Figure 2.21: The MTT assay principle. Blue culture indicates for the presence of the cells before being exposed to the treatment, while the yellow culture indicates for the cells being exposed to the treatment. The purple culture indicates for the presence of MTT within the culture.

Figure 2.22: A descriptive diagram of the western blot technique principle. Adapted from (creativebiomart.net, n.d.).

Chapter 3
Figure 3.1: The surface morphology of blank PLGA fibres prepared by different PLGA MWs showing the presence of beads and pores. Form. 1: 25% PLGA (17 kg/mol), form. 2-4: 20%, 25%, 30% PLGA (44 kg/mol), respectively, form. 5: 12.5% PLGA (153 kg/mol), form. 6-10: 15% PLGA (153 kg/mol) in different applied voltage to test the effect of this parameter on the yielded fibres.

Figure 3.2: The surface morphology of pirfenidone-loaded PLGA fibres prepared by 15% PLGA (153 kg/mol) showing the presence of pores. Form. 11: 0.25% pirfenidone, form. 12: 0.5% pirfenidone, form. 13: 1% pirfenidone, form. 14: 2% pirfenidone.

Figure 3.3: The surface morphology of blank PVA fibres prepared by different PVA MWs showing the presence of beaded or flattened fibres. Form. 15 and 16: 15% PVA (31-50 kg/mol), form. 17-19: 7.5% PVA (146-186 kg/mol) in different distance and flow rate, form. 20: 7.5% PVA (146-186 kg/mol) dissolved in 10% ethanol, form. 21: 7.5% PVA (146-186 kg/mol) dissolved in 20% ethanol.

Figure 3.4: The surface morphology of moxifloxacin-loaded PVA fibres prepared by 7.5% PVA (146-186 kg/mol) showing the presence of drug crystals. Form. 22: 1% moxifloxacin, form. 23: 2% moxifloxacin.
Figure 3.5: The surface morphology of blank coaxial fibres showing the presence of pores. Form. 24-29: prepared by 15% PLGA (MW 153 kg/mol) and 7.5% PVA (MW 146-186 kg/mol) in different core to shell flow rate ratio, form. 30: prepared by 15% PLGA (MW 153 kg/mol) and 7.5% PVA (MW 146-186 kg/mol) dissolved in 10% ethanol, form. 31: prepared by 15% PLGA (MW 153 kg/mol) and 7.5% PVA (MW 146-186 kg/mol) dissolved in 20% ethanol. C-to-S: core-to-shell flow rate.

Figure 3.6: The surface morphology of drug-loaded coaxial fibres prepared by 1% pirfenidone loaded into 15% PLGA (MW 153 kg/mol) and 1% moxifloxacin loaded into 7.5% PVA (MW 146-186 kg/mol) showing its rough surface.

Figure 3.7: PLGA electrospinning jet splitting due to the increase of the applied voltage. 1: PLGA solution droplet (at 0 kV), 2: PLGA solution droplet elongated prior to jet stabilisation (at 13 kV), 3: PLGA stable jet at optimum parameters (at 20 kV), 4: PLGA stable jet start to elongate and split (at 20 kV), 5: PLGA jet split, 6: PLGA jet split into 3 visible jets (at 22 kV), 7: PLGA jet split into multiple jets with the main jet is unstable (at 24 kV), 8: PLGA jet split into multiple jets with the main jet is stable (at 24 kV). The red arrow is pointed at the PLGA main stream (jet).

Figure 3.8: The influence of the applied voltage on the formation of the Taylor cone. Increasing the voltage gradually will form initially narrower fibres and then larger diameter fibres. The Taylor cone is the black coloured cone. Adapted from (Bhattarai et al., 2019).

Figure 3.9: TEM images of the blank monoaxial and coaxial fibres. A: 15% PLGA fibres, B: 7.5% PVA fibres, C: 15% PLGA and 7.5% PVA coaxial fibres.

Figure 3.10: Light microscopic images of the blank and drug-loaded monoaxial and coaxial fibres. A1: 15% PLGA fibres, B1: 7.5% PVA fibres, C1: 15% PLGA and 7.5% PVA coaxial fibres, D1: 1% pirfenidone loaded into 15% PLGA fibres, E1: 1% moxifloxacin loaded into 7.5% PVA fibres, F1: 1% pirfenidone loaded into 15% PLGA and 1% moxifloxacin loaded into 7.5% PVA coaxial fibres.

Figure 3.11: UV microscopic images of the blank and drug-loaded monoaxial and coaxial fibres. A2: 15% PLGA fibres, B2: 7.5% PVA fibres, C2: 15% PLGA and 7.5% PVA coaxial fibres, D2: 1% pirfenidone loaded into 15% PLGA fibres, E2: 1% moxifloxacin loaded into 7.5% PVA fibres,
F2: 1% pirfenidone loaded into 15% PLGA and 1% moxifloxacin loaded into 7.5% PVA coaxial fibres.

Figure 3.12: TGA (top image) and DSC (bottom image) traces of moxifloxacin.

Figure 3.13: TGA (top image) and DSC (bottom image) traces of pirfenidone.

Figure 3.14: TGA (top image) and MTDSC (middle image) traces of high MW PLGA, and MTDSC (bottom image) trace of middle MW PLGA.

Figure 3.15: TGA (top image) and MTDSC (middle image) traces of high MW PVA, and MTDSC (bottom image) of trace low MW PVA which shows the full cycle of heat-cool-heat analysis.

Figure 3.16: MTDSC trace of the 15:1 PLGA-pirfenidone physical mixture.

Figure 3.17: MTDSC trace of the 7.5:1 PVA-moxifloxacin physical mixture.

Figure 3.18: UV absorbance scan for moxifloxacin (left image) and pirfenidone (right image) dissolved in water.

Figure 3.19: Representative HPLC chromatograms using the aqueous extraction method. Moxifloxacin (50 µg/ml) in water (top image), pirfenidone (50 µg/ml) in water (middle image) and moxifloxacin (50 µg/ml) and pirfenidone (50 µg/ml) in combination (bottom image).

Figure 3.20: Standard curves for HPLC aqueous extraction of moxifloxacin in three successive days. All curves show fair linearity ($R^2 \geq 0.998$).

Figure 3.21: Standard curves for HPLC aqueous extraction of pirfenidone in three successive days. All curves show excellent linearity ($R^2 \geq 0.9999$).

Figure 3.22: Standard curves for HPLC aqueous extraction of moxifloxacin and pirfenidone in combination in three successive days. Moxifloxacin curves show fair linearity ($R^2 \geq 0.998$), while pirfenidone curves show excellent linearity ($R^2 \geq 0.9999$).

Figure 3.23: Representative HPLC chromatograms using the organic extraction method. Moxifloxacin (18.75 µg/ml) in ACTN (top image), pirfenidone (18.75 µg/ml) in ACTN (middle image) and moxifloxacin (18.75 µg/ml) and pirfenidone (18.75 µg/ml) in combination (bottom image).

Figure 3.24: Standard curves for HPLC organic extraction of moxifloxacin in three successive days. All curves show good linearity ($R^2 \geq 0.9995$).

Figure 3.25: Standard curves for HPLC organic extraction of pirfenidone in three successive days. All curves show excellent linearity ($R^2 \geq 0.99997$).
Figure 3.26: Standard curves for HPLC organic extraction of moxifloxacin and pirfenidone in combination in three successive days. All curves excellent good linearity ($R^2 \geq 0.9996$).

Figure 3.27: Representative HPLC chromatograms using the buffer extraction method. Moxifloxacin (12.5 µg/ml) and pirfenidone (12.5 µg/ml) in combination.

Figure 3.28: Standard curves for HPLC buffer extraction of moxifloxacin and pirfenidone in combination in three successive days. All curves show good linearity ($R^2 \geq 0.999$).

Figure 3.29: PLGA polymeric gel-like formation due to unstable electrospinning jet. 1: stable PLGA jet at optimum parameters, 2: stable jet elongation, 3: the initiation of gel-like PLGA product formation, 4: jet continue to elongate, 5: further jet elongation, 6: gel-like PLGA product has formed.

Chapter 4

Figure 4.1: The surface morphology of blank PLGA fibres prepared by different solvent showing the presence of beaded fibres. Form. 1: 12.5% PLGA in ethyl acetate, form. 2: 15% PLGA in acetone, form. 3: 12.5% PLGA in DCM, form. 4: 15% PLGA in DCM, form. 5: 25% PLGA in ACTN, form. 6: 20% PLGA in ACTN:ethanol (2:1).

Figure 4.2: The surface morphology of drug-loaded PLGA fibres prepared using different solvents showing the presence of drug crystals. Form. 7: 15% PLGA in DCM and 1% pirfenidone, form. 8: 15% PLGA in DCM and 2% pirfenidone, form. 9: 25% PLGA in ACTN and 1% pirfenidone, form. 10: 25% PLGA in ACTN and 2% pirfenidone, form. 11: 20% PLGA in ACTN:ethanol (2:1) and 2% pirfenidone and 1% moxifloxacin.

Figure 4.3: The surface morphology of blank PVP fibres (10%) prepared using different solvents showing the lack of beads. Form. 12: in ethanol, form. 13: in ethanol:acetone (8:1), form. 14: in ethanol:acetone (2:1), form. 15: in ethanol:DCM (1:1), form. 16: in ethanol:ACTN (1:1).

Figure 4.4: The surface morphology of drug-loaded PVP fibres (10%) prepared using different solvents showing the presence of very fine fibres as an indication of jet instability. Form. 17: in ethanol:DCM (1:1) and 0.5% moxifloxacin, form. 18: in ethanol:DCM (1:1) and 1% moxifloxacin, form. 19: in ethanol:DCM (1:1) and 1.5% moxifloxacin, form. 20: ethanol:ACTN (1:1) and 1% moxifloxacin.
Figure 4.5: The surface morphology of blank coaxial fibres prepared by different solvent showing the lack of beads. Form. 21: 15% PLGA in acetone and 10% PVP in ethanol (C-to-S: 1:6), form. 22: 15% PLGA in acetone and 10% PVP in ethanol (C-to-S: 1:4), form. 23: 15% PLGA in acetone and 10% PVP in ethanol:acetone (8:1), form. 24: 15% PLGA in acetone and 10% PVP in ethanol:acetone (2:1), form. 25: 15% PLGA in DCM and 10% PVP in ethanol:DCM (1:1), form. 26: 15% PLGA in ACTN and 10% PVP in ethanol:ACTN (1:1). C-to-S: core-to-shell flow rate.

Figure 4.6: The surface morphology of drug-loaded coaxial fibres prepared by different solvent showing the presence of very fine fibres as an indication of jet instability. Form. 27: 15% PLGA and 1% pirfenidone in DCM and 10% PVP and 0.5% moxifloxacin in ethanol:DCM (1:1), form. 28: 15% PLGA and 2% pirfenidone in DCM and 10% PVP and 1% moxifloxacin in ethanol:DCM (1:1), form. 29: 15% PLGA and 2% pirfenidone in DCM and 10% PVP and 1.5% moxifloxacin in ethanol:DCM (1:1), form. 30: 25% PLGA and 2% pirfenidone in ACTN and 10% PVP and 1% moxifloxacin in ethanol:ACTN (1:1).

Figure 4.7: The surface morphology of the blank and drug-loaded triaxial fibres showing the lack of beads and drug crystals. Form. 31: 10% PVP in ethanol:ACTN (1:1), 25% PLGA in ACTN and 10% PVP in ethanol:ACTN (1:1), form. 32: 10% PVP and 1% moxifloxacin in ethanol:ACTN (1:1), 25% PLGA and 2% pirfenidone in ACTN and 10% PVP and 1% moxifloxacin in ethanol:ACTN (1:1).

Figure 4.8: TEM images of the drug-loaded fibres showing the distinctive inner layer(s) of the coaxial and triaxial fibres compared to the monoaxial fibres. Form. 8: PLGA in DCM, form. 10: PLGA in ACTN, form. 20: PVP in ethanol:ACTN, form. 28: coaxial fibres prepared using DCM, form. 30: coaxial fibres prepared using ACTN, form. 32: triaxial fibres prepared using ACTN.

Figure 4.9: Light microscope images of the blank and drug-loaded monoaxial, coaxial and triaxial fibres. PLGA monoaxial fibres, PLGA/PVP coaxial fibres and PVP/PLGA/PVP triaxial fibres showed a tube-like appearance compared to the PVP monoaxial fibres which have a thread-like appearance.
Figure 4.10: UV microscopic images of the blank and drug-loaded monoaxial, coaxial and triaxial fibres. The presence of moxifloxacin allows the fibres to fluoresce in the drug-loaded PVP monoaxial fibres (form. 18 and 20), coaxial fibres (form. 28 and 30) and triaxial fibres (form. 32) compared to the blank PLGA monoaxial fibres (form. 4 and 5), drug-loaded PLGA monoaxial fibres (form. 8 and 10), blank PVP monoaxial fibres (form. 15 and 16), blank coaxial fibres (form. 25 and 26) and blank triaxial fibres (form. 31).

Figure 4.11: Confocal microscope image showing moxifloxacin and MB the fluorescence in (a) PVP moxifloxacin monoaxial fibres and (b) PLGA MB monoaxial fibres. A; channel one Ex. 300 nm and Em. 500 nm, B; channel two Ex. 600 nm and Em. 710 nm, T; transmitted light channel, M; merge of channels A, B and T.

Figure 4.12: Confocal microscope image showing moxifloxacin and MB the fluorescence in (a) blank coaxial fibres and (b) moxifloxacin-MB loaded coaxial fibres. A; channel one Ex. 300 nm and Em. 500 nm, B; channel two Ex. 600 nm and Em. 710 nm, T; transmitted light channel, M; merge of channels A, B and T.

Figure 4.13: Confocal microscope image showing moxifloxacin and MB the fluorescence in (a) blank triaxial fibres and (b) moxifloxacin-MB loaded triaxial fibres. A; channel one Ex. 300 nm and Em. 500 nm, B; channel two Ex. 600 nm and Em. 710 nm, T; transmitted light channel, M; merge of channels A, B and T.

Figure 4.14: TGA thermograms of (a) moxifloxacin, (b) pirfenidone, (c) PLGA and (d) PVP raw materials, showing their degradation onset temperature(s).

Figure 4.15: TGA thermograms of (a) PLGA pirfenidone PM, (b) PVP moxifloxacin PM, (c) blank PLGA and PVP PM (coaxial), (d) blank PLGA and PVP PM (triaxial), (e) drug-loaded PLGA and PVP PM (coaxial) and (f) drug-loaded PLGA and PVP PM (triaxial), showing their degradation onset temperature(s).

Figure 4.16: TGA thermograms of (a) blank PLGA fibres, (b) pirfenidone-loaded PLGA fibres, (c) blank PVP fibres and (d) moxifloxacin-loaded PVP fibres, showing their degradation onset temperature(s).
Figure 4.17: TGA thermograms of (a) blank coaxial fibres, (b) drug-loaded coaxial fibres, (c) blank triaxial fibres and (d) drug-loaded triaxial fibres, showing their degradation onset temperature(s).

Figure 4.18: MTDSC traces of the free form PVP (powder form), showing its Tg temperature.

Figure 4.19: MTDSC traces of (a) PLGA pirfenidone PM, (b) PVP moxifloxacin PM, (c) PLGA PVP PM and (d) PLGA pirfenidone - PVP moxifloxacin PM. The melting point and Tg of the drugs and polymers, respectively, were all detected.

Figure 4.20: MTDSC traces of (a) blank PLGA fibres, (b) pirfenidone-loaded PLGA fibres, (c) blank PVP fibres and (d) moxifloxacin-loaded PVP fibres. The Tg of the polymers were detected, while the drugs melting points were absent.

Figure 4.21: MTDSC thermograms of (a) blank coaxial fibres, (b) drug-loaded coaxial fibres, (c) blank triaxial fibres and (d) drug-loaded triaxial fibres. The Tg of the polymers were detected, while the drugs melting points were absent.

Figure 4.22: FTIR spectra of the pure PLGA, PVP, pirfenidone and moxifloxacin, showing each material distinctive peaks.

Figure 4.23: FTIR spectra of (a) blank and pirfenidone-loaded PLGA fibres and PM (b) blank and moxifloxacin-loaded PVP fibres and PM, showing pirfenidone and moxifloxacin distinctive peaks in the drug-loaded monoaxial fibres and the PMs.

Figure 4.24: FTIR spectra of (a) blank and drug-loaded coaxial fibres and PM and (b) blank and drug-loaded triaxial fibres and PM, showing pirfenidone and moxifloxacin distinctive peaks in the drug-loaded coaxial and triaxial fibres and their PMs.

Figure 4.25: XRD patterns of pure PLGA, PVP, pirfenidone and moxifloxacin, showing that both drugs are in the crystalline form (distinct peaks), while the both polymers are in the amorphous form (broad halos).

Figure 4.26: XRD patterns of (a) blank and pirfenidone-loaded PLGA fibres and PM (b) blank and moxifloxacin-loaded PVP fibres and PM. It shows that both the blank and drug-loaded monoaxial fibres are in the amorphous form (broad halos), while there are distinct peaks in the PM.

Figure 4.27: XRD patterns of (a) blank and drug-loaded coaxial fibres and PM (b) blank and drug-loaded triaxial fibres and PM. It shows that the blank
and drug-loaded coaxial and triaxial fibres and their blank PM are in the amorphous form (broad halos), while there are distinct peaks in the drug-loaded PM.

Figure 4.28: The release profile of 2% of pirfenidone-loaded into 25% PLGA monoaxial fibre against and equivalent amount of pirfenidone powder using dialysis bags. The drug-loaded fibres showed a first burst release after 24 hours followed by sustained release profile for 15 days and finally, a second burst release compared to a 24 hours complete release of the powder form drug. The large graph represents the full release duration (30 days), while the small graph represents the release after 24 hours.

Figure 4.29: SEM image of 2% pirfenidone-loaded into 25% PLGA monoaxial fibres after 30 days release. Fibres are entangled and the fibres’ surfaces are smooth.

Figure 4.30: The release profile of 1% moxifloxacin-loaded into 10% PVP monoaxial fibre over 24 hours compared to an equivalent amount of moxifloxacin powder using dialysis bags. The drug-loaded fibres showed a complete release after 24 hours in a sustain release profile similar to the powder form drug.

Figure 4.31: The release profile of 2% pirfenidone-loaded into 25% PLGA and 1% moxifloxacin-loaded into 10% PVP from the drug-loaded coaxial fibres using dialysis bags. The drug-loaded fibres showed a burst release of pirfenidone after 2 hours followed by ~97% compared to a sustained release profile of moxifloxacin for 30 days. The large graph represents the full release duration (30 days), while the small graph represents the release after 24 hours.

Figure 4.32: SEM image of 2% pirfenidone-loaded into 25% PLGA and 1% moxifloxacin into 10% PVP drug-loaded coaxial fibres after 30 days release. Fibres are entangled and the fibres’ surface are smooth.

Figure 4.33: The release profile of 1% and 2% w/v pirfenidone-loaded into 25% PLGA monoaxial fibre against an equivalent amount of pirfenidone powder using custom made cages after 15 days release. The drug-loaded fibres showed a similar profile of a burst release after 1 hour followed by sustained release profile for 15 days compared to the complete release of the powder form drug after the first time point. The large graph represents
the full release duration (15 days), while the small graph represents the release after 24 hours.

Figure 4.34: The release profile of 1% moxifloxacin-loaded into 10% PVP monoaxial fibre against an equivalent amount of moxifloxacin powder using custom made cages after 24 hours release. The drug-loaded fibres showed a complete release of moxifloxacin after 4 hours compared to the full release of the powder form drug after the first time point.

Figure 4.35: The release profile of 2% pirfenidone- and 1% moxifloxacin-loaded into 20% PLGA monoaxial fibre using custom made cages after 4 days release. The drug-loaded fibres showed a fast release of both drugs after 4 hours followed by a complete release after 24 hours.

Figure 4.36: SEM image of 2% pirfenidone- and 1% moxifloxacin-loaded into 20% PLGA monoaxial fibres after 4 days release. Fibres are entangled and the fibres’ surface are smooth.

Figure 4.37: The release profile of 2% pirfenidone-loaded into 25% PLGA and 1% moxifloxacin-loaded into 10% PVP from the drug-loaded coaxial fibres using custom made cages. The drug-loaded fibres showed a burst release of pirfenidone after 30 minutes followed by a complete release after 1 day compared to a burst release of moxifloxacin after 30 minutes followed by a plateau release up to 4 days. The large graph represents the full release duration (4 days), while the small graph represents the release after 24 hours.

Figure 4.38: SEM image of 2% pirfenidone-loaded into 25% PLGA and 1% moxifloxacin-loaded into 10% PVP from drug-loaded coaxial fibres after 4 days release. Fibres are entangled and the fibres’ surface are smooth.

Figure 4.39: The release profile of 1% moxifloxacin-loaded into 10% PVP, 2% pirfenidone-loaded into 25% PLGA and 1% moxifloxacin-loaded into 10% PVP from the drug-loaded triaxial fibres using custom made cages. The drug-loaded fibres showed a complete release of moxifloxacin and pirfenidone after 2 hours and 6 hours, respectively. The large graph represents the full release duration (4 days), while the small graph represents the release after 24 hours.

Figure 4.40: SEM image of 1% moxifloxacin-loaded into 10% PVP, 2% pirfenidone-loaded into 25% PLGA and 1% moxifloxacin-loaded into 10% PVP from drug-loaded triaxial fibres after 4 days release. Fibres are entangled and the fibres’ surface are smooth.
Figure 4.41: Swelling behaviour of pirfenidone-loaded PLGA monoaxial fibres, drug-loaded coaxial and triaxial fibres, showing their swelling % over time in the release medium.

Chapter 5

Figure 5.1: MIC test against S. aureus (A) and P. aeruginosa (B). The minimum concentrating (red circled) which the turbidity (grey or yellow coloured) disappears is the MIC. M: moxifloxacin, P: pirfenidone, MP: moxifloxacin and pirfenidone in 1:1 combination. The combined drugs (MP) was tested twice to verify the absence of any synergistic or inhibitory effects.

Figure 5.2: Zone of inhibition against S. aureus (A) and against P. aeruginosa (B). The diameter of the area of no growth is consider as the zone of inhibition. Blank fibre (upper left), moxifloxacin disc (upper right), drug-loaded coaxial fibre (lower left) and drug-loaded triaxial fibre (lower right).

Figure 5.3: Sterility test after 1 day incubation in FTG medium (left image) and SBC medium (right image). The absence of a change in the medium colour or a visible turbidity or fungus growth is an indication for the medium sterility.

Figure 5.4: Sterility test after 7 days incubation in FTG medium (left image) and SBC medium (right image). The absence of a change in the medium colour or a visible turbidity or fungus growth is an indication for the medium sterility.

Figure 5.5: Sterility test after 14 days incubation in FTG medium (left image) and SBC medium (right image). The absence of a change in the medium colour or a visible turbidity or fungus growth is an indication for the medium sterility. The FTG contaminated medium (in the left image) showed a change in the medium colour and a visible turbidity. The SBC contaminated medium (in the right image) showed a visible growth of a red coloured fungus.

Figure 5.6: A light microscope image of SIRC cell line showing the adherence of the cells on the bottom surface of the culture flask after 24-hour incubation.
Figure 5.7: IC20 and IC50 determination after 24-hour exposure to different concentrations of the free drugs showing the dose response curves (top image) and MTT plate pictures (bottom image). The left curve and top MTT plate pictures represent moxifloxacin, the middle curve and MTT plate pictures represent pirfenidone, the right curve and bottom MTT plate pictures represent moxifloxacin and pirfenidone in 1:1 combination.

Figure 5.8: IC20 and IC50 determination after 48-hour exposure to different concentrations of the free drugs showing the dose response curves (top image) and MTT plate pictures (bottom image). The left curve and top MTT plate pictures represent moxifloxacin, the middle curve and MTT plate pictures represent pirfenidone, the right curve and bottom MTT plate pictures represent moxifloxacin and pirfenidone in 1:1 combination.

Figure 5.9: IC20 and IC50 determination after 72-hour exposure to different concentrations of the free drugs showing the dose response curves (top image) and MTT plate pictures (bottom image). The left curve and top MTT plate pictures represent moxifloxacin, the middle curve and MTT plate pictures represent pirfenidone, the right curve and bottom MTT plate pictures represent moxifloxacin and pirfenidone in 1:1 combination.

Figure 5.10: Cell viability % after 24 and 48 hours cells exposure to free drugs, blank and drug-loaded coaxial and triaxial fibres in equivalent concentrations of 0.08 mg/mL (80 µg/mL).

Figure 5.11: MTT plate pictures after 24 and 48 hours cells exposure to free drugs, blank and drug-loaded coaxial and triaxial fibres in equivalent concentrations of 0.08 mg/mL (80 µg/mL).

Figure 5.12: SEM image for cell growth and proliferation on fibrous membranes. Blank coaxial fibre (upper left), blank triaxial fibre (upper right), drug-loaded coaxial fibre (lower left) and drug-loaded triaxial fibre (lower right). Red arrows indicate the presence of cells growth within the fibrous network.

Figure 5.13: BCA protein standard curve to quantify the amount of proteins from each sample in order to load equivalent amounts of proteins from the tested samples into the wells of the Western blot gel. The curve shows fair linearity ($R^2 \geq 0.996$).

Figure 5.14: The top image shows the Western blot band intensities of β-Actin (experimental control) and α-SMA (protein of interest), while the bottom image shows Western blot quantification using the band intensities.
of both proteins. The bands were obtained after 24 and 48-hour exposure to TGF-β1 alone or in combination with the free drugs, blank or drug-loaded coaxial and triaxial fibres in equivalent concentrations of 40 µg/mL compared to the untreated group. B: blank and DL: drug-loaded.

Chapter 6

Figure 6.1: Representative UPLC chromatograms: (a) blank rabbit aqueous humour, (b) aqueous humour spiked with 100 ng/mL of moxifloxacin and pirfenidone solutions, (c) aqueous humour sample 2 hours after topical application of nanofibres and (d) aqueous humour sample 2 hours after topical application of combined drug solution.

Figure 6.2: UPLC standard curve of moxifloxacin by plotting the area under the curve against the corresponded concentration. The curve shows good linearity ($R^2 \geq 0.9997$).

Figure 6.3: UPLC standard curve of pirfenidone by plotting the area under the curve against the corresponded concentration. The curve shows fair linearity ($R^2 \geq 0.991$).

Figure 6.4: The aqueous humour concentrations of (a) moxifloxacin and (b) pirfenidone versus time profiles following topical ocular application of both drugs either in solution form (ED-Soln) or coaxial nanofibres (NF) to the rabbits. Each data was represented as mean ± SD (n=3).

Figure 6.5: Eye swabs for the growth of S. aureus on MSA plates; (a) pre infection (no bacterial growth), (b) 24-hour post infection, (c) 3-day post treatment and (d) 7-day post treatment. A: Moxifloxacin eye drops, B: untreated control group, C: drug-loaded coaxial fibres, D: blank coaxial fibres.

Figure 6.6. Photographs of the eyes; (a) pre infection (no bacterial growth), (b) 24-hour post infection, (c) 3-day post treatment and (d) 7-day post treatment. A: Moxifloxacin eye drops, B: untreated control group, C: drug-loaded coaxial fibres, D: blank coaxial fibres.
List of Tables

Chapter 1
Table 1.1: Factors affecting the fabrication of electrospinning technique. Adapted from (Chakraborty et al., 2009; Williams et al., 2018).

Chapter 2
Table 2.1: Physiochemical properties of different solvents.

Chapter 3
Table 3.1: Different grades of PLGA polymer and their characteristics according to manufacturer (Corbion, Netherlands).
Table 3.2: Electrospinning parameters for the preparation of blank and drug-loaded PLGA monoaxial fibres.
Table 3.3: Electrospinning parameters for the preparation of blank and drug-loaded PVA monoaxial fibres.
Table 3.4: Electrospinning parameters for the preparation of blank and drug-loaded PLGA/PVA coaxial fibres.
Table 3.5: Mean diameter, standard deviation (SD) and maximum and minimum diameters of the blank and drug-loaded (DL) monoaxial and coaxial fibres.
Table 3.6: Mean peak areas and retention times for day 1, 2 and 3 using HPLC aqueous extraction of moxifloxacin.
Table 3.7: Mean peak areas and retention times for day 1, 2 and 3 using HPLC aqueous extraction of pirfenidone.
Table 3.8: Mean peak areas and retention times for day 1, 2 and 3 using HPLC aqueous extraction of moxifloxacin and pirfenidone combined.
Table 3.9: Moxifloxacin and pirfenidone inter- and intra-day variations in HPLC aqueous extraction.
Table 3.10: Mean peak areas and retention times for day 1, 2 and 3 using HPLC organic extraction of moxifloxacin.
Table 3.11: Mean peak areas and retention times for day 1, 2 and 3 using HPLC organic extraction of pirfenidone.
Table 3.12: Mean peak areas and retention times for day 1, 2 and 3 using HPLC organic extraction of moxifloxacin and pirfenidone combined.
Table 3.13: Moxifloxacin and pirfenidone inter- and intra-day variations in HPLC organic extraction.
Table 3.14: Mean peak areas and retention times for day 1, 2 and 3 using HPLC buffer extraction of moxifloxacin and pirfenidone combined.

Table 3.15: Moxifloxacin and pirfenidone inter- and intra-day variations in HPLC buffer extraction.

Table 3.16: LOD and LOQ of moxifloxacin and pirfenidone in all HPLC methods.

Table 3.17: Formulation 32 - DL and EE% of pirfenidone and moxifloxacin in the outer and inner layer of the coaxial fibres.

Chapter 4

Table 4.1: Electrospinning parameters for the preparation of blank and drug-loaded PLGA monaxial fibres.

Table 4.2: Electrospinning parameters for the preparation of blank and drug-loaded PVP monaxial fibres.

Table 4.3: Electrospinning parameters for the preparation of blank and drug-loaded PLGA/PVP coaxial fibres.

Table 4.4: Electrospinning parameters for the preparation of blank and drug-loaded PVP/PLGA/PVP triaxial fibres.

Table 4.5: The viscosity and conductivity measurements of the monoaxial PLGA and PVP fibres’ polymeric solutions. The viscosity and conductivity values of each formulation are presented as mean ± standard deviation (SD) (n=3).

Table 4.6: Mean diameter, standard deviation (SD), and maximum and minimum diameters of the blank and drug-loaded monoaxial, coaxial and triaxial fibres.

Table 4.7: DL, EE% and Y% for the drug-loaded monoaxial, coaxial and triaxial fibres. Form. 8: PLGA in DCM, form. 10: PLGA in ACTN, form. 18: PVP in ethanol:DCM, form. 20: PVP in ethanol:ACTN, form. 28: coaxial fibres prepared using DCM, form. 30: coaxial fibres prepared using ACTN, form. 32: triaxial fibres prepared using ACTN.

Table 4.8: Various models for fitting drug release data. $F$ is the fraction (%) of drug released in time (t), $k_0$ is the zero-order release constant, $k_1$ is the first-order release constant, $k_H$ is the Higuchi release constant, $k_{KP}$ is the release constant from polymeric matrices, $n$ is the exponent of the release in function of time $t$ indicating the drug release mechanism, $k_{HB}$ is the Hopfenberg model release constant while the $n$ value is 1, 2, and 3 for film, cylinder or sphere, respectively, $\alpha$ value is the timescale of the process.
while $\beta$ defines the type of curve ($\beta=1$ for an exponential curve, $\beta >1$ for a sigmoid curve and $\beta <1$ for parabolic curve). Adopted from Siepmann and Siepmann (2008), Dash et al. (2010), Siepmann and Peppas (2012), Ramteke et al. (2014) and Bruschi (2015).

Table 4.9: Release kinetic modelling showing the coefficient of determination ($r^2$) and the release rate constant ($k$) for pirfenidone-loaded PLGA monoaxial fibres, moxifloxacin-loaded PVP monoaxial fibres, pirfenidone- and moxifloxacin-loaded PLGA monoaxial fibres, drug-loaded coaxial fibres and drug-loaded triaxial fibres. Pir: pirfenidone, moxi: moxifloxacin, $k_0$: zero-order release constant, $k_1$: first-order release constant, $k_H$: Higuchi release constant, $k_{HB}$: Hopfenberg model release constant, $\beta$: defines the type of curve, $k_{KP}$: is the release constant from polymeric matrices, $n$: the exponent of the release in function of time (t) indicating the drug release mechanism.

Table 4.10: Korsmeyer-Peppas release kinetic modelling for pirfenidone-loaded PLGA monoaxial fibres, moxifloxacin-loaded PVP monoaxial fibres, pirfenidone- and moxifloxacin-loaded PLGA monoaxial fibres and drug-loaded coaxial fibres. Pir: pirfenidone, moxi: moxifloxacin, $r^2$: coefficient of determination, $k_{KP}$: is the release constant from polymeric matrices, $n$: the exponent of the release in function of time (t) indicating the drug release mechanism.

Table 4.11: Weight loss behaviour of pirfenidone-loaded PLGA monoaxial fibres, drug-loaded coaxial and triaxial fibres, showing their average weight loss % after 24-hour incubation in the release medium.

Chapter 5

Table 5.1: The zone of inhibition diameters of moxifloxacin disc, blank and drug-loaded coaxial and triaxial fibres against S. aureus and P. aeruginosa.

Chapter 6

Table 6.1: Scores for grading the severity of ocular lesions in the ocular irritation studies.

Table 6.2: Linear regression data for the calibration curves and sensitivity (LOD and LOQ) parameters for moxifloxacin and pirfenidone.

Table 6.3: Intra- and inter-day precision of the developed UPLC method for moxifloxacin and pirfenidone showing the RSD% and recovery% for 3 different concentrations.
Table 6.4: Robustness of the UPLC method to analyse 200 ng/mL of moxifloxacin and pirfenidone following changing the mobile phase ratio by ± 2 mL each, pH by ± 0.2 and flow rate by ± 0.2 mL/minute.

Table 6.5: Ocular pharmacokinetic parameters of moxifloxacin and pirfenidone in aqueous humour after topical application of the drug-loaded coaxial fibres and both drugs in solution form. Each data was represented as mean ± SD (n=3) for each time point.

Table 6.6: Weighted individual scores for ocular irritation studies by blank fibres.

Table 6.7: Weighted individual scores for ocular irritation studies by drug-loaded coaxial fibres.

Table 6.8: Obtained weighted scores for ocular irritation studies by blank and drug-loaded coaxial fibres (combined form of Tables 6.6 and 6.7).

Table 6.9: Kay and Calandra (1962) classification of the ocular irritation scores.

Table 6.10: The MMTS calculations for the blank and drug-loaded coaxial fibres according to the scores from Table 6.9.
### List of Abbreviation

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>pH</td>
<td>A measure of Hydrogen ion concentration</td>
</tr>
<tr>
<td>ACTN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
</tr>
<tr>
<td>α-SMA</td>
<td>Alpha-Smooth Muscle Actin</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>Å</td>
<td>Angstrom</td>
</tr>
<tr>
<td>AUC</td>
<td>Area Under the Curve</td>
</tr>
<tr>
<td>ATR</td>
<td>Attenuated total reflection</td>
</tr>
<tr>
<td>BP</td>
<td>British Pharmacopoeia</td>
</tr>
<tr>
<td>ºC</td>
<td>Celsius degree</td>
</tr>
<tr>
<td>cm</td>
<td>Centimetre</td>
</tr>
<tr>
<td>cP</td>
<td>Centipoise</td>
</tr>
<tr>
<td>$r^2$</td>
<td>Coefficient of Determination</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
</tr>
<tr>
<td>Conc.</td>
<td>Concentration</td>
</tr>
<tr>
<td>R²</td>
<td>Correlation Coefficient</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>DH</td>
<td>Degree of Hydrolysis</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential Scanning Calorimeter</td>
</tr>
<tr>
<td>pKa</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>DL</td>
<td>Drug Loading</td>
</tr>
<tr>
<td>EE</td>
<td>Entrapment Efficiency</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FTG</td>
<td>Fluid Thioglycollate</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier-transform infrared spectroscopy</td>
</tr>
<tr>
<td>F</td>
<td>Fraction of drug released</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>Tg</td>
<td>Glass Transition Temperature</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>Cp</td>
<td>Heat Capacity</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
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<tr>
<td>HPMC</td>
<td>Hydroxypropylmethyl Cellulose</td>
</tr>
<tr>
<td>HEC</td>
<td>Hydroxyl Ethyl Cellulose</td>
</tr>
<tr>
<td>HPC</td>
<td>Hydroxyl Propyl Cellulose</td>
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<tr>
<td>IC</td>
<td>Inhibitory Concentration</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>kV</td>
<td>Kilovolt</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of Detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit of Quantitation</td>
</tr>
<tr>
<td>MMTS</td>
<td>Maximum Mean Total Score</td>
</tr>
<tr>
<td>MSA</td>
<td>Mannitol Salt Agar</td>
</tr>
<tr>
<td>MB</td>
<td>Methylene Blue</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µL</td>
<td>Microlitre</td>
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<tr>
<td>µm</td>
<td>Micrometre</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>mAU</td>
<td>Milli Absorption Units</td>
</tr>
<tr>
<td>mA</td>
<td>Milliampere</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
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<td>mL</td>
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<td>mm</td>
<td>Millimetre</td>
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<tr>
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<td>Millimolar</td>
</tr>
<tr>
<td>mV</td>
<td>Millivolt</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum Inhibitory Concentration</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>MTDSC</td>
<td>Modulated Temperature Differential Scanning Calorimetry</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>-------------</td>
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</tr>
<tr>
<td>mol</td>
<td>Mole</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular Weight Cut-Off</td>
</tr>
<tr>
<td>M. tuberculosis</td>
<td>Mycobacterium tuberculosis</td>
</tr>
<tr>
<td>HPMA</td>
<td>N-(2-hydroxypropyl) Methacrylamide</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>NPs</td>
<td>Nanoparticles</td>
</tr>
<tr>
<td>NLC</td>
<td>Nanostructured Lipid Carriers</td>
</tr>
<tr>
<td>NCTC</td>
<td>National Collection of Type Cultures</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Non-Steroidal Anti-Inflammatory Drugs</td>
</tr>
<tr>
<td>n</td>
<td>Number of measurements</td>
</tr>
<tr>
<td>o/w</td>
<td>Oil in water</td>
</tr>
<tr>
<td>%</td>
<td>Percent sign</td>
</tr>
<tr>
<td>PHMB</td>
<td>Phenyl Hexyl Methylene Biguanide</td>
</tr>
<tr>
<td>PM</td>
<td>Physical Mixture</td>
</tr>
<tr>
<td>PEDF</td>
<td>Pigment Epithelium Derived Factor</td>
</tr>
<tr>
<td>PHBV</td>
<td>Poly (3-hydroxybutyric acid-co-3-hydroxyvaleric acid)</td>
</tr>
<tr>
<td>PACA</td>
<td>Poly(Alkylcyanoacrylate)</td>
</tr>
<tr>
<td>PBCA</td>
<td>Poly(Butylcyanoacrylate)</td>
</tr>
<tr>
<td>PGA</td>
<td>Poly-l-Glutamic Acid</td>
</tr>
<tr>
<td>PLA</td>
<td>Poly Lactic Acid</td>
</tr>
<tr>
<td>coPLA</td>
<td>Poly-l-Lactide-Co-d,l-Lactide</td>
</tr>
<tr>
<td>PCL</td>
<td>Poly ε-Caprolactone</td>
</tr>
<tr>
<td>PAMAM</td>
<td>Polyamidoamine</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene Glycol</td>
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<tr>
<td>PEI</td>
<td>Polyethyleneimine</td>
</tr>
<tr>
<td>PEO</td>
<td>Polyethylene Oxide</td>
</tr>
<tr>
<td>PHB</td>
<td>Polyhydroxybutyrate</td>
</tr>
<tr>
<td>PPI</td>
<td>Polypropylenimine</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
</tr>
<tr>
<td>PVA</td>
<td>Polyvinyl Alcohol</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinyl Pyrrolidone</td>
</tr>
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</table>
PLGA          Poly Lactic-co-Glycolic Acid
PBS          Phosphate Buffer Saline
P. aeruginosa  Pseudomonas aeruginosa
RSD          Relative Standard Deviation
k            Release Constant
Rs           Resolution
RT or Rt     Retention Time
RPE          Retinal Pigment Epithelium
rpm          Revolutions Per Minute
SEM          Scanning Electron Microscope
s or sec     Second
S. aureus    Staphylococcus aureus
SDS          Sodium Dodecyl Sulfate
SLN          Solid Lipid NPs
SBC          Soybean Casein digest
SD           Standard Deviation
SIRC         Statens Seruminstitut Rabbit Cornea
t.i.d.       Three times daily
TGA          Thermogravimetric Analysis
t           Time
TNF-α        Tumor Necrosis Factor-α
TGF-β        Transforming Growth Factor-β
UV           Ultraviolet
UPLC         Ultra Performance Liquid Chromatography
UK           United Kingdom
US           United State
USP          United State Pharmacopoeia
VEGF         Vascular Endothelial Growth Factor
w/o         Water in oil
w/v          Weight/Volume
WHO          World Health Organisation
XRD          X-Ray Diffraction
Y            Yield
Scientific Presentations

Oral Communications:


Dual Drug Loaded Electrospun Coaxial Nanofibres: Demonstrating The Antimicrobial And Anti-scarring Effects. **Oral Presentation.** UCL School of Pharmacy Research Day, 21st September 2018 (London, UK)

Chapter 1

Introduction
1.1. General Introduction

The current research project concerns the development of dual drug-loaded, compartmentalised nanofibre formulations for the treatment of corneal injuries and infections. Corneal diseases may be due to infection, inflammation, degeneration or trauma (Hassell and Birk, 2010; McCluskey and Powell, 2004). In order to maintain a healthy and correctly-functioning cornea, its avascularity and transparency should both be preserved following an injury to the eye (Wilson et al., 2007; Cintron et al., 1973). These factors are crucial in allowing the light to penetrate through the cornea, and ultimately to the retina. Therefore, corneal wound healing plays an essential role to ensure visual acuity and the integrity of the eye.

Corneal abrasion is a scratch at the surface of the eye caused by a foreign body, which can range from superficial to deep (Shahid and Harrison, 2013). Since corneal abrasion is an open wound, infection and fibrosis can be considered as potential risk factors that may lead to visual loss if not treated effectively (Cheng et al., 1999, Bourcier et al., 2003, Schaefer et al., 2001, Vajpayee et al., 2000; Wong et al., 2003). Visual loss due to infection is associated with Gram negative bacteria found in contact lens wearers and Gram positive bacteria in the general population, respectively (Bourcier et al., 2003, Schaefer et al., 2001, Vajpayee et al., 2000, Wong et al., 2003; Keay et al., 2006). *Pseudomonas aeruginosa* (*P. aeruginosa*) is the most common bacterium that is isolated from contact lenses and it is the leading cause of corneal ulceration, which is a visible erosion on the cornea resulted from a serious infection (Stapleton and Carnt, 2012). It was reported in study conducted in the United Kingdom (UK) that 23% of the 772 isolates collected from patients contained *P. aeruginosa* (Sueke et al., 2010). Furthermore, according to the World Health Organization (WHO) corneal ulcers are a leading cause of blindness for 1.5-2.0 million people every year (Ong and Corbett, 2015). Other studies have estimated that bacterial infections are the most common cause of microbial ophthalmic infections in the UK (Manzouri et al., 2001; Dart et al., 2008), while the remaining cases are being caused either by fungi, protozoa, or parasites (Dart et al., 2008; Keay et al., 2006). Corneal fibrosis (also known as corneal scarring) is a condition whereby corneal
epithelial cells form new tissue following corneal abrasion (Kinoshita et al., 2001, Wilson et al., 1999, Salomao et al., 2011; Kuwabara et al., 1976). Infection associated with corneal wound requires an immediate treatment to avoid more severe complications, including corneal ulceration (Lyczak et al., 2000).

It is apparent that eliminating the underlying infection and maximising the healing process are both vital to ensure that corneal ulceration does not occur as a post-infection damage, thus preserving and protecting the patient’s vision as much as possible. Hence, dual therapy with an antibiotic and an anti-fibrotic would be a sensible solution. In this thesis, a proposed delivery platform of a compartmentalised nanofibre system is loaded with moxifloxacin hydrochloride (antibiotic) and pirfenidone (anti-fibrotic). This is to accelerate the healing process of corneal scarring, along with the treatment of the associated corneal infection upon the release of both drugs. The following introduction aims to describe the essential concepts and foundations that are presented throughout this thesis.

1.2. The Anatomy and Physiology of the Eye

The eye is a sensitive and sophisticated organ of the human body (Martini et al., 2015). The main constituents of the human eye are shown in Figure 1.1. In order to visualise an object, light is reflected from that object into the eye of the viewer (Martini, et al., 2015; McGeow, 1999). The cornea is effectively the outermost lens of the eye and serves to control and focus the light entering the eye onto the lens in conjunction with the iris that controls the amount of that light reaching the lens and ultimately the back of the eye (the retina) via the pupil which is essentially a circular gap in the iris, the diameter of which may be controlled. In the process of accommodation, the ciliary muscles surrounding the lens alters its shape to allowing the eye to focus on (accommodate) an object at different distances. However, light leaving the lens reaches the retina via the vitreous humour. The macula that is located at the centre of the retina contains light sensitive nerve endings known as photoreceptors. There are two types of these photoreceptors, rods and cones,
which are responsible to convert the light into electro-chemical signals. These signals then travel to the brain by the optic nerve. Eventually, the brain will receive such collected information from the eye, interpret it, and hence vision occurs. Interpretation of the signals will take place in the occipital cortex at the back of the brain, which is responsible for image visualisation (Martini, et al., 2015; McGeow, 1999).

![Figure 1.1: The main constituents of the human eye. Adapted from (medicinenet.com, n.d.).](image)

On the outer surface of the eye, a thin film that coats and moisturises the cornea is recognised as the pre-corneal tear film (McGeow, 1999). It consists of three layers: a lipid layer, composed mainly of cholesterol, phospholipids, and triglycerides, which protects the other layers; the lacrimal layer, which is an intermediate hydrophilic layer containing proteins, electrolytes, and non-electrolytes; and an inner mucin layer, which is the glycocalyx layer (Nichols et al., 1985; Yanez-Soto et al., 2014). This inner layer contains lysozymes which act as a first line of defense by protecting the eye against bacteria and preventing the growth of microcysts on the cornea (Hanes et al., 1999; Huang et al., 2007). In addition to antibacterial properties of this tear film, it also transmits oxygen to the avascular cornea and eliminates the debris from the surface of the eye by tears flow (James and Bron, 2011). The tear film is
renewed with each blink, which will thin during the eye opening (Cui et al., 2012).

Below the tear film lies the cornea. It is composed of five layers: the outermost multilayer of epithelial cells, followed by Bowman’s membrane, and then a middle aqueous layer that is called the stroma, followed by Descemet’s membrane, and finally, the innermost single layer of endothelial cells (Bohnke and Masters, 1999). Both the epithelial and the endothelial layers are lipophilic in nature (Bohnke and Masters, 1999). The cornea protects the internal eye components and focuses the light onto the retina, together with the lens. Figure 1.2 illustrate the structure of the cornea and the tear film.

![Figure 1.2: A schematic diagram of the anatomy of the cornea and pre-corneal tear film (James and Bron, 2011).](image)

1.3. Corneal Diseases

The lack of blood vessels, known as the avascularity, gives the cornea its transparency and provides visual acuity (Klintworth, 1977). Nonetheless, it has a high concentration of nerve fibres which make the cornea, and the eye, extremely sensitive to pain. Corneal transparency can be lost due to corneal diseases, thus vision will be disrupted (Hassell and Birk, 2010; McCluskey and Powell, 2004).
Keratitis is defined as inflammation of the cornea; it is mainly divided according to its causes into infectious and non-infectious. However, it occurs usually after a micro-organism invading the corneal abrasion which may lead to severe visual disability if left untreated (Whitcher and Srinivasan, 1997). Therefore, appropriate corneal healing is critical in order to regain normal physiological conditions of the cornea. As mentioned before, pre-corneal tear film components, such as lysozyme, can be considered as the first line of defense and are able to protect the eye from being infected (Huang et al., 2007). These antimicrobial components can inhibit or even prevent the growth of the invading micro-organism along with blinking reflex and tearing which wash out pathogens (James and Bron, 2011). Any change in the surface epithelial cells and/or the reduction of the tear film components' concentrations can lead to infections (Jeng and McLeod, 2003). Long term wearing of contact lenses, tear deficiency, ocular trauma, and certain medications, such as corticosteroids, are other risk factors that can also predispose serious infections (Bourcier et al., 2003, Green et al., 2008; Sirikul et al., 2008).

When injuries such as infections take place or ophthalmic surgery is performed, cytokines and growth factors will be triggered in excess. As a result, a series of events in the cornea will occur as part of the normal biological healing mechanisms, such as corneal epithelial stromal interactions, extracellular matrix (ECM) remodeling, and myofibroblast generation (Wilson et al., 2007, Cintron et al., 1973; Gabison et al., 2005). This may lead to a corneal complication known as a corneal fibrosis or scar (Wilson et al., 2007; Cintron et al., 1973).

Corneal epithelial cells have the ability to renew every three to four days (Kinoshita et al., 2001; Kuwabara et al., 1976). In the presence of deep corneal abrasion reaching the stroma, healthy epithelial cells start to migrate to the injured site and replace the damaged cells. Consequently, the healing process will be delayed (Wilson et al., 1999, Salomao et al., 2011; Kuo, 2004). Stromal abrasion can trigger the release of several inflammatory mediators, such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) cytokines (Yamada et al., 2003; Rosenbloom, 2013). TGF-β is found as the isoforms TGF-β1, TGF-β2, and TGF-β3 in humans (Tandon et
The release of IL-1 will convert the keratocytes, which are located beneath the epithelium layer in the stroma, into fibroblasts. Then the fibroblasts will differentiate to myofibroblasts under the influence of TGF-β. Normally, myofibroblasts undergo apoptosis, and thus the cornea will heal as shown in Figure 1.3. However, the presence of an injury or micro-organisms will allow the secretion of irregular matrix (a network of extracellular macromolecules, such as collagen, enzymes, and glycoproteins) by the myofibroblasts, which will lead to hazy vision as shown in Figure 1.3 (Jester et al., 1994, Fini, 1999; Zieske, 2001). Some micro-organisms are opportunistic and can lead to severe infection, for instance *P. aeruginosa* can grow rapidly and cause corneal ulceration (Lyczak et al., 2000). Consequently, corneal infection and fibrosis will develop after corneal abrasion due to the presence of an open wound that could lead to an infection and then trigger cytokines, in excess, facilitating the scar production.

![Figure 1.3: Corneal wound healing process. 1) Corneal basement membrane will be lost by an injury 2) Cytokines will be released into the stroma 3) Keratocytes will proliferate and migrate to form fibroblasts 4) Fibroblasts will trans-differentiate to myofibroblast by TGF 5) In normal conditions, myofibroblasts will undergo apoptosis to repair the cornea 6) In pathological conditions, myofibroblasts secrete irregular matrix 7) This will lead to hazy vision. Adapted from Chaurasia et al. (2015).](image-url)
In some cases, such as eye infection or injury, a more serious illness called corneal neovascularization (an in-growth of new blood vessels) can occur. Lack of oxygen (hypoxia), as well as inflammation, can trigger this condition (Azar, 2006; Chan et al., 2005). The release of cytokines, growth factors, immune cells and pro-angiogenic factors such as vascular endothelial growth factor (VEGF) will eventually cause visual acuity disturbance through stromal edema, haemorrhage, or scarring (Ogawa et al., 1999; Li et al., 2012). Conversely, there are other types of growth factors that contribute to maintaining corneal avascularity, and thus prevent corneal neovascularisation, for instance pigment epithelium derived factor (PEDF), which can inhibit PEDF-derived peptide, and/or VEGF expression (Tombran-Tink et al., 1991; Matsui et al., 2012). Several other molecules such as thrombospondins (Cursiefen et al., 2004), cornea derived transcript-6 (Peek et al., 1998) and inhibitory PAS-domain transcription factor, have shown similar effects in inhibiting corneal neovascularisation (Makino et al., 2001).

1.4. Pharmacological Management of Corneal Diseases

1.4.1. Corneal Infection Management

Antimicrobial agents can be used for corneal infections (McDonald et al., 2014, Waller et al., 2010; Moroi and Lichte, 2001). The most commonly used antibiotics are quinolones, such as ciprofloxacin, levofloxacin, ofloxacin and moxifloxacin; aminoglycosides, such as gentamicin and tobramycin; macrolides, such as azithromycin; cephalosporins, such as cefuroxime and ceftazidime; and miscellaneous antibiotics, such as chloramphenicol, fusidic acid, neomycin and polymyxin B (McDonald et al., 2014, Waller et al., 2010; Moroi and Lichte, 2001). The common antiviral agents than can be used are aciclovir, especially for the treatment of herpes simplex infections, and ganciclovir, particularly for the treatment of sight-threatening cytomegalovirus retinitis (Waller et al., 2010; Moroi and Lichte, 2001). Amphotericin B, nystatin or natamycin are good candidates for treating fungal keratitis (Waller et al., 2010; Moroi and Lichte, 2001). Acanthamoeba keratitis, which is common in soft contact lens wearers, can be treated with anti-amoebic agents such as
PHMB (phenyl hexyl methylene biguanide) and chlorhexidine (Waller et al., 2010; Moroi and Lichte, 2001). Most of these antimicrobial agents can be administered locally, as eye drops or ointments, as well as orally in some cases, as in trachoma treatment (Tabbara et al., 1996). Antibiotic resistance is the major concern in the treatment with any of these agents, and thus using a combination of two or more drugs and/or altering the resistant drug, is the ultimate solution for this obstacle (Waller et al., 2010; Moroi and Lichte, 2001). Although eye drops are relatively easy for patients to use, there are some issues associated with them, such as the requirement for frequent instillations (3 to 4 times daily) of highly concentrated drug solutions/suspensions, and the possibility of significant systemic absorption which can occur when excess drug solution reaches the nasal cavity through the nasolacrimal duct, which can then potentially cause systemic side effects (Thompson, 2007; Donahue et al., 1996). Rapid pre-corneal loss by drainage, blinking reflex, and high tear fluid turnover result in only 5% of the applied drug reaching or penetrating the cornea (Bourlais et al., 1998). Unpleasant effects associated with the use of eye ointments such as blurring of vision make this dosage form preferable at bed time.

1.4.2. Corneal Fibrosis Management

Corneal abrasion can be a superficial or a deeper scratch. The superficial abrasion would affect the corneal outer layer (epithelium). Generally, epithelial cells have the ability to self-renew every 7 to 10 days (Bukowiecki et al., 2017). Therefore, this type of scratch can be healed after 2 to 3 days by the eye’s natural healing process (Wilson and Last, 2004), as described in Figure 1.3. A deeper scratch that reaches to the stroma or the endothelium layer can take a long time to heal naturally. Therefore, a medical intervention might accelerate the healing rate (Bukowiecki et al., 2017; Ljubimov and Saghizadeh, 2015; Wilson and Last, 2004). The presence of this open wound can allow microorganisms to invade leading to an infection. In addition, this injury will trigger the deposition of the ECM elements such as collagen, fibronectin and smooth muscle actin by the myofibroblasts. The activation of myofibroblasts along with
the deposition of the matrix elements can cause the development of a scar (Bukowiecki et al., 2017; Ljubimov and Saghi-zadeh, 2015).

When corneal scarring occurs due to an injury or a complication of an infection, its treatment depends on the severity of the symptoms. Usually, topical antibiotics and anti-inflammatory drugs are used to reduce the risk of infection and inflammation, respectively. Unfortunately, many anti-inflammatory corticosteroids may induce ocular vasodilatation and increased vascular permeability. These effects will lead to pain, increased intraocular pressure, and even impaired vision (Donnenfeld, 2012). Moreover, they can reduce the host’s immune response and thus will worsen some infections such as fungal keratitis (Thomas and Geraldine, 2007; Allan and Dart, 1995). The fact that the non-steroidal anti-inflammatory drugs (NSAIDs) are weak acids make them less favourable to be used as they will be ionised when in contact with the tear fluid, pH 7.4, which will then restrict their corneal permeability (Donnenfeld, 2012). The use of anti-scarring drugs may be a suitable option in the case of corneal fibrosis. Reducing the expression of mediators that produce matrix proteins can accelerate the healing of this fibrosis (Rosenbloom et al., 2010, Wei et al., 2011; Wynn, 2008). Deactivation of myofibroblasts and the resultant production of ECM macromolecules leads to the healing of fibrosis. Therefore, triggering any of these pathways can help to heal the fibrosis. Unfortunately, the limited number of anti-scarring agents, due to lack of methods of measuring the effectiveness of such drugs, make the selection of a suitable agent more challenging (Rosenbloom, et al., 2013).

1.4.3. Corneal Neovascularisation Management

Treatment of the more serious condition of corneal neovascularisation can be difficult. The recently used strategies include topical antimicrobials, corticosteroids, or NSAIDs, laser photocoagulation, photodynamic therapy, and tissue transplantation (Chang et al., 2001, Mirabelli et al., 2014; Shakiba, et al., 2009). Unfortunately, the systemic side effects, such as elevated intraocular pressure that many agents exhibit, as well as the high cost that can be seen in some interventions such as in tissue transplantation, can raise many questions regarding the use of these therapeutic approaches.
1.5. Ophthalmic Dosage Forms

1.5.1. An Overview of Ocular Dosage Forms

There are different dosage forms used to deliver drugs either to the anterior part of the eye (the front of the eye), or to the posterior region (the back of the eye). In order for a drug to reach the posterior region after topical administration, it should be retained at the cornea long enough to permeate through the corneal layers reaching the interior of the eye (Lang et al., 2002).

One of the most used ocular dosage forms is topical ocular drops, which represent 90% of the marketed ocular formulations (Dubald et al., 2018). Eye drop solutions are homogenous products that give uniformity of dosing. Drugs can be absorbed through the cornea to the intraocular tissue or through the conjunctiva, the sclera or the vitreous body (Weng et al., 2017). Despite the ease of manufacturing solutions on a large scale, the short residence time on the eye and the drainage through the lacrimal duct to the systemic circulation have been the main drawbacks of this system (Gaudana et al., 2010). Additionally, due to the multiple doses required by this approach, a secondary ocular infection may potentially developed by the contamination of this dosage form due to the recurrent use (Dubald et al., 2018).

Increasing the drug residence time and permeation across the corneal layers can overcome above mentioned disadvantages. Using permeation enhancers (e.g. polyoxyethylene glycol ester) or viscosity enhancers (e.g. hydroxyl methyl cellulose) may improve the permeation by modifying the corneal integrity and the corneal retaining time by increasing the solution viscosity, respectively (Patel et al., 2013). Therefore, a gel-forming solution has been developed, which will form a gel when it is in contact with the tear fluid (Le Bourlais et al., 1998; Rajoria and Gupta, 2012). This will improve the corneal contact time and reduce the dosing frequency. An example of this system is the United States Food and Drug Administration (US FDA) approved timolol maleate gel-forming solution, which is approved for the treatment of glaucoma. It consists of the
polysaccharide xanthan gum (gelling agent) that will form a gel when it is in contact with the tear protein lysozyme (Schenker and Silver, 2000).

Another way to increase the contact time is the use of suspensions which are suitable for poorly water-soluble drugs, in a micro-fine form, to enhance their stability, bioavailability and efficacy (Aulton and Taylor, 2013). According to the European Pharmacopoeia, in a sample corresponding to 10 µg of drug, not more than 20 particles should exceed 25 µm and not more than 2 of these particles can exceed 50 µm in order to prevent any irritation, as well as to deliver a more uniform dosage to the eye (Aulton and Taylor, 2013). Furthermore, this system may suffer from a short shelf-life due to the sedimentation of the drug, therefore, an alternative way to prepare suspensions has been developed. This developed suspension has the ability to keep the insoluble drug particles (about 95% of the total particles) without precipitation for many months, and any settled particle can be resuspended by gentle shaking (Lang et al., 2002). An example of this approach is the use of a charged water soluble polymer, such as an anionic carbomer polymer, with an oppositely charged counter-ion, such as sodium or potassium ions. The polymer will have the ability to control any flocculation of the insoluble particles (Lang et al., 2002). Drugs that have limited stability in the liquid form may be prepared as sterile powders for reconstitution via lyophilisation of these drugs in individual glass vials, for instance, Miochol® (acetylcholine powder) (Catford and Millis, 1967). Using this technique will prolong the shelf-life of these drugs compared to preparing them in a solution form.

Another dosage form that can be used topically are the semi-solid formulations. These are classified into ointments and gels (Dubald et al., 2018). Ointments are mostly anhydrous with a petrolatum base. The ointment vehicle is often a homogeneous mixture of mineral oil, which is used to reduce the melting point and to modify the consistency, and white petrolatum, which is smooth, anhydrous and inert in nature. This combination can make the vehicle suitable for moisture sensitive drugs and to provide ocular lubrication (Lang et al., 2002). The main drawback of this system is their fatty nature that will blur the vision, and thus will reduce patient compliance. Consequently, it is advised to apply this system at bed time (Dubald et al., 2018).
Hydrogels are used in order to prolong the residence time and to increase drug bioavailability and therapeutic effect due to its mucoadhesive properties and the reduced frequency of administration (Le Bourlais et al., 1998; Kirchhof et al., 2015). They contain approximately 95% water and a high molecular-weight cross linked polymer that provides optimum rheological and viscosity properties (Lang et al., 2002). These aqueous gels are more tolerable on the eye than the ointments due to low side-effects that can be caused from the systemic absorption. The main drawbacks of this dosage form could be the stability, the viscosity of these gels over time and the homogeneity of the lipophilic drugs in this system (Dubald et al., 2018). Hydrogels are mainly composed of mucoadhesive polymers such as methyl cellulose, hydroxyl ethyl cellulose and sodium hyaluronate. Sodium hyaluronate is a polysaccharide that is might be used in ocular gel formulation due to its water retention and viscoelastic properties (Lai et al., 2010, Widjaja et al., 2014; Bora et al., 2016). This hydrogel is marketed for the application of dry eye syndrome as Vismed® (Horus Pharma, Saint-Laurent-du-Var, France), Aqualarm® (Bausch + Lomb, New Jersey, USA) Hylo™ (Candorvision, Montreal, Canada) (Dubald et al., 2018).

The ocular insert, discovered in 1971 (Higuchi, 1971), is a type of solid dosage form that is applied topically on the eye. These inserts are inert and non-allergic and may also sustain the release of a drug, resulting a high bioavailability and low systemic absorption (Dubald et al., 2018). The difficulty of insertion, high production cost and patient discomfort are the main limitations of this dosage form (Kumari et al., 2010). Ocular inserts can be soluble, insoluble, bioerodible or non-bioerodible inserts. These inserts are applied and placed into the cul-de-sac or conjunctival sac (under the eye lid). Soluble inserts are made using water soluble polymers that will dissolve gradually upon absorbing the tear fluid and finally release the drug. An example of a marketed soluble insert that can be used for dry eyes is Lacrisert® (Idis Limited, Weybridge, UK) (Dubald et al., 2018). Alternatively, bioerodible inserts are made by biodegradable hydrophilic polymers that will erode and degrade allowing the drug to slowly release from the matrix. The degradation of the polymer will overcome the need to check or remove the
insert at the end of use (Lang et al., 2002). Recently, a formulation of diclofenac sodium and hydroxyl propyl methyl cellulose has been formulated into an insert, allowing an increase contact time and a decrease in the dosing frequency (Ara et al., 2015). Another example is the development of lidocaine incorporated into hydroxyl propyl methyl cellulose, polyvinyl alcohol (PVA) and cyclodextrin insert for ocular anesthesia (Shukr, 2014). The advantages which might be observed with the soluble and bioerodible inserts are the ability to deliver a precise dose, reduce local and systemic side effects of the administered drug, and finally, in some cases, they may not require the use of preservatives, thus reducing the risk of eye irritation (Devhadrao and Siddhaia, 2018; Dubald et al., 2018).

The insoluble inserts consist of a reservoir that is a matrix or a drug carrier in which the medication can be dissolved or dispersed (Devhadrao and Siddhaia, 2018). This reservoir can be enclosed in a rate controller which can be either semi-permeable membranes or water-permeable polymeric matrix and a semi permeable membrane. The drug can be diffused across the reservoir at a precise rate by the penetration of the tear fluid through the membranes where it will form a sufficient internal pressure that can allow the drug out of the reservoir (Devhadrao and Siddhaia, 2018). An alternative approach is for the tear fluid to permeate through the polymeric matrix. This will produce a hydrostatic pressure against the polymer matrix allowing its bursting and thus the release of the drug (Devhadrao and Siddhaia, 2018).

Non-bioerodible inserts have a design that consists of a drug, a drug delivery unit and a platform (Gurtler and Gurny, 1995; Kumari et al., 2010; Kumar et al., 2013). The delivery unit consists of a drug reservoir with a carrier material (e.g. alginic acid), a rate controlling material (e.g. ethylene vinyl acetate copolymer membrane), an energy source (the concentration of the drug in the reservoir) and a delivery portal (e.g. the copolymer membrane). The platform consists of the housing (e.g. ethylene vinyl acetate copolymer membrane), and an annular ring of membrane soaked with titanium dioxide to form a white visible border. The advantages of this delivery system are the ability to reduce the amount of lost and drained drug and thus prevent local and systemic side effects compared to the eye drops. In addition, it can provide patient
compliance by reducing the frequency of administration whilst maintaining the efficacy (Hajare et al., 2014). However, disadvantages include the cost of designing such inserts and the need to use preservatives. Yet another concern is the prolonged duration of contact with eye surface which may cause irritation, as well as the need to periodically check its place within the eyes (Lang et al., 2002).

Sometimes it is essential to bypass the ocular surface and to deliver drugs directly to the posterior segment of the eye. This can occur by using intraocular injections, whereby a drug is injected into the anterior chamber, or intravitreal injections or implants, which can be injected or inserted directly into the vitreous cavity (Weng et al., 2017), as it can be seen in Figure 1.4.

![Figure 1.4: A schematic diagram of an intravitreal injection bypassing the anterior segment of the eye. Adapted from Ochakovski et al. (2017).](image)

Intravitreal injection is the most commonly used topical ophthalmic injection, in which the drug in solution or suspension is injected through a 27- or 30-gauge needle at a volume ranging from 20 to 100 μL (Ahmed, 2003). This dosage form can be effective for the treatment of back of the eye diseases (Weng et al., 2017). Major disadvantage of this route of administration is the drug distribution patterns through the vitreous cavity gel-like structure that can limit the drug from spreading out the vitreous body. It has been reported that large
molecular-weight drugs (more than 40 kDa) have longer retention time than the small molecular-weight drugs (Weng et al., 2017). A good example of this system is fomivirsen sodium, a phosphorothioate oligonucleotide, which inhibits the replication of cytomegalovirus (CMV) (The Vitravene Study Group, 2002). However, the frequency of injection, in this case, is every 2 to 4 weeks. Avastin® (Roche, Basel, Switzerland), a bevacizumab injection, and Lucentis® (Novartis, Basel, Switzerland), a ranibizumab injection, are commonly used in the treatment of the age-related macular degeneration (Dubald et al., 2018). Another example is Vitrasert®, a ganciclovir implant that is used for local treatment of CMV retinitis in acquired immune deficiency syndrome (AIDS) patients (Musch et al., 1997). This can release the drug for a period of 4 to 8 months, then it can be removed or replaced surgically.

Intracameral injection can bypass the corneal and systemic absorption by injecting drugs directly in the anterior eye chamber (Gaudana et al., 2010). This will eventually provide a high drug concentration localised in the anterior segment of the eye. Periocular injection can deliver drugs in the intraocular tissue with a limited systemic absorption through the subconjunctival or retrobulbar cavities (Gaudana et al., 2010). Drugs that penetrate the corneal choroid or sclera could reach to the back part of the eye. However, the main limitation of this dosage form is the long retention time of the drugs to reach to the site of action (Raghava et al., 2004 Amrite and Kompella, 2005; Amrite et al., 2008). There are other systems that can deliver different materials by injection. For instance, a viscoelastic high purified sodium hyaluronate is used as a surgical adjunct due to its lubricant and viscoelastic properties that can provide a mechanical barrier between tissues to allow more space for manipulation with less trauma to the surrounding tissues (Pouyani and Prestwich, 1994). All of these intraocular systems should be preservative free, to prevent tissue irritation. Therefore, sterilisation must be performed before using these dosage forms (Dubald et al., 2018).

Contact lenses are transparent, thin and curved lenses that are made mostly by silicon hydrogels and can be placed on the surface of the eye to correct visual defects or for cosmetic purposes (Stapleton et al., 2006). Recently, several studies have reported the ability to use these lenses for therapeutic
purposes (Dubald et al., 2018). These lenses would be used to increase drug residence time in the eye (Baranowski et al., 2014). Drugs can be incorporated by imprinting or simply soaking the lenses in drugs’ solution (Guzman-Aranguez et al., 2013). The drawbacks of this system may include the high production cost, the way of handling/cleaning the lenses, and lack of oxygen permeation to the front of the eyes. Two types of contact lenses have been reported for therapeutic uses; the soft lenses and the scleral rigid gas permeable lenses. These lenses are thin and have a large diameter (18 to 24 mm) (van der Worp et al., 2014). They have been reported as a potential treatment of glaucoma, dry eyes syndrome and ocular infections (Glisoni et al., 2013) along with other indications (van der Worp et al., 2014, Harthan, 2014, Rathi et al., 2016, Severinsky et al., 2014; Kramer and Boshnick, 2015).

The use of colloidal dispersions of micro- or nano-carrier systems that enable drugs to be incorporated either to the inside or on the surface will be discussed later in section 1.6. These systems are able to deliver drugs topically on the surface or to the back of the eye for the treatment of the anterior or posterior segment of the eye, respectively.

1.5.2. Barriers to Ocular Absorption

Owing to the nature of the pre-corneal tear film, it is a barrier to the ocular absorption of many drugs. Eye drops can be removed by this tear film, resulting in an ocular absorption of approximately 5% (Jarvinen et al., 1995). In addition to this, and due to the increased volume of cul-de-sac following the administration of an eye drop, more eye blinking and tears secretion may occur. This occurs until the cul-de-sac restores its normal volume (7 to 9 µL) (Bachu et al., 2018). Consequently, the blinking reflex (5-7 blinks/minute) with or without the rapid lacrimal fluid renewal rate (1 to 3 µL/minute) could restrict the drug residence time which is approximately 1 to 2 minutes (Jarvinen et al., 1995; Gaudana et al., 2010).

If the drug is successful in being retained at the cornea or conjunctiva, systemic absorption of this drug can occur via the lacrimal duct (Urtti and Salminen, 1993; Ambati et al., 2000). The nasolacrimal drainage is considered as another barrier owing to the elimination of approximately 95% of the administered dose
through the conjunctiva and nasolacrimal duct (Bachu et al., 2018). This drainage system allows the drug (or the tear) to flow to the nasal cavity. The wall of nasolacrimal duct and the lacrimal sac are vascularised, which enable the systemic drug absorption. In addition, the volume of the eye drop could also influence drug absorption. Small volumes might be eliminated from the lacrimal sac, while large volumes may flow to the nasal cavity through the nasolacrimal duct (Bachu et al., 2018).

The tight lipophilic corneal epithelium and hydrophilic stroma can be another barriers for the ocular absorption of drugs (Tsai et al., 2018). The intercellular tight junctions may hinder the permeation of drugs through the paracellular route (Gumbiner, 1987). Additionally, due to the calcium ions that are present in the extracellular and intracellular tight junctions, the drug permeation might be effected (Bhat et al., 1993). It was reported that by removing the calcium (by the use of EDTA), more drug could permeate through these junctions (Grass et al., 1985). At a physiological pH, the corneal epithelium are negatively charged. Therefore, positively charge drugs can be retained more in the cornea, hence more permeation will occur (Rojanasakul and Robinson, 1989).

Drugs can also suffer from degradation by the metabolic enzymes that are present in the glycocalyx (Duvvuri et al., 2004). A study by Hayakawa et al. (1992) reported that the administration of peptide drugs suffer from low absorption due to metabolism during conjunctival permeation in rabbits' eyes. The transport of drugs by passive diffusion occurs in the cornea, along with the transport by the influx and efflux transporters (Riley, 1977, Bonanno, 1990, Urtti, 2006; Dey et al., 2003). These could either restrict or enhance the drug absorption (Mannermaa et al., 2006). Briefly, there is two main efflux pumps that are responsible for drug resistance, the P-glycoprotein and the multi-drug resistant protein (Mannermaa et al., 2006; Sharom, 2008). The presence of these proteins in the conjunctival epithelial cells (Saha et al., 1998) and the corneal epithelium (Dey et al., 2003; Vellonen et al., 2006) were reported. Therefore, drugs that could avoid these efflux proteins might enhance their ocular absorption (Zhang et al., 2008).
The physiochemical properties of the administered drugs are important in determining their disposition after topical ocular administration (Jarvinen et al., 1995). An understanding of this will aid in the selection of an appropriate formulation to maximise ocular absorption. Low molecular-weight lipophilic drugs permeate via the corneal epithelia cells and into the stroma and then to the intraocular areas of the eye (Urtti, 2006). Therefore, they should be formulated as suspensions or emulsions, which might lead to patient discomfort (Jarvinen et al., 1995). On the other hand, low molecular-weight hydrophilic drugs can be formulated as eye drop solutions, but they suffer from low corneal diffusion and massive drug loss (Huang et al., 1989). The attention on high molecular weight hydrophilic molecules, such as proteins, peptides, nucleic acids, or antibodies, is increasing in many fields including ophthalmology (Urtti, 2006). Unfortunately, these agents have many challenges regarding their rapid degradability by extracellular enzymes, as well as their delivery to the inner eye (Khar et al., 2010, Apaolaza et al., 2014; Yin et al., 2014).

One or more of the aforementioned anterior segment of the eye barriers can hinder the absorption of the administered drug, as it illustrated in Figure 1.5. Consequently, there is a need to either modify the drugs chemically, or to enhance their permeability and retention in the cornea. Another approach is to load those drugs into suitable carrier systems, rather than the conventional systems such as eye drops, to overcome the abovementioned challenges, as well as to improve their ocular bioavailability leading to better treatment.
1.6. Nanotechnology for the Treatment of Eye Disorders

Nanotechnology is the utilisation of materials that are in the nanometre length scale, which can range from 1 nm to 1 µm (Tsai et al., 2018). Nanomedicine can be defined as the application of nanotechnology in order to resolve difficulties associated with medicine in diagnosis, therapy and prevention of diseases (Webster, 2006; Jain, 2010). Many studies predicted that nanomedicine would completely alter the way of making and/or taking drugs (Feng, 2006; Feng, 2008). Nano-carriers have been widely used for the treatment of many diseases including disorders of the eyes (Tsai et al., 2018). These submicron particulate systems are made of several materials including polymers and can act as drug carriers to the targeted sites by protecting the drug during its transportation. They can be applied to more serious conditions such as regeneration of the optic nerve and counteracting neovascularisation (Jain, 2010). Conventional topical ophthalmic drug formulations show low
ocular bioavailability due to the massive loss of the administered drug and the permeability obstruction caused by the corneal tight epithelial cells (Kimura and Ogura, 2001). Nevertheless, the use of nano-carriers can enhance ocular drug delivery even when administered as topical eye drops due to the prolonged residence time in the corneal tissues which prevents rapid drug clearance (Paolicelli et al., 2009; de la Fuente et al., 2010). In addition to this, the controlled drug delivery system can reduce the administered dose; hence the side effects will ultimately be reduced (Singh and Jones, 2014). Owing to the lipophilic nature of several nano-carries, they tend to permeate the epithelial cell layers that can be found in different parts of the human eyes such as in the cornea, conjunctiva, and in some cases, blood-retinal barriers, making them suitable systems for corneal and intraocular delivery (Souza et al., 2014).

There are many advantages provided by nano-carrier based drug delivery systems, which include increasing the drug dissolution rate by increasing the surface area as the particle size reaches the nano dimension, improving drug solubilisation, increasing stability and extending a drug’s shelf-life, enhancing the bioavailability of insoluble/impermeable drugs and macromolecules by enhancing their absorption, improving the absorption of the drugs by the bioadesion on the targeted cell membranes, reducing the required therapeutic dose and/or frequency of administration, thereby increasing and promoting its safety (Dubald et al., 2018). Nevertheless, nano-carriers can interact with cells, body fluids, and proteins by the influence of particle’s size, charge, shape and surface structure, chemical composition and aggregation, as well as the route of administration of these particles leading the way to play an important role in the biological safety and efficacy of drugs (Jain, 2010; Dubald et al., 2018).

1.6.1. Overview of Nano-carriers

Nano-carriers can be produced via different methods and from different materials, thus will have diverse physicochemical and biological properties. They can be broadly classified into metallic, non-metallic, polymeric and hybrid nano-carriers (Cho et al., 2008; Jain, 2010). Among the most widely discussed examples of nano-carriers are polymeric nanoparticles (NPs), micelles,
dendrimers, liposomes, solid lipid NPs and carbon nanotubes as it can be shown in Figure 1.6. Metallic NPs, such as gold and silver, are suitable for use in humans due to their inertness, compatibility, and ease of fabrication. Inorganic NPs, such as calcium phosphate, are commonly used due to their high biodegradability and biocompatibility. Polymeric NPs can be classified into nanospheres or nanocapsules; in which the former can entrap or adsorb a drug whereas the majority of the later ones contain an oil core where the hydrophobic drug is dispersed (Dubald et al., 2018). Finally, hybrid NPs are designed by conjugating metallic and polymeric NPs (Cho et al., 2008; Jain, 2010).

Figure 1.6: Several types of NPs used in drug delivery. Adapted from Maximilien et al. (2015).

Polymeric micelles are formed by the self-assembly of amphiphilic block copolymers in aqueous media. They are able to hold both lipophilic and hydrophilic drugs in their hydrophobic core or hydrophilic shell, respectively. The hydrophilic shell can also be useful to stabilise the core (Adams et al., 2003). The drug is mostly loaded into the micelle core by physical encapsulation (Batrakova et al., 1996) or chemical conjugation (Nakanishi et al., 2001). Polymeric gels are three-dimensional polymeric hydrophilic
networks composed of a polymer, water and crosslinking agents that undergo swelling once they interact with water (Qiu and Park, 2001; Gupta et al., 2002).

Liposomes are spherical, self-assembling closed colloidal structures, which are composed of phospholipid bilayers that surround a central aqueous core (Nii and Ishii, 2005). They are biodegradable and biocompatible systems that can carry both hydrophilic and hydrophobic materials in their internal aqueous core or outer bilayers, respectively (Fresta et al., 1993). However, low drug loading capacity, poor batch-to-batch reproducibility, difficulty in sterilisation and physical instability are considered the main limitations of this system. Due to the versatile nature of liposomes, their surface can be altered to produce a multifunctional delivery system (Wu et al., 2006). On the other hand, Niosomes are self-assembling closed non-ionic amphiphiles (surfactant-based), which form bilayer vesicles in aqueous media. They have greater stability and lower cost of production than liposomes (Muzzalupo et al., 2007). High-density lipoproteins, low-density lipoproteins and very-low-density lipoproteins were introduced as drug carriers due to their ability to stabilise the loaded drug (Kader and Pater, 2002).

Solid lipid NPs (SLN) are NPs containing lipids that are solid at room and body temperatures, hence they are more stable than liposomes. The ability to formulate these particles without the use of organic solvents on a large scale makes them an attractive delivery system, as a result of the recent focus on conserving the environment (Mehnert and Mader, 2001, Puglia and Bonina, 2012, Attama, 2011, Muller et al., 2002; Saupe and Rades, 2006). Nanostructured lipid carriers (NLC) have been introduced with the advantage of enhancing the drug-loading capacity and stability compared to solid lipid NPs. They are composed of solid and liquid lipids (Puglia and Bonina, 2012, Iqbal et al., (2012); Tian et al., 2013). However, they can both be stabilised by different types of surfactants (Puglia and Bonina, 2012; Muller et al., 2002).

Nanoemulsions can be produced by the heterogeneous dispersion of two immiscible liquids to form either oil in water (o/w), or water in oil (w/o) emulsions with nano-sized dispersion (Tadros et al., 2004). Particularly, it has the ability to carry either hydrophilic or lipophilic drugs and to enhance the stability of these drugs by extending their shelf-life (Date et al., 2010; Solans...
Nanoemulsions have low concentration of surfactants, normally between 3 and 10% (Date et al., 2010).

Dendrimers consist of multiple highly branched monomers that occur from a central core (Svenson and Tomalia, 2005). They are hydrophilic multivalent particles that can be easily modified on the surface. This will allow them to carry several molecules such as drugs, targeting ligands or imaging contrast agents which make them a multifunctional delivery system (Svenson and Tomalia, 2005; Malik et al., 1999).

Viral-based NPs’ structures usually resemble protein cages (Cho et al., 2008), which can carry macromolecular materials such as antibodies and peptides (Manchester and Singh, 2006). Viruses such as canine parvovirus, cowpea mosaic virus, cowpea chlorotic mottle virus, and bacteriophages have been studied as drug carriers, due to their natural affinity for different types of receptors (Manchester and Singh, 2006; Singh et al., 2006).

Carbon nanotubes are nano-scaled carbon cylinders having the capacity to deliver different drugs, vaccines or proteins (Bianco et al., 2004). However, they are poorly soluble in all types of solvents, therefore, chemical modification is required to allow them to be more soluble in water. Additionally, this modification can also ease their association with more peptides, proteins and nucleic acids (Bianco et al., 2005). The surface of the carbon nanotubes enables them to carry several molecules at once (Pastorin et al, 2006).

Polymeric NPs can be made of natural or synthetic polymers. They can either entrap a drug inside the NP, or conjugate with it at the surface of these particles (Cho et al., 2008). Naturally occurring polymers, such as alginate, chitosan and gelatin have been used for the delivery of drugs, proteins and nucleic acids (Duncan, 2003). Alginate is a naturally occurring copolymer of glucuronic and manuronic acids, commonly used for food, cosmetic, and medical purposes (Tonnesen and Karlsen, 2002). It has the ability to form a gel in the presence of multivalent cations such as calcium (Tonnesen and Karlsen, 2002). Chitosan is a hydrophilic, biocompatible, biodegradable and mucoadhesive polymer that has been used for cosmetic and medicinal applications (Yan et al., 2006). Gelatin is another hydrophilic and biocompatible polymer which has
been used as well in the food, cosmetic and pharmaceutical manufacturing, mainly as a stabiliser (Narayani and Rao, 1996).

Different synthetic polymers have been used for the delivery of many drugs, proteins, peptides and genetic materials. The most commonly used polymers are polyvinyl alcohol (PVA), polyvinyl pyrrolidone (PVP), poly-L-glutamic acid (PGA), poly-lactic acid (PLA), poly-lactic-co-glycolic acid (PLGA), and poly-ε-caprolactone (PCL), polyethyleneimine (PEI), polyethylene oxide (PEO), polyethylene glycol (PEG) and N-(2-hydroxypropyl)-methacrylamide copolymer (HPMA). The advantage of using these polymers are the ease of chemical modifications, high stability and reproducibility, controlled biodegradability and biocompatibility. The first used biodegradable polymer was PGA (Li, 2002), while PEG is widely used as a non-biodegradable polymer (Vasey et al., 1999). Methacrylate/methacrylic acid polymers (Eudragit® S and L) can be dissolved in aqueous media at pH 6 and 7, respectively and are used for oral administration (Lamprecht et al., 2005). PCL is a semi-crystalline biodegradable, biocompatible, stable, and highly hydrophobic polyester that has been used in slow/sustained release delivery (Sinha et al., 2004).

1.6.2. Nano-carriers for Ocular Delivery

Previous studies, particularly since the 1980s, have demonstrated that nano-carriers drug delivery systems may overcome the ocular barriers discussed above (Wood et al., 1985, Harmia et al., 1986, Harmia et al., 1986, Losa et al., 1991, Losa et al., 1993, Calvo et al., 1996, Paolicelli et al., 2009, de la Fuente et al., 2010, Singh, Jones, 2014; Souza et al., 2014).

Polymeric micelles have shown to improve drug corneal penetration. In fact, Polyoxyethylene-polyoxypropylene block copolymers were firstly used for this purpose in the late 1980s (Saettone et al., 1988). Polymers such as acrylic acid, PLA, N-isopropylacrylamide, vinylpyrrolidone, N’-methylene bis-acrylamide and Pluronic® have been studied in micelle formulations (Gupta et al., 2000; Pepic et al., 2004; Tommaso et al., 2011). In order to prolong the corneal residence time of the micelles, their shell can be labelled with cationic materials, or incorporated with reactive groups, such as thiol groups, which have the ability to interact with the mucin layer that is found in the cornea.
(Khutoryanskiy, 2011). A study by Prosperi-Porta et al. (2016) demonstrated the mucoadhesive effect of micelle system composed of cyclosporine A-loaded into PLA-b-poly(methacrylic acid-co-3-acrylamidophenylboronic acid) block copolymer. An in vitro cell culture and an in vivo studies showed that this system has a low cytotoxicity profile and eye irritation against human corneal epithelial cells and Sprague-Dawley rats, respectively (Prosperi-Porta et al., 2016).

Topical delivery of hydrogels to the eyes is likely to have higher patient compliance as it reduces the necessity for repeated application. This is because of their viscoelastic properties offering high corneal retaining characteristics (Desai and Blanchard, 1998; Nanjwade et al., 2012). Different polymers have been used in these gel systems, and the choice of the polymer is essential as it will affect the properties of the final product (Gel) (Nanjwade et al., 2012; Anumolu et al., 2009). An Example of timolol maleate gel formulation using chitosan and poly(N-isopropylacrylamide) showed good corneal penetration in vivo and improved therapeutic effect compared to the conventional eye drop formulation (Cao et al., 2007). A recent study by Deepthi and Jose (2018), were able to prepare a poloxamer 407 and chitosan hydrogel system incorporating the combination of neomycin sulphate and betamethasone sodium phosphate. The ex vivo permeation study showed the ability of this hydrogel to retain in the corneal tissues, allowing the permeation of both drugs through the goat cornea in a sustain release manner (up to at least 12 hours) (Deepthi and Jose, 2018). This was due to the use of the positively charged polymer (chitosan) which enhanced the corneal residence time by the interaction with the negatively charged (sialic acid) residues of cornea mucin (Schaeffer and Krohn, 1982). A mucoadhesive polymer, sodium alginate, which undergoes gelation by divalent ions, such as calcium that is present in the tears, was also used in association with hydroxyl propyl methyl cellulose (HPMC) to produce a sustained-release formulation of moxifloxacin hydrochloride (Mandal et al., 2012, Rupenthal et al., 2011, Liu et al., 2006; Liu et al., 2010). As a result, there are some ocular gel products on the market which are prescribed for different eye conditions, such as Timoptic-XE® (Merck & Co., Inc., Whitehouse Station, NJ, USA); a timolol gel for the
treatment of glaucoma and Zirgan® (Bausch & Lomb Incorporated, Rochester, NY, USA); a ganciclovir gel for the treatment of herpes simplex keratitis.

Liposomes are a favorable ocular delivery approach due to their lipid nature and hence, the similarity with the cornea and the pre-corneal film. They have been investigated since the 1980s in order to improve the corneal permeability of drugs (Stratford et al., 1982; Stratford et al., 1983). The first ocular liposome formulation loaded with idoxuridine was indicated for the treatment of herpes keratitis and showed enhanced efficiency of this drug (Smolin et al., 1981). As a result of the drawbacks of liposomes, such as instability, degradability and aggregation, dispersing liposomes with polymers was proposed in order to overcome these limitations. It has been found that by using cationic liposomes or coating the liposomes with a cationic polymer, such as chitosan, the corneal penetration will be enhanced along with the residence time (Mehanna et al., 2010, Nagarsenker et al., 1999; Abdelbary, 2011) and thus, the overall therapeutic efficiency will be increased (Hathout et al., 2007). For instance, a cationic liposomal formulation of flurbiprofen was stabilised within a thermosensitive gel to enhance the corneal permeability as compared to the cationic liposomes alone (El-Gazayerly and Hikal, 1997). This is due to the interaction between positively charged liposomes with the negatively charged sialic acid residues of the corneal mucin (Schaeffer and Krohn, 1982). An anionic liposomal system was recently used to deliver small peptides to the human corneal epithelial cells due to its low cytotoxicity profile compared to the cationic liposomes (Neves et al., 2016). The anionic liposomes were taken up by the epithelial cells after binding on the targeted cells, allowing the release of the loaded peptides inside these cells.

Improving corneal permeability and stability of liposomes by associating them with different polymers were further investigated. For instance, liposomes loaded with diclofenac were incorporated with PVA derivatives bearing a hydrophobic anchor (C_{16}H_{33}S) to result in a more efficient formulation than the PVA-liposomes for delivering diclofenac to the retina by eye drop administration (Aggarwal et al., 2007). Moreover, due to the nanometre size of liposomes, they can serve as effective transporters across the cornea to the back of the eye (the retina) (Hironaka et al., 2009). For example, a liposome
formulation for the delivery of nucleic acid to the retina by intravitreal injection has showed promising results (Sanders et al., 2007). Due to the ability of liposomes to carry different types of materials, liposomes loaded with vitamins A and E were released to the market to relieve and/or treat dry eye conditions; examples are Lipo Nit® (Optima Pharmazeutische GmbH, Wittibsmuhle, Wang, Germany), OPTO lipo® (Optox, Montegiorgio, AP, Italy), Tears Again® (OCuSOFT, EUA, Rosenberg, TX, USA), Optrex Actimist Eye Spray® (Optrex, Kingston-upon-Thames, UK).

Similarly to the cationic liposomes, cationic niosomes have shown the same results at the ocular surface (Maiti et al., 2011; Fujisawa et al., 2012). Additionally, certain niosomes are able to enhance the ocular bioavailability of some drugs by opening the epithelial cells tight junctions of the cornea due to the presence of surfactants in their compositions, which act as penetration enhancers (Kaur et al., 2004).

Ocular delivery using SLNs or NLCs has shown potential because of the ability of these formulations to be retained at the surface of the eye, along with improving drug permeability through the cornea (Cavalli et al., 1995, Cavalli et al., 2002, Abul-Kalam et al., 2012; Seyfoddin, Al-Kassas, 2012). A very recent study by Khames et al. (2019) demonstrated the sustained release activity of natamycin from SLNs as a potential antifungal delivery system to treat fungal keratitis. The antimicrobial test “zone of inhibition” against Aspergillus fumigatus and Candida albicans and the ex vivo test on goat cornea showed a higher fungus inhibition and corneal permeation compared to the unformulated drug. Shrivastava et al. (2018) were able to prepare NLCs loaded with timolol maleate and brinzolamide. This dual-drug system showed a high ex vivo corneal penetration and can be considered as a promising delivery system for the treatment of glaucoma. It has been suggested that the reasons behind these effects are the small particle size that will increase the surface area of these particles and the adhesive properties that the lipids possess which will enhance their absorption through lipid based membranes. Additional coating or modifying the surface of these NPs with adhesive polymers can cause a further enhancement in their adhesive characteristics. For instance, by altering the surface of negatively charged NLCs with a
thiolated non-ionic surfactant, the pre-corneal attachment was improved compared to the non-thiolated formulation (Shen et al., 2009; Shen et al., 2010). By coating flurbiprofen NLCs with cationic chitosan, the drug bioavailability and corneal permeability were increased compared to the free drug and the uncoated formulation (Basaran et al., 2010; Luo et al., 2011).

Nanoemulsion have been investigated as an ocular delivery system since the early 1990s, (Muchtar et al., 1992, Muchtar and Abdulrazik, 1997, Klang et al., 1999, Sznitowska et al., 1999, Zurowska-Pryczkowska et al., 1999, Klang et al, 2000, Sznitowska et al., 2000, Ammar et al., 2009, Gallarate et al., 2013; Daull et al., 2013). The first ocular delivery nanoemulsion contained tetrahydrocannabinol for the treatment of glaucoma, and the in vivo study showed that there was an efficient intraocular pressure reduction without any irritation (Muchtar et al., 1992). In fact, the use of surfactants, especially the non-ionic ones, gave this system an advantage similar to niosomes, in that they can overcome the epithelial tight junctions (Jiao, 2008). Recently, Mahboobian et al. (2018) were able to demonstrate the enhanced drug penetration into excised bovine cornea of brinzolamide nanoemulsion compared to the marketed drug suspension. It suggested that the use of nonionic surfactants or oils in the preparation of the nanoemulsion system was able to affect the corneal epithelial cell membrane, hence improving the drug permeation. Cationic surfactants or polymers can prolong the residence time at the cornea serving as drug depots (Klang et al, 2000; Alany et al., 2006). For instance, an indomethacin nanoemulsion coated with chitosan resulted in a higher concentration of indomethacin in the cornea than a non-coated nanoemulsion (Yamaguchi et al., 2009). Due to these efforts, the US FDA approved, in 2002, the first ophthalmic anionic nanoemulsion drug Restasis® (Allergan Inc., Irvine, CA, USA), indicated for the treatment of chronic dry eye. Another anionic nanoemulsion formulation, Durezol® (Alcon Laboratories, Fort Worth, TX, USA) was approved by US FDA in 2008 for the treatment of ocular inflammation (de la Fuente et al., 2010; Lallemand et al., 2012). Two drug-free nanoemulsion formulations were approved for the restoration of the pre-corneal film lipid layer, i.e. Lipimix® (Tubilux Pharma, Italy) and a cationic nanoemulsion Soothe XP Emollient® (Bausch and Lomb, Rochester, NY,
USA) (de la Fuente et al., 2010). An additional drug-free cationic nanoemulsion was also approved to treat symptoms of dry eye, called Cationorm® (Novagali Pharma, Evry, France), composed of poloxamer 188, tyloxapol, cetalkonium chloride and mineral oils. Other products include Cyclokat®, Vekacia® (de la Fuente et al., 2010), and Catioprost® are all indicated for the treatment of glaucoma (Ismail et al., 2011).

Dendrimers have been recently developed as an ocular delivery system, as the first studies were conducted in 2005 (Vandamme and Brobeck, 2005), the most commonly used ones being poly-(amidoamine) (PAMAM), polypropylenimines (PPI), and phosphorus dendrimers (Vandamme and Brobeck, 2005; Stasko et al., 2007). Attributing to their branched structure, they have the ability to carry more than one molecule, such as nucleic acid based drugs (Chaplot and Rupenthal, 2014), as well as both hydrophilic and lipophilic drugs (Lopez et al., 2009, Spataro et al., 2010, Durairaj et al., 2010, Yang et al., 2012; Holden et al., 2012). Additionally, dendrimers have resulted in an increase in the therapeutic efficiency by enhancing the drug bioavailability owing to their ability to retain drugs in the pre-corneal film (Vandamme and Brobeck, 2005, Yang et al., 2012; Holden et al., 2012). For instance, when compared to the drug solutions, PAMAM dendrimers demonstrated higher bioavailability profile when loaded with pilocarpine nitrate and tropicamide, and tested in vivo for their miotic and mydriatic effect, respectively (Vandamme and Brobeck, 2005). Another study has shown that PAMAM dendrimers, with carboxyl or amine groups on their surface, can improve the corneal permeability and retention of puerarin in vivo relative to the drug solution (Yao et al., 2010). Furthermore, the use of anionic or cationic dendrimers have shown enhanced drug permeability through the cornea due to opening of its tight junctions, as well as an increase in the corneal residency by interacting with the mucin layers in the pre-corneal tear film (Kitchens et al., 2006; Kambhampati and Kannan, 2013). A promising dendrimer application, by incorporating nucleic acids such as siRNA, for gene therapy have been investigated for ocular delivery (Zhou et al., 2006, Bielinska et al., 1996, Marano et al., 2004, Marano et al., 2005; Parekh et al., 2006).
Polymeric NPs were investigated in the mid-1980s for ophthalmic delivery, with poly(alkylcyanoacrylate) (PACA) as the first polymer to be formulated (Gurny et al., 1985). Other polymers that have been used include chitosan, gelatin, hyaluronic acid (HA), Eudragit®, Carbopol®, poly-(butylcyanoacrylate) (PBCA), PCL and PLGA (Zimmer et al., 1991, De Campos et al., 2001, Pignatello et al., 2002, Yuan et al., 2006, Valls et al., 2008, de La Fuente et al., 2008, Ibrahim et al., 2010, Gupta et al., 2010, Gupta et al., 2011, Ahuja et al., 2011, Aksungur et al., 2011; Zhang et al., 2013). The application of polymeric NPs for ocular delivery is due to their controlled release, corneal retaining ability, stability and bioavailability improvement of their loaded drugs (Kompella et al., 2003, Kompella et al., 2006; Jwala et al., 2011). It has been suggested that smaller size particles have better corneal retention and permeability due to their higher surface area and solubility (Rafie et al., 2010). Additionally, NPs have shown less drainage and fewer ophthalmic irritation effects than microparticles, especially above 10 μm (Rafie et al., 2010, Kassem et al., 2007; Ali and Lehmussaari, 2006). An example of indomethacin-loaded PCL NPs have shown higher corneal permeability than the microparticles (Aksungur et al., 2011).

By designing NPs associated with cationic components, such as chitosan and/or mucoadhesive polymers, such as PEG, the corneal residence time will be improved (Yuan et al., 2008, Wadhwa et al., 2010, Nagarwal et al., 2011, Jain et al., 2013, Katiyar et al., 2014, De Campos et al., 2004, De Salamanca et al., 2006, Diebold et al., 2007, de la Fuente et al., 2008, Contreras-Ruiz and de la Fuente, 2010; Contreras-Ruiz et al., 2011). A more recently study by Taghe and Mirzaeei (2018) reported a higher permeation of chloramphenicol when loaded into chitosan NPs through excised sheep cornea compared to the drug solution formulation. This was due to the interaction with the mucin's negative charges of the cornea. Furthermore, by formulating or coating NPs with a cationic surfactant or polymer, respectively, the pre-corneal retention will be improved due to the interaction with the negatively charged biological membrane (Bhatta et al., 2012, Nagarwal et al., 2010; Giannavola et al., 2003). For instance, after coating sodium alginate-chitosan NPs with chitosan to deliver 5-fluorouracil to the eye, the bioavailability of the drug was increased.
compared to the free drug solution and the uncoated sodium alginate-chitosan NPs (Nagarwal et al., 2012). The conjugation of ligands, such as folic acid, can directly target some tissues like the retinal pigment epithelium (RPE) cells (Suen and Chau, 2013). Polymeric nanocapsules were used for ophthalmic delivery in the early 1990s (Calvo et al., 1994; Calvo et al., 1996). It has been suggested that by coating nanocapsules with PEG, these capsules will internalise into the deeper layer of the epithelium that is found in the cornea (about 50 μm deep), while by coating these nanocapsules with chitosan, the capsules will retain in the cornea for a longer time (De Campos et al., 2003).

The first hybrid NPs for corneal delivery were designed in 2011 (Sharma et al., 2011). These particles were fabricated by loading therapeutic genes into gold blended with PEI polymer. It has been found that these NPs are safe to be used in the cornea, as well as they have a good corneal residence and rapid uptake profiles into the corneal tissue (Sharma et al., 2011). Non-metallic NPs of calcium phosphate loaded with plasmid DNA are also efficient systems where it can transf ect corneal endothelial cells with only moderate toxicity (Hu et al., 2012).

Ideally, an ophthalmic delivery system should provide sustained and/or controlled drug release, and PLGA NPs have been extensively used for this purpose. More particularly, PLGA NPs loaded with diclofenac have been investigated as an anti-inflammatory delivery system. The results showed that there was no irritation in vivo, with a sustained release profile (Agnihotri and Vavia, 2009). Another PLGA formulation, which was loaded with flurbiprofen, showed better pre-corneal retention, corneal permeability, and anti-inflammatory effects than the marketed eye drop product (Ocuflur®) (Araujo et al., 2009; Vega et al., 2008). PLGA NPs have also been loaded with the antibiotics sparfloxacin and levofloxacin for sustained ocular delivery. The outcomes illustrated that these NP formulations improved the pre-corneal residence time and corneal permeability (Gupta et al., 2010; Gupta et al., 2011). In addition to that, pilocarpine-loaded PLGA NPs were also investigated for the treatment of glaucoma. The in vivo study showed the potential of these NPs to reduce the intraocular pressure, due to their sustained release and enhanced bioavailability effects, compared to the commercially available
pilocarpine eye drops (Nair et al., 2012). A recent study by Sah et al. (2017) demonstrated a higher ex vivo permeation profile in isolated goat cornea for PLGA NPs loaded with the steroidal anti-inflammatory drug loteprednol etabonate compared to the drug suspension. Owing to the nano size and lipophilic nature of these particles, higher corneal retention and bioadhesion allowed the penetration across the corneal layers.

In the development of NPs, PLGA can be mixed with or coated by other polymers to enhance its properties. For example, although melatonin-loaded PLGA-PEG NPs were larger in size than the uncoated PLGA particles, the coated particles had longer corneal retention and higher efficiency than the uncoated ones (Musumeci et al., 2012). Ciprofloxacin has been loaded into NPs composed of PLGA and Eudragit® RL, resulting in a higher drug concentration in the pre-corneal tear layer and a more sustained drug release profile than the marketed emulsion formulation (Dillen et al., 2006). Cyclosporine A was loaded in PLGA associated with Eudragit® RL or coated with Carbopol® for the treatment of dry eye syndrome. Both preparations showed very high drug concentrations in the pre-corneal tear film and promising autoimmune ocular disease management (Aksungur et al., 2011). Additionally, pranoprofen-loaded PLGA NPs were dispersed into carbomer hydrogels, with and without the addition of azone, to form semi-solid formulations suitable for ocular delivery (Abrego et al., 2015). Both hydrogels showed a sustained drug release profile. However, the azone-containing hydrogel showed greater efficiency for the treatment of ocular surface oedema compared to the azone-free hydrogels (Abrego et al., 2015). Another study explored the use of hydrocortisone butyrate-loaded PLGA NPs suspended in PVA, Pluronic F-108, or chitosan to form thermosensitive gels for ocular application (Yang et al., 2015). The chitosan-containing gel resulted in a higher cellular uptake in vitro and greater cytotoxic effect than the other gels. These thermosensitive gels were able to eradicate the burst drug release which is often observed with the drug-loaded PLGA NPs, and thus, they maintain a controlled release profile which will be suitable for an inner ocular delivery (Yang et al., 2015). An anti-VEGF drug, bevacizumab, was successfully loaded into albuminated-PLGA NPs to overcome its short half-life in the
vitreous humor (Varshochian et al., 2015). This formulation was able to maintain a controlled release of the drug over a week after a single intravitreal injection, hence, better retinal and choroidal neovascularisation therapy can be obtained by these NPs (Varshochian et al., 2015).

1.6.3. Overview of Nanofibres

The previously mentioned categories of NP, delivered via the ocular route, have attracted attention owing to their advantages over conventional dosage forms such as eye drops and ointments. However, another nanomaterial has arisen as a possible alternative to NPs, namely nanofibres. These are thread-like nanostructures with a diameter of less than 1 µm; the material may be coiled and collected as a fibrous network (Garg et al., 2015). Figure 1.7 shows a PVA fibrous network that was collected in a random orientation. This nano system has shown significant potential in the fields of drug delivery, wound healing, tissue engineering and antimicrobial dressings. This is due to their relative ease of fabrication, large surface area, tensile strength and flexibility, as well as the similarity that they have to the elements of tissue ECM (Murugan and Ramakrishna, 2007; Garg et al., 2015). ECM plays an important role in wound healing. In the presence of a wound, different cytokines and growth factors will be released due to platelet degranulation, usually 3 days after the injury (Holloway et al., 2016). These will regulate the cell proliferation, migration and promote the formation of the elements of ECM. This matrix is composed of proteins and polysaccharides. The proteins provide the elasticity, which helps maintain the tissue shape, and the structural and metabolic support to other cells. The polysaccharides provide the resilience to the formed tissue. The ECM of the wound will facilitate pulling the undamaged wound margin, allowing what is called “wound contraction” (Holloway et al., 2016). This usually starts 5 days after the injury. The myofibroblast cells enable the contraction occur owing to the movement of microfilament bundles which is a muscle-like movement. Scar formation is the end product of the wound healing that is formed due to the enhance collagen deposition that occur after wound contraction (Holloway et al., 2016). This scar is avascular and acellular mass that consist of collagen and act to reestablish tissue continuity and tensile
strength. Therefore, applying the fibres after an injury might promote cell proliferation and migration. This can enhance the wound healing process by mimicking the ECM, hence reduce the duration of healing.

Previous research has demonstrated the ability of the fibres to be loaded with many types of material including drugs, proteins, nucleic acids and, less commonly, biological cells. Additionally, more than one material can be loaded into different compartments of these nanofibres, making them more attractive in the drug delivery field for combination therapies (Chakraborty et al., 2009). The potential for compatibility of the nanofibres with biological tissues allows the use of these systems as scaffolds for tissue regeneration applications in addition to their application as drugs delivery platforms (Garg et al., 2015). The following studies exemplify the various applications of these nanofibres.

### 1.6.4. Antimicrobial Application of the Nanofibres

The antibacterial application of the nanofibres have been previously investigated in several studies. Figure 1.8 illustrates the nanofibres application in order to accelerate wound healing by preventing bacterial growth. Tetracycline hydrochloride-loaded in either PLA or Poly(ethylene-co-vinyl
acetate) and PLA polymer blend was investigated by He et al. (2009) and Kenawy et al. (2002), respectively. Both fibrous systems were able to sustain the release of this antibiotic for at least 6 days and 5 days, respectively.

Figure 1.8: The application of fibres on an open wound to accelerate its closure with (a) showing the effect of bacteria on retarding the wound closure, (b) showing bacterium entry prevention by applying the fibrous membrane, (c) showing wound closure facilitated by the application of the fibrous membrane. Adapted from (Augustine et al., 2014).

A study by Hwang and Jeong (2011) tested PVP loaded silver nanofibres against S. aureus and E. coli. After 24 hours of incubation, the fibrous system was able to significantly inhibit both bacterial strains compared to 1 hour incubation, as more silver particles were released after 24 hours compared to 1 hour. Dashdorj et al. (2015) successfully loaded silver into the natural occurring protein zein. The nanofibres showed a good cytocompatibility with mouse embryo fibroblasts, as well as an antibacterial efficacy against S. aureus and E. coli bacteria after 24 hour incubation compared to the plain zein. Furthermore, Nguyen et al. (2011) was able to load silver NPs into PVA
nanofibres. This system showed an effective antibacterial activity against *E. coli* and *S. aureus* bacteria compared to the blank fibres after 24 hours of incubation. Another study by Nejad *et al.* (2015) has demonstrated the antibacterial activity of silver NPs loaded into poly(dopamine methacrylamide-co-methyl methacrylate) copolymer (MADO). The nanofibres exhibited a sustained release profile up to 7 days with a bacterial inhibition efficacy against *P. aeruginosa,* *E. coli* and *S. aureus* bacterial strains. In addition, the *in vivo* wound healing experiment was performed after creating a skin wound on the back of rats. The findings showed that the drug-loaded and blank fibres were able to accelerate the wound healing after 15 days compared to the untreated group, with the drug-loaded fibres treated group showing minimal scarring compared to the blank fibres treated group (Nejad *et al.*, 2015).

Parwe *et al.* (2014) successfully loaded ciprofloxacin into PLA. These nanofibres demonstrated a 55% release of the loaded ciprofloxacin after 48 hours compared to 12 hours full release of the free antibiotic. Additionally, the drug-loaded fibres showed a microbial inhibition against *S. aureus* compared to the unloaded fibres. Tammaro *et al.* (2015) reported the antibacterial activity of linezolid-loaded in PCL fibres. This system showed an effective dose dependent antimicrobial efficacy against *S. aureus.* Ignatova *et al.* (2008) were able to prepare iodine-loaded into PVP or PEO and PVP blend fibrous systems. Both nanofibres exhibited a significant antimicrobial activity against *S. aureus* and *E. coli,* as well as the fungus *Candida albicans* compared to the blank nanofibres. A study by Kim *et al.* (2004) demonstrated that the antibacterial efficacy of cefoxitin sodium-loaded in PLGA fibres. An initial burst release of about 65% of the total drug amount was seen after 6 hours with 10% more was released after 1 week (i.e. 10% more after the burst release). The effectiveness of cefoxitin sodium was tested against *S. aureus.* The drug-loaded fibres were able to inhibit the growth of this bacterial strain compared to the blank fibres. Said *et al.* (2011) demonstrated similar results with their PLGA loaded with fusidic acid against *S. aureus,* methicillin-resistant *S. aureus* and *P. aeruginosa.* Additionally, it was shown that increasing the dose of the antibiotic has improved the antimicrobial activity of the fibres against the three bacterial strains (Said *et al.*, 2011). Hilal Algan *et al.* (2016) study showed that
ofloxacin-loaded in PCL: poly(butylene succinate) was able to successfully release and retain the antibiotic activity against different bacteria. Their findings also concluded that an increment in the zone of inhibition against *P. aeruginosa* < *S. epidermidis* < *S. aureus* < *Escherichia coli*, due to the increased susceptibility against these microorganisms (Hilal Algan *et al.*, 2016).

The antimicrobial effect of the naturally occurring compound, orange essential oil, were observed by Yao *et al.* (2017). This oil was loaded in zein prolamin protein fibres and the bacterial growth inhibition was tested against *E. coli*. The result indicated that this essential oil has an antibacterial efficiency compared to the blank zein fibres.

1.6.5. Drug Delivery Application of Nanofibres

Yuan *et al.* (2014) studied the delivery of bovine serum albumin using a chitosan-graft-poly(N-isopropylacrylamide) as the fibre matrix for its application in the drug delivery and tissue regeneration fields. This system showed *in vitro* cytocompatibility, as well as a sustained released profile up to 50 hours. The rate of the protein released was dependent on the pH (2.2 or 7.4) and the temperature (25 or 37ºC), since the grafted copolymer is a temperature and a pH responsive polymer.

In addition, PLGA fibres loaded with chitosan NPs containing siRNA was demonstrated by Chen *et al.* (2012), as a nucleic acid delivery platform. The fibrous systems allowed sustained release of siRNA up to 50 days while showing a 50% gene silencing activity, indicating the retention of the bioactivity of the nucleic acid.

Montero *et al.* (2012) successfully loaded basic fibroblast growth factor into gelatin. Their findings showed that this cytokine delivery was able to promote angiogenesis using a fibrous system as a scaffold. Similarly, Sheng *et al.* (2013) studied the antioxidant delivery of vitamin E using silk fibroin with a view to promoting fibroblast growth. The fibres extended the released of this vitamin up to 80 hours, allowing the mouse skin fibroblast cells to proliferate on the fibrous network; the vitamin E was able to protect the cells against oxidative
stress due to its antioxidant activity. Another study by Li et al. (2014) loaded green tea polyphenols and dexamethasone into PLGA. This nanofibrous system was able to sustain the release of both components up to 25 days. An in vitro cytotoxicity test demonstrated the biocompatibility of the fibres when they were co-cultured with mouse embryonic fibroblast cells, as well as a high suppression of human keloid fibroblasts. The keloid symptoms were significantly reduced by using these drug-loaded PLGA fibres system compared to the traditional treatment with silicone gel sheeting on nude mice after 3 months treatment (Li et al., 2014).

The anticancer applications of the nanofibres have been reported in numerous studies. Zhang et al. (2016) were able to successfully load 5-fluorouracil and oxaliplatin into PLA fibres. This fibrous system resulted in an initial burst release of 35.2% and 33.5% of 5-fluorouracil and oxaliplatin, respectively, after 10 hours. The in vitro cytotoxicity of the fibres against human colorectal cell lines was lower after 36-hour exposure but slightly exceeded the free drugs after 72-hour exposure due to the sustained release profile of these fibres. In addition, an in vivo test against colorectal cancer induced mice showed that the local implantation of the drug-loaded fibres was able to reduce the tumour volume significantly compared to the intravenous administration of both drugs in equivalent doses after 9 days of treatment (Zhang et al. 2016). Another study by Sundar and Sangeetha (2012) loaded with 5-fluorouracil into fibres composed of a blend of collagen, chitosan and poly(N-isopropylacrylamide). The in vitro release study demonstrated a sustained release of the drug for over 72 hours, in which it was completely released. The in vitro cytotoxicity study showed that the drug-loaded fibres clearly inhibited three different cancerous cell lines after 72 hours exposure compared to the untreated group.

Singh et al. (2015) studied the anticancer delivery of PVA fibres loaded with docetaxel. A complete release of this anticancer agent was observed after 7 hours. The drug-loaded nanofibres were able to improve the cytotoxicity against breast carcinoma cell line compared to the free drug. This was explained by the higher solubilisation and permeation of the drug due to the PVA polymer Singh et al. (2015). Recently, Lin et al. (2018) demonstrated the potential application of imiquimod-loaded PCL nanofibres in the treatment of
melanoma. The *in vitro* release study exhibited a burst release after 24 hours followed by a sustained release for at least 10 days. A cell culture *in vitro* test showed a time dependent melanoma proliferation inhibition, indicating more drug was released after 48 hours compared to 24 hours cells exposure.

A range of further drug delivery applications have been outlined in the literature. The local anesthetic effects of PLGA loaded bupivacaine fibres was examined by Weldon *et al.* (2012). The *in vitro* release study exhibited a complete release of bupivacaine after 12 days. An *in vivo* test showed a local analgesic effect of the drug-loaded fibres was achieved after 1 day of an incision made on rat skin, which lasted for a week compared to the blank fibres. However, the wounds of both groups were able to heal normally. The antipsychotic delivery of haloperidol was investigated by Fathi-Azarbajani and Chan (2010). Drug-loaded fibres made from PVA or PVA and β-cyclodextrin blend were able to release haloperidol more rapidly compared to the fibres made of PLA, PLGA or their blends with PVA and β-cyclodextrin which were able to sustain the release for at least 72 hours. These results demonstrated the flexibility of the nanofibre approach in terms of the wide range of polymers that may be used (i.e. PVA is a hydrophilic polymer and PLA and PLGA both are hydrophobic polymers). The healing of pulmonary venous obstruction by using tacrolimus-loaded PLGA fibres was examined by Mutsuga *et al.* (2009). An *in vivo* rat model testing the healing of a venous anastomotic stricture in the rat inferior venous cava showed that the drug-loaded fibres were able to reduce the intimal hyperplasia which caused the obstruction compared to the control group.

1.6.6. Wound Healing Application of the Nanofibres

The previous studies illustrate the diversity of the application of nanofibres as a drug delivery platform loaded with drugs, antioxidants, proteins or nucleic acids. However, these fibrous systems can also be used for wound healing purposes, as shown in Figure 1.9. Ramalingam *et al.* (2015) tested poly(2-hydroxyethyl methacrylate) loaded with curcumin for the treatment of infected wounds. The release study exhibited a release of 72% curcumin after 10 days. A microbiological test showed that these fibres were effective against *S.*
aureus, methicillin-resistant *S. aureus* (MRSA), *E. coli*, and *K. pneumonia*. In addition, this fibrous system has demonstrated an even spread of hepatocytes on the fibres scaffold indicating their good biocompatibility. Another study by Merrell *et al.* (2009) investigated the antioxidant and anti-inflammatory effect of curcumin-loaded PCL fibres. The results indicated that these fibres have a sustained release profile (about 35% has been released) after 3 days. In addition, the oxygen radical absorbance capacity test showed that the drug-loaded fibres were able to inhibit the free oxygen radicals compared to the blank PCL fibres. The anti-inflammatory effect of the curcumin-loaded PCL fibres were demonstrated through a significant expression reduction of IL-6 compared to the blank fibres. Finally, the drug-loaded fibres were able to accelerate the wound closure rate of diabetic mice by 80% compared to 60% for the blank fibres after 10 days of treatment. The wound healing of an infected wound by fusidic acid-loaded into PLGA was reported by Said *et al.* (2012). The drug-loaded fibres were able to sustain the release of fusidic acid for at least 14 days with an initial burst release showing after 2 days. An *in vivo* study on rats’ skin showed that the drug-loaded fibres were able to completely heal an open wound that was infected by *S. aureus* after 14 days of treatment compared to the untreated group. Additionally, the blank fibres demonstrated a better rate of wound closure than the untreated group.
Figure 1.9: Wound closure after treating with silver NPs loaded MADO fibres, blank MADO fibres and untreated control group at 0, 5, 10, and 15 days showing the accelerated wound closure of silver NPs loaded MADO fibres compared to the other groups. Adapted from (Nejad et al., 2015).

The combination of a local anesthetic agent with an antibiotic was also explored for accelerating the wound healing by the nanofibres. Chen et al. (2012) successfully prepared PLGA blended with collagen fibres that is loaded with a combination of vancomycin, gentamicin and lidocaine. An in vivo study on rats’ skin that are infected by S. aureus and E. coli infected wound showed that the drug-loaded fibres were able to close the wound and treat the infection after 21 days of treatment more effectively than the untreated group, which died within the first week, as well as the group treated with the blank fibres. The addition of collagen promoted wound healing due to its natural presence in the tissues which may also stimulate cell proliferation. An Indian plant-extract Memecylon edule was loaded into PCL and investigated by Jin et al. (2013). The in vitro cell culture study on human dermal fibroblasts exhibited a low cytotoxicity and higher cellular proliferation on the loaded fibrous scaffold compared to the blank PCL after 9 days of exposure. Hosseinzadeh et al. (2016) prepared fibres consisting of a blend of natural occurring compounds, hyaluronic acid and gelatin for burn wound healing. A thermal skin burn on rats were treated by the nanofibres and compared with the commercially available
wound dressing product, ChitoHeal gel® (chitosan-based gel), and an untreated control group. The results indicated that both the nanofibres and the gel were able to accelerate the wound closure by 81.9% and 77.8%, respectively, compared with 65% of the untreated control group after 14 days of treatment. A study by Lu et al. (2012) exhibited the wound healing effect of graphene-loaded chitosan and PVA blend fibres. A skin wound on mice and rabbits showed that the graphene-loaded fibres were able to accelerate the wound healing compared to the untreated control group after 15 days and 10 days of treatment, respectively. In addition, the blank fibres showed a better wound closure than the control group but lower than the graphene-loaded fibres.

The wound healing application of the fibres was further investigated by loading different types of proteins. A study by Gil et al. (2013) demonstrated the wound healing effect of epidermal growth factor and silver sulfadiazine antimicrobial agent loaded into silk fibroin protein. The growth factor was used to promote cellular proliferation, while silver sulfadiazine was used to prevent wound infection and to promote rapid re-epithelialisation. An in vivo study on mice skin wounds illustrated that both drug-loaded and blank fibres were able to accelerate the wound healing more than the commercially available wound dressing, 3M Tegaderm™ hydrocolloids, and the untreated group after 12 days of treatment. Another study by Wang et al. (2015) investigated PCL and hyaluronic acid blended fibres loaded with epidermal growth factor for accelerated wound healing effect. An initial burst release of epidermal growth factor (11%) and hyaluronic acid (35%) was seen after 4 days of release followed by a sustained release profile for both components (29.8% and 43.5% epidermal growth factor and hyaluronic acid, respectively) after 25 days of release. Human primary fibroblast cells and human epidermal cells were exposed to the growth factor-loaded PCL and hyaluronic acid fibres, the blank blended fibres and PCL alone fibres. The results indicated that both cell lines were able to proliferate significantly when exposed to the epidermal growth factor-loaded fibres compared to the blank PCL and hyaluronic acid blended fibres and blank PCL fibres after 5 days of exposure. Furthermore, an in vivo wound healing study on rats exhibited a significant smaller wound size for
epidermal growth factor-loaded fibres compared to the blank PCL and hyaluronic acid blended fibres, blank PCL fibres and the untreated control group after a week of treatment. However, after 4 weeks, the wounds had completely closed in all groups with the epidermal growth factor-loaded fibres having the thickest epidermal layer, suggesting that this group had a greater accelerating epidermis regeneration than the other groups (Wang et al. 2015).

Another example of growth factor-loaded fibres was given by Yang et al. (2011) for wound healing application, using basic fibroblast growth factor-loaded into PEG and PLA. A burst release of 14% of the growth factor was seen after 12 hours of release followed by a sustain release of about 90% of after 25 days of release. An in vitro study on mouse embryo fibroblasts showed that the growth factor-loaded PEG and PLA fibres had the highest cellular proliferation compared to the blank fibres, the basic fibroblast growth factor alone and the untreated cells after 3 and 7 days of incubation. An in vivo test on diabetic rat demonstrated the smallest wound area for the growth factor-loaded fibres, compared to the blank fibres and the untreated group after 1 and 2 weeks of treatment. However, both fibrous systems showed a reduction in wound size compared to the untreated group after 4 weeks of treatment (Yang et al., 2011). Lai et al. (2014) successfully prepared two fibrous systems. One consisting of VEGF in gelatin NPs along with basic fibroblast growth factor-loaded in hyaluronic acid fibres. The second contained platelet-derived growth factor in gelatin NPs, along with endothelial growth factor-loaded in collagen fibres. Both fibrous systems were able to sustain their growth factors after 25 days of release. An in vivo study on diabetic rat wounds demonstrated a significant reduction of the wound area after treating the wounds with the growth factor-loaded fibres compared to the untreated control group after 2, 4 and 6 weeks of treatment (Lai et al., 2014). In addition, the blank fibres showed a decrease in the wound size compared to the control group.

A recent study by Gnawi et al. (2018) demonstrated the use of gelatin and chitosan fibres as promising scaffolds for peripheral nerve damage reconstruction. This fibrous system showed a high adhesion on the surface and proliferation of Schwann cells due to the topography of the fibres’ network that facilitated cell adhesion (Gnawi et al., 2018).
In conclusion, the application of fibres in the fields of antimicrobial dressings, drug delivery, tissue engineering and wound healing have attracted interests owing to their resemblance to the tissue ECM. This can accelerate wound healing by applying the fibres as a scaffold, or by loading the fibres with one or more wound healing promoting agent. The large surface area, tensile strength and flexibility of these fibres, allow them to be applied directly and adopt the shape of the injured site. Finally, the ease of incorporating different therapeutic drug classes enables the use of the fibres as a drug delivery platform.

1.7. Nanofibre Fabrication by Electrospinning

A widely used technique for fabricating the nanofibres is electrospinning (Chakraborty et al., 2009). This process involves the application of a high voltage on a liquid solution or suspension to form fibres. In this method, the applied voltage will overcome the viscous polymer liquid surface tension, allowing the ejection of an elongated charged fibres, in which the solvent has been evaporated during the flight from the tip (Doshi and Reneker, 1995). Upon collection, fibres may be oriented in a regular (aligned) or a random pattern, which results in the formation of a fibrous mat (Williams et al., 2018). Figure 1.10 illustrates an example of a PVP fibrous mat that was collecting in aluminium foil. The continuous production of fibres will control the thickness, where a thicker mat can be obtained by collecting for a longer duration.
Figure 1.10: PVP fibrous network forming a mat after 2 hours of collection on aluminium foil.

Although electrospinning has attracted considerable attention in the drug delivery field over recent decades, the generation of fibres by this technique was reported for the first time long ago by Cooley in 1900 (Cooley, 1900). The principles of designing nanofibres by electrospinning are as follows: a polymer liquid, with or without a drug, flows out at a constant rate from a syringe attached to a pump, with the needle being connected to a high voltage electrical force. This electrostatic force will overcome the surface tension of the liquid, forming a droplet into a Taylor cone, as it can be seen in Figure 1.11. Then the liquid will be ejected from the tip of that cone to form a jet in a grounded collector. The jet will be interrupted and result in highly charged elongated fibres. Throughout the ejection process, the solvent will evaporate (Doshi and Reneker, 1995).
1.7.1. The Principle of Monoaxial Electrospinning

The electrospinning process requires four main components, as shown in Figure 1.12; a high voltage source, a syringe pump, a syringe with a fitted needle (emitter) and a collector surface (Williams et al., 2018). The voltage source is connected to the collector at one end and to the emitter at the other, as the dispensed solution would yield droplets in the absence of voltage. Upon the application of a voltage (charge) the surface tension is balanced by the electrostatic forces, which will allow the repulsive forces at the droplet surface to elongate and developed into a cone like shape (Taylor cone) (Pillay et al., 2013). The magnitude of the charge depends on the solution electric conductivity. As the solution droplet’s surface tension is overcame by the strength of applied voltage, a jet of fibre is ejected from the cone tip in a continuous elongated fashion and the solvent will start to evaporate (Huang et al., 2003).

When the jet reaches the oppositely charged grounded collector, it will be discharged and a solid thread-like structures will form, in which the solvent is almost completely evaporated and the fibres will deposit as a fibrous mat (Figure 1.10) (Williams et al., 2018).
Figure 1.12: A schematic diagram of the electrospinning process illustrating its main components and the Taylor cone. The use of monoaxial, coaxial or triaxial needle system can produce single-, double- or triple-layered fibres, respectively.

When suboptimal conditions are used, the fibres produced may not be satisfactory. An unstable polymer jet can be created by changing the electrospinning parameters such as changing the flow rate, voltage or tip-to-collector distance. This unstable jet formation is known as whipping (Williams et al., 2018). This will affect the fibre diameter and the solvent evaporation rate which will either form pores (at higher evaporation rates) on the surface of these fibres, or develop wet fibres (at lower evaporation rates). When the polymer solution viscosity and charge repulsion are insufficiently overcome by the surface tension of the charged solution, this might lead to the formation beaded fibres. These defected fibres resulting a fibre integrated by a droplet (Williams et al., 2018). Therefore, optimising the electrospinning solution and process parameters are essential in order to avoid the fabrication of defected fibres. The parameters affecting electrospinning will be elaborated later in section 1.7.3.
1.7.2. The Principle of Multi-layered Electrospinning

Multi-layered electrospinning, which can be coaxial or triaxial, is a technique which can produce nanofibres that are coated, i.e. they are bi-layered or tri-layered in the case of coaxial or triaxial, respectively. It allows the incorporation of drugs into either the core, the intermediate or the shell layer of these fibres (Greiner et al., 2006). This approach allows more than one material to be loaded into the fibres, and confers the possibility of carrying either hydrophilic and lipophilic drugs, protein, or nucleic acids with different release patterns (Greiner et al., 2006). It has been reported that fibres that are fabricated by the monoaxial method and hydrophobic polymers may release their cargos in an initial burst release followed by a sustained release pattern (Buschle-Diller et al., 2007, Xie and Wang, 2006, Xie et al., 2008, Katti et al., 2004, Kumbar et al., 2006; Chew et al., 2005). Care is required with proteins or nucleic acids that are loaded in these electrospun fibres, as they might suffer from a decrease in bioactivity due to their degradation as a result of the burst release effect (Chew et al., 2005; Chew et al., 2007). On the other hand, multi-layered electrospun fibres have the advantages of potentially improving the stability of the loaded substances whilst achieving a high drug loading. In addition to controlling the release of these materials with the elimination of the initial burst release effect, where it can be prevented due to the additional coating of the fibres (Yu et al., 2013; Wu et al., 2011). The principles of coaxial or triaxial electrospinning are similar to the monoaxial technique, except that there are two or three needles, respectively. The different gauge sizes will allow dispensing two or three distinctive liquids simultaneously, as can be seen in Figure 1.12 (Dror et al., 2007).

It is worth noting that a similar technique existing, known as electrospraying will produce particles instead of fibres. Here, the electric force will allow the polymer solution to flow out of a capillary nozzle and the formed cone will be broken up and dispersed into fine droplets. This is due the high applied voltage and the difference in the physical properties of the polymer solution (i.e. a less viscous solution) (Doshi and Reneker, 1995). The residual charges on the particles along with the drying effect, which occurs during the process, can prevent aggregation of the produced particles (Wu and Clark, 2008; Jaworek
and Sobczyk, 2008). This system can form droplets that are uniformed with particle size that might reach down to less than 10 nm.

The advantages of using electrospinning is the elimination of high energy shearing forces that can affect thermolabile materials and the removal of any solvent remaining, which can have a degree of toxicity. In addition, the achievement of high drug loading and uniform drug distribution, the ease of operation and the cost-effectiveness make this technique more favourable method of preparation than other conventional approaches (Chakraborty et al., 2009). Nevertheless, the low fibre production rate (i.e. scale up), the small volume of liquid that can be spun at one time, the jet instability, and the limited number of studies that can translate these fibres into successful delivery systems for clinical application can be the main drawbacks of electrospinning (Chakraborty et al., 2009, Teo and Ramakrishna, 2006; Kumbar et al., 2008). Thus, some of these disadvantages can be easily overcome, such as the issue of the low levels of fibre production, which might be simply resolved by increasing the number of spinnerets (Teo and Ramakrishna, 2006).

1.7.3. Factors Affecting Electrospinning

There are several parameters that affect the fabrication of electrospun fibres (Chakraborty et al., 2009). Table 1 illustrates these different parameters with their effects on the final product. These can be related to the solution, process or the environmental parameters (Huang et al., 2003).
Table 1.1: Factors affecting the fabrication of electrospinning technique. Adapted from (Chakraborty et al., 2009; Williams et al., 2018).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>↓ Flow rate</td>
<td>↓ diameter size and ↑ drug release rate</td>
</tr>
<tr>
<td>↓ Liquid viscosity</td>
<td>↓ diameter size</td>
</tr>
<tr>
<td>↑ Liquid conductivity</td>
<td>↓ diameter size</td>
</tr>
<tr>
<td>↑ Liquid surface tension</td>
<td>↓ diameter size</td>
</tr>
<tr>
<td>↓ Polymer molecular weight</td>
<td>↓ diameter size and ↑ drug release rate</td>
</tr>
<tr>
<td>↓ Polymer concentration</td>
<td>↓ diameter size and ↑ drug release rate</td>
</tr>
<tr>
<td>↑ Voltage</td>
<td>↓ diameter size initially, then ↑ size</td>
</tr>
<tr>
<td>↑ Solvent volatility</td>
<td>↓ diameter size</td>
</tr>
<tr>
<td>↓ Needle diameter</td>
<td>↓ diameter size</td>
</tr>
<tr>
<td>↑ Distance to collector</td>
<td>↓ diameter size</td>
</tr>
<tr>
<td>↑ Temperature</td>
<td>↓ diameter size</td>
</tr>
<tr>
<td>↓ Humidity</td>
<td>↓ diameter size</td>
</tr>
</tbody>
</table>

Polymer solution viscosity is an essential solution parameter in which the lower viscosity can lead to the formation of particles, while an intermediate viscosity can result beaded fibres (Pillay et al., 2013). An optimum viscosity of the polymer solution can yield unbeaded fibres. However, increasing the viscosity can increase the diameter of the fibres. Ultimately, the very high viscosity leads to polymer accumulation and/or solidification on the tip of the needle (blockage) (Pillay et al., 2013). The solution viscosity is related to the polymer concentration in a solution and to the molecular weight of the polymer, which are affected by the molecular chain entanglement (Bhattarai et al., 2019). When this entanglement occurs, the elongation force outweighs the surface tension of the polymer solution which enables the electrospinning process to take place. Solvent type is another solution parameter due to the high impact of the solvent on the yielded fibres. The use of a high evaporating solvent can accelerate the evaporation of the solvent during the flow of the jet to the collector, allowing the formation of porous fibres (Bhattarai et al., 2019). A low evaporating solvent can result flattened fibres due to the entrapment of
residual solvent in the developed fibres that will be flat upon hitting the ground collector (Williams et al., 2018). In addition, each solvent can influence the polymer solution due to the physiochemical properties of that solvent (viscosity, conductivity, surface tension and volatility). Low to moderate surface tension solvent is preferable in electrospinning, as a low applied voltage can easily overcome the attractive forces within the polymer solution, hence fibres can be produced. Increasing the solution conductivity may also allow for a lower applied voltage, as the greater charge accumulation of the jet enables fibre elongation to take place (Williams et al., 2018).

A sufficient applied voltage is an important process parameter to initiate the jet formation by overcoming the surface tension of the polymer solution (Chakraborty et al., 2009). The application of high voltage (more than the threshold) can produce larger fibre diameter due to the ejection of more fluid in the jet that will eventually result jet instability, i.e. jet whipping (Huang et al., 2003). Nevertheless, this high voltage application can reduce the flight time from the tip of the needle to the collector; this can lead to incomplete solvent evaporation (Williams et al., 2018). This tip-to-collector distance can also affect the resulting fibres. It was reported that the use of high distance can form thin fibres due to the increase in the flight time, while very high distance (above 20 cm) may lead to larger and/or defective fibres (such as beaded fibres) (Bhattarai et al., 2019). An adequate flight time is necessary in order to permit the molecular chain entanglement to overcome the surface tension of the solution preventing the jet from splitting. Optimum flow rate and needle inner diameter are vital to yield uniform fibres. Higher flow rate can produce larger fibres diameter owing to lowering the flight time. In addition, the solution jet will be insufficiently charged, leading to the ejection of polymer droplets (Pillay et al., 2013). The use of smaller needle diameter can produce narrow fibres, though, it is more prone to get blocked, particularly when using a high solution viscosity (Williams et al., 2018).

Temperature and humidity are essential factors to ensure the reproducibility of the yielded fibres. High temperature can increase the polymer solution conductivity yet decrease its viscosity and surface tension, producing thinner fibres. In addition, high temperatures (above 40ºC) may enhance the solvent
evaporation rate resulting larger fibres diameter, owing to the reduction of the
time needed for the fibres to elongate (Chakraborty et al., 2009). High humidity
(above 60%) can increase the water absorption of the hydrophilic polymer to
produce wet fibres, or precipitate the hydrophobic polymer on the needle tip
which will lead to blockage (Williams et al., 2018).

All the aforementioned parameters are related to each other and should be
taken into consideration to yield a reproducible evenly-distributed batch of
fibres. Nevertheless, each polymeric system will require an optimisation
(according to these factors) in order to yield a successful fibrous system.

1.7.4. Alternative Nanofibre Production Methods

Electrospinning is the most commonly used technique for the preparation of
nanofibres. However, there is a wide range of other techniques reported for
the fabrication of fibres and the following methods are those most frequently
used.

“Melt electrospinning” is an alternative technique for the conventional
electrospinning method, which the use of organic solvents is eliminated and a
polymer solution melt is used instead (Nagy et al., 2013; Brown et al., 2016).
This melt solution need to be heated and maintained throughout the process.
The temperature used is a critical parameter, since many drugs can be
affected by high temperatures (Brown et al., 2016). The collected fibres were
reported to have larger diameter than the ones which have been prepared by
the conventional electrospinning (Nagy et al., 2013).

“Centrifugal spinning” is a technique that involves rotating a polymer at a very
high speed (above 2000 rpm) until it melts and produces fibres (Sarkar et al.,
2010). This method can be useful for polymers, such as PCL, and sugars, such
as sucrose. The yielded fibre diameters were reported to be in the μm range
(Marano et al., 2016).

Another alternative technique to electrospinning, but less commonly used, is
the “solution blowing” method. Here, the polymer solution is ejected through a
needle which is surrounded by an outer nozzle containing a pressurised gas
(Souza et al., 2014). Additionally, a polymer melt can be used, which is known
as “melt blowing”. It was reported that both the solution and melt blowing techniques were able to produce fibres of lower diameters and greater uniformity compared to electrospinning and melt electrospinning, respectively (Balogh *et al*., 2015).

“Electroblowing” is a technique that involves both electrospinning and a solution or melt blowing methods. The polymer solution or melt is ejected through a similar needle to that used in the blowing methods, with a potential difference applied between this needle and the collector (Jiang *et al*., 2014; Balogh *et al*., 2015). This technique eradicated the needle clogging problem that is usually experienced in the conventional electrospinning (Balogh *et al*., 2015).

A combination of centrifugal spinning and blowing techniques is known as “pressurised gyration”. Here, a pressurised gas is applied to a polymer solution being rapidly rotated (36,000 rpm) in a cylinder that contains a number of small holes, through which the polymer solution is forced. This allows the solvent to be evaporated and the fibres to be collected on a standing collector, in which the cylinder sits inside (Mahalingam and Edirisinghe, 2013). This technique was reported as a potential scale up for fibre production. The yielded fibre diameters were reported to be between 60 to 1000 nm (Mahalingam and Edirisinghe, 2013).

### 1.7.5. Electrospun Nanofibres for Ocular Drug Delivery

Although there have been only limited studies into using electrospun nanofibres for drug delivery to the ocular system, these efforts have shown very promising results. One of these studies loaded ofloxacin into PCL/polybutylene succinate nanofibres for the treatment of ocular infection (Karatas *et al*., 2015). The results showed that this formulation was effective against different Gram positive and Gram negative bacteria in terms of inhibiting the growth of these strains. Another study by Taghe and Mirzaeei (2017) successfully loaded ofloxacin into a blend of PVA and chitosan. This fibre system was able to prolong the release of ofloxacin up to 18 hours in rabbit eyes. A study by Baskakova *et al.* (2016) successfully loaded aciclovir, ciprofloxacin and cyanocobalamin, and combinations of these drugs into both
water soluble and insoluble polymers (PVP and PCL, respectively). The drug release results showed that these fibres were able to prolong drug release compared to the pure drug solution in a validated in vitro outflow model of the eye, suggesting that this fibrous system can be a potential intravitreal implant (Baskakova et al., 2016). A study by Sun et al. (2016) investigated the incorporation of voriconazole in a blend of PVA and hydroxypropyl-β-cyclodextrin. This fibre system significantly prolonged the half-life and improved the bioavailability of this antifungal drug in rabbit tears after surface ocular administration, with no irritation being observed (Sun et al., 2016).

Mirzaeei et al. (2018) studied triamcinolone acetonide-loaded into four different polymer blends consisting of chitosan, PVP, PVA, Eudragit S100 or zein. The results indicated that the fibres made from PVP and chitosan possessed the best quality and prolonged the release of triamcinolone acetonide compared to the other formulations, and was recommended to be used as ocular inserts (Mirzaeei et al. 2018). Bhattarai et al. (2017) compared an electrospun fibre insert to a solvent cast polymeric insert loaded with dexamethasone, using a PLA and PVA polymer blend. The fibres showed a better release profile than the solvent casted inserts. In addition, the fibres lacked any cytotoxicity after 24-hour exposure to bovine corneal endothelial cells, suggesting a potential delivery system for the anterior part of the eyes (Bhattarai et al., 2017). Gagandeep et al. (2014) investigated timolol maleate and dorzolamide hydrochloride-loaded PVA/PCL nanofibres for the treatment of glaucoma. The formulation showed a significant reduction in the intraocular pressure compared to the marketed available eye drops. Furthermore, Lancina et al. (2017) were able to prepare polyamidoamine dendrimer nanofibres loaded with the anti-glaucoma drug brimonidine tartrate. The in vitro and in vivo studies showed that the dendrimer-based nanofibres had no cytotoxicity and caused no ocular irritation in the cultured cells and the rat eyes, respectively. The study also concluded that this system was more efficient to deliver brimonidine tartrate compared to the eye drop formulation when both are administered once daily for three weeks.

Angkawinitwong et al. (2017) demonstrated the incorporation of bevacizumab (a VEGF antibody) in a buffer as the core and PCL as the shell of a coaxial
fibre system for the treatment of age related macular degeneration. The *in vitro* release result suggested that this fibrous system was able to sustain the release of bevacizumab, which can reduce the frequent administration of this antibody compared to the commercial available eye drops.

### 1.7.6. Electrospun Nanofibres as Ocular Scaffolds

The application of electrospun fibres as a scaffold to facilitate the growth of corneal cells was reported. The study by Deshpande *et al.* (2010) showed that PLGA fibres can be used as a scaffold alternatively for the human amniotic membrane. This was due to the ability of this system to carry limbal epithelial cells while retaining their proliferation rate for the treatment of limbal stem cell deficiency. Azari *et al.* (2015) successfully prepared electrospun fibre scaffolds using PCL, polyhydroxybutyrate (PHB) or poly (3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV) polymers. All three systems showed a high attachment and proliferation rate for corneal keratocytes. In addition, the PCL scaffold was able to up-regulate the expression of some proteins such as collagen type 1, most probably due to the morphological resemblance between the fibres and the ECM proteins. Another study by Da Silva *et al.* (2015) demonstrated the *in vitro* and *in vivo* ocular biocompatibility of a PCL nanofibre scaffold. The *in vitro* (cell culture) and *in vivo* (rat eye) results have proven a high cellular biocompatibility which led to the possibility of using this system as ocular drug carrier. Sharma *et al.* (2014) investigated the use of PCL fibres as ocular surface scaffolds due to their biocompatibility and improved transparency after treating this system with helium–oxygen plasma discharge. This enabled the surface to be hydrophilic, hence, showed better human limbal epithelial cell adhesion and proliferation compared to the plasma-untreated scaffold.

The differences of the cellular behaviour on aligned and randomly oriented fibrous scaffolds has been also investigated. Yan *et al.* (2012) have studied such difference using a blend of gelatin and PLLA. Their findings suggested that fibre alignment benefited cell proliferation due to the higher elasticity and breaking strength than the randomly oriented scaffold. However, each scaffold showed a different response to the growth of different rabbit corneal cell lines.
Aligned fibre scaffolds had a better proliferation of corneal keratocytes, which favoured the fusiform shape of cells. Nevertheless, randomly oriented scaffolds showed a better proliferation of corneal epithelial cells that tended to guide the cell in a polygon shape (Yan et al., 2012). Another study by Zhang et al. (2015) demonstrated the growth effect of rabbit corneal stroma cells on aligned gelatin-PLA fibre scaffolds. The results indicated that this scaffold had a good compatibility with this corneal cell line and can be a promising application to repair cornea stroma. A study by Wray and Orwin (2009) demonstrated the growth of rabbit corneal fibroblasts on aligned collagen type I fibre scaffolds. The findings proposed that this cell line was able to grow on the scaffold and down-regulate alpha-smooth muscle actin (α-SMA) protein expression compared to the unaligned scaffold leading to the possibility of being used as a corneal tissue replacement. Phu et al. (2011) further confirmed the application of collagen type I fibres scaffolds on rabbit corneal fibroblasts. These cells were able to grow on the aligned fibres, as well as reduce the expression of α-SMA compared to the cells which were grown on tissue culture plates and the randomly oriented fibres.

The application of electrospun fibre scaffolds as an alternative option for corneal transplantation was investigated as well. Chen et al. (2015) fabricated fibrous scaffolds made from a blend of silk fibroin and PLA-PCL using different ratios, in order to overcome the poor mechanical properties of the silk fibroin. The scaffold that was made from silk fibroin and PLA-PCL at a ratio of 25:75 showed good light transmission, good biocompatibility for cell adherence and cell proliferation using the human corneal endothelial cell line. Therefore, this scaffold was suggested for corneal endothelial transplantation. More recently, Hong et al. (2018) successfully prepared PLGA fibre scaffolds cultured with human conjunctival epithelial cells for corneal epithelium regeneration on burned rabbit corneas. Their findings suggested that the PLGA-based epithelial membranes enabled the reduction of the defective epithelium, which could be a potential strategy to regenerate corneal epithelium by transplantation.
1.8. Clinical Use of Moxifloxacin and Pirfenidone

1.8.1. Clinical Use of Moxifloxacin

Moxifloxacin is a fourth generation fluoroquinolone antibiotic which has a high potency and low bacterial resistance compared to the older generation fluoroquinolones (Miller, 2008). This is due to its high affinity to two bacterial enzymes, DNA gyrase and topoisomerase IV (Miller, 2008; Wise, 1999).

The marketed moxifloxacin 0.5% eye drops (Vigamox®, or Moxeza®) are widely used due to their effectiveness against a broad spectrum of Gram positive and Gram negative ocular bacteria, as well as some resistant strains such as MRSA (Miller, 2008). The recommended daily dose for the treatment of conjunctivitis is 1 drop three times a day (t.i.d.) for 7 days (Eltis, 2011). However, the dosage regimen for the treatment of corneal ulcers, caused mainly by *P. aeruginosa*, needs to be more aggressive to ensure a rapid eradication of the very antagonistic bacteria (Shovlin, 2004). Here, the initial dose of moxifloxacin 0.5% eye drops is one drop every 30 minutes for the first 6 hours, then every hour for the rest of the day. The frequency will be reduced to one drop every one to two hours in the second day. Patients needed close monitoring, especially in the first couple of days. Depending on the progression of the treatment, patients were asked to administer one drop 6 to 8 times a day, and then 4 times daily for 14 weeks (Shovlin, 2004). Consequently, several studies have reported on the NP delivery of moxifloxacin in order to sustain this antibiotic release (to reduce the frequency of administration), while maintaining high efficacy and good safety profiles.

Kisich *et al.* (2007) investigated moxifloxacin-loaded PBCA NPs against *Mycobacterium tuberculosis* (*M. tuberculosis*) that exist in lung macrophages (Kisich *et al.*, 2007). These NPs were able to accumulate in the macrophages more significantly than the free drug, and remained there for longer periods. Despite the burst release effect, these NPs showed a sustained drug release for more than two days and inhibited the *M. tuberculosis* in a much lower concentration (0.1 μg/mL) than the free drug (1 μg/mL), due to the high uptake and retention of these particles by macrophages. A similar study by Shipuo *et al.* (2008) examined PBCA NPs loaded with moxifloxacin to inhibit *M.*
tuberculosis. The formulated NPs were capable of reducing the mycobacteria count in the lungs, when given by intravenous administration, compared to the standard drug formulation. Ahmad et al. (2008) prepared PLGA NPs loaded with econazole and moxifloxacin individually against multidrug resistant M. tuberculosis mice. After a single oral dose of these NPs, econazole and moxifloxacin were detected in the plasma for up to 5 days and 4 days, respectively, while both drugs were detected in other organs such as the lungs, liver, and spleen for up to six days. However, free econazole was detectable in plasma for up to 3 to 4 hours and 12 hours in other organs, whereas free moxifloxacin was detected after 12 hours in the plasma and 24 hours in the other organs. Interestingly, the combination of econazole and moxifloxacin resulted in a significant therapeutic improvement compared to the individual drugs (Ahmad et al., 2008).

Srinivas and Pragna, (2012) investigated the ophthalmic delivery of moxifloxacin hydrochloride-loaded Eudragit RL 100 NPs which exhibits a sustained drug release profile. An in vitro microbial study, as well as an in vivo study suggested that these particles are effective and safe to be used in ocular delivery. Kesarla et al. (2014) were able to successfully design moxifloxacin Eudragit RL100 NPs loaded in an in situ gelling system. The formulation was capable of changing the liquid into a gel in the presence of cations. It was less viscous before ocular administration and then formed a strong gel following the cul-de-sac instillation. The estimated drug loading was about 86% w/w. This formulation prolonged the adhesion time in the eye, enhanced the corneal permeation and sustained the release of the drug over a period of 12 hours compared to the marketed eye drop formulation. Therefore, this NP system can be used as a drug delivery system due to its ability to reduce the dosage frequency and may improve patient compliance (Kesarla et al., 2014).

Additionally, Mahor et al. (2016) investigated the antibacterial efficacy of gelatin loaded with moxifloxacin NPs against a Gram positive Bacillus subtilus (in vitro study) and a rabbit antibacterial activity against the Gram positive S. aureus (in vivo study). The results illustrated that these NPs were able to inhibit both bacterial strains more effectively than the commercial product. Kaskoos (2014) reported that moxifloxacin-loaded chitosan/dextran NPs have better
ocular bioavailability, lower dosing frequency and systemic side effects, thus better patient compliance than the drug eye drops. This formulation had an \textit{in vitro} sustained release profile with approximately 24\% of this drug content released within the first hour, followed by a gradual drug release for up to a day. In contrast to the marketed eye drops which showed a very fast release of more than 90\% in 30 minutes. The loaded NPs increased the corneal permeation and retention on an \textit{ex vivo} goat eye compared to the eye drops. Additionally, both the eye drops, and the NPs system showed an \textit{in vitro} antibacterial activity against \textit{P. aeruginosa} and \textit{S. aureus} bacteria (Kaskoos, 2014). Liu \textit{et al.} (2018) were able to successfully prepare moxifloxacin-loaded into hybrid NPs from a lipid, chitosan polymer and hyaluronic acid. These NPs exhibited a higher \textit{in vivo} rabbit pre-corneal retention, corneal permeability and ocular bioavailability compared to the eye drop formulation.

Mudgil and Pawar (2013) fabricated PLGA moxifloxacin NPs for corneal delivery. These NPs have a biphasic drug release profile (burst release followed by extended drug release up to 24 hours) performed in a modified USP dissolution apparatus 1 containing a two-sided open glass cylinder. Moreover, this system enhanced the corneal permeability and retention on a freshly excised goat cornea compared to the marketed eye drops. In addition, the \textit{in vitro} microbial efficacy against \textit{S. aureus} and \textit{P. aeruginosa} was also improved due to this system prolonged effect compared to the marketed moxifloxacin eye drops. Garg \textit{et al.} (2014) successfully prepared moxifloxacin-loaded PLGA NPs for ocular delivery. The \textit{in vitro} release showed an initial burst release of 44\% after 24 hour followed by a sustained release of about 90\% after 35 days. An \textit{in vivo} study on rabbit eyes suggested that these NPs improved moxifloxacin ocular bioavailability and reduced its ocular drainage compared to the eye drops formulation. Similarly, Gadad \textit{et al.} (2012) fabricated moxifloxacin-loaded PLGA NPs. The \textit{in vitro} drug release study showed that the NPs system was able to release 86.1\% of the loaded antibiotic after 24 hours compared to the marketed eye drop formulation. This formulation has better \textit{ex vivo} corneal permeation on excised goat cornea and \textit{in vitro} antibacterial efficacy against \textit{S. aureus} and \textit{P. aeruginosa} than the marketed eye drops.
Mandal *et al.* (2012) investigated the antimicrobial activity and the rabbit eye irritation of a moxifloxacin ocular gel using sodium alginate and HPMC. This gel was able to inhibit the growth of Gram positive *S. aureus* and Gram negative *E. coli* bacteria, and there was no signs of eye irritation or tissue damage. This suggested that this formulation can be a promising ophthalmic delivery approach for this antibiotic. Another study by Nanjwade *et al.* (2012) evaluated the *in situ* hydrogel production by the use of polyox (a pH-sensitive gelling agent), sodium alginate (an ion-sensitive gelling agent) and poloxamer (a temperature-sensitive gelling agent) along with HPMC (increases the residence time) loaded with moxifloxacin. All three gelling systems demonstrated an *in vivo* sustained release profiles, for 8 hours, on rabbit eyes compared to the marketed eye drops. Shastri *et al.* (2010) were able to fabricate a thermo-sensitive mucoadhesive hydrogel that consisted of a combination of poloxamer 407 and poloxamer 188 with the mucoadhesive polymers xanthan gum or sodium alginate and loaded with moxifloxacin. The mucoadhesive polymers were applied to increase the gel strength and corneal bioadhesion force. The drug release data suggested a prolonged release profile of moxifloxacin with a more rapid release from the xanthan gum gelling system than from the sodium alginate system.

Bharti and Kesavan (2017) developed a moxifloxacin microemulsion to sustain its release and to improve its pre-corneal retention and thus, its bioavailability. Their findings suggested that this system was able to retain the antibacterial activity of moxifloxacin against Gram positive *S. aureus* and Gram negative *E. coli* bacteria. An *in vivo* bacterial keratitis study on rabbits showed that this emulsion was able to treat the infection in significantly less time than the marketed eye drops (Bharti and Kesavan, 2017). Sebastian-Morello *et al.* (2018) successfully prepared a moxifloxacin ocular insert made of HPMC, PVP and PEG. A higher moxifloxacin transcorneal diffusion was observed on rabbit corneas (*ex vivo*) from the insert compared to the eye drop formulation. A moxifloxacin ocular insert, which was made by solvent casting from sodium alginate and PVA and coated with Eudragit RL-100 was investigated by Pawar *et al.* (2012). This insert was able to prolong the *in vitro* release of moxifloxacin.
up to 7 hours with a maximum *ex vivo* permeation on excised goat corneal tissue.

More recently, Abbas *et al.* (2016) demonstrated the incorporation of moxifloxacin in hydroxyl propyl cellulose (HPC) nanowires or hydroxyl ethyl cellulose (HEC) NPs. Both systems showed similar release profiles of 15% in pH 1.2 after 6 hours. However at pH 7.4, 49% of the loaded drug was release from HPC and 39% from HEC after the same amount of time (6 hours). These approaches were able to sustain the release of moxifloxacin orally up to 36 hours compared to the free drug suspension using male albino rabbits. Fu *et al.* (2016) produced sodium alginate/PVA/moxifloxacin hydrochloride nanofibrous membranes by electrospinning for wound dressing applications. The results showed a very promising antibacterial effect of these nanofibres on *S. aureus* and *P. aeruginosa* with a sustained release profile. In addition, they were able to accelerate the skin wound healing rate in rat treated with these drug-loaded fibres, compared to the blank fibres, the commercially available treatment Woundplast® or the untreated group (Fu *et al.*, 2014).

Giram *et al.* (2018) successfully prepared Eudragit L-100 fibre mats loaded with moxifloxacin with a pH-dependent drug release profile. The fibres showed a slow release profile at pH 1.2, in which 7.5% to 17.5% released after 60 hours. In addition, this system exhibited an *in vitro* antibacterial activities against Gram positive *S. aureus* and Gram negative *E. coli* bacteria strains. Another study by Shawki *et al.* (2010) demonstrated the antimicrobial performance of dextran nanofibres loaded with moxifloxacin. This fibrous system showed an *in vitro* sustained release profile with an inhibiting effect against Gram positive *S. aureus* and Gram negative *E. coli* bacteria. Cheng *et al.* (2015) evaluated the *in vitro* antibacterial activity of ciprofloxacin and moxifloxacin-loaded in a chitosan and PEO blend (9:1) fibres against Gram positive *S. aureus* and Gram negative *E. coli* bacteria. This nanofibre system indicated good antimicrobial activity against both bacterial strains and a prolonged release rate up to 168 hours with no signs of burst release in the first hours (Cheng *et al.*, 2015). Toncheva *et al.* (2012) fabricated electrospun microfibres consisting of Poly-l-Lactide-Co-d,l-Lactide (coPLA) and a blend of coPLA and PEG containing ciprofloxacin hydrochloride, levofloxacin...
hemihydrate or moxifloxacin hydrochloride antibiotics. Antibacterial activity against *S. aureus* was demonstrated in all these fibre systems. However, the presence of PEG accelerated the release of the antibiotic within the first 2 hours compared to the solo coPLA polymer (Toncheva *et al.*, 2012).

Interestingly, moxifloxacin has promoted wound healing after topical application on a rat burn infection model, in addition to its bacterial susceptibility against MRSA and *P. aeruginosa* (Jacobsen *et al.*, 2011). The histological analysis of the burned wound observed a wound healing ability of moxifloxacin with no signs of bacterial resistance during the treatment period.

**1.8.2. Clinical Use of Pirfenidone**

As a consequence of an injury, the activation of myofibroblasts and the trigger of ECM elements such as collagen, fibronectin and smooth muscle actin by myofibroblasts can lead to the formation of a scar (Bukowiecki *et al.*, 2017; Ljubic and Saghizadeh, 2015). Therefore, an agent which is able to reduce or prevent the release of these fibrotic markers might hinder the development of this fibrosis.

The anti-fibrotic drug pirfenidone is approved for the treatment of idiopathic pulmonary fibrosis in Japan (Pirespa®), Europe and the United States (Esbriet®) due to its ability to prevent or reduce a new or already existent fibrotic lesions, respectively. This is by down-regulating the expression of fibrotic markers (cytokines), such as TGF-β and TNFα (European Medicines Agency, 2010, Hirano *et al.*, 2006, Nakazato *et al.*, 2002, Oku *et al.*, 2002, Oku *et al.*, 2008, Iyer *et al.*, 1998 and Iyer *et al.*, 1999).

Although pirfenidone is highly promising for the treatment of many fibrotic cases, the oral treatment protocol is inconvenient and not patient friendly, especially for elderly patients, whom are usually the most vulnerable and require treatment for fibrosis. More precisely, pirfenidone has a recommended dose for idiopathic pulmonary fibrosis of one capsule (267 mg) t.i.d., then after a week, it is increased to two capsules (534 mg) t.i.d., and after the second week, it will rise to the full dose of three capsules (801 mg) t.i.d. for 72 weeks (Dailymed, n.d). This dosage regimen is due to the short half-life (~ 2.5 hours)
and low body accumulation of the drug. This extremely high dose (more than 2 g/day) is likely to result in unpleasant side effects. Therefore, it is necessary to deliver this drug in a delivery system that can sustain its release, as well as maintain its therapeutic effect. Consequently, Jose *et al.* (2015) examined the transdermal delivery of pirfenidone liposomes incorporated in an HPMC hydrogel. The hydrogel formulation showed a more sustained *in vitro* release profile (12 hours) compared to the pirfenidone-loaded liposomes (4 hours) and the free drug (2 hours). Additionally, the hydrogel had greater skin penetration than the other formulations due to the presence of permeation enhancers such as oleic acid and isopropyl myristate.

Abnoos *et al.* (2018) demonstrated the transdermal delivery of pirfenidone using a sodium alginate and chitosan nanogel. This system showed an *in vitro* pirfenidone sustained release profile with an enhanced skin penetration compared to the free drug solution. Another study by Mandapalli *et al.* (2016) evaluated the delivery of this drug in a multi-layered film consisting of chitosan and sodium alginate. The results suggested that this system enabled the contraction of an excisional wound in 9 days compared to the blank formulation (more than 12 days) and the commercial povidone-iodine gel (12 days) with more reduction in the expression of the TGF-β cytokines.

Armendariz-Borunda *et al.* (2011) investigated the topical application of 8% pirfenidone gel for the treatment of burns-related hypertrophic scars in children. The results indicated that this gel was more effective in improving the scar features and a safer treatment option when applied t.i.d. for 6 months compared to the standard pressure therapy after the same duration. Janka-Zires *et al.* (2016) further studied the topical application of pirfenidone in combination with the conventional treatment (such as ulcer cleansing with saline and covering with sterile gauzes) in non-infected chronic diabetic foot ulcer patients. The patients were divided into 2 groups, one of which received topical pirfenidone with the conventional treatment for 8 weeks then switched to the conventional treatment only for 8 more weeks, while the other group received the opposite treatment order. The findings suggested that 52% of ulcers treated with pirfenidone healed before 8 weeks compared to 14.3% treated with the conventional treatment. This study demonstrated the
significant improvement of the healing of diabetic foot ulcer by the addition of pirfenidone to the conventional treatment regimen (Janka-Zires et al., 2016). Another study, by Gasca-Lozano et al. (2017) examined the topical application of pirfenidone with modified diallyl disulfide oxide (antiseptic agent) or ketanserin (serotonin antagonist approved for wound healing by the Federal Commission for the Protection against Sanitary Risk) in the treatment of non-infected chronic diabetic foot ulcer. The relative ulcer volume which was obtained from both treatment options suggested that the addition of the antiseptic agent with pirfenidone completely healed the ulcer after 3 months compared to 6 months with ketanserin alone (Gasca-Lozano et al., 2017).

Interestingly, Morris et al. (2017) have prepared coaxial electrospun fibres of hyaluronic acid (as the core) and PCL (as the shell). Pirfenidone-loaded PCL spheres were embedded on the coaxial electrospun fibres by directly electrospraying them on the fibrous mat, in order to prolong the release of such drug. This system exhibited a sustained pirfenidone release for at least 6 weeks with the ability to reduce the expression of TGF-β cytokine (Morris et al., 2017). Pirfenidone-loaded PLGA NPs have been examined as well for the in vivo treatment of idiopathic pulmonary fibrosis (Trivedi et al., 2012). The results demonstrated that the NPs have a drug loading of about 19.2% w/w, and were able to sustain the release of the drug up to 6 days in comparison to the free drug solution which was released after an hour. Eventually, these particles succeeded in enhancing pirfenidone's anti-scarring efficacy (Trivedi et al., 2012). PLGA loaded with pirfenidone NPs have also been compared with pirfenidone eye drops for the treatment of corneal scarring (Chowdhury et al., 2013). This system showed approximately 102 µg/mg drug loading and a similar in vitro effect to the free drug which was due to the continuous drug availability in the media. However, the in vivo results on rats’ eyes showed superiority of the NPs in prolonging corneal retention, enhancing the corneal permeability and reducing the dose frequency compared to the free drug. Ultimately, these NPs ensured the improvement of corneal wound healing, as well as the prevention of fibrosis development. However, in order to prevent any bacterial growth, a drop of ciprofloxacin was also instilled twice daily (Chowdhury et al., 2013).
1.9. Thesis Aims

Based on the published data, it is likely that a combination of the antibiotic moxifloxacin and the anti-scarring agent pirfenidone, administered by the ocular route in a combined but compartmentalised nanofibre formulation, will result in an improved patient outcomes for corneal diseases. The commercial available moxifloxacin eye drops (0.5% w/v) is widely used for the treatment of the infection at the anterior part of the eyes. Pirfenidone was extensively used experimentally for the treatment of scars that originated at different organs such as the lung, heart, kidney and skin. The use of 0.5% w/v pirfenidone eye drops was also reported to be an effective ocular anti-fibrotic drug (Zhong et al., 2011). Corneal scarring and infection are normally developed after an abrasion (Kinoshita et al., 2001, Wilson et al., 1999; Salomao et al., 2011). These require accelerated healing and prevention (or inhibition) of bacterial growth in order to increase the patient compliance by reducing the treatment duration. Additionally, some opportunistic bacterium can invade this wound leading to a more sight threatening condition, known as a corneal ulcer (Lyczak et al., 2000; Ong and Corbett, 2015). Therefore, the use of the nanofibres as a delivery platform can inhibit any existing bacteria and promote cell proliferation and migration, hence wound healing will be facilitated upon the release of both drugs. This project addresses several obstacles regarding the preparation and characterisation of the electrospun fibres loaded with two different drugs by using different pharmaceutical polymers.

The main aim of this work is to fabricate and characterise dual drug-loaded fibres, loaded with moxifloxacin and pirfenidone in separate compartments using a multi-layered electrospinning technique. To achieve this, different polymers are used in order to sustain the release of both drugs which can allow for a reduction in the dosing frequency to a single daily dose. After the successful preparation of the dual drug-loaded electrospun nanofibres, an in vitro release of moxifloxacin and pirfenidone is evaluated and the efficacy of these drugs are assessed by microbiological “zone of inhibition” and molecular biological “western blot” studies. Zone of inhibition is a test that measures the effect of an antibiotic on the growth of bacteria. While, Western blotting is a technique that quantifies a protein of interest to assess the protein expression
after treating a certain cell line with an anti-scarring agent. The final aim of this project is to test the drug-loaded fibres on an animal model eye to appraise the \textit{in vivo} performance of this delivery system.
1.10. Thesis Overview

An overview of the remaining chapters of this thesis is provided below.

Chapter 2 – Materials and Methods

This chapter reviews the drugs, polymers and solvents used throughout this project. Additionally, instrumental background and some experimental details are given. Detailed experimental parameters are placed in each experimental chapter.

Chapter 3 – Experimental Chapter

This chapter focuses on preparing and characterising pirfenidone-loaded PLGA monoaxial fibres and moxifloxacin-loaded PVA monoaxial fibres using the electrospinning technique. Then, attempts were made to produce coaxial fibres from an immiscible polymer solutions. Finally, the drug loading of the prepared fibres was measured after developing HPLC methods which separated the two drugs using different solvent systems.

Chapter 4 – Experimental Chapter

This chapter focuses on preparing and characterising monoaxial, coaxial and triaxial fibres made of two miscible polymeric solutions, in which PLGA is loaded with pirfenidone and PVP is loaded with moxifloxacin. For the coaxial system, pirfenidone was located in the outer layer, while moxifloxacin was in the inner layer. For the triaxial fibres, an additional layer of moxifloxacin was added as the outer layer while keeping the pirfenidone and moxifloxacin in the intermediate and the inner layers, respectively. This is to enhance the dose of moxifloxacin and to allow its burst release. Finally, the drug loading and the release of these fibres were assessed using the developed HPLC methods.

Chapter 5 – Experimental Chapter

This chapter focuses on investigating the microbiological activity of the coaxial and triaxial fibres that were prepared in Chapter 4 against the Gram positive S. aureus and Gram negative P. aeruginosa bacterial strains. In addition, the anti-scarring effect of the fibres was evaluated by assessing the down regulation of α-SMA protein after TGF-β exposure to rabbit corneal fibroblast
cell line. This cytokine is generally over expressed in response to an injury (scar).

Chapter 6 – Experimental Chapter

This chapter focuses on the in vivo evaluation of the coaxial fibres, which were prepared in Chapter 4. An irritation study, Draize test, and a pharmacokinetics study were performed on New Zealand Albino rabbits. A microbiological testing of these fibres was tested after infecting one rabbit eye with a Gram positive S. aureus bacterium against an untreated group, blank fibres (negative control) and the marketed moxifloxacin eye drops (0.5% w/v).

Chapter 7 – Conclusions and Future Work

This chapter provides a summary of the main conclusions throughout this project and suggests ideas for future work.
Chapter 2

Materials and Methods
2.1. Materials

2.1.1. Moxifloxacin

Moxifloxacin, an 8-methoxy quinolone (Figure 2.1), belongs to a class of antibiotics known as fluoroquinolones and is considered to be a fourth generation fluoroquinolone. The chemical structure of moxifloxacin salt form, moxifloxacin hydrochloride, is illustrated in Figure 2.1. The molecular formula of moxifloxacin hydrochloride is $C_{21}H_{24}FN_3O_4\cdot HCl$ with a molecular weight of 437.98 g/mol (Moxifloxacin, 2008). It is a yellow crystalline powder with a melting point of 238 to 242°C (Moxifloxacin, 2008). It is soluble in water (24 mg/mL) and has dissociation constants (pKa) of 6.4 and 9.5 (MacGowan, 1999). Moxifloxacin has a high binding affinity to the bacterial enzymes DNA gyrase (also known as topoisomerase II) and topoisomerase IV, which inhibit bacterial DNA replication, transcription and recombination, thereby blocking DNA synthesis (Miller, 2008; Wise, 1999). Consequently, it has a higher potency and a lower probability of bacterial resistance than the older fluoroquinolones due to its dual enzyme affinity, as well as the 8-methoxy group addition to the fluoroquinolone molecule (Miller, 2008).

![Figure 2.1: Moxifloxacin hydrochloride chemical structure.](image)

Moxifloxacin is active against a broad spectrum of Gram positive, and Gram negative bacteria including *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, *Mycobacterium tuberculosis*, methicillin-sensitive *S. aureus*, methicillin-resistant *S. aureus* (MRSA), ciprofloxacin-resistant *S. aureus*, most *Enterobacteriaceae*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *P.
*aeruginosa* and many other bacterial species (MacGowan, 1999; Appelbaum and Hunter, 2002).

Moxifloxacin is available for oral, parenteral, and ophthalmic administration. The pharmacokinetic profile has shown that doses from 100 to 400 mg, orally or parenterally, have high bioavailability (more than 85%), long half-life (t½ ≈ 11.5 to 15.5 hours), a large volume of distribution (Vd ≈ 1.7 to 3.6 L/kg), a high degree of tissue penetration (more than 2:1 tissue: serum ratio) and 45% of the total dose is excreted unchanged, with 20 to 30% excreted in the urine and approximately 25% in the faeces (MacGowan, 1999, Neumann, 1988; Keating and Scott, 2004). It can undergo metabolism via sulfate conjugation and glucuronidation to produce metabolites that were identified as M1 (≈38% of the dose, excreted in the faeces) and M2 (≈14% of the dose, excreted in the urine) which lack antimicrobial activity (MacGowan, 1999; Keating and Scott, 2004).

The most common side effects that are associated with the oral administration of moxifloxacin are nausea, diarrhea and dizziness (Elsevier, 2008; Wise, 1999). However, there are other less common side effects that are also reported, which include abdominal pain, vomiting, dyspepsia, dry mouth, flatulence, headache, allergic reactions, tachycardia, insomnia, anxiety, sweating and rash (*Moxifloxacin*, 2008; Wise, 1999).

It has been reported that the ocular commercial product of moxifloxacin (known as Vigamox® or Moxivig® 0.5% eye drops) has a better bioavailability, penetration into ocular tissues, and ultimately, efficacy than the other marketed ocular fluoroquinolone eye drops, such as Zyman® (0.3% gatifloxacin), Oculoflox® (0.3% ofloxacin), and Ciloxan® (0.3% ciprofloxacin). This was due to moxifloxacin high lipophilicity and aqueous solubility at physiologic pH (Robertson *et al.*, 2005, Garcia-Saenz *et al.*, 2001, Callegan *et al.*, 2003, Donnenfeld *et al.*, 2004; Holland *et al.*, 2008). Therefore, it has been approved for the treatment of conjunctivitis and keratitis.

Mechanisms of resistance to the fluoroquinolones in general can include the mutation of the genes of targeted enzymes, DNA gyrase or topoisomerase that alter the drug's binding affinity to them. This may block the drug's entry or accumulation, by an efflux pump for instance, or the presence of a unique gene, which induces specific resistance to protect the bacteria from the drug
There are two fluoroquinolone resistant classes: low level resistance that is caused by a single mechanism, and high level resistance that is caused by multiple mechanisms (Miller, 2008). Unfortunately, when a low level resistance exists for a fluoroquinolone drug, such as ciprofloxacin, the administration of insufficient amounts of a newer generation compound, such as moxifloxacin, will lead to the generation of high levels of resistance (cross-resistance) (Miller, 2008).

2.1.2. Pirfenidone

Pirfenidone (Figure 2.2) is an anti-fibrotic, anti-inflammatory, antioxidant drug, which was first reported in a patent in the 1970s, but introduced in Japan in 2008 (Pirespa®), and then in the European market in 2011 (Esbriet®), for the treatment of idiopathic pulmonary fibrosis (Raghu et al., 2011, du Bois, 2010, Iyer et al., 1999; European Medicines Agency, 2010). In 2014, the US FDA approved Esbriet® for the same purpose. It is able to prevent the development of fibrotic lesions, reduce existing lesions and remove any excessive lesions. This can be achieved through the inhibition of fibroblast proliferation and collagen matrix synthesis, reducing the production of fibrosis markers (fibrotic proteins and cytokines) such as TGF-β and TNFα, and the improvement of ECM biosynthesis (European Medicines Agency, 2010, Hirano et al., 2006, Nakazato et al., 2002, Oku et al., 2002; Oku et al., 2008; Iyer et al., 1998; Iyer et al., 1999). Pirfenidone has the ability to suppress the synthesis of TNFα, compared to the US FDA approved TNF-α binding proteins infliximab (Remicade®), adalimumab (Humira®), and Etanercept (Enbrel®) which can either hinder the secretion of TNF-α, or absorb it (King Jr. et al., 2014).

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Figure 2.2: Pirfenidone chemical structure.
Pirfenidone is a white to pale yellow, non-hygroscopic, stable crystalline powder with a molecular formula of C$_{12}$H$_{11}$NO and a molecular weight of 185.2 g/mol (European Medicines Agency, 2010; Macias-Barragan et al., 2010). It is freely soluble in methanol, ethanol, acetone and chloroform, while sparingly soluble in water, 1.0 N HCl, and 1.0 N sodium hydroxide (NaOH) (European Medicines Agency, 2010; Macias-Barragan et al., 2010). The pKa was estimated at -0.2 and the melting point between 106 and 112°C (European Medicines Agency, 2010). Pirfenidone can be administered orally and has demonstrated significant anti-inflammatory and anti-fibrotic activities in several major organs, such as the lung, liver, and kidneys (Raghu et al., 1999, Azuma et al., 2005, Di Sario et al., 2002, Hewitson et al., 2001; Kim et al., 2010), as well as in different animal and human models (Giri et al., 2002, Bruss et al., 2004, Braim et al., 2008, Bruss et al., 2008, Shi et al., 2007; Taniyama et al., 1997). It can be easily absorbed from the gastrointestinal tract and cross the blood brain barrier (Macias-Barragan et al., 2010). Pirfenidone undergoes metabolism mainly by the enzyme CYP1A2 to yield 5-carboxy-pirfenidone (primary metabolite). The $t_{1/2}$ of pirfenidone is 2 to 2.5 hours, with 80 to 85% being excreted in the urine, of which 95% is as the primary metabolite (European Medicines Agency, 2010; Macias-Barragan et al., 2010). The most frequent side effects of pirfenidone are nausea, vomiting, abdominal pain, gastro-oesophageal reflux, dyspepsia, loss of appetite, rash, headache, dizziness, fatigue, insomnia, sinusitis and loss of weight (Grattendick et al., 2008).

It has been reported that pirfenidone 0.5% w/v eye drops applied six times daily is an effective ophthalmic anti-scarring therapy (Zhong et al., 2011). It prevents the proliferation and migration of cultured human Tenon capsule fibroblasts, cultured human pterygium fibroblasts and TGFβ-stimulated collagen production strabismus, respectively (Lin et al., 2009, Zhong et al., 2011, Sun et al., 2011, Lee et al., 2014; Jung et al., 2012). Consequently, pirfenidone has been shown to be an efficient healing agent after glaucoma filtration, pterygium and strabismus surgeries due to its postoperative anti-inflammatory and anti-fibrotic action via decreasing TGF-β expression. In
addition, pirfenidone has shown a safe and effective inhibition against TGF-β1 induced equine corneal fibrosis *in vitro* (Fink *et al.*, 2015).

### 2.1.3. Poly Vinyl Alcohol (PVA)

PVA has been broadly used in many different applications, especially in the medical and food industries, since 1930. It is a semi-crystalline, synthetic, linear and non-halogenated aliphatic polyhydroxy polymer that has been approved by the US FDA for human consumption (DeMerlis and Schoneker, 2003). PVA is one of the most commonly used polymers for industrial and commercial purposes. It is water soluble and biodegradable polymer that undergoes degradation by a combination of oxidase and hydrolase enzymes into acetic acid (DeMerlis and Schoneker, 2003). PVA can be prepared by the polymerisation of vinyl acetate to polyvinyl acetate (Hassan and Peppas, 2000). The polyvinyl acetate is either partially hydrolysed to yield partially hydrolysed PVA, or completely hydrolysed to produce fully hydrolysed PVA as shown in Figure 2.3, under alkaline or acidic catalysts (DeMerlis and Schoneker, 2003). The commercially available partially hydrolysed PVA grades depend on the amount of the acetate group available in the polymer backbone (Zhang, 2005). The degree of hydrolysis (DH) can influence the solubility of PVA. It was reported that PVA with DH above 98% can be soluble in hot water, while PVA with DH 80% and less can be soluble in cold water (Zhang, 2005).

The physiochemical characteristics of PVA depend on the synthetic process. For instance, by altering the duration of the process, or the degree of hydrolysis, the formed PVA will have different molecular weights (between 20,000 and 400,000), crystallinity index (between 60 and 70%), tensile strength (between 65 and 120 MPa), aqueous solubility, flexibility and bio-adhesiveness (DeMerlis and Schoneker, 2003, Guirguis and Moselhey, 2012; Jelinska *et al.*, 2010). Based on the physical properties, some PVA characteristics, such as viscosity, melting point, etc. may be varied. It has been reported that the glass transition temperature (Tg) for the dry powder PVA can range from 70 to 85°C, while the Tg of PVA fibres can fall to 66°C (Jelinska *et al.*, 2010; Magalhaes *et al.*, 2013). Several thermal analysis studies suggested
that the melting point of PVA can start from 180°C and reach up to 235°C; however, at 239°C PVA degradation will initiate (Jelinska et al., 2010, Magalhaes et al., 2013; Mallapragada and Peppas, 1996).

Figure 2.3: Partially and fully hydrolysed PVA structures in which the former one is the most commonly used type of PVA.

PVA is involved in the manufacture of papers, fabrics, sheets, covers and hospital laundry bags. It is also used in products that are in contact with food, for example in packaging meat products, as well as in latex paints as a thickening agent. Numerous medical products have incorporated PVA in their manufacture, such as transdermal patches, tablet formulations, surgical devices, sutures, contact lenses and artificial organs, due to its non-toxicity, non-carcinogenicity, biocompatibility, thermal stability, chemical resistance and film forming ability (Guirguis and Moselhey, 2012, Peterson et al., 2006; Kobayashi et al., 2005). Due to its dispersion and coating properties, it has been used in artificial tear products, such as Hypotears®, Refresh® and Liquifilm®, to treat dry eye symptoms such as itching, redness and soreness (Cederstaff and Tomlinson, 1983; Sorbara et al., 2004). There are several studies suggesting that PVA may be used as the basis of a drug delivery system due to its rapid drug release profile and the production of a less acidic environment compared to PLGA (DeMerlis and Schoneker, 2003; Alhosseini et al., 2012), especially for ophthalmic use (Beck-Broichsitter et al., 2012; Choy et al., 2011). For instance, PVA was incorporated with chitosan for pilocarpine ocular delivery, whereas it was mixed with PLGA for nasal delivery (Cadinoiu et al., 2015; Tafaghodi et al., 2004).
Interestingly, when PVA aqueous solution is kept at room temperature, it will slowly turn into an elastic gel form. However, PVA can produce hydrogels by using a technique called freeze-thawing. The resultant PVA gel will have some distinctive physiochemical properties compared to the raw PVA properties, such as the extent of aqueous solubility, the degree of swelling in water, elasticity and mechanical strength (Peppas and Stautffer, 1991). The gel-form of PVA will be insoluble but will have a high degree of swelling in water most probably due to the formed polymer network after PVA crosslinking (Yokoyama et al., 1986; Peppas and Stautffer, 1991). These hydrogels have been applied in the pharmaceutical field due to their non-toxicity, non-carcinogenicity and biocompatibility. A number of studies demonstrated that the properties of PVA hydrogels, such as elasticity, depend on many factors, for example, the polymer molecular weight, the aqueous solution concentration, the temperature and time of freezing and the number of freezing-thawing cycles (Yokoyama et al., 1986). Finally, release studies have shown that these gels typically follow a zero-order profile. An example of this system is indomethacin loaded into PVA hydrogels (Morimoto et al., 1989).

For the purpose of preparing PVA electrospun fibres (Chapter 3), two PVA grades were used. A high MW of 146,000-186,000 Da (87-89% hydrolysed) and a low MW of 31,000-50,000 Da (87-89% hydrolysed). Both PVA were purchased from Sigma Aldrich Company Ltd (Sigma Aldrich, Dorset, UK). It was reported by Zhang et al. (2005) that the DH can affect the morphology of these fibres. Three different DH were investigated; 80%, 88%, and 99%. It was observed that the PVA with 99% DH produced fibres that are beaded due to the increase of the solution conductivity upon increasing the DH. This resulted an unstable jet, which contributed to the bead formation. The PVA with 80% DH formed ribbon-like shape fibres owing to the lack of fibres dryness during their collection. However, PVA with 88% DH produced the most uniform fibres that lack any defect (beads or pores). Therefore, the 88% DH has been taken into consideration in this study.
2.1.4. Poly Vinyl Pyrrolidone (PVP)

PVP, also known povidone or polyvinyl pyrrolidone, is a white to yellowish white powder which was first discovered in 1938 (Haaf et al., 1985). It is a water soluble synthetic polymer that is commonly used in various medical and nonmedical applications. This is due to its excellent physiochemical properties such as good biocompatibility, chemical inertness, temperature-resistance, pH-stability, nontoxicity, and solubility in water and a wide range of organic solvents such as methanol, ethanol, propanol, chloroform, dichloromethane, propylene glycol, glycerol and acetic acid (Folttmann and Quadir, 2008; Raimi-Abraham et al., 2014). PVP is mixed with iodine to form a disinfectant complex, povidone-iodine (Betadine®), which is widely used as a solution, ointment, or in liquid soaps and surgical scrubs (Kariduraganavar et al., 2014). This polymer is extensively used as a binder for orally administered tablets (Kariduraganavar et al., 2014). In addition to the tablet manufacturing, this polymer is involved in the making of powders, granules, soft gelatin capsules, gels and hydrogels, syrups, oral or injectable solutions, contact lenses and many others (Khan et al., 2017; Teodorescu and Bercea, 2015). The dry polymer powder is light and hygroscopic, and can absorb water up to 40% of its weight. PVP solution has good wettability properties and can easily form films, which can be used as coating agents (Khan et al., 2017; Kariduraganavar et al., 2014). Other applications for PVP can be seen in water purification, wastewater treatment, food processing and optical and electrical applications, owing to its good electrical properties, as well as in the manufacturing of papers, fibres, textiles, membranes, adhesives, inks and ceramics (Khan et al., 2017; Zhi et al., 2013).

PVP can be synthesised from the monomer N-vinyl pyrrolidone by the free radical polymerisation with Azobisisobutyronitrile (or AIBN) as an initiator catalyst, as shown in Figure 2.4 (Kariduraganavar et al., 2014). This can yield polymer with a wide range of molecular weight ranging from $10^3$ to $10^6$ g/mol, in which higher and lower molecular-weight PVP can be obtained by polymerisation in aqueous and organic solvents, respectively (Haaf et al., 1985). The lower molecular weights are more stable due to the presence of a stable end group compared to the carbonyl and hydroxyl end groups which
result in the aqueous solvent polymerisation (Teodorescu and Bercea, 2015). PVP is amorphous in nature with a Tg (ranging from 54 to 175°C) directly proportional to the molecular weight (Turner and Schwartz, 1985). It has been reported that low molecular weight PVP (2500 g/mol) has a Tg of 100°C, while the high molecular weight polymer (more than $10^6$ g/mol) has a Tg of 175°C (Zabska et al., 2011; Teodorescu and Bercea, 2015).

![Figure 2.4: Polymerisation of PVP from N-vinyl pyrrolidone.](image)

In this study, one PVP grade Kollidon® 90F with a high MW of 1,000,000-1,500,000 was used to prepare electrospun PVP fibres (Chapter 4). This grade was kindly donated by BASF (Frankfurt, Germany).

### 2.1.5. Poly-Lactic-co-Glycolic Acid (PLGA)

PLGA is one of the most widely used synthetic polymers due to its approval by the US FDA for human use (Faraji and Wipf, 2009). It has been involved in the manufacture of fibres and sutures since the late 1960s and the early 1970s (Gilding and Reed, 1979). It has been used in a wide range of biomedical applications, in particular as a carrier for different therapeutic drugs (Lee et al., 2004 and Lewis, 1990), including moxifloxacin and pirfenidone (Mudgil and Pawar, 2013, Garg et al., 2014, Gadad et al., 2012, Trivedi et al., 2012; Chowdhury et al., 2013). PLGA is a biodegradable, biocompatible and mechanically strong polymer (Wu, 1995), which can be easily formulated into various delivery systems, such as NPs, microcapsules, microspheres or implants (Ravivarapu et al., 2000). The interaction of this polymer with water will hydrolyse it into lactic and glycolic acid monomers (Figure 2.5) (Jalil and Nixon, 1990), which will then be consumed by the citric acid cycle and
subsequently eliminated from the body as water and carbon dioxide (Wu, 1995). Since PLGA is a copolymer, it is normally followed by a ratio of lactic acid to glycolic acid. For instance, PLGA 70:30 consist of 70% lactic acid and 30% glycolic acid. PLGA 50:50 is the most commonly used PLGA copolymer due to its fast degradation rate of approximately two months (Park, 1994). However, PLGA 65:35, 75:25, or 85:15 (lactide-rich PLGA) have slower degradation times than PLGA 50:50, owing to the lower hydration of the ester linkage of the lactic acid (Park, 1994). PLGA 30:70 and 10:90 (glycolide-rich PLGA) have faster degradation rates than the lactide-rich PLGA as a result of the low crystallinity of these PLGA copolymers, hence the hydration rate is improved (Makadia and Siegel, 2011).

Figure 2.5: Hydrolysis of PLGA to D, L lactic acid and glycolic acid monomers.

PLGA can be synthesised by either a polycondensation reaction or ring-opening polymerisation of cyclic diesters (Astete and Sabliov, 2006). The ring-opening method is the preferable one due to shorter reaction times and higher monomer conversion rates (Astete and Sabliov, 2006). PLA can be found in two forms: poly-L-lactic acid (optically active form) and poly-D, L-lactic acid (optically inactive form). The former is considered to be semi-crystalline, with a crystallinity of approximately 37%, while the latter is amorphous (Wu, 1995, Kaihara et al., 2007; Lewis, 1990). Due to the lack of the methyl side group of PLA, PGA is more crystalline than PLA (around 46 to 52%) (Wu, 1995; Makadia and Siegel, 2011). Consequently, PLGA that is prepared from poly-L-lactic acid and polyglycolic acid is considered to be crystalline, while PLGA prepared from poly-D, L-lactic acid and polyglycolic acid is considered as amorphous in nature (Wu, 1995; Lewis, 1990). It has been reported that PLGA
has a Tg between 40 and 60°C, depend on the ratio of lactic acid to glycolic acid (Wu, 1995, Lewis, 1990; Loo et al., 2005). However, the melting point is also related to the ratio between lactic acid and glycolic acid (Wang et al., 2000). For example, PLGA 5:95 has a melting point of 173°C, while PLGA 90:10 is 201°C.

In this study, three different PLGA 50:50 grades were used to fabricate the PLGA fibres. Purasorb® PDLG 5002A (MW 17 kg/mol), 5004A (MW 44 kg/mol) and 5010 (MW 153 kg/mol) were obtained from Corbion (Purac Biomaterials, Gorinchem, Netherlands). More details about these grades are presented in Chapter 3 - Table 3.1.

### 2.1.6. Solvents

Table 2.1 summarises some physiochemical properties of the solvents that have been used in the preparation of the electrospun fibres.

**Table 2.1: Physiochemical properties of different solvents.**

<table>
<thead>
<tr>
<th>Properties</th>
<th>Water</th>
<th>Ethanol</th>
<th>Acetone</th>
<th>Acetonitrile</th>
<th>Dichloromethane</th>
<th>Ethyl Acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Molecular Weight (g/mol)</strong></td>
<td>18.20</td>
<td>46.07</td>
<td>58.08</td>
<td>41.05</td>
<td>84.93</td>
<td>88.11</td>
</tr>
<tr>
<td><strong>Density (g/mL) at 25°C</strong></td>
<td>1</td>
<td>0.79</td>
<td>0.79</td>
<td>0.79</td>
<td>1.33</td>
<td>0.90</td>
</tr>
<tr>
<td><strong>Viscosity (cP) at 25°C</strong></td>
<td>1 (at 20°C)</td>
<td>1.095</td>
<td>0.306</td>
<td>0.369</td>
<td>0.413</td>
<td>0.423</td>
</tr>
<tr>
<td><strong>Vapour Pressure (mm Hg) at 20°C</strong></td>
<td>17.5</td>
<td>44.60</td>
<td>184</td>
<td>72.80</td>
<td>353</td>
<td>73</td>
</tr>
<tr>
<td><strong>Dielectric Constant (at 20°C)</strong></td>
<td>80.10</td>
<td>24.30</td>
<td>21.01</td>
<td>36.64</td>
<td>8.93 (at 25°C)</td>
<td>6.08</td>
</tr>
<tr>
<td><strong>Boiling Point (°C)</strong></td>
<td>100</td>
<td>78.37</td>
<td>56</td>
<td>81-82</td>
<td>39.8 - 40</td>
<td>76.5 - 77.5</td>
</tr>
<tr>
<td><strong>UV cutoff (nm)</strong></td>
<td>191</td>
<td>210</td>
<td>330</td>
<td>190</td>
<td>235</td>
<td>255</td>
</tr>
</tbody>
</table>
2.2. Methods

2.2.1. Electrospinning Techniques

The electrospinning process requires four main components, as shown in Figure 2.6; a high voltage source, a syringe pump, a syringe with a fitted needle (emitter) and a collector surface. The voltage source is connected to the collector at one end and to the emitter at the other, as the dispensed solution would yield droplets in the absence of voltage. However, with the application of a voltage (charge) the solution droplet’s surface tension can be overcome, allowing the evaporation of the solvent and thus, the formation of fibres (Williams et al., 2018).

![Electrospinning instrumentation showing its main components (Spraybase.com, n.d.)](image)

Figure 2.6: Electrospinning instrumentation showing its main components (Spraybase.com, n.d.).

Monoaxial electrospinning is a technique that can produce a single-layered fibres, while multi-layered electrospinning, which can be coaxial or triaxial, is a technique that can produce a coated fibres, i.e. they are bi-layered or tri-layered in the case of coaxial or triaxial, respectively. The principle of these electrospinning techniques were described in details in Chapter 1 - Sections 1.7.1. and 1.7.2.
Monoaxial and multi-layered electrospinning was performed in Chapter 3 and 4 to initially prepare a successful single layered fibres that might facilitate the fabrication of multi-layered fibres using Spraybase® electrospinning instrument (Spraybase®, Dublin 2, Ireland).

2.2.2. Characterisation Methods

2.2.2.1. Light and Fluorescence Microscopy

The light microscope is an instrument that is used to magnify objects which are not visible to the naked eye by the use of visible light and magnifying lenses. It was first discovered around the 1590s by two Dutch spectacle makers, Hans and Zaccharias Jenssen (Goodwin, 2015). In 1670, Antonie van Leeuwenhoek was the first to use the microscope as a biological tool, observing different individual cells, such as bacteria, muscle cells and sperms (Goodwin, 2015). Simply, a microscope consists of a light source, a collector lens (to gather the light from its source), a condenser lens (to emit the collected light to illuminate the sample), an objective lens (to collect the light from the sample), a tube lens (to collect the focused light rays coming from the objective lens) and the oculars (re-project the light to the observer's eyes) (Goodwin, 2015; Kubitscheck, 2017). Figure 2.7 illustrates the principle of light microscopy. Depending on the application, the source of the light can vary based on the sample to be detected, for instance, an incandescent bulb, a light-emitting diode (LED) or a laser. Generally, the object of interest is adhered onto a microscope slide in order to be observed.
Fluorescence is the emission of light that occurs after excitation of a sample by light of a shorter wavelength. Reflected light fluorescence microscopy, also known as episcopic fluorescence or epi-fluorescence, is a type of microscopy that uses two illumination sources: a transmitted light source and an episcopic source (which is a halogen lamp and mercury arc-discharge) (Lichtman and Conchello, 2005). It has a similar concept to the light microscope; however, light of a shorter wavelength is used for sample excitation and specific lenses and filters are fitted in this instrument (Lichtman and Conchello, 2005; Abramowitz et al., n.d.). The Stokes shift is the difference between the excitation and emitted wavelengths (Lichtman and Conchello, 2005). After filtering the excitation light and not blocking the emitted light, the fluorescent component within the object will be observed. This component is known as the fluorochrome (or fluorophore) (Lichtman and Conchello, 2005) and is characterised by energy absorption at the excitation wavelength to generate new photons at a second wavelength (emission wavelength), which enables the object to be observed against the background at that wavelength (Goodwin, 2015). This is achieved by first selectively filtering (an excitation filter) the illumination that is travelling from the excitation wavelength source (Abramowitz et al., n.d.). The filtered excitation light will be reflected by the
dichromatic mirror, while transmitting both shorter and longer wavelengths simultaneously (Abramowitz et al., n.d.). Finally, a second filter (an emission filter) is used to isolate the weaker fluorescence emission that enables the formation of an image on a dark background (Abramowitz et al., n.d.).

Light microscopy was used in Chapter 3 and 4 to observe the successful preparation of the electrospun fibres by observing the morphology of the fibres and detecting the presence and absence of beads using a Nikon Microphot-FXA microscope (Nikon, Japan). The fluorescence microscopy was used in Chapter 3 and 4 to assess the presence of moxifloxacin within the electrospun fibres due to its fluorescent characteristic using the Nikon Microphot-FXA microscope.

2.2.2.2. Confocal Microscopy

Confocal microscopy is a type of fluorescent microscopy that uses a focused laser (argon ion) beam to scan an object, point-by-point, to allow for a 3-dimensional (3-D) reconstruction, which was patented by Marvin Minsky in 1955 (Amos and White, 2003; Paddock, 2000). As shown in Figure 2.8, the laser beam path is then split to separate the excitation from the emitted light via the beam splitter filter. This focused beam will be guided across the sample by a unit of two or more mirrors, in which it will scan the object pixel-by-pixel and line-by-line to reach to the objective lens (Wilhelm et al., 2003). One mirror is used to tilt the beam in the X direction, while the other in the Y direction. The compelling part of this microscope is the Z-control, which allows the user to focus on any focal plane (the plane through the focus perpendicular to the axis of a lens) within the sample allowing the 3-D reconstruction (Wilhelm et al., 2003). After reaching to the objective lens, if the sample is fluorescent, part of the light will travel back into that lens through the same path that the laser travels, which will determine the image formation, as well as its resolution. This light will pass via a semi-transparent mirror that reflects it away from the laser and towards the detection system. The out-of-focus light from the image will be excluded before it reaches to the detector by the pinhole, which is a unique feature in this type of microscope (Wilhelm et al., 2003). The presence of a fluorescent light will be in a different colour than the laser light.
that has been reflected from the sample and it will be separated by the emission filter. The detector should be a highly sensitive one, such as a photomultiplier tube, in order to collect the photons that are emitted by the samples by transforming the light signals into electrical signals to be recorded by a computer (Wilhelm et al., 2003; Paddock, 2000).

Figure 2.8: Confocal microscopy principle showing the laser pathway. Adapted from (Fellers and Davidson, n.d.).

This microscopy approach was used in Chapter 4 to evaluate the presence of moxifloxacin and methylene blue (MB) in the inner and outer layers of coaxial fibres, respectively using Zeiss LSM 710 confocal microscope (Carl Zeiss Meditec, Germany). The presence of MB in the intermediate layer and moxifloxacin in the inner and the outer layers of the triaxial fibres were also assessed. MB was used here instead of pirfenidone due to its fluorescent characteristic and solubility in organic solvents. The confocal imaging was performed under the supervision of Dr David Gathercole at the Confocal Microscopy Unit at UCL School of Pharmacy.
2.2.2.3. Scanning Electron Microscopy (SEM)

This type of microscopy is a non-destructive technique which focuses a high energy electron beam at the surface of a sample to generate a variety of signals resulting from the interaction between the electron and the sample (Swapp, 2012; Gree, 2015). The first SEM system was put together by Charles Oatley and his students at Cambridge University's Engineering Department in 1948, which produced a 3-D image four years after (Swapp, 2012). The signals can include secondary electrons, diffracted backscattered electrons, photons and heat. The outcome could illustrate the external morphology and the crystalline structure of the materials that make up the sample.

The secondary electrons are generated to produce an image and diffracted backscattered electrons are commonly used for determining the crystal structures of the material (Swapp, 2012). At a selected area on the sample, a range of 1 cm to 5 µm in width can be imaged at a magnification range of 20X to 30,000X. Due to the smaller wavelength of electrons than that of visible light, the optimal resolution and magnification attainable for SEM images are higher than that from a light microscope. One of the highest resolutions that can be obtained by SEM is 0.4 nm at an accelerated beam of 30 kV by a Hitachi S-5500 SEM model.

An intense beam of electrons is emitted by an electron source (also called electron gun), which consists of a cathode and an anode. The cathode is a tungsten filament which emits electrons once heated, and a negative cap directs the electrons into a loosely focused beam. The beam is then accelerated to the sample by the positive anode that is located underneath the cathode. The beam will travel through two condenser lenses, to be directed to the objective lens where the electron beam will be limited before its exposure on the sample (Gree, 2015), as shown in Figure 2.9. A vacuum chamber is installed to prevent the obstruction of the electron beam passing to the sample. After the interaction between the electrons and the surface of the sample, X-rays, primary backscattered electrons, secondary electrons and Auger electrons are emitted (Gree, 2015).
Both the primary backscatter electrons and secondary electrons are used to generate an image by the SEM (Swapp, 2012). The secondary electrons are low energy electrons, which are very difficult to be collected, hence a high voltage is applied to the collector. Finally, a detector is used to generate an image via a display output device (i.e. a computer) (Gree, 2015).

Samples that are metallic in nature are electrical conductors and will interact with electrons so can be studied directly. For non-metallic samples, a metal coat is applied to enable analysis (Gree, 2015). The sample is coated with a thin layer of conducting material, such as carbon or gold, in order to prevent any charge build-up during the imaging (Swapp, 2012). The choice of this material will depend on the data to be developed; for instance, the use of a gold coating is commonly used to obtain a high resolution imaging, while carbon coating is used for elemental analysis (Swapp, 2012). Owing to the purpose of achieving high resolution images, gold is used in an electric field and in the presence of argon gas. The electric field will remove the electrons
from the argon gas, resulting in positively charged ions. These ions are attracted to the negatively charge gold foil, allowing the argon ions to expel the gold atoms, resulting a thin conductive coating around the sample (Gree, 2015).

Certain wet samples can be assessed without the use of a conductive coating, but this will require low vacuum conditions (Swapp, 2012). This is because the water molecules will vapourise in high vacuum, creating difficulties for the electron beams and will reduce the clarity of the image (Gree, 2015).

This microscopy approach was used in Chapter 3 and 4 to analyse the morphology of the electrospun fibres using FEI Quanta 200F (FEI company Ltd, Eindhoven, The Netherlands). The SEM analysis was performed with the assistance of Dr Andrew Weston and Mr David McCarthy at the Electron Microscopy Unit at UCL School of Pharmacy.

2.2.2.4. Transmission Electron Microscopy (TEM)

TEM was first proposed by Knoll and Ruska in 1932 and has similar imaging principles to SEM, in terms of using electrons to generate signals after their interaction with the sample (Williams and Carter, 2009). However, this microscope operates in a different way, revealing the finest details of the internal structure of a sample.

Electrons are initiated from an electron gun, then the electron beam will travel to a magnetic condenser lens and be condensed and adjusted to form a small thin beam of electrons directed to the sample. The interaction of electrons and the sample will yield unscattered electrons (transmitted beam) and scattered electrons (diffracted beam) (Williams and Carter, 2009). The transmitted electrons will travel to a magnetic objective lens (to block the high angle diffracted beam) to an intermediate lens and finally to a projector lens, where it will expand the electron beam onto a fluorescent (also known as phosphor) screen by forming an enlarged image (Williams and Carter, 2009). This screen is usually made of fine (10 to 100 µm) particulate zinc sulphide, which glows upon getting hit by the passed electrons allowing the image to be formed. The
darker areas of the image represent those that have less electrons transmitted compared to the lighter areas.

Figure 2.10 illustrates the principle of TEM. In this microscopy, the electron beam can be accelerated through a potential that can reach up to 1,000 kV and passed through a strong magnetic field that acts as a lens (Williams and Carter, 2009). The faster the electrons travel, the shorter their wavelength, generating a higher resolution image. The resolution can reach to 0.2 nm, with a magnification ability between 50X and 2,000,000X. The whole optical system is enclosed in vacuum to evacuate the air in order to prevent the collision of electrons and air molecules and hence avoid their scattering (Williams and Carter, 2009).

![Figure 2.10: Transmission electron microscopy principle showing the electrons pathway. Adapted from (Bitesizebio.com, n.d.).](image)

The sample properties are crucial, thus requiring particular preparation. The thickness and composition of the sample for instance can play a role in determining the quantity of electrons which will pass through the sample (Williams and Carter, 2009). A thin and porous sample will allow more electrons to pass through compared to a thick and non-porous sample. The
sample can also be stained by an electron dense material and placed in the vacuum, then the electron beam will pass through the sample and be scattered by its internal structure (Williams and Carter, 2009). Thin sample sections can be supported on fine mesh grids, which will remain attached throughout the preparation and imaging. A carbon layer is used to stabilise the grid when it is exposed to the electron beam. Grids can be stained with heavy metals, such as uranyl acetate and lead citrate, in order to either absorb the electrons or to scatter part of the electron beam, thus improving the image contrast (Williams and Carter, 2009).

This microscopy was used in Chapter 3 and 4 to evaluate the presence of the inner fibrous layers of the coaxial and triaxial fibres using FEI CM120 BioTwin TEM (FEI Company Ltd. Eindhoven, The Netherlands). The TEM analysis was performed with the assistance of Dr Andrew Weston and Mr David McCarthy at the Electron Microscopy Unit at UCL School of Pharmacy.

2.2.2.5. Thermogravimetric Analysis (TGA)

This thermal analytical technique is used to assess the sample change in weight measured as a function of temperature or time (Craig and Reading, 2006). It is used to indicate the onset decomposition temperature of materials and to observe any solvent (water or a volatile solvent) loss from the sample during the heating process (Craig and Reading, 2006; Pina et al., 2014).

The TGA instrument is equipped with a high precision balance that allows accurate measurements of the sample weight during heating (Craig and Reading, 2006). The sample is placed in a pan, usually made of aluminium, on a holder that is hung by a piece of metal wire attached to the balance. A furnace then rises around the sample holder allowing the temperature to be controlled while the weight is concurrently measured. An optimised flow of a purge gas, such as N₂, is generally used to remove any moisture or volatile compound from the furnace and to stabilise the environment around the sample and the furnace (Craig and Reading, 2006). Figure 2.11 illustrates the principle of TGA instrument. Data are presented in the form of a thermogram, which quantifies weight loss against temperature.
TGA data can facilitate the interpretation of results of other thermal analytical techniques, such as differential scanning calorimetry (DSC), for example by helping to differentiate thermal events due to solvent loss or melting. These findings might also require a further confirmation by X-ray diffraction in order to understand the structural changes of the sample (Craig and Reading, 2006). Similarly, TGA data can aid in the interpretation of the glass transition temperature (Tg) of amorphous materials with differing levels of residual solvent (Pina et al., 2014).

This technique was used in Chapter 3 and 4 to analyse the degradation temperature of moxifloxacin, pirfenidone and the used polymers using a TA Hi-Res TGA 2950 thermogravimetric analyser (TA Instruments UK, Herts, UK) in Chapter 3 and a TA discovery thermogravimetric analyser (TA Instruments New Castle, DE, USA) in Chapter 4. In addition, the presence of remaining solvent after electrospinning was also investigated by this method.

2.2.2.6. Differential Scanning Calorimetry (DSC)

This is another thermal analytical technique that is used to characterise materials (Thomas, 2005). It measures the temperature and energy associated with a range of thermal events such as melting, Tg, crystallisation and
decomposition by applying heat to a sample (Craig and Reading, 2006). These events can be distinguished by the direction of the resultant peaks; an upward direction indicates an exothermic event (heat released), while a downward direction indicates an endothermic event (heat absorbed) (Craig and Reading, 2006); this orientation may vary with the instrument used.

A heat flow (standard) DSC consist of two thermocouples, one for the sample and another for the reference, which are connected in a back-to-back arrangement and placed symmetrically within a furnace, as shown in Figure 2.12 (Craig and Reading, 2006).

![Figure 2.12: DSC experimental setup showing the reference and the sample pans being placed on the DSC pans’ holders. Adapted from (evitherm.org, n.d.).](image)

Samples are enclosed in a metal (usually aluminium) DSC pan. The furnace is purged with an inert gas (usually N₂ at a flow rate of 50 mL/min) in order to remove any moisture or volatile compound coming out from the sample during the heating process, which can influence the analysis and damage the furnace (Craig and Reading, 2006). The voltage which is developed from these thermocouples can measure the temperature difference between the sample and the reference. The heat flow (dQ/dt) is estimated by the following equation:
\[
\frac{dQ}{dt} = \frac{\Delta T}{R}
\]

where \( Q \) is heat, \( t \) is time, \( \Delta T \) is the temperature difference between the furnace and the pan and \( R \) is the thermal resistance of the heat path between the furnace and the pan. Therefore, the temperature difference between the sample and the reference can be correlated directly to the heat flow difference (Craig and Reading, 2006).

For more accurate measurements, instrument calibration is required and can be achieved by the following steps: furnace cleaning (by heating while empty from -90 to 400 °C at 20 °C/minute), baseline calibration (using sapphire discs and a heating range from -90 to 400 °C at 20 °C/minute), enthalpy (or cell constant) calibration (using indium through its melting transition \( T_m = 156.6 \) °C) and finally, temperature calibration (using high purity materials such as n-octadecane or tin) (Craig and Reading, 2006).

Modulated Temperature DSC (MTDSC) is a technique in which a sinusoidal modulation is superimposed on the linear temperature ramp combined with a mathematical procedure designed to separate different types of sample behaviour (deconvolution) (Craig and Reading, 2006; Thomas, 2005). This is useful to precisely measure the \( T_g \) of an amorphous sample. This will lead to the measurement of the heat flow and the change in heat capacity (\( C_p \), which is the energy required to raise the temperature of the sample by 1 Kelvin) concurrently as follows:

\[
\frac{dQ}{dt} = C_p \left( \frac{dT}{dt} \right) + f(t,T)
\]

where \( dQ/dt \) is heat flow, \( C_p \) is heat capacity, \( dT/dt \) is heating rate which has both a linear and sinusoidal component, \( f(t,T) \) is a function of time and temperature (the kinetic component of the total heat flow which is an expression of a response associated with the physical or chemical transformation) (Thomas, 2005).

Therefore, MTDSC results show three signals in one single heating run, which enables the \( C_p \), kinetic and total heat flow components to be described. Total heat signal (equivalent to the heat flow DSC result), reversing heat flow signal
isolates the heat flow due to the reversing changes in the Cp of the sample and occurs with a change in temperature) and non-reversing heat flow signal (irreversible kinetic changes of the total heat flow such as melting, structural relaxation and crystallisation) can all be detected in the MTDSC trace (Craig and Reading, 2006; Thomas, 2005).

In order to obtain good quality results the heating rate should be low (1 to 5 °C/min) to allow at least six modulation cycles during each thermal event (to enable the complete separation of cycles). Additionally, large modulation amplitudes (ranging from ± 0.1 to ± 1 °C) permit a high sensitivity measurement without affecting the resolution. Finally, the modulation period is typically selected within 30 to 80 seconds (Craig and Reading, 2006).

The MTDSC calibration is similar to the heat flow DSC calibration, except for an additional calibration of the Cp (using a known Cp material such as sapphire). This is to allow a correct measurement of the sample heat capacity and an adequate separation of the total, reversing and non-reversing heat flow signals (Craig and Reading, 2006).

This assay was used in Chapter 3 and 4 to analyse the thermal characteristics and the solid state of moxifloxacin, pirfenidone and the electrospun fibres using a TA Q2000 calorimeter (TA Instruments UK, Herts, UK).

2.2.2.7. Fourier Transform Infrared Spectroscopy (FTIR)

Infrared (IR) spectroscopy is a technique that is used to identify the functional groups of a sample. It also can be used to verify the purity of a compound (i.e. the presence of impurities) resulting from the unique collection of absorption bands. The first IR spectroscopy was reported by Barer et al. in 1949 (Wellner, 2013). Organic molecules can absorb IR radiation between 4000 cm⁻¹ and 400 cm⁻¹ (Doyle, 1992). There are two important regions in the IR spectrum: the functional group region (4000 to 1500 cm⁻¹) and the fingerprint region (1500 to 400 cm⁻¹) (Wellner, 2013). The peaks in the functional group region can be used to identify the presence of a specific functional group. However, the peaks that are located in the fingerprint region may not easily identify an unknown compound. The amount of absorbed energy will initiate the transition
between vibrational states of bonds contained within the molecule, in which the frequency of the radiation will equal the energy required for a particular bond to vibrate (O’Neil and Edwards, 2011).

The IR instrument consists of a light source that generates IR light across the spectrum of interest (Doyle, 1992). An important unique part is the interferometer, consisting of a beam-splitter which splits the passing light in two directions at right angles. One will travel to a mirror then reflect back to the beam-splitter, while the other goes to a moving mirror, as shown in Figure 2.13 (Wellner, 2013).

![Figure 2.13: FTIR principle illustration showing the IR light pathway. Adapted from (instrumentationforum.com, n.d).](image)

When both beams intersect at the beam-splitter, the difference in path lengths creates constructive and destructive interference, which is called the interferogram. The sample will absorb the recombined beam, based on its chemical composition, at all the different wavelengths and subtracts specific wavelengths from the interferogram (Doyle, 1992). Finally, a detector collects the variation in energy versus time that pass through the sample. The energy versus time spectrum is converted to an energy intensity versus frequency spectrum by a mathematical function known as Fourier transform (FT). The FTIR spectrum can be interpreted by examining the functional group and the fingerprint regions, in order to determine the groups that might be present which is shown as peaks (O’Neil and Edwards, 2011).
Some FTIR models are attached to an attenuated total reflection (ATR) crystal, which is made of a high refractive index material such as thallium bromide, thallium iodide or zinc selenide (O’Neil and Edwards, 2011). The main advantage of the use of ATR is to avoid the need for sample preparation. The samples are placed on the opposite side of these transparent crystalline materials. The radiation is set to an angle at which the internal reflections occur between the sample and the crystal interface before it travels to the detector (O’Neil and Edwards, 2011).

This ATR-FTIR technique was used in Chapter 4 to assess the compatibility of moxifloxacin and pirfenidone with the used polymers using Spectrum 100 FTIR spectrometer (Perkin Elmer, Massachusetts, USA). In addition, this assay was also used to determine the presence of both drugs in the drug-loaded electrospun fibres.

2.2.2.8. X-Ray Powder Diffraction (XRD)

This is a non-destructive technique that is used to characterise and identify crystalline materials by the use of X-ray beams to provide information on solid structures, phases and crystal orientations (Bunaciu et al., 2015). The first developed diffractometer was in 1954 by Parrish, Hamacher and Lowitzsch (Chung and Smith, 1999). A continuous monochromatic incident beam of X-rays is directed to a solid material by an X-ray source, where the radiation is scattered at specific angles from each set of lattice planes in the sample. The X-rays are generated by heating a filament (a cathode) to produce electrons, which will be accelerated by a voltage to an anode (usually Cu, Fe, Cr or Ag) to produce a characteristic X-ray spectrum (Bunaciu et al., 2015). This spectrum will then travel through a monochromatic crystal, such as graphite, to ensure that the radiation wavelength remains consistent throughout the analysis. A goniometer is used in order to rotate the sample. The radiation is diffracted and the X-ray diffracted peaks can be detected by a signal processor (Bunaciu et al., 2015).

The atomic distribution within the lattice determines the peak intensities and the arrangement of these atoms in the unit cell of a crystal will form the X-ray diffraction pattern (Chung and Smith, 1999). The incident beam, applied at an
angle \( \theta \), will hit either a lower or an upper surface of an atomic plane and be reflected, with the reflected beams from the lower plane travelling farther than those from the upper surface (Chung and Smith, 1999). When the path difference between these beams equals an integral multiple of their wavelengths, constructive interference from multiple rows of atoms will occur, resulting in strong peaks. Bragg’s Law describes this as follows:

\[
n \lambda = 2d \sin \theta \tag{2.3}
\]

where \( n \) is an integer, \( \lambda \) is the wavelength of the X-rays, \( d \) is the interplanar spacing (or atomic plane distance) generating the diffraction, and \( \theta \) is the diffraction angle (Murthy, 2016). This law relates the wavelength of X-ray radiation to the diffraction angle and the lattice spacing in a crystalline sample (Bunaciu et al., 2015). The sample is scanned by a range of 2\( \theta \) angles (which can be from 2\(^\circ\) to 70\(^\circ\)) in order to attain all possible diffraction directions of the lattice owing to the random orientation of the scanned material (Bunaciu et al., 2015). Therefore, a goniometer is used to maintain the angle while rotating the sample, as it can be seen in Figure 2.14. The X-ray source will determine the radiation wavelength, which will determine the 2\( \theta \) angle at which reflections are measured (Bunaciu et al., 2015 and Murthy, 2016).

![Figure 2.14: A schematic diagram of the XRD goniometer. Adapted from (Husnain and Madhuku, 2017).](image-url)
This analytical approach was used in Chapter 4 to characterise the solid state of moxifloxacin and pirfenidone after spinning them using MiniFlex 600 benchtop diffractometer (RigaKu, Tokyo, Japan).

2.2.2.9. High Performance Liquid Chromatography (HPLC)

HPLC is an analytical technique that is based on chromatographic separation which emerged due to the progression in the development of chromatography and has been the leading chromatographic technique since its wide application in the 1970s (Lough and Wainer, 1995). It consists of a mobile phase (a solvent that is pumped continuously), a pump (to pump the mobile phase in a constant flow), an auto-sampler injector (to inject a certain volume of a sample), a stationary phase (which is packed inside a column to interact with a solute) and a detector (to detect the eluted solute), as illustrated in Figure 2.15. (Valko, 2006).

![Figure 2.15: HPLC instrumentation illustration showing its main components. Adapted from (laboratoryinfo.com, 2019).](image)

The interaction between the solute and the stationary phase is dependent upon their relative polarities. This interaction can be achieved through different techniques, one of them is called reversed phase chromatography. Here, the stationary phase consists of a non-polar material, such as C₈ or C₁₈, while a polar mobile phase is flowing throughout the system (Valko, 2006). If the solute to be analysed is non-polar, it will be eluted more slowly than a polar compound, due to its interaction with the stationary phase material. After the solute is eluted from the column, it can be detected by different types of
detector, such as ultraviolet (UV) or fluorescence detectors. A modern version of UV detection is the diode array, which can analyse a full UV spectrum, unlike the conventional UV detector that uses only a single wavelength. The result is a 3-D chromatogram of the UV absorbance plotted against the wavelength and the analysis time. This detector provides a close view on the outcome peak at various parts to check for any peaks overlapping which can indicate for sample impurities (Valko, 2006).

The resultant chromatograms can be interpreted by the following important features to check for a good quality of separation (Valko, 2006). The ‘retention time’ which is the time from the sample injection to the time the peak maximum leaves the column. The difference between two adjacent peaks is known as the resolution (Rs), in which the larger the difference, the better the separation and the higher the resolution. Another feature is the peak asymmetry (As) which can be calculated by:

\[ As = \frac{b}{a} \]  

Equation 2.4

where \( a \) is the distance from the leading edge of a peak to the peak midpoint, while \( b \) is the distance from the peak midpoint to the trailing edge of the peak. Both \( a \) and \( b \) are measured at 10% of peak height. Peak tailing can be obtained when the As is > 1, while peak fronting is when the As is < 1 (Valko, 2006).

In order to develop an HPLC method, the following parameters should be considered for validation (Lough and Wainer, 1995; Valko, 2006).

Precision: to check the reproducibility of an individual analyte in a sample when the test is applied repeatedly (three times) for the same sample. It is usually expressed as the relative standard deviation. Both intraday and interday variations were reviewed to check whether the peak of the same analyte is changing during the same day and during three consecutive days of storing, respectively.

Selectivity: to test the ability of the method to measure accurately and specifically an analyte in a sample in the presence of other known components. It is usually expressed by the minimum resolution factor, \( Rs \), of two neighbouring peaks.
Linearity: is the ability of the method to produce directly proportional results for a range of concentration points. It is usually assessed by plotting a standard curve of at least six concentration points (concentration versus area under the curve).

Limit of detection: is the lowest concentration of an analyte in a sample that can be detected with an acceptable precision. It is to verify whether the analyte concentration is above or below a certain level.

Limit of quantitation: is the lowest concentration of an analyte in a sample to be quantified with an acceptable precision. It is useful to determine sample impurities or degradation. Both limits represent a signal to noise ratio by comparing the results from a sample of a known analyte concentration with a blank sample. Additionally, it can be mathematically calculated based on the analyte linear regression equation.

Three HPLC methods were developed in Chapter 3 using water, acetonitrile (ACTN) and phosphate buffer saline (PBS) with a pH 7.4 to dissolve moxifloxacin and pirfenidone alone or in combination. In order to quantify both drugs an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, United States) was used. The quantification of these drugs in water and ACTN was performed to measure the drug loading of the electrospun fibres, while the PBS method was used to assess the release of both drugs from the fibres.

2.2.2.10. Drug Release Studies

Dissolution testing is used to assess the release profile of a drug from a formulation. In order for the drug molecules to be absorbed and distributed to the site of action, they must be in solution. Dissolution is an important test to characterise the performance of pharmaceutical systems. This test may be crucial to identify the bioavailability problems of drug formulations. In addition, it is used for quality assurance purpose to ensure the batch-to-batch reproducibility and to evaluate the changes in the manufacturing process or the formulation itself (Hauck et al., 2005).
Dissolution test parameters can be easily manipulated to mimic the conditions of the targeted site of action. Dissolution medium pH and the physicochemical properties of a drug are two important factors that should be considered during performing this test (Hörter and Dressman, 2001). The dissolution process of solid materials involves two steps. First, the wetting and solvation of the solid molecules at the solid-liquid interface, where the solute-solute bonds would be broken and new solute-solvent bonds are formed leading to the detachment of the solute molecules from the solid surface. Second, the solute molecules will diffuse away from the saturated boundary layer at the solid surface (Hörter and Dressman, 2001). The first theories on this dissolution process were described more than a century ago by Noyes and Whitney in 1897, then by Nernst and Brunner in 1904, who applied Fick’s law of diffusion (Klein, 2010). The factors that affect the rate of drug dissolution are described in the following modified equation:

\[
\frac{dX_d}{dt} = \frac{A \times D}{d} \times \left( \frac{C_s - X_d}{V} \right)
\]

Equation 2.5

where X_d is the amount of solute in solution, t is time, A is the surface area of the solute, D is the diffusion coefficient of the solute (related to solvent viscosity), d is the thickness of the diffusion layer at the surface of the dissolving material, Cs is the saturation solubility of the solute in the solvent and V is the volume of the dissolution medium (Klein, 2010). The most commonly used dissolution apparatus and conditions are listed in the United State Pharmacopoeia (USP) guidelines, which depend on the dosage form (Stage 6 harmonisation. 711 DISSOLUTION, 2011).

Most conventional formulations, such as tablets and capsules, can be tested using apparatus 1 (basket apparatus, as shown in Figure 2.16) or 2 (paddle apparatus), while other assemblies such as apparatus 3 (reciprocating cylinder) or apparatus 4 (flow through cell) might be used. The assembly of apparatus 1 and 2 consists of: a vessel mainly made of glass, a motor, a metallic drive shaft and either a cylindrical basket or a paddle made of blade and shaft, respectively. The assembly of apparatus 3 consists of: a cylindrical flat-bottomed glass vessel, a glass reciprocating cylinder, inert fittings and
screens and a drive assembly motor to reciprocate the cylinder. The assembly of apparatus 4 usually consists of: a reservoir and a pump, a flow-through cell and a water bath.

![Diagram of rotating basket apparatus](image)

*Figure 2.16: A schematic diagram of the rotating basket apparatus for dissolution test. Adapted from (Blaesi, 2014).*

The dissolution media is usually made of water or a buffer system with a specific pH that resembles the route of delivery. For instance, the physiological pH 7.4 is used for a dosage form that is delivered to the blood, while pH 1.2 and 6.8 are used to mimic the gastric fluid and intestinal fluid, respectively (Zhang *et al*., 2012). The medium temperature and the stirring speed are other important factors that should be considered when designing a dissolution experiment (Stage 6 harmonisation. *711 DISSOLUTION*, 2011). For a successful dissolution test, the volume of the dissolution medium must be sufficient in order to quantify the drug when it is released from the tested formulation and dissolved in the medium (Qureshi, 2012; Klose *et al*., 2011). Sink conditions could be defined as the ‘volume of dissolution medium that is at least 3-10 times the saturation volume to provide a complete drug dissolution’, otherwise, it is considered as a non-sink condition (Sun *et al*., 2016; Liu *et al*., 2013).
Due to the preparation of a non-conventional formulation (electrospun fibres) that will be delivered to the front of the eye (non-conventional route of administration) in this thesis, this dissolution test guidance may not be applicable. Due to the lack of precedent for assessing the drug release from the electrospun fibres in this application, a bespoke dialysis method was used to measure the release of the drug-loaded electrospun fibres based on the intended site of therapy, namely the cornea.

It has been reported that the average corneal temperature is 34.3°C (Efron et al., 1989), hence this temperature was used for the release experiment. Previous studies on ocular delivery have suggested that the stirring (or shaking) speed is between 50 to 100 rpm, in order to mimic some eye functions, such as blinking (Garg et al., 2014, Hilal et al., 2016, Sun et al., 2016; Mirzaeei et al., 2018). Therefore, a middle point of 75 rpm was chosen in order to avoid changes in the release rate of the drugs from the fibres. Scheubel et al. (2010) reported that by increasing the speed of the paddle in small vessel USP apparatus, the release of prednisone tablets was increased. Another observation was by reducing the volume used in the release vessels, lower percent of drug was dissolved compared to the one L vessel at constant stirring speed (Scheubel et al., 2010).

Consequently, a volume of 30 mL of PBS (pH 7.4) was used, while the weight of the fibres was calculated in order to fit the sink condition (i.e. volume of dissolution medium which is at least 3-10 times the saturation volume). All fibres were kept inside either dialysis bags or custom-made cages, as shown in Figure 2.17. The use of the bag was to achieve the permeation of the drug across it. This was suggested in order to mimic the permeation across the corneal layers and to prevent the fibres from floating to the surface. However, the use of the cage was to examine the actual release of the drug from the fibres matrix without its permeation across the bag and to avoid the fibres from floating.
These release studies were performed in Chapter 4 to measure the cumulative drug release % of moxifloxacin and pirfenidone, individually or combined, which are loaded into the monoaxial, coaxial or triaxial fibres.

2.2.2.11. Minimum Inhibitory Concentration (MIC)

The MIC test is considered as the ‘gold standard test’ to evaluate the effectiveness of an antibiotic on microorganisms, which can assess the performance of other susceptibility tests (Andrews, 2001). MIC is the lowest drug concentration that can cause an inhibition of the growth of an organism after incubation for a certain time point (Andrews, 2001).

To determine the MIC of an antibiotic, iso-sensitest broth was used to culture the bacterium after it was sterilised by autoclave. A stock solution of an antibiotic was prepared and a serial dilution was performed in a range one step higher than the required final dilution range. This is to compensate the addition of an equal volume of an inoculum that contains 1X10⁶ CFU/mL of the microorganism dissolved in the sterilised broth. A sterilised cell culture well plate was used. After incubation, the absence of solution turbidity is the indication of the bacterial growth inhibition, with the lowest concentration at
which there is no growth is considered as the MIC, as shown in Figure 2.18. This value is then compared with the standard MIC values presented in Andrews (2001).

![MIC determination assay](image)

**Figure 2.18:** The antibiotic MIC determination assay. The yellow colour indicates for the turbidity that occur due to the bacterial growth, while the white colour indicates the absence of bacterial growth.

This test was performed in Chapter 5 to determine the MIC of moxifloxacin against a Gram positive *S. aureus* and a Gram negative *P. aeruginosa* bacteria, as well as to check if pirfenidone has an antibacterial effect against these bacterial strains.

### 2.2.2.12. Zone of Inhibition

This microbiological test, also known as Kirby Bauer test, is to determine the antibiotic efficacy against certain microorganisms (Tóth *et al.*, 2013). A petri dish containing a specific nutrient agar is infected by a bacterium strain. A filter paper disc containing a known antibiotic concentration is placed onto the infected agar plate to be incubated to a certain time point, usually 24 hours at 37 °C. The agent will start diffusing from the disc creating a zone of no growth, known as ‘the zone of inhibition’, as seen in Figure 2.19. The diameter of this zone is measured and the value is compared to a standard reference value to determine whether the microorganism is resistant or sensitive to the antibiotic.
Sensitivity is determined based on the organism inhibition by the tested agent (Tóth et al., 2013).

![Diagram of antibiotic disc diffusion assay](image)

**Figure 2.19: The antibiotic disc diffusion assay.** A, B and C are antibiotic discs with different bacterial susceptibility.

This test was performed in Chapter 5 to assess the performance of the electrospun fibres against a Gram positive *S. aureus* and a Gram negative *P. aeruginosa* bacteria.

### 2.2.2.13. Cellular Sub-Culturing

Rabbit corneal fibroblast (SIRC - Statens Seruminstitut Rabbit Cornea, purchased from ATCC® CCL-60™) cell line was used in this thesis. Upon receiving the cell line, the cells were preserved in liquid nitrogen. Cells were initially thawed until a small quantity of ice is left (Invitrogen, 2014; Geraghty et al., 2014). This was transferred directly into a centrifuge tube that contains a certain volume of a pre-warmed complete medium (i.e. contains serum). This cell suspension was centrifuged at about 1500 rpm for 5 minutes until a pellet of cells is visible (precipitated). Then the supernatant was discarded and the cells were resuspended with a fresh complete medium. The cells were eventually transferred into a 25 cm² culture flask and incubated at 37 °C and 5% CO₂ (ATCC® CCL-60™, 2019, Invitrogen, 2014; Geraghty et al., 2014).

After reaching 90% of cell confluency, the consumed medium was replaced with a pre-warmed PBS to wash any remained medium containing serum.
Then the PBS was replaced with a certain volume of trypsin and incubated (at 37 °C and 5% CO₂) for 3-5 minutes in order to detach the attached cells. After incubation, the trypsin was neutralised by a similar volume of fresh medium and the formed solution was centrifuged 1500 rpm for 5 minutes at 20°C. The supernatant was discarded and a certain volume of the complete medium was added to the cell pellet to dissolve it (ATCC® CCL-60™, 2019, Invitrogen, 2014; Geraghty et al., 2014).

After dissolving the cells, cell counting was performed using haemocytometer (Invitrogen, 2014). A 10 µL of cells suspension was mixed with an equivalent volume of trypan blue and this was loaded into a haemocytometer. The blue stained cells were observed under a microscope and the cells viability was calculated based on the following equation:

\[
\text{Number of Cells/mL} = \text{Mean Number of Viable Cells} \times 10,000 \times 2 \quad \text{Equation 2.6}
\]

where the average number of viable cells is the number of counted blue stained cells that are located in the 4 gridded squares located in each corner of the haemocytometer, as shown in Figure 2.20 (ATCC® CCL-60™, 2019, Invitrogen, 2014; Geraghty et al., 2014).

![Figure 2.20: The haemocytometer gridded squares located in each corner (A-D). Blue stained cells that are located in these corners were visually counted. Adapted from (weberscientific.com, n.d).](image-url)
After counting the number of cells per mL, a certain volume was added to 75 cm$^2$ culture flask and the cells were diluted by fresh complete medium to be incubated at 37 °C and 5% CO$_2$. This sub-culture assay is known as a ‘passage’ (ATCC® CCL-60™, 2019, Invitrogen, 2014; Geraghty et al., 2014).

This sub-culturing assay was performed in Chapter 5 in order to assess the cytotoxicity of moxifloxacin, pirfenidone and the blank and drug-loaded electrospun fibres using the colorimetric (MTT) assay.

### 2.2.2.14. MTT Assay

MTT, which is (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide), is a yellow powder that will be converted to a formazan crystal by the cell’s mitochondrial activity (Van Meerloo et al., 2011; Hansen and Bross, 2010). This conversion is an indication of the living viable cells and hence this test is used to quantitatively measure viable count. The formazan concentration, which can be detected by a UV plate reader that measures the optical density, is related to a high or low number of living cells (Van Meerloo et al., 2011). The values of the cells exposed to drugs will be compared to the drug free cells. This colorimetric assay is appropriate to determine cytotoxicity of a drug, in which the concentration that inhibits the growth of these cells by 50% compared to the growth of the untreated cells is known as the 50% inhibitory concentration (IC$_{50}$) (Van Meerloo et al., 2011; Hansen and Bross, 2010).

The principle of the MTT assay, shown in Figure 2.21, is to culture a certain type of cell line in a sterile cell culture well plate (Tawfik, et al., 2017; Van Meerloo et al., 2011). After reaching 50-60% confluency upon incubation (at 37 °C and 5% CO$_2$), the consumed media was replaced by fresh media that contains the tested drugs in a serial dilution range. After incubation for a certain time point, the consumed media was replaced with a fresh media containing the MTT in a concentration of 0.5 mg/mL (Tawfik, et al., 2017). A further incubation (at 37 °C and 5% CO$_2$) for 2 to 4 hours was required to allow the reduction of the tetrazolium salt to the formazan crystal by the mitochondria of the viable cells. After this conversion, the formed crystals were dissolved by
using either isopropanol or Dimethyl sulfoxide (DMSO) and the optical density was measured by a plate reader at 570 nm (Tawfik, et al., 2017).

**MTT assay**

Figure 2.21: The MTT assay principle. Blue culture indicates for the presence of the cells before being exposed to the treatment, while the yellow culture indicates for the cells being exposed to the treatment. The purple culture indicates for the presence of MTT within the culture.

This assay was performed in Chapter 5 to measure the IC$_{20}$ and IC$_{50}$ of moxifloxacin and pirfenidone on rabbit corneal fibroblast cell line. In addition, these cells were exposed to the blank and drug-loaded electrospun fibres to assess their compatibility with the fibroblasts.

**2.2.2.15. Western Blot**

Western blotting has been a commonly used technique since it was firstly described by Towbin et al. in 1979 to detect and analyse proteins based on their ability to bind to specific antibodies (Yang and Ma, 2009). The specificity of the antibody-antigen interaction enables the targeted protein to be identified.
in a protein mixture. This is accomplished by an electrophoresis technique (on SDS-PAGE gel) followed by an electro-transfer to a membrane that can be detected using specific primary and secondary enzyme labelled antibodies. Generally antibodies will bind to a specific amino acids sequence (known as an epitope), which can recognise specific proteins within a group of many (Mian et al., 1991). Therefore, a single protein can be identified and quantified through this technique. The main principle of Western blotting can be categorised into three parts: the proteins can be separated based on their size, then antibodies are used to detect the protein of interest and finally, a substrate which reacts with an enzyme is used to view the antibody-protein complex (Jensen, 2012; Yang and Ma, 2009).

Electrophoresis is the technique that separates proteins from each other based on their molecular weights, by moving through an SDS polyacrylamide gel (SDS-PAGE) toward an anode (Yang and Ma, 2009). The smaller sized proteins will migrate faster than the larger ones. Sample proteins are boiled with Laemmli sample buffer which contains SDS and 2-mercaptoethanol (which breaks the protein disulphide bonds) to denature the protein and enable the negatively charged SDS to bind to the protein. An equal amount of the protein in solution is loaded into a gel lane for separation and a molecular weight marker (also called a ‘ladder’) is usually loaded in one of the lanes, as a reference (Yang and Ma, 2009). A current is applied and since the proteins are attached to SDS (negative charged), they are pulled down through the gel to the positive pole. Then, the gel is placed over a sheet of polyvinylidene difluoride (PVDF) or nitrocellulose membrane to transfer the proteins electrophoretically onto the membrane (Jensen, 2012; Yang and Ma, 2009).

After the transfer is completed, the membrane is soaked in a blocking buffer to block the non-specific proteins that are bound to the membrane surface, then it is incubated with a specific antibody for the desired protein using the same blocking buffer (Jensen, 2012). This will prevent the antibody giving a non-specific signal instead of binding to a specific amino acid sequence of a particular protein. After incubation with the primary antibody, a wash is conducted to remove any remaining unbound primary antibody then the membrane is further incubated with a second antibody that recognises the first
antibody (Jensen, 2012). Another wash is performed to remove any unbound secondary antibody, as well as, the non-specific binding of both the primary and secondary antibodies, which might occur. The secondary antibody usually has a covalently conjugated enzyme, such as HRP (Horseradish Peroxidase), which will be detected upon its reaction with a chemiluminescent substrate (Jensen, 2012). The light that is emitted due to the enzyme-substrate interaction can be observed by an imaging system and the size and the amount of the protein of interest will be distinguished eventually from the protein mixture (Jensen, 2012; Yang and Ma, 2009). Figure 2.22 illustrates the principle of western blot technique.

This assay was performed in Chapter 5 in order to measure the expression of α-SMA protein after exposing rabbit corneal fibroblasts to TGF-β1 cytokine to mimic the immune response of these cells after an injury. The protein expression was quantified after treating the cells with moxifloxacin, pirfenidone and the blank and drug-loaded electrospun fibres.
Chapter 3

Preparation of PLGA-Pirfenidone/PVA-Moxifloxacin Coaxial Fibres
3.1. Introduction

Corneal abrasion is a scratch wound on the surface of the anterior segment of the eye, which can penetrate to the deep corneal layers (Shahid and Harrison, 2013). Patients usually suffer from pain, redness, itching (foreign body sensation) and blurred vision. Superficial scratches usually heal within several days. However, the healing might be longer (for at least 2 weeks) for deeper cuts (Salomao et al., 2011, Kuo, 2014), leading to a greater possibility of infection. Several micro-organisms could invade this open wound and cause mild corneal infections. However, certain types of bacteria such as *P. aeruginosa* can cause a more sight-threatening condition known as corneal ulcer, which is an erosion on the cornea that might lead to loss of vision if it was not treated efficiently (Sirikul et al., 2008, Dart et al., 2008, Keay et al., 2006).

Currently, the treatment of all the above mentioned problems involves the use of broad-spectrum antibiotic eye drops (Shahid and Harrison, 2013, Keay et al., 2006, Jeng and McLeod, 2003). An anti-scarring agent pirfenidone, has been approved for the treatment of idiopathic pulmonary fibrosis (Raghu et al., 2011, du Bois, 2010, Iyer et al., 1999, European Medicines Agency, 2010). It has been reported that pirfenidone 0.5% w/v eye drops is an effective ocular anti-scarring treatment (Lin et al., 2009, Zhong et al., 2011, Sun et al., 2011, Lee et al., 2014, Jung et al., 2012), although this is not currently a licensed indication for pirfenidone. However, it is recommended to apply the eye drops six times daily due to the short half-life of pirfenidone in the ocular tissues (Zhong et al., 2011), which was reported to be 18 to 72 minutes (Sun et al., 2011).

Therefore, to accelerate the scar healing and to prevent or inhibit any bacterial infection, a combined surface ocular treatment with both an anti-scarring agent (pirfenidone) and an antibiotic (moxifloxacin) would be a sensible therapeutic approach. In order to prolong the residence time on the eye and to reduce the dose frequency of the applied dosage form, a polymeric delivery system has been suggested in this study. This will allow the incorporation of moxifloxacin in a separate compartment to pirfenidone, in addition to extending their release.
compared to the eye drop solutions. Moxifloxacin 0.5% w/v marketed eye
drops (applied 3 to 4 times daily) and pirfenidone 0.5% w/v experimentally
tested eye drops (applied 6 times daily) showed successful antibacterial (Elitis,
2011) and anti-scaring effects (Sun et al., 2011; Zhong et al., 2011),
respectively. However, in this study, it was proposed to use a higher drug
concentration (> 0.5% w/v) in order to achieve a high drug loading profile due
to the fabrication of a solid dosage form (i.e. electrospun fibres) that will be
administered once a day.

Electrospun nanofibres are relatively novel as drug delivery systems but have
been used for several years in the field of tissue engineering and wound
healing (Chakraborty et al., 2009). This is due to their resemblance to the
elements of tissue extracellular matrix (ECM) which are trigged as a response
to an injury (Murugan and Ramakrishna, 2007). As described earlier in chapter
1, the structural similarity between the electrospun fibres (a filament-like
network made of polymers) and the ECM (a filament-like network consists of
proteins and polysaccharides) may promote cell migration and proliferation,
which in turn can improve the wound healing process.

These fibres can be prepared by electrospinning whereby a high voltage
source is applied to overcome the surface tension of a viscous polymeric
solution, which will allow the evaporation of the solvents, thus forming an
elongated fibrous mat (Williams et al., 2018). Coaxial electrospinning is a
technique that involves the preparation of bi-layered fibres which can
capsulate two different drugs into separate compartments by running two
polymer solutions simultaneously (Chakraborty et al., 2009).

In this chapter, the preparation of coaxial fibres has been assessed, involving
the incorporation of pirfenidone in the hydrophobic polymer, PLGA, as the
outer layer and moxifloxacin in the water soluble polymer PVA, as the inner
layer. This has been suggested to prolong the release of pirfenidone from the
PLGA that will occur either after the degradation of the PLGA layer or by the
penetration of the tear fluid into this layer. In addition, the release of
moxifloxacin will be delayed until the penetration of the tear fluid into the PLGA
layer reaches the PVA core layer, allowing the fast disintegration of PVA and
the release of this antibiotic. The morphology of these fibres has been evaluated using a range of microscopic techniques. Thermal analysis of the raw materials using TGA and DSC has been performed to measure the degradation, melting or glass transition temperatures of these materials in order to compare the thermal behaviour of the fibrous system accordingly to the raw materials. Finally, two HPLC methods have been developed to analyse the two drugs separately for the quantification of the drug loading into the fibres. A third HPLC method has been developed to allow the simultaneous detection of both drugs during drug release studies.
3.2. Materials and Methods

3.2.1. Materials

Different PLGA 50:50 grades (as shown in Table 3.1), Purasorb® PDLG 5002A, 5004A, and 5010, have been obtained from Corbion (Purac Biomaterials, Gorinchem, Netherlands). High molecular weight (MW) PVA 146,000 to 186,000 (87-89% hydrolysed) and low MW PVA 31,000 to 50,000 (87-89% hydrolysed), dichloromethane (DCM), ethanol, HPLC grades of water and acetonitrile (ACTN) and phosphate buffer saline (PBS) tablets, were all obtained from Sigma Aldrich Company Ltd (Sigma Aldrich, Dorset, UK). Pirfenidone was purchased from Tokyo Chemical Industry UK Ltd (The Magdalen Centre, Oxford, UK), while moxifloxacin hydrochloride, which will be referred to as moxifloxacin henceforth, was purchased from Cambridge Bioscience Ltd, (Munro House, Cambridge, UK). Distilled water was generated by an ELGA Option 4 Water Purifier (Veolia Water Technologies, High Wycombe, UK).

Table 3.1: Different grades of PLGA polymer and their characteristics according to manufacturer (Corbion, Netherlands).

<table>
<thead>
<tr>
<th>Polymer grade</th>
<th>Polymer MW (kg/mol)</th>
<th>Polymer inherent viscosity (d/g)</th>
<th>Polymer degradation time (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5002A</td>
<td>17</td>
<td>0.2</td>
<td>0.5 to 1</td>
</tr>
<tr>
<td>5004A</td>
<td>44</td>
<td>0.4</td>
<td>0.75 to 1.5</td>
</tr>
<tr>
<td>5010</td>
<td>153</td>
<td>1</td>
<td>3 to 4</td>
</tr>
</tbody>
</table>

3.2.2. Methods

3.2.2.1. Preparation of Monoaxial PLGA Fibres

Electrospun PLGA fibres have been prepared by modifying the electrospinning parameters given by Said et al. (2012) and Deshpande et al. (2010) using a Spraybase® electrospinning instrument (Spraybase®, Dublin 2, Ireland). Three PLGA 50:50 grades have been used. DCM (3 mL) was used to dissolve a specified amount of this polymer, dependent upon the concentration. Table 3.2 illustrates the electrospinning parameters which have been used with and
without the addition of pirfenidone. Four concentrations of pirfenidone were loaded into the PLGA fibres which are 0.25%, 0.5%, 1% and 2% w/v in order to evaluate the effect of increasing the drug concentration on the morphology of the fibres and the electrospinning processing. The voltage was varied until a stable jet was achieved by visually observe this stability using an attached camera. The needle diameter of 0.9 mm was kept constant throughout the entire preparation of PLGA fibres in order to mimic the outer diameter of the coaxial needle. In addition, the flow rate and collector distance were also constant at 1 mL/h and 15 cm, respectively. The end product fibres were collected on aluminum foil. All formulations (1 to 14) were fabricated at ambient temperature (range of 21.5 to 25.5°C) and humidity (range of 34 to 51%).

Table 3.2: Electrospinning parameters for the preparation of blank and drug-loaded PLGA monoaxial fibres.

<table>
<thead>
<tr>
<th>Form. #</th>
<th>PLGA grade</th>
<th>PLGA conc. in solution (% w/v)</th>
<th>Pirfenidone conc. in solution (% w/v)</th>
<th>Voltage (kV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5002A</td>
<td>25</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>5004A</td>
<td>20</td>
<td>0</td>
<td>9-10</td>
</tr>
<tr>
<td>3</td>
<td>5004A</td>
<td>25</td>
<td>0</td>
<td>8-10</td>
</tr>
<tr>
<td>4</td>
<td>5004A</td>
<td>30</td>
<td>0</td>
<td>19-20</td>
</tr>
<tr>
<td>5</td>
<td>5010</td>
<td>12.5</td>
<td>0</td>
<td>19-20</td>
</tr>
<tr>
<td>6</td>
<td>5010</td>
<td>15</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>5010</td>
<td>15</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>8</td>
<td>5010</td>
<td>15</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>9</td>
<td>5010</td>
<td>15</td>
<td>0</td>
<td>22</td>
</tr>
<tr>
<td>10</td>
<td>5010</td>
<td>15</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>11</td>
<td>5010</td>
<td>15</td>
<td>0.25</td>
<td>20</td>
</tr>
<tr>
<td>12</td>
<td>5010</td>
<td>15</td>
<td>0.5</td>
<td>21</td>
</tr>
<tr>
<td>13</td>
<td>5010</td>
<td>15</td>
<td>1</td>
<td>23</td>
</tr>
<tr>
<td>14</td>
<td>5010</td>
<td>15</td>
<td>2</td>
<td>24</td>
</tr>
</tbody>
</table>
3.2.2.2 Preparation of Monoaxial PVA Fibres

PVA nanofibres were fabricated by modifying the electrospinning methods of Taepaiboon et al. (2006) and Felice et al. (2015) using the same Spraybase® electrospinning instrument as for the PLGA nanofibres. High MW PVA (146,000 to 186,000), and low MW PVA (31,000 to 50,000) have been used, both with a degree of hydrolysis of 87-89%. PVA solutions were prepared by dissolving PVA in distilled water at 90°C for 1 hour in order to obtain concentrations of 7.5% w/v and 15% w/v for the high and low PVA MW, respectively. Table 3.3 shows the preparation parameters of PVA electrospun nanofibres, with and without the addition of moxifloxacin. Two moxifloxacin concentrations, 1% and 2% w/v, were loaded into the PVA fibres. The needle diameter of 0.45 mm has been kept constant during the PVA fibre preparation to mimic the inner diameter of the coaxial needle. The flow rate and collector distance were varied in order to observe any morphological changes in the obtained fibres. The end product nanofibres have been collected on a Parafilm Wrap PM996, 4 inches wide. All formulations (15 to 23) were fabricated at ambient temperature (range of 23 to 24°C) and humidity (range of 40 to 54%).
Table 3.3: Electrospinning parameters for the preparation of blank and drug-loaded PVA monoaxial fibres.

<table>
<thead>
<tr>
<th>Form. #</th>
<th>PVA MW (kg/mol)</th>
<th>PVA conc. in solution (% w/v)</th>
<th>Moxifloxacin conc. in solution (% w/v)</th>
<th>Ethanol (% v/v)</th>
<th>Flow rate (mL/h)</th>
<th>Voltage (kV)</th>
<th>Distance (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>31-50</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>1.5</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>16</td>
<td>31-50</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>1.5</td>
<td>28.5</td>
<td>15</td>
</tr>
<tr>
<td>17</td>
<td>146-186</td>
<td>7.5</td>
<td>0</td>
<td>0</td>
<td>1.5</td>
<td>23</td>
<td>15</td>
</tr>
<tr>
<td>18</td>
<td>146-186</td>
<td>7.5</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>19</td>
<td>146-186</td>
<td>7.5</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>16.5</td>
<td>10</td>
</tr>
<tr>
<td>20</td>
<td>146-186</td>
<td>7.5</td>
<td>0</td>
<td>10</td>
<td>1</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>21</td>
<td>146-186</td>
<td>7.5</td>
<td>0</td>
<td>20</td>
<td>1</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>22</td>
<td>146-186</td>
<td>7.5</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>26</td>
<td>15</td>
</tr>
<tr>
<td>23</td>
<td>146-186</td>
<td>7.5</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>29</td>
<td>15</td>
</tr>
</tbody>
</table>
3.2.2.3. Preparation of Coaxial Fibres

Electrospun coaxial fibres have been prepared by modifying the procedure described in Tiwari et al. (2010). These fibres have been fabricated by preparing an organic phase consisting of 15% w/v PLGA (MW of 153 kg/mol), with and without pirfenidone, dissolved in DCM, and an aqueous phase of 7.5% w/v PVA (MW of 146-186 kg/mol), with and without moxifloxacin, dissolved in distilled water. The effect of the addition of 10% v/v and 20% v/v ethanol into the aqueous phase has been assessed. All the fibres were prepared using the Spraybase® electrospinning instrument. A coaxial needle was used with an inner diameter of 0.45 mm and an outer diameter of 0.9 mm. The organic phase, which is the shell, has been emitted at a higher flow rate than the core aqueous phase. Table 3.4 shows the electrospinning parameters of these fibres. The end product were collected on an aluminum foil at a distance of 15 cm. All formulations (24 to 32) were fabricated at ambient temperature (range of 22.1 to 24°C) and humidity (range of 32 to 39%).
Table 3.4: Electrospinning parameters for the preparation of blank and drug-loaded PLGA/PVA coaxial fibres.

<table>
<thead>
<tr>
<th>Form. #</th>
<th>Pirfenidone (% w/v)</th>
<th>Moxifloxacin (% w/v)</th>
<th>PVA Ethanol (% v/v)</th>
<th>Outer flow rate (mL/h)</th>
<th>Inner flow rate (mL/h)</th>
<th>Voltage (kV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0.5</td>
<td>15</td>
</tr>
<tr>
<td>25</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4.5</td>
<td>0.5</td>
<td>16</td>
</tr>
<tr>
<td>26</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4.5</td>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td>27</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>19</td>
</tr>
<tr>
<td>28</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0.75</td>
<td>25</td>
</tr>
<tr>
<td>29</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0.75</td>
<td>24</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>4</td>
<td>0.75</td>
<td>18</td>
</tr>
<tr>
<td>31</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>4</td>
<td>0.75</td>
<td>17</td>
</tr>
<tr>
<td>32</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2.25</td>
<td>0.75</td>
<td>18</td>
</tr>
</tbody>
</table>
3.2.2.4. Size and Shape of Fibres

The morphological characteristics of the prepared fibres were examined using scanning electron microscopy (SEM), transmission electron microscopy (TEM), and light and fluorescence microscopy.

3.2.2.4.1. Scanning Electron Microscopy

A 0.5cm x 0.5cm piece of foil, on which the fibres were collected, was adhered onto an SEM stub, using double sided carbon tabs (Agar Scientific, Stansted, UK). The prepared stub was then given a thin coating of gold (10 nm) using a Quorum Q150T Sputter Coater (Quorum Technologies Ltd. East Sussex, UK) in an argon atmosphere. The coated stub was then transferred and imaged under FEI Quanta 200F (FEI company Ltd, Eindhoven, The Netherlands), at an acceleration voltage of 5 kV. Fibre size analysis was performed by measuring the diameter of these fibres using ImageJ software (National Institutes of Health, Maryland, USA).

3.2.2.4.2. Transmission Electron Microscopy

Fibres were collected on a copper grid directly during fabrication. The prepared grids were stained with 2% aqueous uranyl acetate solution prior to imaging under a FEI CM120 BioTwin TEM (FEI Company Ltd. Eindhoven, The Netherlands) at an accelerating voltage of 120 kV.

3.2.2.4.3. Light and Fluorescence Microscopy

Fibres were assessed under both light and UV fluorescence microscopy using a Nikon Microphot-FXA microscope (Nikon, Japan). Fibres were collected directly on microscope slides, and examined using total magnifications of 40X, 100X and 400X. The fluorescence wavelength is in the range of 320 to 340 nm.
3.2.2.5. Thermal Analysis

Differential scanning calorimetry (DSC) has been performed for PLGA 5004A and 5010 grades, PVA low and high MWs and pirfenidone and moxifloxacin as raw materials. In addition, physical mixtures of 15% PLGA 5010 and 1% pirfenidone, as well as 7.5% PVA high MW and 1% moxifloxacin have been analysed. Thermogravimetric analysis (TGA) has been performed for the raw materials only. Data analysis for both experiments has been performed on TA Instruments Universal Analysis 2000 software.

3.2.2.5.1. Differential Scanning Calorimetry

Conventional heat flow DSC was performed using a TA Q2000 calorimeter (TA Instruments UK, Herts, UK). Temperature calibration using indium, tin and octadecane was performed before running the samples. An aliquot of each sample (weight range from 3 to 5 mg) was weighed into a 40 µL aluminum DSC pan (Perkin-Elmer) which was then sealed by an aluminum lid. Three holes have been punched through the lid after sealing. Each sample was analysed in triplicate to check the reproducibility of the thermal analysis. For pirfenidone and moxifloxacin samples, a standard DSC analysis was performed by equilibrating the samples at 0°C and then heating at a rate of 10°C/minute to 180°C and 250°C, respectively.

Modulated temperature DSC (MTDSC) was also performed using the TA Q2000 instrument. Calibration by sapphire disc was performed before running the samples. Here, the underlying heating rate was adjusted to 2°C/minute with modulation parameters of amplitude ± 0.212°C and period 40 seconds. For PLGA Tg determination, the samples were equilibrated at 0°C and heated to 100°C. However, for PVA samples, the analysis was performed using the heat-cool-heat modulated method. These samples were equilibrated at 0°C, heated to 180°C, cooled to 0°C, and then reheated to 235°C. The data from the third cycle was used to determine the Tg and the melting point.
3.2.2.5.2. Thermogravimetric Analysis

TGA was performed using a TA Hi-Res TGA 2950 thermogravimetric analyser (TA Instruments UK, Herts, UK). An aliquot of the sample (weight range from 5 to 10 mg) was placed into an open aluminum pan. Each sample was analysed in triplicate to check the reproducibility of the thermal analysis. The samples were equilibrated at 30°C and heated to 400°C at a rate of 10°C/minute.

3.2.2.6. HPLC Assay Methods for Determination of Pirfenidone and Moxifloxacin in the Fibres

In order to assess the encapsulation and the release of each drug in its corresponding layer, for example pirfenidone in PLGA, pirfenidone and moxifloxacin were analysed by high performance liquid chromatography (HPLC) using three solvents; ACTN, distilled water and PBS with a pH 7.4. The use of the ACTN is to dissolve PLGA and the water is used to dissolve PVA for measuring the drug loading. The PBS was used to test the release of both drugs in a medium that resembles the tear fluid. The HPLC system consisted of an Agilent model which contains Agilent Technologies 1200 G1329A ALS autosampler, G1322A degasser, G1311A quat pump, G1316A diode array detector, and Agilent ChemStation for LC and LC/MS systems software. Quantification was achieved by isocratic elution.

3.2.2.6.1. Development of the HPLC Assay Using Water as the Dissolution Solvent

This assay has been developed by combining the parameters described in Parmar et al. (2014), Dewani et al. (2011) and Ravisankar et al. (2014). An acidic phosphate buffer 20 mM [2.72 g Potassium dihydrogen orthophosphate (MW 136.02) in 1 L of HPLC grade water and adjusted to pH 3.3 using 4-5 drops of phosphoric acid] and ACTN were used as the mobile phase in a ratio of 65:35 and delivered at a flow rate of 1 mL/minute at ambient temperature. A ZORBAX Eclipse plus C18 analytical column (C18, 5 µm, 4.6 mm x 250 mm) was used. The injection volume was 50 µL. Ultraviolet (UV) detection was
performed at a wavelength of 310 nm, at which both drugs have showed an absorbance. For standard curve preparation, serial dilutions were performed to obtain solutions of 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.78, 0.39 µg/mL of each drug individually and then combining 0.5 mL from each matching drug dilution into one vial to obtain mixed solutions containing 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.78, 0.39 µg/mL of each of the two drugs.

3.2.2.6.2. Development of the HPLC Assay Using Acetonitrile as the Dissolution Solvent

This assay has been developed by combining the parameters described in Parmar et al. (2014), Dewani et al. (2011) and Ravisankar et al. (2014). An acidic HPLC grade water (1 L adjusted to pH 3.3 by 4-5 drops of phosphoric acid) and acetonitrile were used as the mobile phase in a ratio of 70:30 and delivered at a flow rate of 1 mL/minute at ambient temperature. A ZORBAX Eclipse plus C18 analytical column (C18, 5 µm, 4.6 mm x 250 mm) was used. The injection volume was 20 µL. UV detection was performed at a wavelength of 310 nm. For standard curve preparation, serial dilutions were performed to obtain solutions of 75, 37.5, 18.75, 9.375, 4.688, 2.344, 1.172, 0.586, 0.293, 0.147 µg/mL of each drug individually and then combining 0.5 mL from each matching drug dilution into one vial to obtain mixed solutions containing 37.5, 18.75, 9.375, 4.688, 2.344, 1.172, 0.586, 0.293, 0.147 µg/mL of each of the two drugs.

3.2.2.6.3. Development of the HPLC Assay Using PBS (pH 7.4) as the Dissolution Solvent

This assay has been developed by combining the parameters described in Parmar et al. (2014), Dewani et al. (2011) and Ravisankar et al. (2014). An acidic HPLC grade water (1 L adjusted to pH 3.3 by 4-5 drops of phosphoric acid) and ACTN were used as the mobile phase in a ratio of 65:35 and delivered at a flow rate of 1 mL/min at ambient temperature. A ZORBAX Eclipse plus C18 analytical column (C18, 5 µm, 4.6 mm x 250 mm) was used. The injection volume was 50 µL. UV detection was performed at a wavelength
of 310 nm. For standard curve preparation, serial dilutions were performed to obtain solutions of 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.78, 0.39 µg/mL of each drug individually and then combining 0.5 mL from each matching drug dilution into one vial to obtain mixed solutions containing 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.78, 0.39 µg/mL of each of the two drugs.

3.2.2.7. Drug Loading [DL], Entrapment Efficiency [EE%] and Yield [Y%]

For direct determination of the drug loading and entrapment efficiency of loaded fibres, a certain volume of ACTN for PLGA fibres, or HPLC grade water for PVA fibres, was added to a known amount of the formulation until complete dissolution of the relevant polymer was noted. Then 1 mL of the formed solution was withdrawn and centrifuged for 5 minutes at 10,000 rpm at room temperature. The drug content in the solution was determined by HPLC using the methods detailed above and by comparison to a standard curve. The HPLC will provide the area under the curve (AUC) of the injected drug after eluting from the column. This AUC can be converted to concentration by using the constructed calibration curve. This concentration is then converted to an actual amount by multiplying it by the total volume.

The drug loading (DL) in the formulation was calculated using the following theoretical equation:

\[
DL = \frac{\text{Entrapped drug amount}}{\text{Yield of fibres amount}}
\]

Equation 3.1

The entrapment efficiency (EE) was determined by the following equation:

\[
EE\% = \frac{\text{Actual drug amount}}{\text{Theoretical drug amount}} \times 100
\]

Equation 3.2
The yield of the fabricated fibres was calculated by the following equation:

\[ Y\% = \frac{\text{Actual amount of fibres}}{\text{Theoretical amount of fibres}} \times 100 \]  

Equation 3.3

where the theoretical amount of fibres was calculated by estimating the amount of the solid materials polymer ± drug in the total volume that was injected in the electrospinning system.
3.3. Results and Discussion

As reported by Chakraborty et al. (2009), Tiwari et al. (2010) and Williams et al. (2018), in order to fabricate a coaxial system, the two polymer solutions should have a different miscibility degree or even be immiscible. This will allow the encapsulation of the inner layer within the outer layer. Beaded fibres might be an indicator for an unstable jet due to insufficient viscosity or conductivity, which would require either increasing the polymer concentration or altering the applied voltage. In addition, the surface of the monoaxial or coaxial fibres should be smooth and free from pores. This will avoid the fast release of the loaded drugs due to the enhance penetration of the dissolution medium. This can be a limitation as the suggested treatment regimen is a single daily dose. Owing to the larger surface area of the nanofibres compared to microfibres, fibre diameters which are in the nano range are preferable and can contribute to a high dissolution rate (Dubald et al., 2018). As mentioned earlier, both moxifloxacin and pirfenidone were used as eye drops in a concentration of 0.5% w/v (Eltis, 2011; Zhong et al., 2011). However, it was suggested to load a higher drug concentration (> 0.5% w/v) to achieve a higher drug loading which will decrease the weight (size) of the final dosage form, hence reducing eye irritation.

Therefore, it was proposed that the PLGA will be the outer layer to sustain the release of pirfenidone, while the PVA will be the inner layer in order to compensate for the fast release of moxifloxacin (within few minutes or hours) which under usual dosage regimes necessitates multiple daily doses.

For the fabrication of the final coaxial formulation of PLGA-pirfenidone/PVA-moxifloxacin fibres, optimisation of PLGA and PVA loaded and unloaded fibres, as well as blank coaxial fibres, has been performed. The production parameter (needle diameters) of the monoaxial fibres were kept the same as they would be in the coaxial fibres, i.e. the PLGA is intended to be the outer layer of the coaxial fibres, so the PLGA monoaxial fibres were produced with a 0.9 mm needle. Conversely, the PVA is intended to be the inner layer of the coaxial fibres, so the PVA monoaxial fibres were produced with a 0.45 mm needle.
3.3.1. Morphological Characteristics of Fibres

3.3.1.1. Blank Monoaxial PLGA Fibres

Three PLGA 50:50 grades have been assessed with the purpose of optimising PLGA monoaxial fibres, using a variety of production parameters as an initial exploratory study. Figure 3.1 shows the SEM images taken of representative samples of the fibres produced following the parameters listed in Table 3.2.

PLGA 50:50 MW 17 kg/mol electrospun at an initial solution concentration of 25% w/v produced particles rather than fibres (Figure 3.1 - formulation 1). This was due to low solution viscosity which was enough to break the electrospinning jet and disperse into fine droplets (Wu and Clark, 2008; Jaworek and Sobczyk, 2008). It has been reported in the literature that it is possible to produce fibres using this grade of PLGA 50:50, but with the concentration above 30% w/v (Katti et al., 2004). In the current system, such a high polymer concentration was thought to be unfavourable as it may decrease the drug loading potential in the solution, due to the high percentage of the polymer used, and was therefore not studied.

Three initial concentrations of a higher MW of PLGA 50:50, 44 kg/mol, were examined (parameters are outlined in Table 3.2 - formulations 2, 3, and 4). Using this grade of PLGA 50:50, the initial solution concentrations of 20% w/v and 25% w/v both produced beaded fibres, while the 30% w/v initial concentration formed unbeaded fibres, as shown in Figure 3.1 - formulation 2-4. Said et al. (2012) showed unbeaded fibres when they used slightly higher MW PLGA 50:50 grade (50 kg/mol) in a concentration of 25% w/v than the grade that was used in this study (44 kg/mol) in a concentration of 30% w/v using the same solvent (DCM). In the current study, upon increasing the concentration to 35% w/v, the polymer solution viscosity visually appeared to be too high for the electrospinning process and therefore was not considered further.

Solutions of 12.5% w/v and 15% w/v of the highest MW of PLGA 50:50, 153 kg/mol produced unbeaded fibres (Figure 3.1 - formulations 5 and 8). Consistently, Tiwari et al. (2010) were able to prepare an unbeaded fibrous system from a high MW PLGA 80:20 (120 kg/mol), slightly lower than the used
PLGA, from a polymer solution of 12.5% w/v dissolved in a cosolvent system of chloroform and dimethyl formamide (80:20).

<table>
<thead>
<tr>
<th>Form. #</th>
<th>SEM image</th>
<th>Form. #</th>
<th>SEM image</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25% PLGA (17 kg/mol)</td>
<td>6</td>
<td>The use of 10 kV in 15% PLGA (153 kg/mol)</td>
</tr>
<tr>
<td>2</td>
<td>20%, PLGA (44 kg/mol)</td>
<td>7</td>
<td>The use of 13 kV in 15% PLGA (153 kg/mol)</td>
</tr>
<tr>
<td>3</td>
<td>25% PLGA (44 kg/mol)</td>
<td>8</td>
<td>The use of 20 kV in 15% PLGA (153 kg/mol)</td>
</tr>
<tr>
<td>4</td>
<td>30% PLGA (44 kg/mol)</td>
<td>9</td>
<td>The use of 22 kV in 15% PLGA (153 kg/mol)</td>
</tr>
<tr>
<td>5</td>
<td>12.5% PLGA (153 kg/mol)</td>
<td>10</td>
<td>The use of 24 kV in 15% PLGA (153 kg/mol)</td>
</tr>
</tbody>
</table>

Figure 3.1: The surface morphology of blank PLGA fibres prepared by different PLGA MWs showing the presence of beads and pores. Form. 1: 25% PLGA (17 kg/mol), form. 2-4: 20%, 25%, 30% PLGA (44 kg/mol), respectively, form. 5: 12.5% PLGA (153 kg/mol), form. 6-10: 15% PLGA (153 kg/mol) in different applied voltage to test the effect of this parameter on the yielded fibres.
Based on these initial results, the lower MW PLGA 50:50 sample was considered to be poor performer and removed from future experiments. PLGA 50:50 with a MW of 153 kg/mol and a concentration of 15% w/v has been used for further optimisation due to its ability to produce fibres at a lower concentration compared to other grades. The polymer solution viscosity can be influenced by the polymer MW and the solvent to be used. Therefore, adjusting the solution concentration to the minimum solution viscosity that undergo polymer chain entanglement is essential in order to produce non-defective fibres (Qasim et al., 2018). This preliminary work demonstrated the inverse relationship between the polymer MW and the polymer concentration and solution viscosity that can result successful fibres (i.e. unbeaded). As observed, increasing the MW of PLGA enabled the production of a high viscosity solution using a lower concentration, which was also reported in Williams et al. (2018) and Qasim et al. (2018). This facilitated the fabrication of unbeaded fibres at a lower polymer concentration, while the fibre diameter increased due the effective polymer chain entanglement that resulted from a high viscous spinning solution (Haider et al., 2018).

Using a fixed concentration of 15% w/v of PLGA 50:50 (MW 153 kg/mol), the effect of changing the voltage on the electrospun product was studied. Five voltages have been used: 10, 13, 20, 22, and 24 kV as seen in Table 3.2 - formulations 6, 7, 8, 9, and 10, respectively. The produced fibres showed a decrease in the formation of pores on the fibre surface as the voltage increased (≥ 20 kV), suggesting that the higher the voltage, the more stabilised the jet and the lower chance of DCM evaporation (Figure 3.1 - formulations 6-10). It was reported by Williams et al. (2018) that by increasing the applied voltage, the flight time to reach the collector is reduced allowing less solvent to evaporate. This lowers the likelihood of producing porous fibres. In addition, Bognitzki et al. (2001) found that the use of a quickly evaporating solvent (i.e. one with a high vapour pressure) such as DCM, is likely to produce more porous fibres of PLA, PCL and Poly-vinylcarbazole due to the accelerated phase separation during the manufacturing process. It seems that the solvent-rich regions of the sample are converted into pores during the evaporation process. Furthermore, increasing the voltage will tend to reduce the diameter
of the fibres, although further increases will yield higher diameter fibres (Demir et al., 2002; Bhattarai et al., 2019). This observation will be explored more in the next section 3.3.2.

This initial work showed the influence of the applied voltage on the stability of the jet, allowing lower pore formation as the jet is more stable. Overall, the effect of increasing the applied voltage and the MW of a polymer on producing unbeaded and non-porous fibres using a lower concentration was successfully investigated. Following this exploratory study, PLGA 50:50 MW 153 kg/mol was selected for further development and for the drug loading studies, using an initial solution concentration of 15% w/v and a voltage range from 20-24 kV (parameters are outlined in Table 3.2 - formulation 8-10). This was due to the lower degree of surface pores formation (Figure 3.1 - formulation 8-10). In all further discussion ‘PLGA’ refers to this grade.

3.3.1.2. Pirfenidone-loaded Monoaxial PLGA Fibres

Pirfenidone was loaded into the 15% w/v PLGA solution at four different concentrations: 0.25%, 0.5%, 1%, and 2% w/v as seen in Table 3.2 formulations 11, 12, 13 and 14, respectively. The resultant fibres were porous with rough surface (Figure 3.2 - formulation 11-14). This can be a limitation in this study, as the release of this drug will be accelerated due to the presence of the pores. However, there was no visible evidence of drug crystals at the surface of these fibres, suggesting that a pirfenidone concentration of up to at least 2% w/v can be used with 15% w/v PLGA to prepare pirfenidone-loaded PLGA fibres, in order to obtain a high drug loading profile.
Figure 3.2: The surface morphology of pirfenidone-loaded PLGA fibres prepared by 15% PLGA (153 kg/mol) showing the presence of pores. Form. 11: 0.25% pirfenidone, form. 12: 0.5% pirfenidone, form. 13: 1% pirfenidone, form. 14: 2% pirfenidone.

3.3.1.3. Blank Monoaxial PVA Fibres

In the evaluation of the manufacture of PVA fibres, two MWs have been examined: a low MW of 31 to 50 kg/mol and a high MW of 146 to 186 kg/mol. For the low MW PVA, an initial solution concentration of 30% w/v has been prepared, but the viscosity was visually too high to be electrospun. Lowering the concentration to 15% w/v produced unbeaded nano-sized fibres with smooth surfaces, as seen in Figure 3.3 - formulation 15. However, slightly beaded nanofibres were observed by increasing the needle diameter from 0.45 to 1.2 mm, as observed in Figure 3.3 - formulation 16. It was reported by Kizildag et al. (2012) and Abunahel et al. (2018) that using a small needle diameter can produce unbeaded and smaller diameter fibres due to the high surface tension of the small solution drop that has formed at the tip of the needle. This would require high applied voltage to overcome the surface tension.
tension to form a jet, which allow the stretching and thinning of the jet. For the high MW PVA, a concentration of 15% w/v has been prepared, but again it was difficult to be spun due to the high viscosity. By decreasing the concentration to 7.5% w/v and reducing the needle diameter to 0.45 mm, it was able to produce nanofibres (Figure 3.3 - formulation 17). This formulation is consistent with that used by Tiwari et al. (2010) who used high MW PVA of 124 to 186 kg/mol in a concentration 8% w/v. Because of the high drug loading requirement in our system, the high MW PVA was chosen for further assessments.

The effect of varying the distance between the tip and the collector (from 15 cm to 10 cm) has been assessed using this formulation (parameters are outlined in Table 3.3 - formulation 18 and 19, respectively). By decreasing the distance, the findings suggested that the nanofibres were still wet on the collector resulting the fibres to flattened upon hitting the collector plate, as seen in Figure 3.3 - formulations 18 (15 cm) and 19 (10 cm). Ding et al. (2002) have showed that PVA fibres’ diameter was decreased from 330 to 260 nm by lowering the distance from 14 to 6 cm, while keeping the polymer concentration and voltage constant at 10% and 19 kV, respectively. In addition, Williams et al. (2018) reported that due to lowering the travel distance which allowed less residual solvent to evaporate, flattened (ribbon like shape) fibres were more likely to be obtained. Despite the shorter distance (10 cm), the use of a lower voltage (16.5 kV) to stabilise the jet allowed the production of unbeaded fibres. Demir et al. (2002) reported that by increasing the distance or decreasing the voltage, less beaded fibres can be obtained due to the increase of the flight time which allow the stretching of the electrospinning jet. This work demonstrates the effect of increasing the tip-to-collector distance on lowering the fibres’ residual solvent due to increasing the flight time, which allow more solvent to evaporate (Williams et al., 2018).

The addition of ethanol to the electrospinning solution was investigated, due to the reported works of producing un-flattened PVA fibre when ethanol is added to the PVA aqueous solution as observed in Figure 3.3 - formulations 20 and 21 (Sukyte et al., 2012; Asawahame et al., 2015). However, it produced a very dry fibrous mat which was difficult to peel from the surface of the
collector. By keeping the tip-to-collector distance constant at 15 cm, the voltage needed was reduced to 6 kV and 5 kV when 10% v/v and 20% v/v ethanol has been added, respectively, as shown in Table 3.3 - formulations 20 and 21. This distance (15 cm) produced unbeaded and un-flattened fibres but the collected fibres were very dried (Figure 3.3 - formulations 20 and 21). The addition of ethanol reduced the voltage that was used due to decrease the conductivity and surface tension of the water, as well as increase the evaporation rate of the PVA solution, as reported by Sukyte et al. (2012) and Asawahame et al. (2015). In addition, the ethanol enabled these nanofibres to lose the ribbon-like shape that has been shown in formulation 18. Koski et al. (2004) were able to prepare flattened PVA fibres from a wide range of MWs without the addition of ethanol. Therefore, the addition of a low % of ethanol can be an alternative way to the abovementioned increase in the tip-to-collector distance, which can overcome the production of wet fibres (i.e. residual solvent is still entrapped).

Following this exploratory study, PVA MW 146 to 186 kg/mol with no ethanol addition (Table 3.3 - formulation 18) was selected for further development and for the drug loading studies, using an initial solution concentration of 7.5% w/v. In all further discussion ‘PVA’ refers to this grade.
**Figure 3.3:** The surface morphology of blank PVA fibres prepared by different PVA MWs showing the presence of beaded or flattened fibres. Form. 15 and 16: 15% PVA (31-50 kg/mol), form. 17-19: 7.5% PVA (146-186 kg/mol) in different distance and flow rate, form. 20: 7.5% PVA (146-186 kg/mol) dissolved in 10% ethanol, form. 21: 7.5% PVA (146-186 kg/mol) dissolved in 20% ethanol.

### 3.3.1.4. Moxifloxacin-loaded Monoaxial PVA Fibres

Moxifloxacin at concentrations of 1% and 2% w/v (parameters are outlined in Table 3.3 - formulations 22, and 23, respectively) was incorporated into the
7.5% PVA solutions and the distance was 15 cm as the blank fibres. However, the voltage was increased to 26 kV and 29 kV for the 1% and 2% moxifloxacin, respectively, compared to 20 kV for the blank fibres. This was due to the use of the salt form of moxifloxacin, moxifloxacin hydrochloride, which required higher voltage to stabilise the electrospinning jet. Owing to the increased number of ions in the polymer solution, the solution surface charge and the electrostatic force that is produced by the applied voltage will enhanced, as suggested by Haider et al. (2018). It was reported that the solution conductivity is essential in forming the Taylor cone, hence the electrospinning jet is initiated (Haider et al., 2018). This is due to the movement of the free charges on the surface of this conductive polymer solution droplet by the electrostatic force of the applied voltage, which will be sufficient to form the Taylor cone.

In addition, the resultant fibres were smooth and non-porous (Figure 3.4 - formulation 22 and 23), as seen in the equivalent blank fibres (Figure 3.3 - formulation 18). The fibres produced using the solution containing 2% w/v moxifloxacin showed evidence of drug crystals on the surface (Figure 3.4 - formulation 23), which were not observed in the fibres produced with lower concentrations of the drug (Figure 3.4 - formulation 22). The presence of drug crystals on the surface of the fibres could be a drawback in some formulation that require a sustained release profile with low burst release. Owing to the hydrophilic polymer (PVA) that is used in this system, the presence of drug crystals on the surface will not have a major effect on the release, since this polymer is expected to disintegrate within few minutes. However, due to the aim of fabricating a multi-layered fibrous system that can sustain the release of the inner drug (moxifloxacin) for at least a day, the solution containing 1% w/v of moxifloxacin will be used to prepare moxifloxacin-loaded PVA fibres.
3.3.1.5. Blank Coaxial PLGA/PVA Fibres

The electrospinning concentration and the grade of both PLGA and PVA were chosen based on the above studies and were used to prepare blank coaxial fibres. The critical parameter that has been investigated is the difference between the outer and inner flow rates. The outer flow rate was maintained at 3 mL/h and the inner flow rate was varied from 0.5 to 1 mL/h, as shown in Table 3.4 - formulations 24 and 27 respectively. This high core to shell flow rate ratio (1:3 and 1:10) was previously recommended for the preparation of coaxial fibrous systems (Chakraborty et al., 2009). This was to ensure the encapsulation of the core solution and the formation of a stable jet that lead to a consistent coaxial fibres.

The resulting fibres were rough and porous. With a higher outer flow rate of 4.5 mL/h and varying the inner flow rate from 0.5 to 1 mL/h, a similar observation resulted, as shown in Figure 3.5 - formulations 25 and 26,
respectively. Finally, experiments with an intermediate inner flow rate (0.75 mL/h) and variable outer flow rates (2 and 4 mL/h) and higher voltages produced very porous fibres, as shown in Figure 3.5 - formulations 28 and 29, respectively. The addition of ethanol to the PVA solution produced highly porous nanofibres due to the increase in the evaporation rate of the PVA solution, allowing more solvent to evaporate before reaching the collector, as shown in Figure 3.5 - formulations 30 and 31. The blank coaxial fibres resembled the PLGA monoaxial fibres, both blank and drug-loaded, in their external appearance which was expected as the outer layer is PLGA.

Changing the flow rate of the core and the shell solutions, in the recommended ratio range (1:3 to 1:10), was performed to ensure the encapsulation of the core solution within the shell and to stabilise the electrospinning jet in order to produce a reproducible coaxial fibrous system. Owing to the immiscibility between the core solution (PVA in water) and the shell solution (PLGA in DCM), achieving a stable jet was difficult. In addition, the use of a high volatile solvent (DCM) with the outer PLGA layer, allowed the formation of a rough and porous surface fibre. This was due to the faster evaporation of DCM than the water during the flight distance (i.e. from the needle tip to the collector). This difference in the evaporation rate of the two solvents allowed the formation of porous fibres after the complete evaporation of both solvents, as reported by Haider et al. (2018). Therefore, pirfenidone will be loaded into 15% PLGA (dissolved using DCM) as the outer layer while moxifloxacin will be loaded into 7.5% PVA (dissolved using water with no addition of ethanol) as the inner layer with a core to shell flow rate ratio of 1:3. These parameters were used due to the absence of any visible pores on the surface of the produced fibres (Figure 3.5 - formulation 27).
<table>
<thead>
<tr>
<th>Form. #</th>
<th>SEM image</th>
<th>Form. #</th>
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<td>28</td>
<td><img src="image2" alt="SEM image" /></td>
</tr>
<tr>
<td>Coaxial fibres C-to-S: 1:6</td>
<td></td>
<td>Coaxial fibres C-to-S: 1:4</td>
<td></td>
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<tr>
<td>25</td>
<td><img src="image3" alt="SEM image" /></td>
<td>29</td>
<td><img src="image4" alt="SEM image" /></td>
</tr>
<tr>
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<td></td>
<td>Coaxial fibres C-to-S: 1:5</td>
<td></td>
</tr>
<tr>
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<td><img src="image5" alt="SEM image" /></td>
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<td>Coaxial fibres with 20% ethanol in PVA</td>
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</table>

Figure 3.5: The surface morphology of blank coaxial fibres showing the presence of pores. Form. 24-29: prepared by 15% PLGA (MW 153 kg/mol) and 7.5% PVA (MW 146-186 kg/mol) in different core to shell flow rate ratio, form. 30: prepared by 15% PLGA (MW 153 kg/mol) and 7.5% PVA (MW 146-186 kg/mol) dissolved in 10% ethanol, form. 31: prepared by 15% PLGA (MW 153 kg/mol) and 7.5% PVA (MW 146-186 kg/mol) dissolved in 20% ethanol. C-to-S: core-to-shell flow rate.
3.3.1.6. Dual Drug-loaded Coaxial PLGA/PVA Fibres

Dual drug-loaded coaxial fibres were prepared incorporating 1% w/v pirfenidone in 15% w/v PLGA solution and 1% w/v moxifloxacin in the 7.5% w/v PVA solution. This required an outer flow rate of 2.25 mL/h in order to stabilise the jet, less than was used for the outer PLGA layer of the blank coaxial fibres, and an intermediate inner flow rate (0.75 mL/h). The prepared fibres exhibited rough surfaces, as seen in Figure 3.6.

![Figure 3.6: The surface morphology of drug-loaded coaxial fibres prepared by 1% pirfenidone loaded into 15% PLGA (MW 153 kg/mol) and 1% moxifloxacin loaded into 7.5% PVA (MW 146-186 kg/mol) showing its rough surface.](image)

When the monoaxial PLGA and PVA fibres were fabricated, the PLGA fibre demonstrated a rough and porous surface compared to the PVA fibre’s surface which was smooth and non-porous. The rough and porous surface may potentially enable the fibres to burst release its loaded drug, which might be a limitation if a sustained release system was required, as reported in Chakraborty et al. (2009) and Williams et al. (2018). Due to the presence of PLGA in the outer layer of the coaxial fibres, these fibres were similar to the surface morphology to the PLGA monoaxial fibres.

3.3.2. Fibre Size and Microscopic Analysis

Using ImageJ software on the SEM images, the fibre diameters were measured for blank and pirfenidone-loaded 15% PLGA monoaxial fibres, blank
and moxifloxacin-loaded 7.5% PVA monoaxial fibres and blank and drug-loaded PLGA/PVA coaxial fibres. These correspond to formulations 9, 13, 18, 22, 29 and 32, respectively, as described in Tables 3.2, 3.3, and 3.4, with the results presented in Table 3.5. About 70 fibres have been assessed per batch, and the mean diameters along with the standard deviations (SDs), the maximum and the minimum diameters have been calculated.
Table 3.5: Mean diameter, standard deviation (SD), maximum and minimum diameters of the blank and drug-loaded (DL) monoaxial and coaxial fibres.

<table>
<thead>
<tr>
<th>Form. #</th>
<th>Mean diameter (nm)</th>
<th>SD</th>
<th>Maximum diameter (nm)</th>
<th>Minimum diameter (nm)</th>
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<tr>
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<td>1856</td>
<td>605</td>
<td>3009</td>
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<tr>
<td>(Blank PLGA fibres)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>13</td>
<td>1958</td>
<td>582</td>
<td>3541</td>
<td>387</td>
<td></td>
</tr>
<tr>
<td>(DL PLGA fibres)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>18</td>
<td>354</td>
<td>129</td>
<td>822</td>
<td>161</td>
<td></td>
</tr>
<tr>
<td>(Blank PVA fibres)</td>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td>22</td>
<td>372</td>
<td>082</td>
<td>580</td>
<td>202</td>
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<tr>
<td>(DL PVA fibres)</td>
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<tr>
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<td>2580</td>
<td>873</td>
<td>4376</td>
<td>1012</td>
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</tr>
<tr>
<td>(Blank coaxial fibres)</td>
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<td></td>
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<tr>
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<td>3531</td>
<td>928</td>
<td>4959</td>
<td>1643</td>
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</tr>
<tr>
<td>(DL coaxial fibres)</td>
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As shown in Table 3.5, the blank and drug-loaded PLGA fibres exhibited a larger diameter (1856 and 1958 nm, respectively) than the blank and drug-loaded PVA fibres (354 and 372 nm, respectively). This was due to the higher viscosity of the PLGA polymer solution that can be visually seen than PVA solution. In addition, the large size distribution of PLGA fibres (SD of ~ 590 nm) is suggested to be due to the instability and splitting of the processing jet to multiple jets (Zargham et al., 2012). This allowed the production of a range of fibre diameters with a minimum diameter of 387 nm and a maximum diameter of 3541 nm which are directly proportional to the diameter of the jet.

However, PVA nanofibres have been spun by a more stable jet which produced a low diameter distribution range (SD of ~ 105 nm). Splitting is considered to be a defect of the electrospinning jet that involves the single stream jet being split into multiple thinner jets. This will produce fibres with smaller diameters (as a result of the thinner jets) along with the main fibres that are produced by the primary jet stream as shown in Figure 3.7. Process parameters such as increasing the applied voltage or solution flow rate and decreasing the tip-to-collector distance could influence the stability of the jet producing splitting. This disturbance in the jet stability is owing to the reduction of the jet flight time which allowed less time to distribute the charge along the jet length and less time to evaporate the solvent. This can cause a smaller jet to eject from the main jet, in order to reduce the charge per unit surface area, as explained in Zargham et al. (2012) and Williams et al. (2018). Consequently, the reproducibility of the resulting fibres will be effected.
Figure 3.7: PLGA electrospinning jet splitting due to the increase of the applied voltage. 1: PLGA solution droplet (at 0 kV), 2: PLGA solution droplet elongated prior to jet stabilisation (at 13 kV), 3: PLGA stable jet at optimum parameters (at 20 kV), 4: PLGA stable jet start to elongate and split (at 20 kV), 5: PLGA jet split, 6: PLGA jet split into 3 visible jets (at 22 kV), 7: PLGA jet split into multiple jets with the main jet is unstable (at 24 kV), 8: PLGA jet split into multiple jets with the main jet is stable (at 24 kV). The red arrow is pointed at the PLGA main stream (jet).

The high MW of the PLGA and PVA used here produced highly viscous solutions when dissolved using the same solvent at the same concentration than the lower MW polymers owing to the effective polymer chain entanglement. This entanglement and the applied voltage will normally overcome the surface tension of a polymer solution that will initiate the formation of electrospun fibres (Haider et al., 2018). The high viscosity solution will resist the electrospinning jet elongation, hence yield larger diameter fibres as described by Beglou and Haghi (2008), Fong et al. (1999), Demir et al. (2002) and Huang et al. (2003). The use of high voltage (≥ 20 kV for both PLGA and PVA monoaxial fibres) may also yield larger diameter fibres. Increasing
the applied voltage will decrease the fibres diameter owing to the reduction in the polymer solution droplet size (as shown in Figure 3.8 - needle capillary 1 and 2) until the Taylor cone is formed (Figure 3.8 - needle capillary 3); this will yield uniform fibres. By further increasing the voltage, the fibre diameter will increase owing to the ejection of more fluid in the processed jet which will result in the deformation of the Taylor cone. This allows the formation of fibres from within the needle tip as shown in Figure 3.8 - needle capillary 4. This explanation of the production of large diameter fibres by high applied voltage was reported by Demir et al. (2002) and Bhattarai et al. (2019).

![Figure 3.8: The influence of the applied voltage on the formation of the Taylor cone. Increasing the voltage gradually will form initially narrower fibres and then larger diameter fibres. The Taylor cone is the black coloured cone. Adapted from (Bhattarai et al., 2019).](image)

The larger diameters of the blank and drug-loaded coaxial fibres (formulation 29 and 32, respectively; > 2500 nm), compared to the blank and drug-loaded PLGA and PVA monoaxial fibres (< 2000 nm) suggests the successful preparation of the coaxial fibres. This result was in agreement with Han and Steck (2013) who showed that the monoaxial PCL fibres have a narrower diameter (360 nm) than the PCL/PVP coaxial fibres (582 nm) due to the
absence of other layers. The PCL/PCL/PVP triaxial fibres exhibited a large diameter of 648 and 702 nm for two different triaxial systems in this same study, owing to the presence of a third PCL layer compared to PCL/PVP coaxial fibres (Han and Steck, 2013). Nevertheless, some studies reported a contrast observation. Zhu et al. (2015) were able to obtain a larger diameter of PLGA monoaxial nanofibres compared to PLGA/PVP coaxial system. This was due to the PVP solution high conductivity which allows more stretching of the electrospinning jet, hence reducing the fibre diameter. However, the diameter results of this current study indicated that the larger diameters (> 2500 nm) of the blank and drug-loaded coaxial fibres were reliant on the solution viscosity rather than conductivity.

The difference between the coaxial and monoaxial fibres has also been assessed by TEM due to the possibility of observing the internal structure of these fibres via the transmitted electron beams. Blank fibres of PLGA, PVA, and PLGA/PVA (formulations 9, 18, and 29) have been examined. The images showed that both monoaxial PLGA and PVA fibres (Figures 3.9 - A, and B, respectively) are single layers compared to the coaxial fibres that showed clearly two layers (Figure 3.9 - C). This observation can also be seen in the TEM results of Zhao et al. (2014) for PLGA monoaxial fibres, Asran et al. (2010) for PVA monoaxial fibres and You and Zhang (2011) for PEO/PLGA coaxial fibres.
Figure 3.9: TEM images of the blank monoaxial and coaxial fibres. A: 15% PLGA fibres, B: 7.5% PVA fibres, C: 15% PLGA and 7.5% PVA coaxial fibres.

Another method of distinguishing between the blank and drug-loaded monoaxial and coaxial fibres is by investigating their appearance under light and UV fluorescence microscopy. Light microscopy images of PLGA fibres, with or without pirfenidone (formulations 13, and 9, respectively), showed that these fibres have a tube-like appearance (Figure 3.10 A1 and D1) compared to PVA fibres, with or without moxifloxacin (formulations 22, and 18, respectively), which looked like solid threads (Figure 3.10 B1 and E1). This observation is in agreement with Grafahrend et al. (2011) and Song et al. (2012) who showed similar images for PLGA fibres and PVA fibres, respectively. For coaxial unloaded and loaded fibres (formulations 29 and 32, respectively), light microscopy showed that these fibres have a similar tube-like appearance to the monoaxial PLGA fibres, but with larger diameters (Figure 3.10 C1 and F1).
Figure 3.10: Light microscopic images of the blank and drug-loaded monoaxial and coaxial fibres. A1: 15% PLGA fibres, B1: 7.5% PVA fibres, C1: 15% PLGA and 7.5% PVA coaxial fibres, D1: 1% pirfenidone loaded into 15% PLGA fibres, E1: 1% moxifloxacin loaded into 7.5% PVA fibres, F1: 1% pirfenidone loaded into 15% PLGA and 1% moxifloxacin loaded into 7.5% PVA coaxial fibres.
Fluorescence microscopy images indicated that the PLGA fibres, with and without pirfenidone, had no fluorescence appearance when viewed under this microscope due to the lack of any fluorescent molecule within this system (Figure 3.11 A2 and D2). Similarly, monoaxial PVA fibres without moxifloxacin did not show an illuminated appearance (Figure 3.11 B2). However, due to the fluorescent nature of moxifloxacin, which has excitation and emission wavelengths at about 290 and 500 nm, respectively (Chan et al., 2006; Wang et al., 2016), the drug-loaded monoaxial PVA fibres fluoresced under this fluorescence microscopy (Figure 3.11 E2). The presence of the PLGA and PVA as the outer and inner layers, respectively, would be expected to lack any illuminated appearance as seen in the blank monoaxial PLGA and PVA fibres; as it was observed in Figure 3.11 C2. The dual drug-loaded coaxial fibres were much more highly fluorescent than the unloaded coaxial fibres (Figure 3.11 F2), due to the presence of the moxifloxacin in the core of these fibres. The fluorescent intensity of the drug-loaded fibres (Figure 3.11 E2 and F2) can be an indication of the distribution of moxifloxacin within these fibrous systems, which appeared poorly distributed in the coaxial fibres compared to the PVA monoaxial fibres. Tiwari et al. (2010) were able to detect rose bengal dye (a fluorescent and hydrophilic dye) after loading this dye into the PVA inner layer of PLGA/PVA coaxial fibres by the use of confocal microscopy. However, the distribution of this dye appeared to be poor, which was consistent with the above finding.
Figure 3.11: UV microscopic images of the blank and drug-loaded monoaxial and coaxial fibres. A2: 15% PLGA fibres, B2: 7.5% PVA fibres, C2: 15% PLGA and 7.5% PVA coaxial fibres, D2: 1% pirfenidone loaded into 15% PLGA fibres, E2: 1% moxifloxacin loaded into 7.5% PVA fibres, F2: 1% pirfenidone loaded into 15% PLGA and 1% moxifloxacin loaded into 7.5% PVA coaxial fibres.
The effect of electrospinning process parameters such as voltage and solution viscosity on the diameters of electrospun fibres were assessed by SEM and light microscopy. The use of high voltage and high solution viscosity can produce large diameter fibres, in addition to the production of a coaxial system due to the presence of two fibre layers coating one another. The TEM microscopy (Figure 3.9) demonstrated the distinctive inner core of the PLGA/PVA coaxial fibres compared to the PLGA and PVA monoaxial fibres, respectively. The presence of moxifloxacin in the PVA monoaxial fibres and the PVA inner layer of the coaxial fibres was detected by the fluorescence microscopy. This was an indication for the successful encapsulation of moxifloxacin in both monoaxial and coaxial fibres. However, it was observed that moxifloxacin was poorly distributed in the coaxial fibres compared to the PVA monoaxial fibres, as shown in Figure 3.11 F2 and E2, respectively.

3.3.3. Thermal Analysis

3.3.3.1. Thermal Characterisation of the Drugs

Moxifloxacin and pirfenidone were assessed using TGA and standard DSC methods, with the results being shown in Figures 3.12 and 3.13. Moxifloxacin (Figure 3.12) showed a weight loss (3.7%) at around 100°C, most probably due to water loss, since it is the salt form, which is more likely to adsorb the atmospheric water. The onset degradation temperature was about 234°C. DSC results confirmed the water loss at 94.5°C and also demonstrated a melting point of about 227°C with a subsequent polymer degradation at around 236°C that was consistent with the TGA finding. However, this melting point is slightly lower than previously reported (238 to 242°C) in the literature (Moxifloxacin, 2008).
Figure 3.12: TGA (top image) and DSC (bottom image) traces of moxifloxacin.

Pirfenidone (Figure 3.13) showed no water loss on TGA but exhibited almost complete degradation (99.5% weight loss) when it was heated to 400°C, with an onset degradation temperature of about 138°C. The DSC traces showed a sharp melting peak at around 108°C, which was within the published pirfenidone melting point range of 107-111°C (European Medicines Agency, 2010, Mandapalli et al., 2016).
3.3.3.2. Thermal Characterisation of the Polymers

The thermal behaviour of PLGA was examined using TGA and MTDSC, with the results being presented in Figure 3.14. The high MW PLGA (153 kg/mol) showed a degradation onset temperature of 205.5°C with about 98% mass loss. MTDSC exhibited a Tg at around 48°C for this PLGA grade, while it was lower at about 40.5°C, for the middle MW PLGA (44 kg/mol). These findings were consistent with those of Makadia and Siegel (2011) and Passerini and Craig (2001).
PVA’s thermal behaviour was also observed using TGA and MTDSC, with the results being displayed in Figure 3.15. The degradation onset temperature for the high MW PVA (146 to 186 kg/mol) was about 258°C with a weight loss of 75.5%. This may represent the degradation of this polymer side chain, as
suggested by Kim et al. (2008) who reported a similar weight loss at a range of 210 to 400°C. In addition, a mass loss of around 6.7% was detected between 90°C and the degradation onset temperature, which may be due to the weakly physiosorbed water as described in Kim et al. (2008). A third possible mass loss was detected after 320°C which might be due to the degradation of the main chain (Kim et al., 2008). This PVA MW showed a Tg at 73°C and a melting point at 188°C which is in agreement with Jelinska et al. (2010) and Mallapragada and Peppas (1996). This semi-crystalline property was also shown (Figure 3.15) with the low MW PVA (31 to 50 kg/mol) with a Tg of about 75°C and a melting peak at 220°C. This was unexpected as by decreasing the polymer MW, the polymer chains become small and thus a lower melting temperature will required to break these chains (Balani et al., 2015), which will require a further investigation. The heat-cool-heat cycle was performed in order to detect both the Tg and the melting point of a material clearly after eliminating the standard heat history of that material by cooling after the first heat (PerkinElmer, 2014).
Figure 3.15: TGA (top image) and MTDSC (middle image) traces of high MW PVA, and MTDSC (bottom image) of trace low MW PVA which shows the full cycle of heat-cool-heat analysis.
3.3.3.3. Thermal Characterisation of the Polymer-drug Physical Mixtures

Physical mixtures of 15:1 high MW PLGA and pirfenidone, and 7.5:1 high MW PVA and moxifloxacin were analysed using the MTDSC and heat-cool-heat MTDSC methods, respectively. For the PLGA-pirfenidone mixture (Figure 3.16), it has been observed that the Tg of PLGA (48.1°C) appeared at the same point as observed in the raw material (~ 48°C); similarly, pirfenidone in the mixture showed a sharp melting peak (108.3°C) at a similar melting point to the drug when tested alone (~ 108°C). As a result, it is suggested that there is no clear interaction between PLGA and pirfenidone, which was also observed in the DSC results of Gupta et al. (2010) and Mohammadi et al. (2010) on PLGA-sparfloxacin and PLGA-azithromycin, respectively.

Figure 3.16: MTDSC trace of the 15:1 PLGA-pirfenidone physical mixture.

The PVA-moxifloxacin mixture (Figure 3.17), however, exhibited an overall shift in the water loss (117.2°C) and the melting point (218.5°C) peaks for moxifloxacin compared to the raw drug (~ 94.5°C for water loss and ~ 227°C for melting point). This is in contrast with the results of Novoa et al. (2005) who demonstrated no melting point shift of ibuprofen with PVA. Nonetheless, the semi-crystalline property of PVA, with a Tg of 71°C and a melting point of 187°C demonstrated in the physical mixture, were closely similar to the raw polymer. The appearance of the PVA and moxifloxacin separated peaks and
the shift of the melting point of the drug suggested a slight interaction between them, which was interpreted by Novoa et al. (2005). The 9°C decrease of the drug's melting point might suggest a small scale of solid-solid interaction between these two materials, although it does not necessarily indicate any incompatibility.

Figure 3.17: MTDSC trace of the 7.5:1 PVA-moxifloxacin physical mixture.

This thermal analysis study was an initial study to measure the degradation, melting and/or Tg temperatures of the raw PLGA and PVA polymers, as well as the raw pirfenidone and moxifloxacin drugs and to compare them with the reported values. The results will be considered later in the next chapter, Chapter 4, specifically the thermal analysis study on the electrospun fibres and in the interpretation of the solid state characterisation (XRD).

3.3.4. HPLC Analysis

Two HPLC methods were developed for the analysis of the drug-loaded monoaxial and coaxial fibres, one based on an organic extraction solvent and one based on an aqueous extraction solvent. This was to disintegrate the PLGA layer of the drug-loaded coaxial system by using the organic solvent and to detect pirfenidone within this solution. This will follow by using water to disintegrate the remaining PVA layer in order to detect moxifloxacin. A third HPLC method was developed to assess the release of the drug-loaded
monoaxial and coaxial fibres in phosphate buffer saline (PBS) at pH 7.4 that resembles the tear fluid. All HPLC assays have detected both drugs at a mutual wavelength which is 310 nm, as it can be seen in Figure 3.18. These HPLC methods were simple, rapid and sensitive.

Figure 3.18: UV absorbance scan for moxifloxacin (left image) and pirfenidone (right image) dissolved in water.

3.3.4.1. Aqueous Extraction Method

Figure 3.19 shows representative chromatograms for injections of moxifloxacin alone, pirfenidone alone, and moxifloxacin and pirfenidone in combination, using the process described in Section 3.2.2.6.1. The individual peaks generally showed a slight broad appearance that has no influence on the separation, though a degree of asymmetry with a tailing appearance was evident. This might be due to injecting high volume of samples into the column, the drugs being adsorbed strongly onto active sites within the column, or poor trapping of these drugs when injected into the column (Watson, 2005). There was complete separation between both moxifloxacin and pirfenidone in the combined samples, with no overlapping peaks. Additionally, the retention times of the components of the combined solution were similar, but slightly shorter than those of the individual solutions.
Figure 3.19: Representative HPLC chromatograms using the aqueous extraction method. Moxifloxacin (50 µg/ml) in water (top image), pirfenidone (50 µg/ml) in water (middle image) and moxifloxacin (50 µg/ml) and pirfenidone (50 µg/ml) in combination (bottom image).

In order to assess the method performance, standard curves for the individual and combined solutions were generated. Ten standard concentrations (points) ranging from 0.39 to 200 µg/mL containing each drug in separate aqueous solutions were prepared. The solutions were injected into the HPLC column in a volume of 50 µL and the peak areas were recorded at 310 nm. The analysis was repeated over three consecutive days, labelled day 1, day 2 and day 3, using the same solutions.
3.3.4.1.1. Moxifloxacin alone

The obtained peaks for moxifloxacin had a retention time (RT) between 3 and 5 minutes with a slight broad appearance. The standard calibration curves were constructed by plotting the peak area against the respective concentration. The regression equations and correlation coefficients ($R^2$) were $Y = 127.73X + 6.08$ and $R^2 = 0.9992$ for day 1, $Y = 128.00X - 21.09$ and $R^2 = 0.9992$ for day 2, and $Y = 122.19X - 14.30$ and $R^2 = 0.9982$ for day 3, as shown in Figure 3.20. For all three curves, the $R^2$ values prove that the methods were linear in the specified range. Table 3.6 summarises the overall results for moxifloxacin alone.

Figure 3.20: Standard curves for HPLC aqueous extraction of moxifloxacin in three successive days. All curves show fair linearity ($R^2 \geq 0.998$).
Table 3.6: Mean peak areas and retention times for day 1, 2 and 3 using HPLC aqueous extraction of moxifloxacin.

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<th>Moxifloxacin concentration (µg/ml)</th>
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<th>Day 1 RT (min)</th>
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<th>Day 2 Mean Peak area, n=3 (mAU*sec)</th>
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<th>Day 2 RT (min)</th>
<th>SD</th>
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3.3.4.1.2. Pirfenidone alone

The obtained pirfenidone peaks had a slight broad shape with RT between 7 and 9 minutes. The standard calibration curves were constructed by plotting the peak area against the respective concentration. The regression equations and $R^2$ values were $Y = 10.59X - 0.15$ and $R^2 = 1.0000$ for day 1, $Y = 10.35X - 0.23$ and $R^2 = 0.9999$ for day 2, and $Y = 10.14 X - 0.30$ and $R^2 = 0.9999$ for day 3, as shown in Figure 3.21. Again, the $R^2$ values prove the linearity over the specified range. Table 3.7 summarises the overall results for pirfenidone alone.

![Figure 3.21: Standard curves for HPLC aqueous extraction of pirfenidone in three successive days. All curves show excellent linearity ($R^2 \geq 0.9999$).]
Table 3.7: Mean peak areas and retention times for day 1, 2 and 3 using HPLC aqueous extraction of pirfenidone.

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<th>Pirfenidone concentration (µg/ml)</th>
<th>Day 1 Mean Peak area, n=3 (mAU*sec)</th>
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<th>Day 1 RT (min)</th>
<th>SD</th>
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3.3.4.1.3. Moxifloxacin and pirfenidone in combination

The obtained separated peaks for both moxifloxacin and pirfenidone were in similar shapes as the individually obtained drugs’ peaks with moxifloxacin RT between 3 and 4 minutes, and pirfenidone RT between 6 and 8 minutes. The standard calibration curves were constructed by plotting the peak area against the respective concentration. The regression equations and R² values for moxifloxacin were \( Y = 125.57X - 20.17 \) and \( R² = 0.9992 \) for day 1, \( Y = 123.18X - 5.51 \) and \( R² = 0.9976 \) for day 2, and \( Y = 120.61X - 4.06 \) and \( R² = 0.9977 \) for day 3, as shown in Figure 3.22. The regression equations and R² values for pirfenidone were \( Y = 10.47X - 0.04 \) and \( R² = 0.9999 \) for day 1, \( Y = 10.34X - 0.21 \) and \( R² = 0.9999 \) for day 2, and \( Y = 10.06X - 0.18 \) and \( R² = 0.9999 \) for day 3, as shown in Figure 3.22. The regression equations for both drugs separately and in combination are very similar, suggesting that the developed HPLC method was efficient at separating the two compounds after injection within the same solution and was linear over the same range as the separate solutions. Table 3.8 summarises the overall results for moxifloxacin and pirfenidone in combination.
Figure 3.22: Standard curves for HPLC aqueous extraction of moxifloxacin and pirfenidone in combination in three successive days. Moxifloxacin curves show fair linearity \((R^2 \geq 0.998)\), while pirfenidone curves show excellent linearity \((R^2 \geq 0.9999)\).
Table 3.8: Mean peak areas and retention times for day 1, 2 and 3 using HPLC aqueous extraction of moxifloxacin and pirfenidone combined.

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<td>6.63</td>
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<td>31.93</td>
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<td>31.17</td>
<td>0.10</td>
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</tr>
<tr>
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<td>0.30</td>
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<td>15.87</td>
<td>0.20</td>
<td>6.47</td>
<td>0.03</td>
<td>15.57</td>
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</tr>
<tr>
<td>0.78</td>
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<td>0.10</td>
<td>7.70</td>
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<td>3.97</td>
<td>0.10</td>
<td>6.35</td>
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<td>3.83</td>
<td>0.10</td>
<td>7.39</td>
<td>0.02</td>
</tr>
</tbody>
</table>
In order to assess the precision of this separation method, three concentration levels (low, medium, and high) for each drug and for their combinations were analysed for intraday and interday variation. For the intraday investigation, three replicates of solutions of 1.563, 12.5 and 100 µg/mL were analysed on the same day (day 1 of the analysis). Interday analysis was performed by studying the same points every day during three consecutive days, with three replicates at each concentration assayed per day. Table 3.9 demonstrates the intraday and interday variations between these three points by calculating the relative standard deviation percentage (RSD %).

Table 3.9: Moxifloxacin and pirfenidone inter- and intra-day variations in HPLC aqueous extraction.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Moxifloxacin</td>
<td>0.50</td>
<td>14.54</td>
<td>0.14</td>
<td>2.53</td>
<td>0.23</td>
<td>3.00</td>
</tr>
<tr>
<td>Pirfenidone</td>
<td>0.70</td>
<td>2.40</td>
<td>0.78</td>
<td>2.51</td>
<td>0.12</td>
<td>2.17</td>
</tr>
<tr>
<td>Moxifloxacin and Pirfenidone</td>
<td>0.15</td>
<td>1.74</td>
<td>0.28</td>
<td>0.62</td>
<td>0.17</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>1.90</td>
<td>3.07</td>
<td>0.15</td>
<td>1.73</td>
<td>0.19</td>
<td>1.48</td>
</tr>
</tbody>
</table>

The intraday variations between samples are small for all concentrations, less than 2% in all cases. According to Parmar et al. (2014) and Dewani et al. (2011) this demonstrates good precision. On the other hand, all the points have interday variations of more than 2%, except for moxifloxacin and pirfenidone when they are in combination at the middle and high concentrations. As a result, the dissolving of both drugs in water can be precise for intraday analysis, while upon storage over several days, there is a risk of instability.
3.3.4.2. Organic Extraction Method

Figure 3.23 shows representative chromatograms for injections of moxifloxacin alone, pirfenidone alone, and moxifloxacin and pirfenidone in combination, using the process described in Section 3.2.2.6.2. The individual peaks generally showed a slight broad shape that has no influence on the separation, but displayed a degree of asymmetry with a tailing appearance. This degree of asymmetry was also seen in Parmar et al. (2014) and Dewani et al. (2011) who analysed pirfenidone and moxifloxacin in methanol, respectively. This might be due to injecting high volume of samples into the column, the drugs being adsorbed strongly onto active sites within the column or poor trapping of these drugs when injected into the column (Watson, 2005). There was complete separation between both moxifloxacin and pirfenidone in the combined samples, with no overlapping peaks. Additionally, the retention times of the components of the combined solution were similar to those of the individual solutions.
Figure 3.23: Representative HPLC chromatograms using the organic extraction method. Moxifloxacin (18.75 µg/ml) in ACTN (top image), pirfenidone (18.75 µg/ml) in ACTN (middle image) and moxifloxacin (18.75 µg/ml) and pirfenidone (18.75 µg/ml) in combination (bottom image).

In order to assess the method performance, standard curves for the individual and combined solutions were generated. Ten standard concentrations ranging from 0.147 to 75 µg/mL containing each drug in separate organic (ACTN) solutions were prepared. The solutions were injected into the HPLC column in a volume of 20 µL and the peak areas were recorded at 310 nm. The analysis was repeated over three consecutive days, labelled day 1, day 2 and day 3, using the same solutions.
3.3.4.2.1. Moxifloxacin alone

The obtained peaks for moxifloxacin had a slight broad shape with RT of about 2.7 minutes. The standard calibration curves were constructed by plotting the peak area against the respective concentration. The regression equations and R² values were $Y = 40.39X - 4.07$ and $R^2 = 0.9996$ for day 1, $Y = 40.60X - 6.15$ and $R^2 = 0.9995$ for day 2, and $Y = 39.53X - 6.00$ and $R^2 = 0.9995$ for day 3, as shown in Figure 3.24. The R² for the three curves is the evidence of linearity in the specified range. Dewani et al. (2011) were able to obtain an R² of 0.999 for moxifloxacin dissolved in methanol. Table 3.10 summarises the overall results for moxifloxacin alone.

![Figure 3.24: Standard curves for HPLC organic extraction of moxifloxacin in three successive days. All curves show good linearity ($R^2 \geq 0.9995$).](image-url)
Table 3.10: Mean peak areas and retention times for day 1, 2 and 3 using HPLC organic extraction of moxifloxacin.

<table>
<thead>
<tr>
<th>Moxifloxacin concentration (µg/ml)</th>
<th>Day 1 Mean Peak area, n=3 (mAU*sec)</th>
<th>SD</th>
<th>Day 1 RT (min)</th>
<th>SD</th>
<th>Day 2 Mean Peak area, n=3 (mAU*sec)</th>
<th>SD</th>
<th>Day 2 RT (min)</th>
<th>SD</th>
<th>Day 3 Mean Peak area, n=3 (mAU*sec)</th>
<th>SD</th>
<th>Day 3 RT (min)</th>
<th>SD</th>
</tr>
</thead>
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<td>39.83</td>
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<td>2.67</td>
<td>0.04</td>
<td>37.93</td>
<td>0.67</td>
<td>2.67</td>
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<td>0.15</td>
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3.3.4.2.2. Pirfenidone alone

The obtained pirfenidone peaks had a slight broad shape with RT of about 6.1 minutes. The standard calibration curves were constructed by plotting the peak area against the respective concentration. The regression equations and $R^2$ values were $Y = 27.32X + 0.24$ and $R^2 = 0.9999$ for day 1, $Y = 27.39X - 0.23$ and $R^2 = 0.9999$ for day 2, and $Y = 27.49X - 0.59$ and $R^2 = 0.9999$ for day 3, as shown in Figure 3.25. These results agree with those in Parmar et al. (2014) who showed an $R^2$ of 0.999 for pirfenidone dissolved in methanol. Table 3.1 summarizes the overall results for pirfenidone alone.

![Figure 3.25: Standard curves for HPLC organic extraction of pirfenidone in three successive days. All curves show excellent linearity ($R^2 \geq 0.9997$).](image)
Table 3.11: Mean peak areas and retention times for day 1, 2 and 3 using HPLC organic extraction of pirfenidone.

<table>
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<tr>
<th>Pirfenidone concentration (µg/ml)</th>
<th>Day 1 Mean Peak area, n=3 (mAU*sec)</th>
<th>SD</th>
<th>Day 1 RT (min)</th>
<th>SD</th>
<th>Day 2 Mean Peak area, n=3 (mAU*sec)</th>
<th>SD</th>
<th>Day 2 RT (min)</th>
<th>SD</th>
<th>Day 3 Mean Peak area, n=3 (mAU*sec)</th>
<th>SD</th>
<th>Day 3 RT (min)</th>
<th>SD</th>
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</tr>
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<td>6.10</td>
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<tr>
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<td>0.01</td>
<td>31.93</td>
<td>0.06</td>
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<td>0</td>
<td>32.23</td>
<td>0.40</td>
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<tr>
<td>0.586</td>
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<td>0.01</td>
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<td>0.12</td>
<td>6.11</td>
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</tr>
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</table>
3.3.4.2.3. Moxifloxacin and pirfenidone in combination

The obtained separated peaks for both moxifloxacin and pirfenidone were in similar shapes as the individually obtained drugs’ peaks with a moxifloxacin RT of about 2.7 minutes and a pirfenidone RT of about 6.1 minutes. The standard calibration curves were constructed by plotting the peak area against the respective concentration. The regression equations and R² values for moxifloxacin were $Y = 40.47X - 5.04$ and $R^2 = 0.9999$ for day 1, $Y = 40.31X - 5.44$ and $R^2 = 0.9999$ for day 2, and $Y = 39.98X - 5.89$ and $R^2 = 0.9996$ for day 3, as shown in Figure 3.26. However, the regression equations and R² for pirfenidone were $Y = 27.39X + 0.12$ and $R^2 = 1.0000$ for day 1, $Y = 27.40X - 0.33$ and $R^2 = 0.9999$ for day 2, and $Y = 27.39X - 0.44$ and $R^2 = 0.9999$ for day 3, as shown in Figure 3.26. The R² values for this separation confirm the linearity. The regression equations for both drugs separately and in combination are very similar, suggesting that the developed HPLC method was efficient at separating the two compounds after injection within the same solution, and was linear over the same range as the separate solutions. Table 3.12 summarises the overall results for moxifloxacin and pirfenidone in combination.
Figure 3.26: Standard curves for HPLC organic extraction of moxifloxacin and pirfenidone in combination in three successive days. All curves show excellent linearity ($R^2 \geq 0.9996$).
Table 3.12: Mean peak areas and retention times for day 1, 2 and 3 using HPLC organic extraction of moxifloxacin and pirfenidone combined.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Day 1 Mean Peak area, n=3 (mAU*sec)</th>
<th>SD</th>
<th>Day 1 RT (min)</th>
<th>Day 2 Mean Peak area, n=3 (mAU*sec)</th>
<th>SD</th>
<th>Day 2 RT (min)</th>
<th>Day 3 Mean Peak area, n=3 (mAU*sec)</th>
<th>SD</th>
<th>Day 3 RT (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moxifloxacin</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>2.65</td>
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<td>0.01</td>
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<td>2.65</td>
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<td>6.33</td>
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<td>2.65</td>
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<td>0.15</td>
<td>2.66</td>
<td>0.02</td>
<td>2.03</td>
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<td></td>
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<td></td>
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<td></td>
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<td>1024</td>
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<td>1.33</td>
<td>6.10</td>
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<td>0.01</td>
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<td>1.172</td>
<td>31.93</td>
<td>0.49</td>
<td>6.10</td>
<td>0</td>
<td>31.73</td>
<td>0.32</td>
<td>6.10</td>
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<tr>
<td>0.586</td>
<td>15.73</td>
<td>0.35</td>
<td>6.10</td>
<td>0</td>
<td>15.70</td>
<td>0</td>
<td>6.11</td>
<td>0</td>
<td>15.23</td>
</tr>
<tr>
<td>0.293</td>
<td>7.80</td>
<td>0.20</td>
<td>6.10</td>
<td>0.01</td>
<td>7.333</td>
<td>0.12</td>
<td>6.11</td>
<td>0.01</td>
<td>7.27</td>
</tr>
<tr>
<td>0.147</td>
<td>3.73</td>
<td>0.12</td>
<td>6.10</td>
<td>0</td>
<td>3.70</td>
<td>0.10</td>
<td>6.11</td>
<td>0.02</td>
<td>3.73</td>
</tr>
</tbody>
</table>
Three concentration levels (low, medium, and high) for each drug and for their combination were analysed for intraday and interday variability to assess the separation precision. For the intraday investigation, three replicates of 0.586, 4.688, and 37.5 µg/ml were analysed on the same day (day 1 of the analysis). Interday analysis was performed by studying the same points every day during three consecutive days. Three replicates at each concentration were assayed per day. Table 3.13 demonstrates the intraday and interday variations between these three points by calculating the RSD %. Dewani et al. (2011) and Parmar et al. (2014) were able to obtain an intraday and interday variations RSD % less than 2% for both moxifloxacin and pirfenidone, respectively, which are considered as good precision.

Table 3.13: Moxifloxacin and pirfenidone inter- and intra-day variations in HPLC organic extraction.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Moxifloxacin</td>
<td>1.48</td>
<td>3.34</td>
<td>0.98</td>
<td>1.51</td>
<td>0.33</td>
<td>0.96</td>
</tr>
<tr>
<td>Pirfenidone</td>
<td>1.10</td>
<td>3.48</td>
<td>0.08</td>
<td>0.43</td>
<td>0.11</td>
<td>0.44</td>
</tr>
<tr>
<td>Moxifloxacin and</td>
<td>1.41</td>
<td>3.28</td>
<td>0.97</td>
<td>1.70</td>
<td>0.04</td>
<td>0.53</td>
</tr>
<tr>
<td>Pirfenidone</td>
<td>2.23</td>
<td>1.80</td>
<td>0.08</td>
<td>0.14</td>
<td>0.02</td>
<td>0.25</td>
</tr>
</tbody>
</table>

As shown in the table, the intraday variations show very similar results to the aqueous extraction method and were less than 2%, which agree with Parmar et al., 2014 and Dewani et al., 2011, except for low concentration pirfenidone in combination with moxifloxacin. All of the middle and high points have very low intraday variations, while the low concentrations showed some intraday deviations. The interday variations were greater than the intraday variations, especially for the low concentrations and may reflect a degree of instability.
3.3.4.3. Phosphate Buffer Extraction Method

Figure 3.27 shows representative chromatograms for an injection of moxifloxacin and pirfenidone in combination, using the process described in Section 3.2.2.6.3. The individual peaks generally showed a slight broad appearance, which has no influence on the separation but displayed a degree of asymmetry with a tailing. This might be due to injecting high volume of samples into the column, the drugs being adsorbed strongly onto active sites within the column, or poor trapping of these drugs when injected into the column (Watson, 2005). There was complete separation between the moxifloxacin and the pirfenidone in the combined samples, with no overlapping peaks.

Figure 3.27: Representative HPLC chromatograms using the buffer extraction method. Moxifloxacin (12.5 µg/ml) and pirfenidone (12.5 µg/ml) in combination.

In order to assess the method performance, standard curves for the combined drug solution was generated. Nine standard concentrations ranging from 0.39 to 100 µg/mL containing both drugs in PBS were prepared. The solutions were injected into the HPLC column in a volume of 50 µL and the peak areas were recorded at 310 nm. The analysis was repeated over three consecutive days, labelled day 1, day 2 and day 3, using the same solutions.
3.3.4.3.1. Moxifloxacin and pirfenidone in combination

The obtained separated peaks for both moxifloxacin and pirfenidone were slightly broad in shape with a RT of around 2.99 minutes and 5.8 minutes for moxifloxacin and pirfenidone, respectively. The standard calibration curves were constructed by plotting the peak area against the respective concentration. The regression equations and $R^2$ values for moxifloxacin were $Y = 109.62X + 3.16$ and $R^2 = 0.9991$ for day 1, $Y = 105.46X + 4.27$ and $R^2 = 0.9994$ for day 2, and $Y = 106.15X + 6.69$ and $R^2 = 0.9987$ for day 3, as shown in Figure 3.28. The regression equations and $R^2$ values for pirfenidone were $Y = 94.54X + 2.34$ and $R^2 = 0.9991$ for day 1, $Y = 92.66X + 3.85$ and $R^2 = 0.9999$ for day 2, and $Y = 93.41X + 4.19$ and $R^2 = 0.9999$ for day 3, as shown in Figure 3.28. The regression equations for both drugs in combination suggested that the developed HPLC method was efficient at separating the two compounds after injection within the same solution. Table 3.14 summarises the overall results for moxifloxacin and pirfenidone in combination.

Figure 3.28: Standard curves for HPLC buffer extraction of moxifloxacin and pirfenidone in combination in three successive days. All curves show good linearity ($R^2 \geq 0.999$).
Table 3.14: Mean peak areas and retention times for day 1, 2 and 3 using HPLC buffer extraction of moxifloxacin and pirfenidone combined.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Day 1 Mean Peak area, n=3 (mAU*sec)</th>
<th>SD</th>
<th>Day 1 RT (min)</th>
<th>SD</th>
<th>Day 2 Mean Peak area, n=3 (mAU*sec)</th>
<th>SD</th>
<th>Day 2 RT (min)</th>
<th>SD</th>
<th>Day 3 Mean Peak area, n=3 (mAU*sec)</th>
<th>SD</th>
<th>Day 3 RT (min)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moxifloxacin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>100</td>
<td>10453.37</td>
<td>22.76</td>
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<td>0</td>
<td>10407.13</td>
<td>7.02</td>
<td>2.98</td>
<td>0</td>
<td>10343.23</td>
<td>5.95</td>
<td>2.98</td>
<td>0</td>
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<tr>
<td>50</td>
<td>5287.67</td>
<td>1.72</td>
<td>2.99</td>
<td>0</td>
<td>5306.40</td>
<td>11.62</td>
<td>2.98</td>
<td>0</td>
<td>5305.63</td>
<td>7.11</td>
<td>2.98</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>2727.83</td>
<td>4.06</td>
<td>2.98</td>
<td>0.01</td>
<td>2741.67</td>
<td>3.72</td>
<td>2.98</td>
<td>0</td>
<td>2742.63</td>
<td>3.43</td>
<td>2.98</td>
<td>0</td>
</tr>
<tr>
<td>12.5</td>
<td>1399.73</td>
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<td>2.98</td>
<td>0</td>
<td>1411.47</td>
<td>6.95</td>
<td>2.98</td>
<td>0</td>
<td>1409.17</td>
<td>1.42</td>
<td>2.97</td>
<td>0</td>
</tr>
<tr>
<td>6.25</td>
<td>680.20</td>
<td>0.82</td>
<td>2.97</td>
<td>0.01</td>
<td>681.20</td>
<td>1.92</td>
<td>2.97</td>
<td>0</td>
<td>678.13</td>
<td>1.70</td>
<td>2.97</td>
<td>0</td>
</tr>
<tr>
<td>3.125</td>
<td>341.17</td>
<td>0.21</td>
<td>2.97</td>
<td>0</td>
<td>342.87</td>
<td>0.75</td>
<td>2.97</td>
<td>0</td>
<td>341.23</td>
<td>0.32</td>
<td>2.97</td>
<td>0</td>
</tr>
</tbody>
</table>
| 1.563                 | 173.57                               | 0.29  | 2.97           | 0  | 173.80                               | 0.44  | 2.97           | 0  | 173.97                               | 0.12  | 2.96           | 0.01 |}

| Pirfenidone           |                                      |    |                |    |                                      |    |                |    |                                      |    |                |    |
| 100                   | 9178.07                              | 4.50  | 5.82           | 0.01 | 9241.87                              | 5.26  | 5.78           | 0.01 | 9311.93                              | 5.88  | 5.77           | 0.01 |}

| 50                    | 4575.83                              | 0.93  | 5.82           | 0.01 | 4597.43                              | 16.92 | 5.77           | 0.01 | 4602.43                              | 12.12 | 5.77           | 0.01 |}
| 25                    | 2304.17                              | 1.12  | 5.81           | 0  | 2310.17                              | 3.43  | 5.78           | 0  | 2314.3                               | 4.33  | 5.77           | 0  |
| 12.5                  | 1164.80                              | 0.40  | 5.80           | 0  | 1160.87                              | 5.58  | 5.77           | 0.01 | 1162.53                              | 1.01  | 5.77           | 0  |
| 6.25                  | 600.47                               | 0.50  | 5.80           | 0  | 599.70                               | 1.48  | 5.79           | 0  | 600.90                               | 1.42  | 5.77           | 0  |
| 3.125                 | 298.90                               | 0.26  | 5.81           | 0.01 | 298.07                               | 1.18  | 5.79           | 0.01 | 297.90                               | 0.17  | 5.77           | 0.01 |}
| 1.563                 | 150.03                               | 0.32  | 5.80           | 0.01 | 149.60                               | 0.17  | 5.79           | 0  | 150.20                               | 0.004 | 5.77           | 0  |
| 0.78                  | 76.60                                | 0.004 | 5.79           | 0.01 | 76.47                                | 0.06  | 5.79           | 0.01 | 76.67                                | 0.058 | 5.77           | 0  |
| 0.39                  | 38.70                                | 0.004 | 5.79           | 0.01 | 38.60                                | 0.10  | 5.78           | 0  | 38.50                                | 0.27  | 5.77           | 0  |
Three concentration levels (low, medium, and high) for each drug and for their combination were analysed for intraday and interday variation to assess the method precision (Dewani et al., 2011). For the intraday investigation, three replicates of 0.78, 6.25 and 50 µg/mL were analysed on the same day (day 1 of the analysis). Interday analysis was performed by studying the same points every day during three consecutive days. Three replicates at each concentration were assayed per day. Table 3.15 demonstrates the intraday and interday variations between these three points by calculating the RSD %.

Table 3.15: Moxifloxacin and pirfenidone inter- and intra-day variations in HPLC buffer extraction.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Moxifloxacin and</td>
<td>0.24</td>
<td>0.28</td>
<td>0.12</td>
<td>0.23</td>
<td>0.03</td>
<td>0.20</td>
</tr>
<tr>
<td>Pirfenidone</td>
<td>0.01</td>
<td>0.13</td>
<td>0.08</td>
<td>0.10</td>
<td>0.02</td>
<td>0.31</td>
</tr>
</tbody>
</table>

According to the table, the intraday and interday variations between samples are small for all concentrations, less than 2% in all cases. According to Parmar et al. (2014) and Dewani et al. (2011) this demonstrates good precision. As a result, the dissolving of both drugs in PBS can be precise for intraday and interday analysis, hence, the risk of instability upon storage is negligible.

3.3.4.4. Limit of Detection and Limit of Quantitation

Overall, the three previous extraction methods showed high resolution of the two compounds and a good degree of reliability, both intraday and interday. The limit of detection (LOD) and limit of quantitation (LOQ) can be defined as the lowest concentration of a drug that can be detected or quantified, respectively, with acceptable precision (Ravisankar et al., 2014). They also represent a signal to noise ratio. LOD and LOQ have been determined based
on the linear regression equations for each drug in the three systems as follows:

\[ \text{LOD} = \frac{3.3 \times \text{Standard Deviation of Intercept}}{\text{Slope}} \]  

Equation 3.4

\[ \text{LOQ} = \frac{10 \times \text{Standard Deviation of Intercept}}{\text{Slope}} \]  

Equation 3.5

Table 3.16 shows the LOD and LOQ of both drugs in the three methods. Interestingly, both drugs showed more sensitivity in the buffer method of extraction compared to the aqueous and the organic methods.

**Table 3.16: LOD and LOQ of moxifloxacin and pirfenidone in all HPLC methods. Moxi: moxifloxacin, pir: pirfenidone.**

<table>
<thead>
<tr>
<th>Method</th>
<th>Aqueous extraction</th>
<th>Organic extraction</th>
<th>Phosphate buffer extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug</td>
<td>Moxi</td>
<td>Pir</td>
<td>Moxi</td>
</tr>
<tr>
<td><strong>LOD (µg/mL)</strong></td>
<td>1.05</td>
<td>0.61</td>
<td>0.29</td>
</tr>
<tr>
<td><strong>LOQ (µg/mL)</strong></td>
<td>3.17</td>
<td>1.86</td>
<td>0.87</td>
</tr>
</tbody>
</table>

The development of these HPLC methods was essential in order to measure the DL, EE% and the release of both moxifloxacin and pirfenidone, alone or combined, in the electrospun fibres. The separation of both pirfenidone and moxifloxacin by HPLC using aqueous and organic solvents were used in the next section, Section 3.3.5, as well as, the next chapter. This is to measure the DL and EE% of pirfenidone and moxifloxacin loaded in either a single layered or a multi-layered fibres. The separation of both drugs by HPLC using phosphate buffer was used in the next chapter, Chapter 4, in order to estimate the release amount of pirfenidone and moxifloxacin from the monoaxial and the multi-layered fibrous systems.
3.3.5. Drug loading [DL], Encapsulation Efficiency [EE%] and Yield [Y%]

In order to assess the DL and EE% of the monoaxial and coaxial fibres, samples of PLGA-pirfenidone fibres (formulation 13 - 15% PLGA and 1% pirfenidone), PVA-moxifloxacin fibres (formulation 22 - 7.5% PVA and 1% moxifloxacin), and PLGA-pirfenidone/PVA-moxifloxacin coaxial fibres (formulation 32 - 15% PLGA-1% pirfenidone and 7.5% PVA-1% moxifloxacin), were assessed using the developed HPLC separation assays (aqueous and organic separation assays).

For the pirfenidone loaded PLGA fibres, the obtained yield was 30.83%. This low yield is probably due to the unstable jet (whipping) that produces a polymeric gel, the sticking of the polymer solution in the spraying tube and material loss during the fabrication onto the side walls of the instrument's protection guard. After completely dissolving a measured amount of collected fibres (148 mg) in 120 mL of ACTN, a 1 mL sample was withdrawn, centrifuged at 10,000 RPM for 5 minutes, then analysed using the organic extraction HPLC process and the relevant sample regression equation as described above. The calculated DL was 55.75 µg/mg and the EE% was 89.2%.

The yield of the moxifloxacin loaded PVA fibres was around 16.9%. This might be due to the sticking of these fibres to the collecting material (parafilm) and the spraying tube, as well as loss during the fabrication. The full amount (43 mg) of the PVA-moxifloxacin fibres was dissolved in 300 mL of distilled water, then 1 mL was withdrawn, centrifuged and analysed using the aqueous extraction HPLC process and the relevant sample regression equation as described above. The DL was 107.85 µg/mg and the EE% was 91.7%.

For the coaxial formulation, a yield of 15.5% was obtained. In addition, the amount that is produced as a result of the jet whipping is about 33.8% of the total starting amount. This product had a gel like appearance, which mainly consists of PLGA, and it will be later referred to as 'gel', as it can be seen in Figure 3.29.
The extraction of the two drugs from these fibres has been completed in two phases. The first phase involves the dissolving of fibres (21.2 mg ± 1.2 mg) in 30 mL ACTN, then after the complete dissolution of the outer layer of PLGA, both drugs can be detected using the organic extraction HPLC process and the relevant sample regression equations as described above. The second phase involves the dissolution of the internal PVA layer, which remained suspended in the first organic solution, in 5 mL distilled water. Both drugs can then be detected using the aqueous extraction HPLC process and the relevant sample regression equations as described above.

Three different regions of the yielded fibres have been examined using this double extraction method. In addition, the gel-like product that resulted due to jet whipping was assessed by dissolving the full amount (363 mg) in 200 mL.
ACTN only, as it was completely dissolved in this phase. Tables 3.17 summarises the results of this analysis.

Table 3.17: Formulation 32 - DL and EE% of pirfenidone and moxifloxacin in the outer and inner layer of the coaxial fibres.

<table>
<thead>
<tr>
<th>Sub-batch no.</th>
<th>DL in PLGA outer layer (µg/mg) ± SD</th>
<th>EE in PLGA outer layer (%) ± SD</th>
<th>DL in PVA inner layer (µg/mg) ± SD</th>
<th>EE in PVA inner layer (%) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pirfenidone (n=3)</td>
<td>57.63 ± 5.89</td>
<td>103.17 ± 10.93</td>
<td>0.04 ± 0.01</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>Moxifloxacin (n=3)</td>
<td>2.13 ± 0.87</td>
<td>16.87 ± 6.84</td>
<td>0.36 ± 0.01</td>
<td>2.80 ± 0.10</td>
</tr>
<tr>
<td>Gel – Pirfenidone</td>
<td>34.29</td>
<td>61.40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gel – Moxifloxacin</td>
<td>5.25</td>
<td>41.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

As it can be seen in Table 3.17, pirfenidone has been successfully loaded in the PLGA outer phase of the coaxial fibres, and there was no diffusion of the drug into the inner PVA core. This compartmentalisation of the pirfenidone into the PLGA outer layer of the coaxial fibres was suggested in order to deliver this drug in a sustain release profile after administration on the site of action.

The variability in the calculated EE values is likely a reflection of the process capability and needs further investigation. In addition, moxifloxacin was also detected in the analysis of the PLGA ACTN solution. It is not clear whether this is as a result of the analytical process, i.e. the moxifloxacin is moving from the core PVA fibre into the ACTN during the testing process, or whether the drug is located on the surface of the inner PVA core so it will be available immediately to the dissolution medium. The apparent lack of moxifloxacin in the coaxial fibres is worrying, especially as it was encapsulated well (EE% of 91.7%) in the monoaxial fibres. This low encapsulation of the core drug, moxifloxacin, can be considered as a limitation of this coaxial system, along with its low yield (15.5%), which will require a further optimisation. This result has further confirmed the fluorescence microscopy image of the drug-loaded coaxial fibres that observed a poorly moxifloxacin distribution compared to
moxifloxacin loaded PVA monoaxial fibres, which are shown in Figure 3.11 F2 and E2, respectively.

A previous study by Gagandeep et al. (2014) observed a high EE % of 95.2% w/w for PCL nanofibres and 96.4% w/w for PVA nanofibres loaded with the combination of timolol maleate and dorzolamide hydrochloride in each fibrous system for the treatment of glaucoma. Sofokleous et al. (2013) successfully prepared amoxicillin loaded PLGA microfibres for wound dressing purposes. These fibres had an EE % of about 90%. Finally, Taepaiboone et al. (2006) have fabricated four PVA nanofibre spun mats, each mat containing a different drug, with the actual DL ranging between 81 and 98%. These previous electrospinning studies have recorded a very high DL and EE% values for their prepared fibres, which indicate the low EE% drawback of the current coaxial fibrous system.
3.4. Conclusions

Pirfenidone-PLGA/moxifloxacin-PVA coaxial fibres were prepared using a coaxial electrospinning technique. In order to fully assess this formulation, monoaxial fibres of PVA and PLGA, both blank and drug-loaded, and blank coaxial fibres were also fabricated. Different processing parameters have been considered during the manufacturing of all the fibres. Polymer MW and concentration, drug concentration, and the voltage of the electrospinning instrument were the main parameters studied. These factors affected the morphological appearance of the produced fibres.

Different microscopic imaging techniques have been used to observe the morphology of the yielded fibres. SEM results illustrate the surface characteristics and how increasing the voltage can decrease the formation of pores that present on the surface of the PLGA fibres and the use of highly evaporating solvent (DCM) could produce a rougher surface on these fibres. In addition, monoaxial fibres have shown a mean fibre diameter of 1850 nm for the blank PLGA fibres and 1950 nm for the drug-loaded PLGA fibres, while the blank and drug-loaded PVA fibres have been 350 nm and 370 nm, respectively. However, the coaxial mean fibre diameter has shown to be 2580 nm for the blank fibres and 3530 nm for the drug-loaded fibres. TEM findings have shown the successful coaxial preparation of the blank fibres. Fluorescence microscopy visually indicated the encapsulation of moxifloxacin, which has fluorescent properties, in the core of the coaxial electrospun fibres rather than the shell.

Finally, the DL and EE% of the monoaxial drug-loaded fibres indicated high encapsulation for both moxifloxacin and pirfenidone. However, the coaxial system demonstrated a successful encapsulation of pirfenidone compared to moxifloxacin that exhibited a very low EE% (~ 20%). This drawback of the coaxial fibres along with the low yield (15.5%) will require further optimisation in order to improve this delivery system, which will take place in the next chapter.
Chapter 4

Preparation of PLGA-Pirfenidone/PVP-Moxifloxacin Multi-Layered Fibres
4.1. Introduction

Coaxial and multiaxial electrospinning are techniques which involve the preparation of fibres comprising two or more layers. This is useful in order to incorporate more than one active compound in separate compartments, or to control the release of that compound by coating the inner layer with one or more additional layers (Tiwari et al., 2010).

In the previous chapter, coaxial fibres loaded with a broad-spectrum antibiotic, moxifloxacin, and an anti-scarring agent, pirfenidone, have been prepared. This combination is intended to accelerate the wound healing of corneal abrasion and to prevent or inhibit any associated infection. The water insoluble polymer PLGA is present as the outer layer in order to load pirfenidone and to sustain its release. On the other hand, the water soluble polymer PVA was used to incorporate moxifloxacin in order to allow for immediate release after the aqueous humour comes into contact with the core of these fibres. The manufacturing optimisation of this system has involved the changing of electrospinning parameters for the monoaxial systems such as; voltage, tip-to-collector distance and the concentration and the solvent used in the PVA spinning solution. The microscopy images have shown that by using a high evaporating solvent, DCM, and increasing the applied voltage, rougher surface and less pores formation on the surface of the PLGA fibres can be produced, respectively. In addition, by lowering the tip-to-collector distance, ribbon shaped PVA fibres are produced, indicating the fabrication of wetter fibres. Finally, the addition of ethanol to the aqueous PVA solution reduced the voltage required to stabilise the jet (Sukyte et al., 2012 and Asawahame et al., 2015). This has eliminated the flattened-like shape of PVA nanofibres due to the eradication of the entrapped residual water, but produced a very dry fibrous mat that stuck on the collecting material (parafilm).

Nevertheless, due to the low encapsulation of moxifloxacin in the inner core of the coaxial fibres, as well as, the low production yield of these fibres, more optimisation is required to overcome the unstable jet (whipping). Therefore, the use of another water soluble polymer, PVP, is suggested, in order to control the jet. This water soluble polymer can be dissolved in water and absolute
ethanol, providing an alternative approach to prepare multiaxial fibres using miscible solvents systems instead of the immiscible solvents system that was used in the previous chapter.

In this chapter, coaxial fibres are prepared by dissolving of pirfenidone in hydrophobic polymer PLGA as the outer layer and moxifloxacin in water soluble polymer PVP as the inner layer. In addition, triaxial fibres composed of moxifloxacin in PVP as the inner and outer-most layer and pirfenidone in PLGA as the intermediate layer, have been investigated. This formulation is proposed in order to obtain a burst release of the antibiotic within the first minutes of the release, while sustaining the release of pirfenidone during the remaining duration of the release. The inner layer of moxifloxacin should delay this antibiotic release to prevent its frequent dosing. The morphology of these fibres has been evaluated using SEM, TEM, light and confocal microscopy. The thermal analysis and physical state characterisation of the raw PVP, the physical mixtures (PM) of drugs and polymers and the blank and drug-loaded fibres have been performed using TGA, DSC, FTIR, and XRD. The drug loading has then been measured using the previously developed HPLC methods that separate both drugs in acetonitrile (ACTN). Finally, the release profiles for the monoaxial, coaxial, and triaxial fibres have been assessed by the developed HPLC method that separates both drugs in PBS pH 7.4. Furthermore, the swelling and weight loss behaviour for the blank and drug-loaded PLGA monoaxial, coaxial and triaxial fibres have been evaluated by using PBS pH 7.4.
4.2. Materials and Methods

4.2.1. Materials

A high MW PLGA 50:50 grade Purasorb® PDLG 5010 (153 kg/mol) was obtained from Corbion (Purac Biomaterials, Gorinchem, Netherlands). High MW PVP (1,000,000-1,500,000) Kollidon® 90F was kindly donated by BASF (Germany). Dichloromethane (DCM), ethanol, ethyl acetate, acetone, HPLC grades of water and acetonitrile (ACTN), PBS tablets with a pH 7.4, and methylene blue (MB) were all obtained from Sigma Aldrich Company Ltd (Sigma Aldrich, Dorset, UK). Pirfenidone was purchased from Tokyo Chemical Industry UK Ltd (The Magdalen Centre, Oxford, UK), while moxifloxacin hydrochloride was purchased from Cambridge Bioscience Ltd (Munro House, Cambridge, UK). BioDesign Dialysis Tubing™ (D002) with a molecular weight cut-off (MWCO) 14,000 daltons was brought from Fisher Scientific (Bishop Meadow Road, Leicestershire, UK).

4.2.2. Methods

4.2.2.1. Preparation of Monoaxial PLGA Fibres

PLGA fibres have been prepared using different solvents by modifying the electrospinning parameters of Said et al. (2012) and Deshpande et al. (2010) using a Spraybase® electrospinning instrument (Spraybase®, Dublin 2, Ireland). 3 mL of a solvent has been used to dissolve a defined quantity of PLGA. Table 4.1 demonstrates the electrospinning parameters that have been used with and without the addition of drugs. The needle diameter of 0.9 mm has remained constant throughout the entire PLGA fibre preparation to mimic the outer needle diameter of the coaxial needle and the intermediate layer of the triaxial needle. In addition, the flow rate was constant at 1 mL/h. The end product fibres have been collected on aluminum foil. Additionally, these fibres were fabricated under conditions of ambient temperature (range from 20 to 25°C) and humidity (range from 30 to 50%).
Table 4.1: Electrospinning parameters for the preparation of blank and drug-loaded PLGA monoaxial fibres.

<table>
<thead>
<tr>
<th>Form. #</th>
<th>Solvent used</th>
<th>Conc. of PLGA (% w/v)</th>
<th>Pirfenidone (% w/v)</th>
<th>Moxifloxacin (% w/v)</th>
<th>Voltage (kV)</th>
<th>Distance (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ethyl acetate</td>
<td>12.5</td>
<td>0</td>
<td>0</td>
<td>18</td>
<td>17.5</td>
</tr>
<tr>
<td>2</td>
<td>Acetone</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>9-10</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>DCM</td>
<td>12.5</td>
<td>0</td>
<td>0</td>
<td>23-24</td>
<td>12.5</td>
</tr>
<tr>
<td>4</td>
<td>DCM</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>10.5</td>
<td>12.5</td>
</tr>
<tr>
<td>5</td>
<td>ACTN</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>14-15</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>ACTN:ethanol (2:1)</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td>7</td>
<td>DCM</td>
<td>15</td>
<td>1</td>
<td>0</td>
<td>13-14</td>
<td>12.5</td>
</tr>
<tr>
<td>8</td>
<td>DCM</td>
<td>15</td>
<td>2</td>
<td>0</td>
<td>12-13</td>
<td>12.5</td>
</tr>
<tr>
<td>9</td>
<td>ACTN</td>
<td>25</td>
<td>1</td>
<td>0</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>10</td>
<td>ACTN</td>
<td>25</td>
<td>2</td>
<td>0</td>
<td>15-16</td>
<td>15</td>
</tr>
<tr>
<td>11</td>
<td>ACTN:ethanol (2:1)</td>
<td>20</td>
<td>2</td>
<td>1</td>
<td>14</td>
<td>15</td>
</tr>
</tbody>
</table>
4.2.2.2 Preparation of Monoaxial PVP Fibres

PVP nanofibres have been fabricated by modifying the electrospinning method of Chuangchote et al. (2009) and Illangakoon et al. (2014) using the same Spraybase® electrospinning instrument as for the PLGA fibres. High MW PVP has been dissolved in different organic solvents to obtain a concentration of 10% w/v. Table 4.2 shows the PVP electrospun nanofibre preparation parameters, with and without the addition of moxifloxacin. The flow rate has remained constant at 1 mL/h, in addition to the needle diameter of 0.45 mm during the preparation to mimic the inner needle diameter of both coaxial and triaxial needles. The collector distance, however, was varied depending on the solvent in use. The end product nanofibres have been collected on aluminum foil. Additionally, these fibres were fabricated under ambient temperature (range from 20 to 23.5°C) and humidity (range from 30 to 48%).

Table 4.2: Electrospinning parameters for the preparation of blank and drug-loaded PVP monoaxial fibres.

<table>
<thead>
<tr>
<th>Form. #</th>
<th>Solvent used</th>
<th>Moxifloxacin (% w/v)</th>
<th>Voltage (kV)</th>
<th>Collector distance (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>Ethanol</td>
<td>0</td>
<td>7</td>
<td>15</td>
</tr>
<tr>
<td>13</td>
<td>Ethanol:acetone (8:1)</td>
<td>0</td>
<td>9</td>
<td>12.5</td>
</tr>
<tr>
<td>14</td>
<td>Ethanol:acetone (2:1)</td>
<td>0</td>
<td>7.5</td>
<td>12.5</td>
</tr>
<tr>
<td>15</td>
<td>Ethanol:DCM (1:1)</td>
<td>0</td>
<td>9.5</td>
<td>12.5</td>
</tr>
<tr>
<td>16</td>
<td>Ethanol:ACTN (1:1)</td>
<td>0</td>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td>17</td>
<td>Ethanol:DCM (1:1)</td>
<td>0.5</td>
<td>10</td>
<td>12.5</td>
</tr>
<tr>
<td>18</td>
<td>Ethanol:DCM (1:1)</td>
<td>1</td>
<td>14</td>
<td>12.5</td>
</tr>
<tr>
<td>19</td>
<td>Ethanol:DCM (1:1)</td>
<td>1.5</td>
<td>10-16</td>
<td>12.5</td>
</tr>
<tr>
<td>20</td>
<td>Ethanol:ACTN (1:1)</td>
<td>1</td>
<td>16</td>
<td>15</td>
</tr>
</tbody>
</table>
Coaxial fibres have been prepared by modifying the described procedure in He et al. (2018), Zhu et al. (2015) and Sun et al. (2006). These fibres consist of different concentrations of PLGA, with or without pirfenidone, dissolved in different solvents, while 10% w/v PVP, with or without moxifloxacin, was dissolved in different solvent mixes. A coaxial needle was used with an inner diameter of 0.45 mm and an outer diameter of 0.9 mm. Table 4.3 shows the electrospinning parameters of these fibres. The end product fibres were collected on aluminum foil. Additionally, these fibres were fabricated under conditions of ambient temperature (range from 20 to 24.8°C) and humidity (range from 30 to 45%).
Table 4.3: Electrospinning parameters for the preparation of blank and drug-loaded PLGA/PVP coaxial fibres.

<table>
<thead>
<tr>
<th>Form. #</th>
<th>PLGA (% w/v)</th>
<th>PLGA solvent used</th>
<th>Pirfenidone (% w/v)</th>
<th>PVP solvent used</th>
<th>Moxifloxacin (% w/v)</th>
<th>Outer flow rate (mL/h)</th>
<th>Inner flow rate (mL/h)</th>
<th>Voltage (kV)</th>
<th>Distance (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>15</td>
<td>Acetone</td>
<td>0</td>
<td>Ethanol</td>
<td>0</td>
<td>3</td>
<td>0.5</td>
<td>9</td>
<td>15</td>
</tr>
<tr>
<td>22</td>
<td>15</td>
<td>Acetone</td>
<td>0</td>
<td>Ethanol</td>
<td>0</td>
<td>2</td>
<td>0.5</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td>23</td>
<td>15</td>
<td>Acetone</td>
<td>0</td>
<td>Ethanol:Acetone (8:1)</td>
<td>0</td>
<td>1</td>
<td>0.1</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>24</td>
<td>15</td>
<td>Acetone</td>
<td>0</td>
<td>Ethanol:Acetone (2:1)</td>
<td>0</td>
<td>1</td>
<td>0.1</td>
<td>9.7</td>
<td>15</td>
</tr>
<tr>
<td>25</td>
<td>15</td>
<td>DCM</td>
<td>0</td>
<td>Ethanol:DCM (1:1)</td>
<td>0</td>
<td>0.2</td>
<td>0.8</td>
<td>11</td>
<td>12.5</td>
</tr>
<tr>
<td>26</td>
<td>25</td>
<td>ACTN</td>
<td>0</td>
<td>Ethanol:ACTN (1:1)</td>
<td>0</td>
<td>0.5</td>
<td>0.5</td>
<td>18-19</td>
<td>15</td>
</tr>
<tr>
<td>27</td>
<td>15</td>
<td>DCM</td>
<td>1</td>
<td>Ethanol:DCM (1:1)</td>
<td>0.5</td>
<td>0.2</td>
<td>0.8</td>
<td>12</td>
<td>12.5</td>
</tr>
<tr>
<td>28</td>
<td>15</td>
<td>DCM</td>
<td>2</td>
<td>Ethanol:DCM (1:1)</td>
<td>1</td>
<td>0.2</td>
<td>0.8</td>
<td>12-13</td>
<td>12.5</td>
</tr>
<tr>
<td>29</td>
<td>15</td>
<td>DCM</td>
<td>2</td>
<td>Ethanol:DCM (1:1)</td>
<td>1.5</td>
<td>0.2</td>
<td>0.8</td>
<td>13</td>
<td>12.5</td>
</tr>
<tr>
<td>30</td>
<td>25</td>
<td>ACTN</td>
<td>2</td>
<td>Ethanol:ACTN (1:1)</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>21-22</td>
<td>15</td>
</tr>
</tbody>
</table>
4.2.2.4. Preparation of Triaxial Fibres

Triaxial fibres have been prepared by modifying the described procedure in Han and Steckl (2013). These fibres are composed of an inner core of 10% w/v PVP dissolved in ethanol:ACTN (1:1), an intermediate layer of 25% w/v PLGA dissolved in ACTN and an outer layer of 10% w/v PVP dissolved in ethanol:ACTN (1:1). Each layer of PVP and PLGA has been prepared with or without the addition of moxifloxacin and pirfenidone, respectively. A triaxial needle has been used with an inner diameter of 0.45 mm, an intermediate diameter of 0.9 mm and an outer diameter of 1.65 mm. Table 4.4 shows the electrospinning parameters of these fibres. The end product fibres have been collected on an aluminum foil from a distance of 15 cm. Additionally, these fibres were fabricated in the ambient temperature (range from 22 to 22.3°C) and humidity (range from 32 to 33%).
Table 4.4: Electrospinning parameters for the preparation of blank and drug-loaded PVP/PLGA/PVP triaxial fibres.

<table>
<thead>
<tr>
<th>Form. #</th>
<th>Pirfenidone (% w/v)</th>
<th>Moxifloxacin (% w/v)</th>
<th>Outer flow rate (mL/h)</th>
<th>Intermediate flow rate (mL/h)</th>
<th>Inner flow rate (mL/h)</th>
<th>Voltage (kV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>0</td>
<td>0</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>19-20</td>
</tr>
<tr>
<td>32</td>
<td>2</td>
<td>1 + 1</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>23-24</td>
</tr>
</tbody>
</table>
4.2.2.5. Conductivity and Viscosity Determination

The conductivity and viscosity of the polymer spinning solutions (PLGA and PVP) were determined using SciQuip benchtop pH and conductivity instrument (SciQuip, Shropshire, UK) and Malvern Bohlin Gemini HR Nanorheometer (Malvern, Worcester, UK), respectively. For conductivity measurement, the probe was immersed directly in the vial that contains the homogenous solution of the polymer (with or without the drug) and the values were recorded. For viscosity determination, 2 to 3 mL of the polymeric solution (with or without the drug) was added to the flat metal plate (sample plate). The geometry used for analysis was a 40 mm diameter steel parallel plate with a fixed gap of 1 mm from the sample plate. All samples were measured at 25ºC and shear rate ranging from 0.1 to 100 1/s. The viscosity was determined according to the change in the shear rate. Viscosity at a shear rate of 1 1/s was recorded. Each sample was analysed in triplicate to check the reproducibility of both analyses.

4.2.2.6. Size and Shape of Fibres

The morphological characteristics of the prepared fibres were examined with scanning electron microscopy (SEM), transmission electron microscopy (TEM), light and fluorescence microscopy, and confocal microscopy.

4.2.2.6.1. Scanning Electron Microscopy

A 0.5 cm x 0.5 cm piece of foil, on which the fibres were collected, was adhered onto an SEM stub, using double sided carbon tabs (Agar Scientific, Stansted, UK). The prepared stub was then given a thin coating of gold (10 nm) using a Quorum Q150T Sputter Coater (Quorum Technologies Ltd. East Sussex, UK) in an argon atmosphere. The coated stub was then transferred and imaged under FEI Quanta 200F (FEI company Ltd, Eindhoven, The Netherlands), at an acceleration voltage of 5 kV. Fibre size analysis was performed by measuring the diameter of these fibres using ImageJ software (National Institute of Health, Maryland, USA).
4.2.2.6.2. Transmission Electron Microscopy

Fibres were collected on a copper grid directly during fabrication. The prepared grids were stained with 2% aqueous uranyl acetate solution prior to imaging under a FEI CM120 BioTwin TEM (FEI Company Ltd. Eindhoven, The Netherlands) at an accelerating voltage of 120 kV.

4.2.2.6.3. Light and Fluorescence Microscopy

Fibres were assessed under both light and UV fluorescence microscopy using a Nikon Microphot-FXA microscope (Nikon, Japan). Fibres were collected directly on microscope slides and examined using total magnifications of 40X, 100X and 400X. The fluorescence wavelength is in the range of 320-340 nm.

4.2.2.6.4. Confocal Microscopy

PLGA and PVP monoaxial fibres loaded with methylene blue (MB) and moxifloxacin, respectively, were prepared according to Table 4.1 - formulation 10 for PLGA fibres and Table 4.2 - formulation 20 for PVP fibres. In addition, coaxial and triaxial fibres loaded with MB in the PLGA layer and moxifloxacin in the PVP layer(s) were prepared according to Table 4.3 - formulation 30 for coaxial fibres and Table 4.4 - formulation 32 for triaxial fibres. Fibres were collected directly on microscope slides and examined at excitation and emission wavelengths according to the added drug or dye using a Zeiss LSM 710 confocal microscope (Carl Zeiss Meditec, Germany) with total magnification of 40X and 100X. Moxifloxacin has an excitation and an emission wavelength around 290 and 500 nm respectively (Chan et al. 2006, Wang et al. 2016), while methylene blue has an excitation and an emission wavelength of 570-690 and 710 nm, respectively (Olmsted, 1979, Tomita, 1968).

4.2.2.7. Thermal Analysis and Physical State Characterisation

Thermogravimetric analysis (TGA), differential scanning calorimetry (DSC), Fourier-Transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD)
were performed to assess the thermal characteristics of raw materials, physical mixtures of drug(s) and polymer(s), blank and drug-loaded fibres.

4.2.2.7.1. Thermogravimetric Analysis

TGA was performed using a TA discovery thermogravimetric analyser (TA Instruments New Castle, DE, USA). An aliquot sample (weight range from 5 to 10 mg) was placed into an open aluminum pan. Each sample was analysed in triplicate to check the reproducibility of the thermal analysis. The samples were equilibrated at 30°C and heated to 400°C at a rate of 10°C/minute.

4.2.2.7.2. Differential Scanning Calorimetry

Modulated temperature DSC (MTDSC) was performed using the TA Q2000 instrument. Calibration by sapphire disc was performed before running the samples. An aliquot of each sample (weight range from 3 to 5 mg) was weighed into a 40 µL aluminum DSC pan (Perkin-Elmer) which was then sealed by an aluminum lid. Three holes have been punched through the lid after sealing. Each sample was analysed in triplicate to check the reproducibility of the thermal analysis. Here, the underlying heating rate was adjusted to 2°C/minute with modulation parameters of amplitude ± 0.212°C and a period 40 seconds. For PVP Tg determination, the samples were equilibrated at 0°C and heated to 190°C. For the physical mixtures of PLGA-pirfenidone and PVP-moxifloxacin, blank and drug-loaded fibres, Tg determination was by equilibrating at 0°C and heating up to 250°C.

4.2.2.7.3. Fourier-Transform Infrared Spectroscopy

Solid state characterisation was performed using Spectrum 100 FTIR spectrometer (Perkin Elmer, Massachusetts, USA). Measurements were performed with a resolution of 4 cm⁻¹ and a duration of 16 scans over a range of 4000-650 cm⁻¹ at ambient temperature. Each sample was analysed in triplicate to check the reproducibility. Spectra were analysed using OriginPro 2016 (OriginLab Corporation, Northampton, MA, USA).
4.2.2.7.4. X-Ray Diffraction

Further solid state characterisation was performed using MiniFlex 600 benchtop diffractometer (RigaKu, Tokyo, Japan). Powder samples of the raw materials or physical mixtures were lightly pressed onto 20 mm aluminium trays and the surface scraped evenly using a glass slide. However, the blank and drug-loaded monoaxial, coaxial and triaxial fibres were placed directly on the aluminium trays. A Cu Kα radiation point source (λ=1.5148 227 Å) was operated at 40 mV and 15 mA. XRD patterns were recorded using diffraction angle (2θ) from 3° to 40° (step size of 0.05° and time per step of 0.2 second). Data was analysed using OriginPro 2016 (OriginLab Corporation, Northampton, MA, USA).

4.2.2.8. Drug Loading [DL], Entrapment Efficiency [EE%] and Yield [Y%]

For direct determination of the entrapment efficiency of loaded fibres, a certain volume of ACTN was added to a known amount of the formulation until complete dissolution. Then 1 mL of the formed solution is withdrawn and centrifuged for 5 minutes at 10,000 rpm at room temperature. The drug content was determined by the previously developed organic extraction HPLC method and the drug loading was calculated using the following theoretical equation:

\[
DL = \frac{\text{Entrapped drug amount}}{\text{Yield of fibres amount}}
\]

Equation 4.1

The entrapment efficiency was calculated by the following equation:

\[
EE\% = \frac{\text{Actual drug amount}}{\text{Theoretical drug amount}} \times 100
\]

Equation 4.2

While the yield of the fabricated fibres was calculated by the following equation:

\[
Y\% = \frac{\text{Actual amount of fibres}}{\text{Theoretical amount of fibres}} \times 100
\]

Equation 4.3
where the theoretical amount of fibres was calculated by estimating the amount of the solid materials polymer ± drug in the total volume that was injected in the electrospinning system.

### 4.2.2.9. Drug Release Profile

For the release determination of the drug-loaded fibres, bespoke dialysis methods were used under sink condition as explained in chapter 2 - section 2.2.2.10., Dissolution Studies. A known amount of the formulation was kept in either a dialysis bag or custom-made cages inside a sealed glass container. A defined volume of preheated PBS, with a pH 7.4, was added to a glass container holding the fibres inside a shaking incubator with a temperature kept constant at 34.3°C and a shaking speed of 75 rpm. After a designated time point, 1 mL of PBS was withdrawn and centrifuged for 5 minutes at 10,000 rpm at room temperature, and 1 mL of fresh buffer was replaced. The drug release content was calculated by the previously developed buffer extraction HPLC method and the cumulative release % was calculated using the following equation:

\[
\text{Cumulative release } \% = \frac{\text{Cumulative drug amount}}{\text{Theoretical drug amount}} \times 100 \quad \text{Equation 4.4}
\]

where the cumulative drug amount is the total amount of drug released at each time point, while the theoretical drug amount is the actual amount of drug-loaded in each formulation.

### 4.2.2.10. Release Kinetic Modelling

After plotting the release profile curves (cumulative release % against time) of the drug-loaded electrospun fibres, suitable release kinetic models that would fit each fibrous system release profile were explored. DDSolver software (China Pharmaceutical University, Nanjing, China) was used to calculate the coefficient of determination \(r^2\) and the release model parameters such as the release constant \(k\) as described in Zhang et al. (2010). This study can identify the appropriate model(s) that fits the release profile of each fibrous system.
However, these models are assumptions of the release mechanism(s) of the fibres.

The obtained released data from the previous section were analysed with the following kinetic models; zero-order, first-order, Higuchi, Korsmeyer-Peppas, Hopfenberg and Weibull. These models will be briefly described later in the results and discussion section 4.3.5. In all models, F is the fraction (%) of drug released in time (t).

4.2.2.11. Swelling and Weight Loss Behavior

The swelling and weight loss properties of the drug-loaded PLGA monoaxial, coaxial and triaxial fibres were assessed by adding a defined amount of the fibres to the release medium (PBS, pH 7.4). Each formulation was kept in a sealed glass container with 30 mL of PBS and incubated at 34.3°C. After 2, 4, 6, and 24 hours, the wetted fibre-mat of each formulation was weighed. The percentage of swelling was calculated using the following equation:

\[ \text{Swelling\%} = \frac{W-W_d}{W_d} \times 100 \]  

Equation 4.5

where W is the weight of wetted fibres and Wd is the dry weight (i.e. after drying) of the fibres of each formulation.

However, the percentage of weight loss was measured after drying the fibres in 34.3°C for 1 day and then reweighed to determine the dry weight using the following equation:

\[ \text{Weight\ loss\%} = \frac{W_i-W_d}{W_i} \times 100 \]  

Equation 4.6

where Wi is the initial weight of the fibres and Wd is the dried weight of the fibres of each formulation.
4.3. Results and Discussion

In order to prepare the final multi-layered formulations (PLGA-pirfenidone/PVP-moxifloxacin coaxial fibres and PVP-moxifloxacin/PLGA-pirfenidone/PVP-moxifloxacin triaxial fibres), optimisation of PLGA and PVP loaded and unloaded fibres, as well as blank coaxial and triaxial fibres, was performed. For the coaxial fibre fabrication, the PLGA is proposed to be the outer layer while PVP is intended to be the inner layer. Hence, needle diameters of 0.9 mm and 0.45 mm have been used to prepare monoaxial PLGA and PVP fibres, respectively. This was to mimic the diameter of the inner and the outer needles of the coaxial emitter and the inner and the intermediate needles of the triaxial emitter. For the triaxial fabrication, PVP is proposed to be in the outer layer, in addition to the inner layer while PLGA is intended to be the intermediate layer. Accordingly, a triaxial needle with diameters 1.65 mm, 0.9 mm and 0.45 mm was used for the outer, intermediate and inner layers, respectively.

4.3.1. Morphological Characteristics of Fibres

4.3.1.1. Blank Monoaxial PLGA Fibres

With the aim of optimising PLGA monoaxial fibres, five different solvents have been used to dissolve the high MW PLGA 50:50 (153 kg/mol). Figure 4.1 shows the SEM images taken of representative samples of the fibres (the blank fibres, formulations 1-6) produced following the parameters listed in Table 4.1, to assess the surface morphology of these fibres. PLGA was able to form a spinnable viscous solution at a concentration 12.5% w/v in ethyl acetate. As shown in Figure 4.1 - formulation 1, beaded fibres were produced, despite using a higher tip-to-collector distance of 17.5 cm. This has been used to allow more time for the solvent to evaporate, in order to overcome the low volatility (vapour pressure of 73 mm Hg and boiling point 76.5-77.5ºC) of this solvent. In addition, the jet stability was poorly controlled by using this solvent.

A higher concentration of 15% w/v was too viscous to be spun probably due to ethyl acetate high relative viscosity (0.423 cP at 25ºC). Therefore an alternative, more environmentally friendly solvent, acetone, was used for the
preparation of PLGA fibres. This solvent has a relative viscosity of 0.306 cP at 25°C and a high volatility (vapour pressure of 184 mm Hg and boiling point 56°C). A concentration of 15% w/v and a distance of 15 cm have yielded smooth non-porous fibres, as shown in Figure 4.1 - formulation 2, while the jet was more controllable than the previously used solvent.

DCM has previously been used in PLGA fibre fabrication (Deshpande et al., 2010, Said et al., 2011, Zhang et al., 2012, Sun et al., 2014). Concentrations of 12.5% and 15% w/v have been assessed, as it shown in Figure 4.1 - formulation 3 and 4, respectively. The 12.5% showed rough porous fibre surfaces compared to the 15%, which showed smoother fibre surfaces, which is consistent with Zhang et al. (2012) and Sun et al. (2014). In spite of using a lower distance (12.5 cm), this porous surface appearance of the 12.5% was due to the very high volatility of DCM (vapor pressure of 353 mm Hg and boiling point 39.8-40°C) and the high applied voltage used (23-24 kV) (Deitzel et al., 2001). This high voltage accelerated the evaporation rate of DCM and thus, enhanced phase separation during the manufacturing process (Bognitzki et al. 2001). Owing to the high relative viscosity of 0.413 cP at 25°C of DCM, as well as, its high volatility, the jet stability was very poorly controlled (visually).

A less commonly used solvent in electrospinning, ACTN, has been used to dissolve PLGA. It has a viscosity between acetone and DCM, 0.369 cP at 25°C, and low volatility (vapor pressure of 72.8 mm Hg and boiling point 81-82°C). Moreover, PLGA has a higher solubility in this solvent than those previously mentioned, and so a concentration of 25% w/v was used. The produced fibres (Figure 4.1 - formulation 5) were more uniform with a non-porous smooth surface appearance, while the jet was the most controlled compared to the fibres produced with the other solvents.

Finally, blank PLGA fibres were prepared using a mixed solvent system of ACTN and ethanol. This is to help in dissolving the water soluble antibiotic moxifloxacin within the PLGA for the purpose of testing the incorporation of both pirfenidone and moxifloxacin into PLGA. Due to the insolubility of PLGA in ethanol, the ratio of 2:1 (ACTN:ethanol) decreased the spin-able concentration from 25% to 20% w/v. The resultant fibres were smooth and
non-porous as can be seen in Figure 4.1 - formulation 6, while the spinning jet was also stable.

Consequently, formulations number 4 (15% PLGA dissolved in DCM), 5 (25% PLGA dissolved in ACTN) and 6 (20% PLGA dissolved in ACTN:ethanol (2:1)) were selected for further drug(s) incorporation studies. This was suggested in order to load different concentrations of pirfenidone in the formulations 4 and 5 to be used for the drug release studies on the PLGA monoaxial system, as well as to use these system for the fabrication of the multi-layered fibrous systems. However, formulation 6 was proposed to incorporate both pirfenidone and moxifloxacin into PLGA monoaxial fibres in order to compare the release profile of this system against the coaxial system, which incorporates both drugs in separate compartments.
<table>
<thead>
<tr>
<th>Form. #</th>
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<th>Form. #</th>
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<td>4</td>
<td><img src="image4" alt="" /></td>
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<tr>
<td>12.5% PLGA in ethyl acetate</td>
<td></td>
<td>15% PLGA in DCM</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><img src="image2" alt="" /></td>
<td>5</td>
<td><img src="image5" alt="" /></td>
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<td>15% PLGA in acetone</td>
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</tr>
<tr>
<td>3</td>
<td><img src="image3" alt="" /></td>
<td>6</td>
<td><img src="image6" alt="" /></td>
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<tr>
<td>12.5% PLGA in DCM</td>
<td></td>
<td>20% PLGA in ACTN: ethanol</td>
<td></td>
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</tbody>
</table>

Figure 4.1: The surface morphology of blank PLGA fibres prepared by different solvent showing the presence of beaded fibres. Form. 1: 12.5% PLGA in ethyl acetate, form. 2: 15% PLGA in acetone, form. 3: 12.5% PLGA in DCM, form. 4: 15% PLGA in DCM, form. 5: 25% PLGA in ACTN, form. 6: 20% PLGA in ACTN:ethanol (2:1).

### 4.3.1.2. Pirfenidone-loaded Monoaxial PLGA Fibres

Pirfenidone with a concentration of 1% or 2% w/v was loaded into the PLGA solution as seen in Table 4.1 - formulations 7-10. The resultant fibre surface morphology of the 15% w/v PLGA dissolved in DCM (Figure 4.2 - formulations 7 and 8) were rougher compared to the 25% w/v PLGA dissolved in ACTN (Figure 4.2 - formulations 9 and 10). This observation was similar to the blank
PLGA fibres in formulation 4 and 5, respectively. In addition, there was no evidence of drug crystals at the surface of these fibres. However, loading both drugs in PLGA dissolved in ACTN:ethanol with concentrations of 2% and 1% for pirfenidone and moxifloxacin, respectively, indicated possible drug crystals at the surface of these fibres (Figure 4.2 - formulation 11). Nevertheless, this formulation will still be considered in the later studies in order to compare a monoaxial system containing both drugs to a coaxial system that compartmentalises each drug in a separated layer.
4.3.1.3. Blank Monoaxial PVP Fibres

Despite being classified as a water soluble polymer, PVP can be dissolved in absolute ethanol. However, for the purpose of preparing multi-layered fibres, mixtures of ethanol and other organic solvents have been examined. A concentration of 10% w/v has been kept constant throughout all PVP formulations. However, the tip-to-collector distance was changed from 15 cm
to 12.5 cm to accommodate for when a solvent with a high evaporation rate (acetone or DCM) has been used. Unbeaded, smooth and non-porous fibres were prepared by using the absolute ethanol, as well as, the cosolvents as shown in Figure 4.3 - formulation 12-16. This observation is consistent with Illangakoon et al. (2014) and Chuangchote et al. (2009), who prepared their PVP fibres by using either ethanol, or ethanol as part of a cosolvent, respectively. In addition, for all the formulations, the spinning jet was stable. The purpose of using a cosolvent consisting of ethanol and either acetone, DCM or ACTN is to make the PVP polymer solution more miscible with the PLGA solution dissolved in acetone, DCM or ACTN, respectively, and to stabilise the electrospinning jet of the coaxial or the triaxial fibres later.
Figure 4.3: The surface morphology of blank PVP fibres (10%) prepared using different solvents showing the lack of beads. Form. 12: in ethanol, form. 13: in ethanol:acetone (8:1), form. 14: in ethanol:acetone (2:1), form. 15: in ethanol:DCM (1:1), form. 16: in ethanol:ACTN (1:1).

4.3.1.4. Moxifloxacin-loaded Monoaxial PVP Fibres

Concentrations of 0.5%, 1%, and 1.5% w/v of moxifloxacin (Table 4.2 - formulations 17, 18 and 19, respectively) have been dissolved in PVP solutions of ethanol:DCM (1:1). The resultant fibres were smooth and non-porous (Figure 4.4 - formulations 17-19), which were similar to the blank fibres and no evidence of drug crystals on the surface (Figure 4.3 - formulation 15). However, the spinning jet was less stable for the 1.5% moxifloxacin PVP solution. This resulted in thinner fibres covering the drug-loaded fibres as
shown in Figure 4.4 - formulation 19. Furthermore, a moxifloxacin concentration of 1% w/v was dissolved in PVP solution of ethanol:ACTN (1:1). This yielded a more controlled jet, resulting in smooth non-porous fibres as can be seen in Figure 4.4 - formulation 20. Therefore, the 1% moxifloxacin concentration was used to prepare the drug-loaded PVP monoaxial fibres, as well as, the multi-layered fibres. This was to achieve a high DL profile in order to reduce the required weight (dose) of the fibres that will be administered topically into the cornea.

<table>
<thead>
<tr>
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<th>SEM image</th>
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<td><img src="image1" alt="SEM image" /></td>
<td>19</td>
<td><img src="image2" alt="SEM image" /></td>
</tr>
<tr>
<td>0.5% Moxi in 10% PVP (Ethanol :DCM)</td>
<td>1.5% Moxi in 10% PVP (Ethanol :DCM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td><img src="image3" alt="SEM image" /></td>
<td>20</td>
<td><img src="image4" alt="SEM image" /></td>
</tr>
<tr>
<td>1% Moxi in 10% PVP (Ethanol :DCM)</td>
<td>1% Moxi in 10% PVP (Ethanol :ACTN)</td>
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</table>

Figure 4.4: The surface morphology of drug-loaded PVP fibres (10%) prepared using different solvents showing the presence of very fine fibres as an indication of jet instability. Form. 17: in ethanol:DCM (1:1) and 0.5% moxifloxacin, form. 18: in ethanol:DCM (1:1) and 1% moxifloxacin, form. 19: in ethanol:DCM (1:1) and 1.5% moxifloxacin, form. 20: ethanol:ACTN (1:1) and 1% moxifloxacin.
4.3.1.5. Blank Coaxial PLGA/PVP Fibres

Both PLGA and PVP concentrations and relevant solvent or cosolvents for the preparation of the coaxial fibres have been chosen based on the above monoaxial preparations. The critical parameter that has been investigated is the miscibility between the core and shell solutions as well as, the flow rate differences between the core and shell solutions. Since both polymers are soluble in organic solvents, this eventually will be considered as miscible electrospinning system, unlike the immiscible coaxial system of PLGA and PVA that was previously mentioned in chapter 3.2.2.3. According to He et al. (2018), Zhu et al. (2015) and Sun et al. (2006), the mixing of a cosolvent with another cosolvent, such as N,N-dimethylformamide(DMF):DCM and DMF:ethanol, was more preferable. However, in this study the mixing of an absolute solvent and a cosolvent, such as DCM and ethanol:DCM, was used. This was proposed in order to allow the separation of the layers and to avoid the margin of both layers into one, while still being miscible.

Another important factor, namely the flow rate of the core solution and the shell was investigated. Generally, coaxial fibres are prepared by using a higher shell flow rate than the core, for instance 0.5 mL/h (shell) and 0.2 mL/h (core), 1 mL/h (shell) and 0.1-0.4 mL/h (core) and 0.1 mL/h (shell) and 0.05 mL/h (core) that can be observed in He et al. (2018), Zhu et al. (2015) and Sun et al. (2006), respectively. Nevertheless, the coaxial fibres in this study have been prepared by varying the shell and core flow rates in order to control the electrospinning jet, which will help in forming more uniform fibres.

A coaxial system was initially proposed consisting of PLGA dissolved in acetone and PVP dissolved in ethanol. Two shell to core flow rate ratios were assessed, 3:0.5 mL/h and 2:0.5 mL/h. The produced fibres were smooth, non-porous and non-uniform, which indicated the production of some monoaxial fibres in the same fibrous mat, as shown in Figure 4.5 - formulations 21 and 22. This was due to the unstable jet, which resulted in a multi-jet formation.

Consequently, the preparation of coaxial fibres consisting of a PLGA shell dissolved in a single solvent and a PVP core dissolved in a cosolvent system was proposed. The dissolving of PLGA in acetone and PVP in an
ethanol:acetone mixture of 8:1 and 2:1 were examined. The yielded fibres were more uniform with a smooth and non-porous appearance, as it can be seen in Figure 4.5 - formulations 23 and 24. The stability of the jet was achieved with flow rates of 1 mL/h for the shell and 0.1 mL/h for the core. Similarly, PLGA dissolved in DCM or ACTN and PVP dissolved in the cosolvent of ethanol:DCM (1:1) or ethanol:ACTN (1:1), respectively were assessed. Both formulations demonstrated smooth non-porous fibres, as shown in Figure 4.5 - formulations 25 and 26, respectively. Interestingly, the jet stability for these formulations were obtained with flow rates that are rarely used, which are 0.2 mL/h (shell) and 0.8 mL/h (core) for formulation 25 and 0.5 mL/h (shell) and 0.5 mL/h (core) for formulation 26. Therefore, formulations 25 (15% PLGA dissolved in DCM and 10% PVP dissolved in ethanol:DCM (1:1)) and 26 (25% PLGA dissolved in ACTN and 10% PVP dissolved in ethanol:ACTN (1:1)) were selected for drug loading purposes.
Figure 4.5: The surface morphology of blank coaxial fibres prepared by different solvent showing the lack of beads. Form. 21: 15% PLGA in acetone and 10% PVP in ethanol (C-to-S: 1:6), form. 22: 15% PLGA in acetone and 10% PVP in ethanol (C-to-S: 1:4), form. 23: 15% PLGA in acetone and 10% PVP in ethanol:acetone (8:1), form. 24: 15% PLGA in acetone and 10% PVP in ethanol:acetone (2:1), form. 25: 15% PLGA in DCM and 10% PVP in ethanol:DCM (1:1), form. 26: 25% PLGA in ACTN and 10% PVP in ethanol:ACTN (1:1). C-to-S: core-to-shell flow rate.
4.3.1.6. Drug-loaded Coaxial PLGA/PVP Fibres

Drug-loaded coaxial fibres were prepared by incorporating 0.5%, 1% and 1.5% w/v moxifloxacin in PVP that is dissolved in ethanol:DCM (1:1) solutions, while keeping the concentration of pirfenidone constant at 2% w/v in the PLGA dissolved in DCM. As expected, formulations 27 and 28 (Figure 4.6) that consisted of 0.5% and 1% moxifloxacin concentrations, respectively, resulted in more uniform fibres. However, the 1.5% moxifloxacin concentration in formulation 29 (Figure 4.6) produced very fine fibres embedded into the coaxial fibres. This was due to jet instability of formulation 29. The flow rate that has been used in formulations 27, 28 and 29 was 0.2 mL/h for the shell solution and 0.8 mL/h for the core solution. This was to achieve the jet stability in order to have reproducible and uniform fibres. However, due to the high flow rate of the core solution, more moxifloxacin-loaded PVP monoaxial fibres were produced, which will be highlighted later in the results.

The dual drug-loaded coaxial fibres were also prepared by incorporating 2% w/v pirfenidone in PLGA that is dissolved in ACTN and 1% w/v moxifloxacin in PVP that is dissolved in ethanol:ACTN (1:1) solution, which yielded fibres that were smooth and non-porous, as shown in Figure 4.6 - formulation 30. A flow rate of 0.5 mL/h for both core and shell solutions was used to stabilise the jet in formulation 30. This was suggested to obtain more coaxial fibres and to reduce the chance of producing monoaxial fibres mixed with the coaxial ones. Therefore, by allowing the two solutions to combine into one droplet, using the same flow rate (0.5 mL/h), the voltage will overcome the surface tension of this droplet, producing a jet and thus a fibrous mat.
Figure 4.6: The surface morphology of drug-loaded coaxial fibres prepared by different solvent showing the presence of very fine fibres as an indication of jet instability. Form. 27: 15% PLGA and 1% pirfenidone in DCM and 10% PVP and 0.5% moxifloxacin in ethanol:DCM (1:1), form. 28: 15% PLGA and 2% pirfenidone in DCM and 10% PVP and 1% moxifloxacin in ethanol:DCM (1:1), form. 29: 15% PLGA and 2% pirfenidone in DCM and 10% PVP and 1.5% moxifloxacin in ethanol:DCM (1:1), form. 30: 25% PLGA and 2% pirfenidone in ACTN and 10% PVP and 1% moxifloxacin in ethanol:ACTN (1:1).

4.3.1.7. Blank and Drug-loaded Triaxial PVP/PLGA/PVP Fibres

Blank triaxial fibres were fabricated by coating the coaxial PLGA/PVP fibres with a layer of PVP. This is to include the antibiotic, moxifloxacin, in the outer layer in order to have a burst release of the antibiotic followed by sustained release of pirfenidone (from the intermediate layer) and a delayed release of moxifloxacin (from the inner layer). The coaxial formulation in Tables 4.3 - formulation 26 (25% PLGA dissolved in ACTN and 10% PVP dissolved in ethanol:ACTN (1:1)) was used to prepare the triaxial system. This is to reduce the monoaxial fibres that can be produced through electrospinning. Therefore, a similar flow rate of 0.3 mL/h was used to pump the three solutions (core,
intermediate and shell). Nonetheless, the voltage used was higher for the drug-loaded formulation due to the high conductivity of the moxifloxacin antibiotic addition. Both the blank and drug-loaded triaxial fibres were uniform with smooth and non-porous surfaces, as shown in Figure 4.7 - formulation 31 and 32, respectively. Han and Steckl (2013) showed reasonably uniform PCL/PCL/PVP triaxial fibres that were smooth and unbeaded. However, this PCL/PCL/PVP triaxial fibres were prepared using different flow rate for each layer (PCL outer layer: 0.2 mL/h, PCL middle layer: 0.8 or 1.2 mL/h, PVP inner layer: 0.04 mL/h).

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<tr>
<td>31</td>
<td>Blank triaxial fibres</td>
<td>32</td>
<td>Drug-loaded triaxial fibres</td>
</tr>
</tbody>
</table>

*Figure 4.7: The surface morphology of the blank and drug-loaded triaxial fibres showing the lack of beads and drug crystals. Form. 31: 10% PVP in ethanol:ACTN (1:1), 25% PLGA in ACTN and 10% PVP in ethanol:ACTN (1:1), form. 32: 10% PVP and 1% moxifloxacin in ethanol:ACTN (1:1), 25% PLGA and 2% pirfenidone in ACTN and 10% PVP and 1% moxifloxacin in ethanol:ACTN (1:1).*

Optimising the electrospinning parameters by varying the solvent used to dissolve PLGA and PVP, the tip-to-collector distance and the flow rate were essential in order to successfully prepare a single-layered fibres, as well as, a multi-layered fibres. In this chapter, the focus is on preparing a coaxial and triaxial fibres that contain PLGA and PVP polymers which have a small proportion of miscibility between each other. This was suggested to overcome the jet instability that was observed in the PLGA and PVA coaxial fibres in the
From the previous chapter, hence, higher collected yield and more uniform fibres can be obtained.

Consequently, blank and drug-loaded PLGA monoaxial fibres that are prepared using DCM (Formulation 4 and 8) and ACTN (Formulation 5 and 10) and blank and drug-loaded PVP monoaxial fibres which are fabricated using ethanol:DCM (Formulation 15 and 18) and ethanol:ACTN (Formulation 16 and 20) were considered for the preparation of the coaxial and triaxial fibres. Blank and drug-loaded coaxial fibres that are prepared using DCM (Formulation 25 and 26) and ACTN (Formulation 28 and 30) and blank and drug-loaded triaxial fibres which are fabricated using ACTN (Formulation 31 and 32) were further characterised. This is to determine the best drug-loaded fibrous system that can be tested later using antibacterial and anti-scarring in vitro assays.

### 4.3.2. Fibre Size and Microscopic Analysis

The set-up of the multi-layered electrospinning instrument requires the operator to prepare the polymeric solution of each layer separately and to load the solutions via individual syringes. Hence, the flow rate of 2 or 3 solutions can be adjusted directly through the pumps. Therefore, the viscosity and conductivity of the polymeric solutions of the successfully prepared monoaxial fibres (PLGA and PVP using either DCM or ACTN), with or without the drugs, were measured. These represent formulations 4 and 5 for the blank PLGA fibres, formulations 8 and 10 for the pirfenidone-loaded PLGA fibres, formulations 15 and 16 for the blank PVP fibres and formulations 18 and 20 for moxifloxacin-loaded PVP fibres, as shown in Table 4.5.
Table 4.5: The viscosity and conductivity measurements of the monoaxial PLGA and PVP fibres’ polymeric solutions. The viscosity and conductivity values of each formulation are presented as mean ± standard deviation (SD) (n=3).

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<thead>
<tr>
<th>Form. #</th>
<th>Solvent used</th>
<th>Conc. of PLGA (% w/v)</th>
<th>Conc. of PVP (% w/v)</th>
<th>Pirfenidone (% w/v)</th>
<th>Moxifloxacin (% w/v)</th>
<th>Viscosity (Pa.s) ± SD</th>
<th>Conductivity (µS/cm) ± SD</th>
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<td>Ethanol:ACTN</td>
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<td>1</td>
<td>0.14 ± 0.01</td>
<td>588.33 ± 18.15</td>
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Fibre diameters were assessed for the PLGA monoaxial (blank formulations 4, 5 and drug-loaded formulations 8, 10) and PVP monoaxial fibres (blank formulations 15, 16 and drug-loaded formulations 18, 20). In addition, coaxial (blank formulations 25, 28 and drug-loaded formulations 26, 30) and triaxial (blank formulation 31 and drug-loaded formulation 32) fibre diameters were measured using ImageJ software. The results are outlined in Table 4.6. About 100 fibres have been measured per batch and the mean diameters along with the standard deviations (SDs), the maximum and the minimum diameters have been calculated.

Table 4.6: Mean diameter, standard deviation (SD), and maximum and minimum diameters of the blank and drug-loaded monoaxial, coaxial and triaxial fibres.

<table>
<thead>
<tr>
<th>Form. #</th>
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<th>SD</th>
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![Graph 1](image1.png)

![Graph 2](image2.png)

![Graph 3](image3.png)

![Graph 4](image4.png)
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(Moxi-loaded PVP fibres using DCM)

(Moxi-loaded PVP fibres using ACTN)

(Blank coaxial fibres using DCM)

(Blank coaxial fibres using ACTN)
The viscosity and conductivity of the monoaxial PLGA and PVP fibres’ polymeric solutions, which are presented in Table 4.5, are recognised as being crucial factors for the determination of fibre diameter. As presented in Table 4.6, PLGA monoaxial fibre diameters varied depending on the solvent used.
For the DCM solvent in formulation 4, the diameter (1799 nm) was larger than formulation 5 (683 nm) that was prepared using ACTN. This is due to the higher viscosity of the formulation 4 solution (29.6 Pa.s) compared to the formulation 5 solution (3.9 Pa.s). However, the 2% w/v pirfenidone incorporation into formulation 8 (1886 nm) and 10 (784 nm) slightly increased the solution concentration and hence viscosity. This allowed the formation of a slight larger fibre diameters compared to the blank fibres in formulation 4 (1799 nm) and 5 (683 nm), respectively. Fong et al. (1999), Demir et al. (2002) and Huang et al. (2003) have reported that by increasing the concentration of the spinning solution, larger diameter fibres and fewer beads can be produced.

Blank PVP monoaxial fibres demonstrated similar results in terms of solvent choice. PVP which was prepared using ethanol:DCM (formulation 15) had a larger diameter (925 nm) than the PVP fibres prepared using ethanol:ACTN (formulation 16, 689 nm). This is due to the higher viscosity of formulation 15’s solution (0.4 Pa.s) compared to formulation 16’s solution (0.13 Pa.s). However, because of the high conductivity resulting from the addition of moxifloxacin in formulations 18 (86.5 µS/cm) and 20 (588.3 µS/cm), the voltage was increased in order to overcome the surface tension of the spinning solutions. This reduced the fibre diameter of moxifloxacin-loaded PVP fibres that are prepared using ethanol:DCM and ethanol:ACTN, formulation 18 (315 nm) and 20 (136 nm), respectively. Chakraborty et al. (2009) and Williams et al. (2018) have reported that by increasing the voltage, narrower fibres diameter can be produced, then by further increasing the voltage, the fibre diameter became larger.

The coaxial blank fibres in formulation 25 prepared using DCM were narrower in diameter (1193 nm) than the blank PLGA fibres (1799 nm) and larger than the blank PVP fibres (925 nm). This was due to the fabrication of these fibres in a flow rate of 0.2 mL/h for the shell solution and 0.8 mL/h for the core solution. Hence, due to the accelerated flow rate of the core solution, more PVP reached the needle tip and thus, more monoaxial PVP fibres were produced. This reduced the overall fibres diameter of the coaxial fibres compared to the monoaxial PLGA fibres. However, due to the inconsistent production of coaxial fibres (i.e. monoaxial fibres mixed with coaxial fibres), the
use of similar flow rate (0.5 mL/h) for the core and shell solutions was proposed. This is suggested to lead to the fabrication of uniform coaxial fibres, while reducing the chance of producing monoaxial fibres. The lower viscosity of the PVP core solution and the difference of the needle internal diameters (0.45 mm for the core and 0.9 mm for the shell) will allow the production of these coaxial fibres, while keeping the flow rate constant for the core and the shell. Therefore, blank coaxial fibres in formulation 26 (prepared using ACTN) were obtained and had a diameter (644 nm), which is almost similar to the PLGA monoaxial fibres (683 nm).

The addition of an inner layer, which is the PVP layer, is expected to produce a higher diameter fibre. Nevertheless, due to the higher voltage used to stabilise the jet, these coaxial fibres were slightly narrower diameter than the PLGA monoaxial fibres. Similar finding was observed in Zhu et al. (2015) study on monoaxial and coaxial fibres of PLGA loaded in flurbiprofen axetil. This study indicated that the drug-loaded PLGA monoaxial fibres had larger diameter (418 nm) than the drug-loaded PLGA/PVP (282 nm) coaxial fibres. This was due to the increase of the solution conductivity by the addition of the PVP solution (Zhu et al., 2015). Furthermore, the addition of the drugs to each layer allowed the PLGA and PVP solutions’ conductivity to increase. This allowed the voltage to be increased, in order to control the stability of the electrospinning jet. This led to the production of thinner fibres, as can be seen in the drug-loaded coaxial fibres that is prepared using DCM - formulation 28 (778 nm) and ACTN - formulation 30 (633 nm), respectively.

Triaxial fibres were fabricated by using the same flow rates for all three layers (core, intermediate and shell). This was to avoid the production of monoaxial fibres by the use of a higher flow rate. In addition, the voltage used to control the jet was increased, while decreasing the flow rate to 0.3 mL/h compared to the coaxial fibres (0.5 mL/h). Consequently, the diameters of the blank triaxial fibres were 683 nm, while the drug-loaded fibres were 677 nm. Both triaxial systems showed to have almost similar diameters to the drug-loaded PLGA monoaxial fibres (683 nm) and coaxial fibres (644 nm) which were prepared by using ACTN. This was in contrast to Han and Steckl (2013) who observed a larger diameter fibres of their PCL/PCL/PVP triaxial system using variable
flow rates (PCL outer layer: 0.2 mL/h, PCL middle layer: 0.8 or 1.2 mL/h, PVP inner layer: 0.04 mL/h) compared to the PCL monoaxial and PCL/PVP coaxial fibres.

Another factor that plays a key role in fibre diameter reduction is the flow rate. Chakraborty et al. (2009) reported that by reducing the flow rate, narrower fibres can be produced. Therefore, due the reduction of flow rate from 1 mL/h (in PLGA monoaxial fibres - formulation 10), to 0.5 mL/h (in coaxial fibres - formulation 30), to 0.3 mL/h (in triaxial fibres - formulation 32), this led to a relatively smaller fibre diameters, which was obtained in the coaxial and triaxial systems compared to the monoaxial. This observation was inconsistent with Han and Steckl's (2013) study which showed that by coating the monoaxial fibres with one (coaxial) or two (triaxial) layers, the diameter should increase accordingly. However in this study, lowering the flow rate and increasing the voltage were essential in order to stabilise the electrospinning jet and thus produce more uniform fibres.

The multi-layers of coaxial and triaxial fibres were observed by TEM. Drug-loaded fibres of PLGA monoaxial (formulation 8 and 10), PVP monoaxial (formulation 20), coaxial (formulation 28 and 30) and triaxial (formulation 32) were assessed. The images in Figure 4.8 showed that both PLGA monoaxial fibre formulations (formulations 8 and 10), as well as PVP fibres (formulation 20) are single layered. However, the coaxial and triaxial fibre formulations showed clearly two (Figure 4.8 - formulation 28 and 30) and three layers (Figure 4.8 - formulation 32), respectively. This was also seen in the TEM images of Zhao et al. (2014), Hwang and Jeong (2011), He et al. (2018), Han and Steckl (2013), for PLGA, PVP, PVP/PLGA, PVP/PCL/PCL fibres, respectively.
Figure 4.8: TEM images of the drug-loaded fibres showing the distinctive inner layer(s) of the coaxial and triaxial fibres compared to the monoaxial fibres. Form. 8: PLGA in DCM, form. 10: PLGA in ACTN, form. 20: PVP in ethanol:ACTN, form. 28: coaxial fibres prepared using DCM, form. 30: coaxial fibres prepared using ACTN, form. 32: triaxial fibres prepared using ACTN.
Light microscopy was used to differentiate between blank and drug(s) loaded fibres as shown in Figures 4.9 and 4.10. Blank and pirfenidone-loaded PLGA monoaxial fibres, which were prepared using DCM solvent, appeared as a tube-like shape with large fibres diameters (Figure 4.9 - formulation 4 and 8, respectively), similar to the blank and drug-loaded PLGA fibres that have been prepared using ACTN (Figure 4.9 - formulation 5 and 10, respectively). This observation is consistent with Grafahrend et al. (2011) who showed similar images for PLGA fibres under light microscope. However, blank and moxifloxacin-loaded monoaxial PVP fibres which were prepared using ethanol:DCM and ethanol:ACTN solvent mix appeared as fine solid threads (Figure 4.9 - formulation 15 and 16 for blank fibres and formulation 18 and 20 for drug-loaded fibres, respectively). This fibre appearance is in agreement with Enculescu et al. (2014) PVP fibrous system under light microscope. For coaxial unloaded and loaded fibres prepared by using DCM (Figure 4.9 - formulations 25, and 28, respectively) and ACTN (Figure 4.9 - formulations 26, and 30, respectively), the light microscopy showed that these fibres have a similar tube-like appearance to the monoaxial PLGA fibres. A similar appearance was observed for the blank and drug-loaded triaxial fibres (Figure 4.9 formulation 31 and 32, respectively).
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<td>Pir-loaded PLGA fibres (DCM)</td>
</tr>
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<td>Blank PLGA fibres (ACTN)</td>
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Blank PVP fibres (ethanol : DCM)

Moxi-loaded PVP fibres (ethanol: DCM)

Blank PVP fibres (ethanol : ACTN)

Moxi-loaded PVP fibres (ethanol: ACTN)
25
Blank coaxial fibres (DCM)

26
Blank coaxial fibres (ACTN)

28
Drug-loaded coaxial fibres (DCM)

30
Drug-loaded coaxial fibres (ACTN)
Figure 4.9: Light microscope images of the blank and drug-loaded monoaxial, coaxial and triaxial fibres. PLGA monoaxial fibres, PLGA/PVP coaxial fibres and PVP/PLGA/PVP triaxial fibres showed a tube-like appearance compared to the PVP monoaxial fibres which have a thread-like appearance.
The UV fluorescence microscopy showed that monoaxial PLGA fibres (blank and pirfenidone-loaded), which have been prepared using DCM (Figure 4.10 - formulation 4 and 8, respectively) and ACTN (Figure 4.10 - formulation 5 and 10) had no illumination appearance. Blank monoaxial PVP fibres that were prepared using ethanol:DCM and ethanol:ACTN solvent mix showed no fluorescence (Figure 4.10 - formulation 15 and 16, respectively). Nevertheless, due to the fluorescent nature of moxifloxacin, the drug-loaded fibres (Figure 4.10 - formulation 18 and 20) fluoresced under the UV microscopy. This finding indicates the presence of moxifloxacin-loaded within the PVP monoaxial fibres. Zhi-Hua et al. (2012) observed a fluorescence appearance of PVP fibres under this microscope due to the successful encapsulation of the fluorescent material, fluorescein.

Blank coaxial fibre formulations (Figure 4.10 - formulations 25 and 26) showed no fluorescence under UV microscopy, unlike the drug-loaded coaxial fibres (Figure 4.10 - formulations 28 and 30) due to the encapsulation of moxifloxacin in the core of these fibres. This fluorescence appearance was also seen in a study by Tiwari et al. (2010) who detected rose bengal fluorescent dye, which was located in the PVA core of PLGA/PVA coaxial fibres, under confocal microscopy. Studies by Zhang et al. (2006) and Romano et al. (2016) also showed that by the incorporation of fluorescent-labelled BSA in the core of PEG/PCL (shell/core) fibres and rhodamine B isothiocyanate in the core of PCL/PEO (shell/core) fibres, respectively, both systems fluoresced under confocal microscopy.

The drug-loaded triaxial fibre formulation (Figure 4.10 - formulation 32) fluoresced under the UV microscope, while the unloaded fibres (Figure 4.10 - formulation 31) lacked this fluorescent appearance. A study by Liu et al. (2013) observed two distinctive fluorescent layers of rhodamine B in the core and shell of gelatine layers which were separated by a middle layer of PCL in the gelatine/PCL/gelatine triaxial fibres by confocal microscopy. The appearance of moxifloxacin that fluoresce under the fluorescent microscopy can be an indication for the successful loading of this antibiotic in the core of the coaxial fibres, and in the core and the shell of the triaxial fibres.
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Figure 4.10: UV microscopic images of the blank and drug-loaded monoaxial, coaxial and triaxial fibres. The presence of moxifloxacin allows the fibres to fluoresce in the drug-loaded PVP monoaxial fibres (form. 18 and 20), coaxial fibres (form. 28 and 30) and triaxial fibres (form. 32) compared to the blank PLGA monoaxial fibres (form. 4 and 5), drug-loaded PLGA monoaxial fibres (form. 8 and 10), blank PVP monoaxial fibres (form. 15 and 16), blank coaxial fibres (form. 25 and 26) and blank triaxial fibres (form. 31).

Due to the limited detection wavelength (320-340 nm) of the UV fluorescent microscope, a confocal microscope was used to detect a higher wavelength compound. This form of microscopy can observe the presence of different dyes.
(or moxifloxacin) throughout different channels (filters) for the same sample. Therefore, methylene blue (MB) dye was used to be incorporated with PLGA in order to fluoresce under this microscope, while moxifloxacin was loaded into PVP. This can indicate the successful preparation of the multi-layered fibres and the distribution of moxifloxacin and MB into these fibres by showing both components separately in different channels and together in a merged channel. After collecting fibres on a microscope slide during the spinning process, the sample was observed through two detection wavelength channels. Specifically, an excitation wavelength of 300 nm and emission wavelength of 500 nm were used to detect moxifloxacin in one channel (A), which was shown as a cyan colour. For methylene blue (MB) dye, an excitation and emission wavelength of 600 and 710 nm, respectively were used as a second channel (B), and the dye was detected as a magenta colour. In addition, transmitted light (T) was used initially to confirm the presence of fibres. As it can be seen in Figure 4.11.a and 4.11.b, cyan and magenta colours were observed, suggesting the presence of moxifloxacin and MB in PVP and PLGA monoaxial fibres, respectively. The black colour indicates the absence of any fluorescent compound.

For coaxial and triaxial loaded fibres, Figures 4.12.a and 4.13.a showed that both channels, A and B, were black, suggesting lack of any fluorescent compound, while the transmitted light showed the presence of fibres. However, in Figures 4.12.b and 4.13.b, channel A exhibited the presence of cyan coloured fibres, indicating the loading of moxifloxacin. In addition, channel B showed the occurrence of magenta coloration, representing the encapsulation of MB dye. By merging both channels (M), a colour mix of cyan-magenta was observed, indicating the successful loading of both components. This was consistent with Wakuda et al. (2018), who loaded Rhodamine B (red) and uranine (green) in collagen (core) and PVP (shell), respectively. After merging the two channels, a red-greenish colour was observed, indicating the presence of both dyes in the coaxial fibres.
Figure 4.11: Confocal microscope image showing moxifloxacin and MB the fluorescence in (a) PVP moxifloxacin monoaxial fibres and (b) PLGA MB monoaxial fibres. A; channel one Ex. 300 nm and Em. 500 nm, B; channel two Ex. 600 nm and Em. 710 nm, T; transmitted light channel, M; merge of channels A, B and T.

Figure 4.12: Confocal microscope image showing moxifloxacin and MB the fluorescence in (a) blank coaxial fibres and (b) moxifloxacin-MB loaded coaxial fibres. A; channel one Ex. 300 nm and Em. 500 nm, B; channel two Ex. 600 nm and Em. 710 nm, T; transmitted light channel, M; merge of channels A, B and T.
Figure 4.13: Confocal microscope image showing moxifloxacin and MB the fluorescence in (a) blank triaxial fibres and (b) moxifloxacin-MB loaded triaxial fibres. A; channel one Ex. 300 nm and Em. 500 nm, B; channel two Ex. 600 nm and Em. 710 nm, T; transmitted light channel, M; merge of channels A, B and T.

The detection of both compounds, the moxifloxacin and the MB, is an indication for their successful loading into the coaxial and triaxial fibres. In addition, the combined cyan-magenta colour can suggest for the uniformity distribution of both compounds in these fibrous systems.

4.3.3. Thermal Analysis and Physical State Characterisation

Previous thermal analysis studies have been outlined in Chapter 3 - Section 3.3.3. In particular, the TGA and standard DSC assessments for both drugs, moxifloxacin and pirfenidone, are described. PLGA was evaluated by TGA and modulated DSC with modulation parameters of amplitude ± 0.212°C and period 40 seconds. In this chapter, thermal analysis for PVP is performed along with the physical mixture of PLGA-pirfenidone and PVP-moxifloxacin using TGA and modulated DSC. Fibres prepared using ACTN have been tested, due to the fabrication of the single and multi-layered fibres using this solvent. This will eliminate the effect of using a different solvent on the results. Therefore,
blank and drug-loaded PLGA monoaxial (Table 4.1 - formulation 5 and 10), PVP monoaxial (Table 4.2 - formulation 16 and 20), coaxial (Table 4.3 - formulation 26 and 30) and triaxial (Table 4.4 - formulation 31 and 32) were assessed using TGA and modulated DSC. In addition, the physical state properties of the pure drugs, polymers and PM, as well as the blank and drug-loaded fibres (the same as to the above formulations) were evaluated by using FTIR and XRD.

4.3.3.1. Thermogravimetric Analysis

The raw materials (moxifloxacin, pirfenidone, PLGA and PVP) were assessed using TGA, with the results being shown in Figure 4.14. Moxifloxacin (Figure 4.14 (a)) exhibited a weight loss (3.5%) between 63°C and 96°C, most probably due to water loss. The onset degradation temperature was about 274°C. This was inconsistency with Al Omari et al. (2014) who reported 3.9% weight loss at around 150°C and a degradation point at approximately 250°C. Pirfenidone (Figure 4.14 (b)) showed no water loss on TGA but exhibited almost complete degradation (99.7% weight loss) when it was heated to 400°C, with an onset degradation temperature of about 199°C. PLGA (Figure 4.14 (c)) displayed a degradation onset of 344°C with a complete mass loss. This result agreed with Khalil et al. (2013) study which reported decomposition weight loss of PLGA from 260 to 380°C. PVP (Figure 4.14 (d)) showed a weight loss of 5.6% between 49°C and 76°C, indicating water loss of the polymer, since PVP is a hygroscopic material. The onset degradation temperature was about 383°C. Salles et al. (2015) reported an initial weight loss of PVP (20%) from 18 to 80°C due water loss and, to a lesser extent, residual solvent. PVP degradation was observed above 330°C that suggests that the weight loss was due to the polymer decomposition.
Figure 4.14: TGA thermograms of (a) moxifloxacin, (b) pirfenidone, (c) PLGA and (d) PVP raw materials, showing their degradation onset temperature(s).
The PMs of PLGA and pirfenidone, PVP and moxifloxacin, PLGA and PVP (representing the blank coaxial and triaxial fibres), and PLGA-pirfenidone/PVP-moxifloxacin (representing the drug-loaded coaxial and triaxial fibres), are presented in Figure 4.15 (a-f). For PLGA and pirfenidone PM (Figure 4.15 (a)), there were two weight loss phases, at approximately 153°C and 348°C, suggesting the degradation of both pirfenidone and PLGA, respectively. For PVP and moxifloxacin PM (Figure 14.5 (b)), there were three detected weight loss phases, at about 53°C, 225°C and 384°C, indicating water loss in the first degradation temperature, and then the degradation of moxifloxacin and PVP, respectively. Both drugs demonstrated lower degradation temperatures in the PM than their pure forms. This could be due to the presence of the polymer and the drug in combination that can enhance the thermal conductivity, which will need further investigation.

For PLGA and PVP PMs, which represent the blank coaxial and triaxial fibres, the thermograms in Figure 4.15 (c) and (d) exhibited two and three weight loss phases, respectively. For the coaxial blank PM, there was no distinct water loss, suggesting the presence of more PLGA than PVP, which corresponds to the coaxial system (Figure 4.15 (c)). The degradation of PLGA was at 352°C, while PVP was at 383°C, which was almost the same as the pure polymers. For the triaxial blank PM, water loss was seen at 42°C, earlier than the water loss in pure PVP. Second and third weight loss phases were seen at 345°C and 378°C, suggesting the degradation of PLGA and PVP, respectively (Figure 4.15 (d)).

Both PLGA-pirfenidone/PVP-moxifloxacin PMs that represent the drug-loaded coaxial and triaxial fibres showed similar thermograms to one another with four weight loss phases (Figure 4.15 (e) and (f)). These involved a water loss at about 52°C, PLGA degradation at 338-340°C and PVP degradation at about 385°C, with the fourth phase corresponding to the degradation of the drug. For the coaxial PM, the degradation appeared at about 174°C (Figure 4.15 (e)), while for the triaxial PM, the degradation was about 204°C (Figure 4.15 (f)), suggesting the presence of more pirfenidone or moxifloxacin, respectively.
Figure 4.15: TGA thermograms of (a) PLGA pirfenidone PM, (b) PVP moxifloxacin PM, (c) blank PLGA and PVP PM (coaxial), (d) blank PLGA and PVP PM (triaxial), (e) drug-loaded PLGA and PVP PM (coaxial) and (f) drug-loaded PLGA and PVP PM (triaxial), showing their degradation onset temperature(s).

The blank and pirfenidone-loaded PLGA fibres (Figure 4.16 (a) and (b), respectively) exhibited similar thermograms to the raw PLGA polymer with a degradation at 344 to 383°C, suggesting no residual solvent was entrapped during the manufacturing. This was consistent with Khalil et al. (2013) and their PLGA fibres which showed a weight loss from 240 to 365°C. Similarly, the blank and moxifloxacin-loaded PVP fibres (Figure 4.16 (c) and (d), respectively) demonstrated equivalent thermograms to the raw PVP polymer. This includes the solvent loss at about 51°C, which might be water, or residual solvents (ethanol or ACTN) that are entrapped within the fibres. This was in contrast to Salles et al. (2015), whose electrospun PVP/gelatine fibres lacked any residual solvents.
Figure 4.16: TGA thermograms of (a) blank PLGA fibres, (b) pirfenidone-loaded PLGA fibres, (c) blank PVP fibres and (d) moxifloxacin-loaded PVP fibres, showing their degradation onset temperature(s).
Blank and drug-loaded coaxial fibres (Figure 4.17 (a) and (b), respectively) demonstrated a weight loss at about 45-47°C which corresponds to entrapped solvent loss. The lost weight (under 2.5%) was less than PVP monoaxial fibres, probably due to the presence of PLGA at the outer layer. A degradation onset was seen at 332 and 318°C for the blank and drug-loaded coaxial fibres suggesting the degradation of PLGA. A third weight loss was observed at about 368°C and 354°C, indicating the degradation of PVP in the blank and drug-loaded triaxial fibres, respectively.

Triaxial fibres blank and drug-loaded (Figure 4.17 (c) and (d), respectively) showed a similar trend in weight loss (above 2.5%) between 41-47°C. A degradation of PLGA at about 320°C and 310°C was seen for the blank and drug-loaded triaxial fibres, respectively. PVP degradation was shown at about 359°C and 346°C for both blank and drug-loaded triaxial fibres.
Figure 4.17: TGA thermograms of (a) blank coaxial fibres, (b) drug-loaded coaxial fibres, (c) blank triaxial fibres and (d) drug-loaded triaxial fibres, showing their degradation onset temperature(s).
It should be noted that all the degradation temperatures for the polymers were below the expected temperatures (compared to the pure polymer degradation temperature). This may be due to the higher surface area of the fibres compared to the raw materials (Khalil et al., 2013). The degradation of the drugs was not observed, as in the PMs (Figure 4.15 (e) and (f)). This means that the thermal stability of the drugs was improved due to the presence of the polymers (Salles et al., 2015). Furthermore, due to the presence of residual solvent in the coaxial and triaxial fibres, a cytotoxicity test of the blank fibres is essential, which will be explored later in the next chapter.

4.3.3.2. Differential Scanning Calorimetry

PLGA, pirfenidone and moxifloxacin DSC results have been discussed previously in Chapter 3 - Section 3.3.3. MTDSC showed a Tg at around 48°C for PLGA, while the standard DSC exhibited a sharp melting peak at around 108°C for pirfenidone and a melting point of about 227°C for moxifloxacin. These results were consistent with the literature (Makadia and Siegel, 2011, Passerini and Craig, 2001, European Medicines Agency, 2010, Mandapalli et al., 2016; Moxifloxacin, 2008). In addition, it has been observed in the DSC traces of moxifloxacin that there was a water loss at 100°C. MTDSC results of the PVP polymer (Figure 4.18) demonstrated an endothermic peak at about 73°C, probably corresponding to water loss. A Tg at around 176°C was observed, which is consistent with the findings of Asawahame et al. (2015) and Suknuntha et al. (2012), who had a similar observation but with different PVP MWs. It is also important to note that the presence of water can act as a plasticiser, which will interact with the polymer backbone chain allowing its flexibility and thus, lowering its Tg temperature. Therefore, pinholes were created in the lids of the pans in order to allow the evaporation of the water, hence the measured Tg is measured after the water is lost (i.e. in the dry state).
For PLGA pirfenidone PM (Figure 4.19 (a)), it was observed that the Tg of PLGA appeared a slightly higher point (51°C) than was observed for the pure PLGA form (48°C). Similarly, pirfenidone in the mixture showed a sharp melting peak at an almost similar melting point (107°C) to the drug when tested alone (108°C). As a result, it is suggested that there is no clear interaction between these materials. Gupta et al. (2010) and Mohammadi et al. (2010) reported a similar finding when tested the PM of PLGA with sparfloxacin and azithromycin, respectively. On the other hand, the PVP moxifloxacin PM (Figure 4.19 (b)) exhibited a shift in the water loss peak (62°C) for both PVP and moxifloxacin compared to the pure polymer (73°C) and drug (100°C). Nonetheless, both the Tg of PVP and the melting point of moxifloxacin in the PM were equivalent to the pure PVP (176°C) and moxifloxacin (227°C). This suggests no interaction occurred between these two materials which is in agreement with the results of Hosny et al. (2015) who tested the PM of PVP and sildenafil citrate. Overall, the data indicates that the materials within the PMs was in the same physical state as the raw materials, with no evidence of incompatibility found.
The PLGA and PVP PMs, which represent the blank coaxial and triaxial fibres (Figure 4.19 (c), demonstrated two Tg's at 45.6°C and 175.7°C that correspond to the PLGA and PVP, respectively. The presence of both polymers' Tgs confirm their immiscibility (i.e. compatibility) (Coleman and Craig, 1996). PLGA-pirfenidone/PVP-moxifloxacin that represent the drug-loaded coaxial and triaxial fibres (Figure 4.19 (d), showed clear endothermic peaks of pirfenidone (105.3°C) and moxifloxacin (237.4°C). In addition, the Tg of PLGA (47.4°C) and PVP (168°C) were indicated. All these temperature points were slightly above or below the raw material temperature points owing to the presence of the hygroscopic drugs and PVP polymer in this PM. The presence of water could act as a plasticiser, hence lowering the Tg temperature of the polymer. Plasticiser molecules enhance the mobility of polymer molecular chain segments, hence the Tg is lowered due to less energy being required by the chain segment to convert the polymer to the rubbery state (Jamarani et al., 2018).
Figure 4.19: MTDSC traces of (a) PLGA pirfenidone PM, (b) PVP moxifloxacin PM, (c) PLGA PVP PM and (d) PLGA pirfenidone - PVP moxifloxacin PM. The melting point and Tg of the drugs and polymers, respectively, were all detected.
Monoaxial fibres of PLGA blank and pirfenidone-loaded (Figure 4.20 (a) and (b), respectively), demonstrated similar PLGA Tg (52°C) for both fibres compared to the PLGA PM (51°C). However, the melting peak of pirfenidone was absent in the drug-loaded monoaxial fibres compared to the PM, for which it was clearly observed. This signified molecular dispersion of the drug in the fibre matrix (i.e. the drug has been converted into an amorphous state in the fibres). This molecular dispersion was also observed in Said et al. (2011) PLGA fibres loaded with fusidic acid, in which the drug endothermic peak was absent from the DSC thermogram. Interestingly, the Tg of PLGA showed no reduction owing to the presence of pirfenidone which is expected to act as a plasticiser (i.e. lower the Tg of the polymer). This probably due to the low loading of pirfenidone into the PLGA fibres (drug:polymer ratio of 2:25).

A different observation was seen with the blank and moxifloxacin PVP monoaxial fibres (Figure 4.20 (c) and (d), respectively). The Tg of the PVP in the blank fibres was similar to the Tg of this polymer in the PM (177°C). An endothermic peak at 59.5°C indicated the evaporation of any remaining solvent. Nevertheless, the Tg of PVP in the moxifloxacin-loaded fibres was shifted by three degrees lower (174°C) than the PM (177°C). This might be due to the molecular dispersion of moxifloxacin which may lower the Tg value of the polymer due to the plasticising effect. The absence of the endothermic peak of moxifloxacin (at 227°C) further supported the suggestion of molecular dispersion. Illangakoon et al. (2014) reported the disappearance of the endothermic peaks of paracetamol and caffeine from the DSC thermogram of the drug-loaded PVP fibres compared to the PM, suggesting their molecularly dispersion in the fibre matrix.
Figure 4.2: MTDSC traces of (a) blank PLGA fibres, (b) pirfenidone-loaded PLGA fibres, (c) blank PVP fibres and (d) moxifloxacin-loaded PVP fibres. The Tg of the polymers were detected, while the drugs melting points were absent.
The MTDSC of the coaxial fibres, blank and drug-loaded (Figure 4.21 (a) and (b), respectively), showed a shift in the Tg of both PLGA and PVP polymers, with the lack of any melting peaks of the drugs. This might be due to the molecular dispersion formation of both drugs, as reported in Zhang et al. (2012) study on captopril loaded into PLGA, PLA or PCL fibrous systems. Similarly, the blank and drug-loaded triaxial fibres (Figure 4.21 (c) and (d), respectively) demonstrated a shift in the Tg of the polymers and the absence of the drugs' endothermic peaks. In addition, the shift of the Tg for both polymers were larger in the drug-loaded coaxial and triaxial fibres compared to the blank fibres. This can be a result of the miscibility of the drugs with the polymers, which has been observed in their PM (Figure 4.19 (d)).
Figure 4.21: MTDSC thermograms of (a) blank coaxial fibres, (b) drug-loaded coaxial fibres, (c) blank triaxial fibres and (d) drug-loaded triaxial fibres. The Tg of the polymers were detected, while the drugs melting points were absent.
This DSC study demonstrated the presence of moxifloxacin and pirfenidone melting peak (endothermic peaks) in the PM while their absence in the drug-loaded monoaxial, coaxial and triaxial fibres thermograms. This indicates a molecular dispersion of both drugs in the fibres matrix and the absence of any recrystallisation that may occur during the electrospinning process.

4.3.3.3. Fourier-Transform Infrared Spectroscopy

The FTIR spectra of the pure polymers and drugs are illustrated in Figure 4.22. The PLGA FTIR spectrum exhibited a C=O stretch at 1746 cm⁻¹ and a C-O-C stretch at 1100 cm⁻¹. These spectra demonstrated carbonyl stretching due to the presence of an ester group. This is in consistent with the PLGA FTIR spectrum of Zhang et al. (2012), Khalil et al. (2012) and Zhu et al. (2015). The characteristic peaks of PVP were showed at 1658 cm⁻¹ (C=O stretching), 1455 cm⁻¹ (C-H bending in aliphatic compound) and 1280 cm⁻¹ (C-N stretching in aromatic amine). Due to the hygroscopic nature of PVP, a broad band was observed at 3418 cm⁻¹ indicating the presence of O-H stretch. This spectrum is in agreement with Illangakoon et al. (2014), Wang et al. (2015) and Asawahame et al. (2015) PVP FTIR spectrums.

The FTIR spectrum for pirfenidone demonstrated stretching at 3050 cm⁻¹ (aromatic C-H stretching), 2929 cm⁻¹ (aliphatic C-H stretching), 1671 cm⁻¹ (C=O stretching in tertiary amide), 1622-1454 cm⁻¹, (aromatic C=C and pyridine ring stretching) and 1273-1260 cm⁻¹ (C-N stretching), which is similar to pirfenidone FTIR spectrum of Soni et al. (2018). In addition, sharp bands between 824 cm⁻¹ and 699 cm⁻¹ are present in the fingerprint region that represent substitutions in the benzene ring. The moxifloxacin FTIR spectrum showed some key peaks at 3523-3470 cm⁻¹ (N-H aromatic amine stretching), 2950 cm⁻¹ (aliphatic C-H stretching), 1703 cm⁻¹ (carboxylic acid C=O stretching), 1621 cm⁻¹ (pyridine ring stretching), 1513 cm⁻¹ (aromatic benzene ring stretching), 1454-1423 cm⁻¹ (carboxylic acid O-H stretching) and 1280-1180 cm⁻¹ (aromatic amine C-N stretching). In addition, characteristic peaks
were observed at the fingerprint region at about 780-720 cm$^{-1}$, which represent the stretch of substitutions in the benzene ring. This spectrum agrees with Sabitha et al. (2012) and Mudgil and Pawar (2013) moxifloxacin FTIR spectrums.

**Figure 4.22:** FTIR spectra of the pure PLGA, PVP, pirfenidone and moxifloxacin, showing each material distinctive peaks.

The main peaks of PLGA and PVP can be observed in both the blank and drug-loaded monoaxial fibres and their PMs, as shown in Figure 4.23 (a) and (b), respectively. Moreover, multiple peaks located between 1671 cm$^{-1}$ and 1455 cm$^{-1}$ were detected in the PM and pirfenidone-loaded PLGA monoaxial fibres, while they were absent in the blank fibres (Figure 4.23 (a)). This suggests the presence of the drug with the polymer. Additionally, the key peaks of PLGA (i.e. C=O stretch at 1746 cm$^{-1}$ and a C-O-C stretch at 1100 cm$^{-1}$) were presented in the blank, drug-loaded and the PM. A similar observation was seen in the fingerprint region, at 772 cm$^{-1}$, of the moxifloxacin PVP monoaxial and the PM, but not in the blank fibres, thus suggesting good compatibility between both compounds (Figure 4.23 (b)). Furthermore, the
main peaks of PVP (i.e. O-H stretching at 3418 cm\(^{-1}\), C=O stretching at 1658 cm\(^{-1}\), C-H bending at 1455 cm\(^{-1}\) and C-N stretching at 1285 cm\(^{-1}\)) were observed in the blank and drug-loaded fibres, as well as the PM.

Figure 4.23: FTIR spectra of (a) blank and pirfenidone-loaded PLGA fibres and PM (b) blank and moxifloxacin-loaded PVP fibres and PM, showing pirfenidone and moxifloxacin distinctive peaks in the drug-loaded monoaxial fibres and the PMs.
The FTIR spectra for the coaxial and triaxial fibres and their PMs suggested the presence of both polymers. This is due to the detection of the characteristic peaks at $1746 \text{ cm}^{-1}$ (C=O stretch) for PLGA and at $3418 \text{ cm}^{-1}$ (O-H stretching) and at $1658 \text{ cm}^{-1}$, (C=O stretching) for PVP, as shown in Figure 4.24 (a) and (b), respectively. In addition, the pirfenidone peak at around $1530 \text{ cm}^{-1}$ and the moxifloxacin peak at approximately $770 \text{ cm}^{-1}$ can be observed in the drug-loaded PMs, coaxial and triaxial fibres but not in the blank PMs and fibres (Figure 4.24 (a) and (b), respectively). This indicates the presence of these drugs within these formulations. Zhu et al. (2015) demonstrated the presence of flurbiprofen axetil characteristic peaks in the FTIR spectrum of drug-loaded PLGA/PVP coaxial fibres.
Figure 4.24: FTIR spectra of (a) blank and drug-loaded coaxial fibres and PM and (b) blank and drug-loaded triaxial fibres and PM, showing pirfenidone and moxifloxacin distinctive peaks in the drug-loaded coaxial and triaxial fibres and their PMs.
This FTIR result indicates the presence of the distinctive peaks of the drugs and polymers in the drug-loaded fibres and their corresponded PM. This suggests the absence of chemical interactions between the drug and the polymer due to the electrospinning process. Numerous studies, such as Zhang et al. (2012) on captopril loaded PLGA, PCL or PLA nanofibres, Khalil et al. (2012) on silver loaded PLGA nanofibres, Wang et al. (2015) on curcumin loaded PVP nanofibres, Asawahame et al. (2015) on propolis extract loaded PVP nanofibres, Sabitha et al. (2012) on moxifloxacin-loaded chitosan NPs, Mudgil and Pawar (2013) on moxifloxacin-loaded PLGA nanosuspension and Soni et al. (2018) on pirfenidone-loaded PVA microspheres, have shown a consistent finding of no interaction between the loaded drug and the polymer after the fabrication process. This suggests the presence of these drugs with the absence of any changes in their structural integrity or any loss of the efficiency of these drugs.

4.3.3.4. X-Ray Diffraction

XRD diffraction patterns for the pure polymers and drugs are presented in Figure 4.25. PLGA and PVP are amorphous polymers and hence will not be expected to show any crystalline peaks. Mudgil and Pawar (2013) and Asawahame et al. (2015) demonstrated broad halos XRD patterns for PLGA and PVP polymers, respectively. However, for the pure pirfenidone and moxifloxacin, a very clear characteristic reflection is observed in their XRD pattern. Distinct peaks at 8.90°, 14.94°, 19.04°, 23.08°, 24.80° and 27.60° were observed in pirfenidone diffractogram, which is similar to the findings of Kashikar et al. (2014) and Soni et al. (2018) pirfenidone diffractograms. The moxifloxacin XRD pattern showed diffraction peaks at 5.90°, 8.62°, 11.81°, 14.48°, 15.58°, 17.34°, 24.18°, 26.60°, 27.46° and 31.54°, which are in agreement with the moxifloxacin XRD pattern of Mudgil and Pawar (2013).
Figure 4.25: XRD patterns of pure PLGA, PVP, pirfenidone and moxifloxacin, showing that both drugs are in the crystalline form (distinct peaks), while the both polymers are in the amorphous form (broad halos).

For the blank and drug-loaded monoaxial fibres and their PMs, both PLGA and PVP retained their amorphous state, as can be seen in Figure 4.26 (a) and (b), respectively. However, pirfenidone showed crystallinity peaks at 8.69° and 27.29° in the PM, while no diffraction peaks were visible in the drug-loaded PLGA fibres (Figure 4.26 (a)). Similarly, moxifloxacin exhibited characteristic peaks at 11.83°, 17.05°, 26.39° and 27.24° in the PM, whereas these peaks were absent in the drug-loaded PVP fibres (Figure 4.26 (b)). This indicates an amorphous distribution of each drug in their corresponding monoaxial fibres. This observation is consistent with the XRD pattern of pirfenidone-loaded microspheres (Kashikar et al., 2014 and Soni et al., 2018) and moxifloxacin-loaded PLGA NPs (Mudgil and Pawar, 2013). In addition, PLGA and PVP fibres that are loaded with different drugs; Zhang et al. (2012) on captopril loaded PLGA, PCL or PLA nanofibres, Illangakoon et al. (2014), on paracetamol and caffeine loaded PVP, Wang et al. (2015) on curcumin loaded PVP nanofibres, Asawahame et al. (2015) on propolis extract loaded PVP.
nanofibres, have shown similar results of amorphous distribution of the loaded drugs within the corresponded systems.

**Figure 4.26:** XRD patterns of (a) blank and pirfenidone-loaded PLGA fibres and PM (b) blank and moxifloxacin-loaded PVP fibres and PM. It shows that both the blank and drug-loaded monoaxial fibres are in the amorphous form (broad halos), while there are distinct peaks in the PM.

Correspondingly, both pirfenidone and moxifloxacin peaks appeared at diffraction angles of 18.45°, 20.99°, 23.21°, 27.27°, 28.45° and 32.37° for the drug-loaded coaxial PM and at 16.59°, 18.33°, 27.17° and 28.05° for the drug-loaded triaxial PM, which were lacking from the blank PMs, as can be seen in Figure 4.27 (a) and (b), respectively. However, the blank and drug-loaded coaxial and triaxial fibres (Figure 4.27 (a) and (b), respectively) exhibited broad halos similar to the PLGA and PVP monoaxial fibres in Figure 4.26 (a) and (b), respectively. This was due to the molecular dispersion of both drugs in the coaxial and triaxial fibrous systems.
Figure 4.27: XRD patterns of (a) blank and drug-loaded coaxial fibres and PM (b) blank and drug-loaded triaxial fibres and PM. It shows that the blank and drug-loaded coaxial and triaxial fibres and their blank PM are in the amorphous form (broad halos), while there are distinct peaks in the drug-loaded PM.

The absence of the crystalline peaks of both drugs in the drug-loaded fibres suggested that these drugs were molecularly dispersed into the fibre matrix and that the electrospinning process did not develop a crystalline structure of these fibres. This is supported by several studies such as Zhang et al., 2012, Illangakoon et al., 2014, Wang et al., 2015 and Asawahame et al., 2015. In addition, this XRD data is consistent with the DSC results in Section 4.3.3.2, which further confirmed this molecular dispersion.

4.3.4. Drug Loading [DL], Entrapment Efficiency [EE%] and Yield [Y%]

The DL, EE% and Y% for pirfenidone-loaded PLGA fibres (Formulations 8 and 10), moxifloxacin-loaded PVP fibres (Formulations 18 and 20), drug-loaded coaxial (Formulations 28 and 30) and triaxial fibres (Formulation 32) are presented in Table 4.7. As can be seen, all monoaxial fibres had an EE higher than 87%, which can be considered high. Sofokleous et al. (2013) were able
to achieve 90% EE of amoxicillin loaded into PLGA fibres for wound dressing purposes. In addition, Illangakoon et al. (2014) were able to load the combination of paracetamol and caffeine in PVP. These fibres had EE of more than 95% for both drugs. The EE of pirfenidone and moxifloxacin was between 80 and 88% within both coaxial fibre formulations, whereas, they were about 96% and 85% for both drugs, respectively, in the triaxial formulation. Furthermore, it is noted that the monoaxial PLGA and PVP fibres that were prepared using ACTN (Formulation 10 and 20, respectively) had higher EE than the PLGA and PVP fibres fabricated using DCM (Formulation 8 and 18, respectively). This is probably due to the stability of the electrospinning jet and the less gel-like polymer formation on the needle tip that can be observed with the ACTN formulations. However, this would need a further investigation for a mechanistic explanation.

Table 4.7: DL, EE% and Y% for the drug-loaded monoaxial, coaxial and triaxial fibres. Form. 8: PLGA in DCM, form. 10: PLGA in ACTN, form. 18: PVP in ethanol:DCM, form. 20: PVP in ethanol:ACTN, form. 28: coaxial fibres prepared using DCM, form. 30: coaxial fibres prepared using ACTN, form. 32: triaxial fibres prepared using ACTN.

<table>
<thead>
<tr>
<th>Form. #</th>
<th>DL (µg/mg) ± SD</th>
<th>EE (%) ± SD</th>
<th>Y (%) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pirfenidone</td>
<td>Moxifloxacin</td>
<td>Pirfenidone</td>
</tr>
<tr>
<td>8</td>
<td>104.39 ± 0.78</td>
<td>n/a</td>
<td>88.76 ± 0.66</td>
</tr>
<tr>
<td>10</td>
<td>74.44 ± 2.25</td>
<td>n/a</td>
<td>100.50 ± 3.04</td>
</tr>
<tr>
<td>18</td>
<td>n/a</td>
<td>79.92 ± 0.10</td>
<td>n/a</td>
</tr>
<tr>
<td>20</td>
<td>n/a</td>
<td>82.45 ± 1.11</td>
<td>n/a</td>
</tr>
<tr>
<td>28</td>
<td>27.38 ± 0.90</td>
<td>52.54 ± 0.80</td>
<td>83.48 ± 2.74</td>
</tr>
<tr>
<td>30</td>
<td>41.87 ± 1.13</td>
<td>23.04 ± 0.89</td>
<td>79.61 ± 2.16</td>
</tr>
<tr>
<td>32</td>
<td>39.33 ± 1.51</td>
<td>34.85 ± 1.89</td>
<td>96.40 ± 3.71</td>
</tr>
</tbody>
</table>

Due to the higher flow rate of the core to the shell (0.8 mL/h of the core and 0.2 mL/h of the shell) in the coaxial fibres which were prepared by using DCM solvent (Formulation 28), more moxifloxacin was loaded (52 µg/mg) than...
pirfenidone (27 µg/mg), despite using twice the concentration of pirfenidone. However, by using a similar flow rate (in Formulation 30), the DL of pirfenidone (42 µg/mg) was almost double the DL of moxifloxacin (23 µg/mg), due to the initial twofold concentration difference of pirfenidone to moxifloxacin. The addition of an outer PVP layer that contained 1% moxifloxacin in the triaxial fibres (Formulation 32) equalised that difference. Eventually, the DL for both drugs in this formulation were almost similar (39 µg/mg for pirfenidone and 35 µg/mg for moxifloxacin).

PLGA fibres that were spun using DCM (Formulation 8) had a low Y (34.37%) compared to the formulation made using ACTN (Formulation 10, Y=82.5%). This may attributed to the higher solubility of PLGA in ACTN and the stability of the electrospinning jet, which was poorly controlled in the case of the DCM spinning solution compared to ACTN spinning solution. For PVP monoaxial fibres, which were prepared using DCM (Formulation 18) and ACTN (Formulation 20), in 1:1 mix with ethanol, showed Y above 80%. However, the coaxial system showed a higher Y of 83.67%, for the fibres that were prepared using DCM (Formulation 28), compared to the fibres prepared using ACTN (64.18%), despite using this solvent. This higher Y was due to the high core flow rate of the PVP solution, which allowed more control to the jet and thus, the formation of more monoaxial PVP superimposed into the coaxial fibres. By using a similar flow rate of the core and shell, more coaxial fibres were produced compared to the monoaxial fibres, while controlling the spinning jet. Due to the close resemblance between the triaxial (Formulation 32) and the coaxial fibres (Formulation 30), in terms of the manufacturing processing, both systems had similar Y. This result showed the improvement of the manufacturing process of PLGA fibres by changing the solvent to ACTN, instead of DCM. In addition, there was a drop in the quantity of the coaxial fibres prepared using ACTN compared to the DCM, probably due to the collection of more moxifloxacin-loaded PVP monoaxial fibres (had the highest Y when spun alone) covering the coaxial ones.

Overall, the electrospinning technique provides the production of fibres in high DL and EE, as reported in Chakraborty et al. (2009) review paper. However, loading drugs in fibres requires a relatively acceptable drug to polymer ratio,
as well as good chemical compatibility between the drug and the polymer after the fabrication process. This will ensure a better encapsulation and avoid an unwanted release profile, such as burst release (Chakraborty et al., 2009). Unfortunately, there is not an absolute figure for the drug to polymer ratio and chemical compatibility. Nevertheless, it has been reported that choosing a high polymer to drug ratio is better in case of less compatible components (i.e. lipophilic polymer and hydrophilic drug). Zamani et al. (2010) study suggested that by increasing the concentration of the lipophilic drug metronidazole benzoate, the EE went down and the release was faster, despite using a water insoluble polymer PCL. Another study by Said et al. (2011) indicated a similar observation, in that by increasing the drug concentration the EE is reduced. Here, the drug used, fusidic acid, was water soluble while the polymer was PLGA. Another observation in this study is by using a higher PLGA concentration and keeping the drug concentration constant, the EE is reduced. These two results were explained by the lower drug saturated solubility in the electrospinning polymer solution.

Accordingly, the DL of the fibres can be considered more important than the EE, since it can measure the amount of the loaded drug within the fibres, while the EE measures the process efficiency. The amount of pirfenidone and moxifloxacin was determined in order to be able to calculate the required dose of both drugs for the microbiological and cell culture studies later. Due to the flexibility of the electrospun fibres, the collected fibrous mat can be cut into a specific weight and shape based on the required final dosage. Finally, only the formulations that are prepared using ACTN solvent (Formulations 10, 20, 30 and 32) were used in the next in vitro studies (the release and swelling tests, as well as, the microbiological and cell culture tests). This was owing to DL results which showed an evenly drug distribution, i.e. a ratio of 2:1 and 1:1 pirfenidone to moxifloxacin for the coaxial and triaxial fibres, respectively, that correspond to the initial drugs’ concentration. This was also confirmed by the confocal microscope images (Figure 4.12 and 4.13 for the coaxial and triaxial fibres, respectively) that showed an even distribution of moxifloxacin and MB dye upon merging the two channels, as previously explained.
In conclusion, the fabrication of a multi-layered fibrous system using the hydrophilic polymer PVP instead of PVA has improved the process reproducibility and the collected fibre yield. Owing to the high solubility of PVP in the organic solvent ethanol, a multi-layered system was fabricated using solvents that are miscible. This eliminated the use of a large different flow rate between the core and the other layers that is recommended in many multi-layered fibres preparation studies, hence an even drug distribution has resulted. PVP monoaxial fibres was successfully prepared with a high Y% of more than 83% compared to the Y% of the PVA fibres (16.9%). This was due to the ease of peeling the PVP fibres of the collecting material (aluminium foil) and the lack of fibres sticking on the spraying tube and the surrounding walls that was observed with the PVA fibres. The use of a high conductive solvent (i.e. water) with the PVA allowed the application of a high voltage (> 20 kV) in order to stabilise the electrospinning jet. This might have produced over-dried fibres that stuck on the collecting material. Eventually, the use of PLGA and PVP solution which are miscible has produced multi-layered fibres with high EE% and Y% compared to the immiscible fibrous system of PLGA and PVA.

4.3.5. Drug Release Profile

It was previously reported by Lu et al. (2011), Said et al. (2011), Weldon et al. (2012), Li et al. (2014) and Jahangiri et al. (2014) that the drug release from a polymeric delivery system can be due to desorption of the weakly bound drug from the surface (adsorbed drug), diffusion of the drug from the polymer matrix and/or erosion of the polymer. The accelerated release of the adsorbed drug on the surface of the large surface area of the polymeric system is known as ‘burst release’ and it occurs at the initial stage of the dissolution study (Lu et al., 2011 and Said et al., 2011). Drug that is uniformly distributed and incorporated into the polymeric matrix often released via a combination of erosion and diffusion processes. However, when the drug diffuses faster than polymer matrix erosion, the release of the drug will depend mainly on the diffusion process (Lu et al., 2011).

The release studies were performed on the fibres that are prepared using ACTN solvent, i.e. PLGA, PVP, coaxial and triaxial fibres - Formulations 10,
20, 30 and 32, respectively. This was due to the pharmaceutical purpose of manufacturing a multi-layered system that is reproducible in the DL, EE% and Y%.

4.3.5.1. Monoaxial Fibres Release using Dialysis Bags

The release of the drug-loaded PLGA and PVP monoaxial fibres via the dialysis bags are shown in Figure 4.28 and 4.29, respectively. The release of pirfenidone from PLGA was a triphasic pattern, which showed an initial burst release after one day, followed by sustained release profile for 15 days and finally, a second burst release (Figure 4.28). This triphasic release profile was consistent with Said et al. (2011) who studied fusidic acid release from PLGA fibres. The initial burst release represents the drug molecules that are located near the surface of the fibres in addition to the large surface area of the fibres and the presence of PBS that readily dissolve the hydrophilic drugs allowing their attraction out of the fibres. The sustained release was due to the diffusion of the drug through the polymer matrix and through the pores that were generated on the surface of the fibres upon the release of that drug. The second burst release resulted after three weeks with a complete release after a month due to the polymer matrix erosion and the formation of the pores. This observation was expected since PLGA is a biodegradable polymer and drug release mechanisms can be due to desorption of the attached drug at the surface, drug diffusion within the polymer matrix and/or the erosion (hydrolysis) of the polymeric matrix by PBS penetration forming channels, as reported in Weldon et al. (2012), Li et al. (2014) and Jahangiri et al. (2014). Another explanation for the second burst release is because of possible drug entrapment inside the dialysis bag and the presence of a visible entrapped air bubble that might hindered the release through the bag. On the other hand, the release of the drug-loaded PLGA fibres was compared with the release of the free drug (powder form), which demonstrated a full drug release after one day from the dialysis bags. The release of the drug in the powder form was expected to be completely released after few minutes to hours due to the significantly smaller MW of the pirfenidone (185.2 daltons) compared to the molecular weight cut-off of the dialysis bag (14,000 daltons). However, this
slow release of the drug from the bag indicates that the drug release may be being controlled by the bag itself.

**Figure 4.28**: The release profile of 2% of pirfenidone-loaded into 25% PLGA monoaxial fibre against and equivalent amount of pirfenidone powder using dialysis bags. The drug-loaded fibres showed a first burst release after 24 hours followed by sustained release profile for 15 days and finally, a second burst release compared to a 24 hours complete release of the powder form drug. The large graph represents the full release duration (30 days), while the small graph represents the release after 24 hours.

SEM image (Figure 4.29) of pirfenidone-loaded PLGA fibres was taken after 30 days of release. The fibres were entangled (fused with each other) with the absence of any microscopic gaps between the fibres in the fibrous network, which was consistent with Said et al. (2011) PLGA fibres loaded with fusidic acid SEM image after 30 days of release. Due to the lack of formed pores on the surface of the fibres, the presence of an entrapped air that was visually observed, or an entrapped drug inside the bag would be the rational explanation for the second burst release.
Moxifloxacin was released from PVP fibres after one day at a rate which was slightly higher than the free drug (powder form), as can be seen in Figure 4.30. This release was expected, since PVP is a water soluble polymer, which will dissolve rapidly in PBS allowing the release of the loaded moxifloxacin. Mirzaeie et al. (2018) reported a complete release of triamcinolone acetonide loaded in PVP blended with chitosan fibres in four days using dialysis bags.
Figure 4.30: The release profile of 1% moxifloxacin-loaded into 10% PVP monoaxial fibre over 24 hours compared to an equivalent amount of moxifloxacin powder using dialysis bags. The drug-loaded fibres showed a complete release after 24 hours in a sustain release profile similar to the powder form drug.

4.3.5.2. Coaxial Fibres Release using Dialysis Bags

The 2% pirfenidone and 1% moxifloxacin-loaded coaxial fibres (Formulation 30) showed a burst release of pirfenidone from the PLGA layer after two hours and about 97% release after one day (Figure 4.31). This unexpected release (compared to the monoaxial fibres) was probably due to the location of the encapsulated drug molecules near the surface of the fibres, allowing less space for the drug to travel through the PLGA polymer matrix. Owing to the presence of pirfenidone and moxifloxacin-loaded into PVP inside the PLGA fibres, this might have improved the penetration of PBS that accelerated the release of pirfenidone. On the other hand, moxifloxacin was released at a steady rate up to a day in which 70.6% of the drug was released. Then a very slight release was shown after 30 days, which reached to approximately 78% (Figure 4.31). This slow release profile of moxifloxacin might be due to the
entrapment of the drug in the core layer (PVP) which required more PBS to penetrate the PLGA layer in order to free this entrapped drug.

Figure 4.31: The release profile of 2% pirfenidone-loaded into 25% PLGA and 1% moxifloxacin-loaded into 10% PVP from the drug-loaded coaxial fibres using dialysis bags. The drug-loaded fibres showed a burst release of pirfenidone after 2 hours followed by ~ 97% compared to a sustained release profile of moxifloxacin for 30 days. The large graph represents the full release duration (30 days), while the small graph represents the release after 24 hours.

The SEM image of this coaxial system in Figure 4.32 was taken after the completion of this release study (30 days). This image showed that the fibres were entangled with a smoother surface appearance compared to the image before the release. The lack of the microscopic gaps in the fibres may hinder the penetration of the PBS into the fibres that contributed to the remaining amount of drug. To confirm this hypothesis, the EE% of the released fibre mat was measured, by the above method of detection (section 4.2.2.8.), and the
EE% for pirfenidone and moxifloxacin were 0% and 18.1%, respectively, demonstrating the continued entrapment of the latter drug.

Figure 4.32: SEM image of 2% pirfenidone-loaded into 25% PLGA and 1% moxifloxacin into 10% PVP drug-loaded coaxial fibres after 30 days release. Fibres are entangled and the fibres’ surface are smooth.

However, since the release rate of the powder form of pirfenidone (Figure 4.28) and moxifloxacin (Figure 4.30) was after 24 hours rather than few minutes, it was suggested that the use of the dialysis bags may have an effect on the release profile of the tested drugs (i.e. control the release rate). This may be due to adsorption effect, of the drugs onto the dialysis bags, or poor stirring within the interior of the bags, but in either case, there is a clear implication that the dialysis bags are inhibiting the release of the drugs. The shaking speed of this release study (75 rpm) was chosen as a mid-point of previous dissolution studies of electrospun fibres on ocular delivery, which used either 50 or 100 rpm (Garg et al., 2014, Hilal et al., 2016, Sun et al., 2016; Mirzaeei et al., 2018). This mid-point shaking speed and the presence of an entrapped air (i.e. mechanical fault) probably would be insufficient to permit an even stirring to the release medium inside the dialysis bags, hence delaying the release of the drug. The data for the fibre systems cannot be regarded as a
true reflection of the release from the fibres themselves, thus another release study was performed, avoiding the use of these bags.

4.3.5.3. Monoaxial Fibres Release using Custom-Made Cages

An alternative release method was used which placed the drug-loaded fibres inside a perforated custom-made cages to prevent the fibres from floating and to eliminate the use of the dialysis bags. Here, the release of the drugs will be through the polymer matrix rather than the matrix followed by the dialysis bag. Both PLGA and PVP monoaxial fibre release profiles were shown in Figures 4.33 and 4.34, respectively. In the case of pirfenidone-loaded in PLGA fibres, both 1% and 2% drug concentrations were used (in an equivalent DL amounts) to demonstrate the effect of increasing the drug concentration on the release study. As it can be seen in Figure 4.33, pirfenidone showed a similar release profile for both drug concentrations. This suggests that by using a higher concentration of 2%, there is no acceleration in the rate of the release which can be expected due to enhancing the drug concentration. Therefore, PLGA loaded with the higher pirfenidone concentration (2%) was taken forward for the coaxial and triaxial fibres preparation. Both fibre systems showed a burst release of approximately 36% after 1 hours of release. However, 91.6% and 87.1% of the drug was released after one day for the 1% and 2% drug concentration, respectively, followed by a sustained release for 15 days. A complete release has been achieved at this time point.
Figure 4.33: The release profile of 1% and 2% w/v pirfenidone-loaded into 25% PLGA monoaxial fibre against an equivalent amount of pirfenidone powder using custom made cages after 15 days release. The drug-loaded fibres showed a similar profile of a burst release after 1 hour followed by sustained release profile for 15 days compared to the complete release of the powder form drug after the first time point. The large graph represents the full release duration (15 days), while the small graph represents the release after 24 hours.

This release profile might be due to the diffusion of the drug from a larger surface area-to-volume ratio in addition to the slow rate erosion of PLGA that allow the full release of pirfenidone after 15 days. Furthermore, this profile demonstrated the effect of the dialysis bags on controlling the release of the drug. Additionally, the pure drug showed a complete dissolution from the first time point up to one day (Figure 4.33), compared to a full pirfenidone release after 24 hour using the dialysis bag (Figure 4.28). This suggest that there is no release controlling step by using the cages in comparison to the dialysis bags.

This release profile was consistent with the release study of captopril loaded PLGA fibres of Zhang et al. (2012), which demonstrated a 90% drug release after 2 hours and a complete release after 250 hours (~ 10.4 days). This release profile was due to the use of a water soluble drug and the swelling of
the fibres (~ 290%) that allowed to loosen the entangled chains of PLGA matrix and hence, more buffer penetrated which accelerated the drug release from within the fibres (Zhang et al., 2012).

A more accelerated release of moxifloxacin was observed in this release method compared to the dialysis bags, as it can be seen in Figure 4.34. Almost complete release was obtained after 4 hours compared to a day in the case of the dialysis bags, with 92% drug release achieved in 30 minutes.

![Figure 4.34](image_url)  
*Figure 4.34. The release profile of 1% moxifloxacin-loaded into 10% PVP monoaxial fibre against an equivalent amount of moxifloxacin powder using custom made cages after 24 hours release. The drug-loaded fibres showed a complete release of moxifloxacin after 4 hours compared to the full release of the powder form drug after the first time point.*

This PVP fibre release profile was consistent with Illangakoon et al. (2014) who observed more accelerated dissolution rates of caffeine and paracetamol from PVP fibres within six minutes. A complete dissolution of moxifloxacin was measured from the first time point up to one day of release (Figure 4.34). This was in contrast to the release of this drug using the dialysis bags, which
showed a complete release after 24 hours (Figure 4.30). Similarly, the use of the dialysis bag controlled the release of the drugs through the dialysis membrane. The very fast release of the drug-loaded PVP fibres was owing to the hydrophilicity of PVP which attributed to the quick disintegration and dissolving of the polymer in buffer solution. Additionally, the high surface area-to-volume ratio of the fibres and buffer solution was able to accelerate the drug release. These release mechanisms for PVP fibres were explained by Illangakoon et al. (2014).

The release of a monoaxial formulation consisting of PLGA loaded with both pirfenidone and moxifloxacin (Table 4.1 - formulation 11) was evaluated. This is to make a comparison between a system that has both drugs separated into two different compartments (layers), which is the coaxial fibres, and a system which carries both drugs into the same layer. Formulation 11 (20% PLGA loaded with 2% pirfenidone and 1% moxifloxacin) demonstrated a fast release of moxifloxacin (87%) and pirfenidone (92%) after 4 hours, with a complete release of both drugs in 24 hours (Figure 4.35).
Figure 4.35: The release profile of 2% pirfenidone- and 1% moxifloxacin-loaded into 20% PLGA monoaxial fibre using custom made cages after 4 days release. The drug-loaded fibres showed a fast release of both drugs after 4 hours followed by a complete release after 24 hours.

This unexpected fast release might be due to the hydrophilicity of both drugs that allowed the penetration of PBS into PLGA and the higher drug-loaded concentration that accounts for 2% pirfenidone and 1% of moxifloxacin which facilitated the diffusion of both drugs out of this system. This explanation was also reported by Chakraborty et al. (2009) and Williams et al. (2018). SEM image of the fibres after 4 days of release, shown in Figure 4.36, exhibited that the fibres were entangled with a smoother surface appearance compared to the fibres image before the release.
4.3.5.4. Coaxial Fibres Release using Custom-Made Cages

The drug-loaded coaxial system showed a burst release of pirfenidone (39%) after 30 minutes then a complete release after 1 day (Figure 4.37). This release rate was similar to the release using the dialysis bags (Figure 4.31). However, moxifloxacin demonstrated 60% release after 30 minutes with a plateau release up to 4 days (Figure 4.37). This burst release of moxifloxacin was in contrast to the sustain release of this drug using the dialysis bags (Figure 4.31). In addition, the EE% of the entrapped moxifloxacin was estimated to be 24.8%. This entrapment was due to the entanglement of the fibres after 4 days of release, as shown in Figure 4.38. This indicated that moxifloxacin can be released either after the erosion of the PLGA outer layer, or the penetration of more PBS into the inner layer, which is a similar finding to the release using dialysis bags.
Figure 4.37: The release profile of 2% pirfenidone-loaded into 25% PLGA and 1% moxifloxacin-loaded into 10% PVP from the drug-loaded coaxial fibres using custom made cages. The drug-loaded fibres showed a burst release of pirfenidone after 30 minutes followed by a complete release after 1 day compared to a burst release of moxifloxacin after 30 minutes followed by a plateau release up to 4 days. The large graph represents the full release duration (4 days), while the small graph represents the release after 24 hours.
This release profile of moxifloxacin from the coaxial fibres was consistent with Zhu et al. (2015) who were able to fabricate a PLGA/PVP coaxial system loaded with flurbiprofen axetil in the core, which has an initial burst release (40%) after 6 hours followed by a sustained release (70%) after 204 hours. In addition, Han and Steckl (2013) demonstrated approximately 50% release of the core drug after 24 hours from PCL/PVP immiscible coaxial system (PCL was dissolved in chloroform and PVP was dissolved in water). However, both the core and the shell drugs were released completely after 24 hours and 2 hours, respectively, from the miscible coaxial system (PCL was dissolved in trifluoroethanol and PVP was dissolved in water). Another study by He et al. (2018) reported a sustained release of PLGA-metronidazole/PVP-naringin coaxial system, with the shell drug (metronidazole) exhibited a faster release profile than the core drug (naringin). A complete release of both drugs was seen after 5 days. These studies demonstrated the ability to fabricate coaxial systems that can slow the release of the core drug compared to the shell,
except for the PCL/PVP miscible coaxial system (Han and Steckl, 2013), which showed an accelerated release of both the core and the shell drugs.

The accelerated release of pirfenidone from the coaxial fibres (compared to the pirfenidone-loaded PLGA monoaxial fibres) was attributed to the hydrophilicity of this drug in addition to moxifloxacin and PVP. The incorporation of several hydrophilic materials within the PLGA matrix may have changed its nature into a hygroscopic layer allowing the penetration of PBS, hence accelerate the release of pirfenidone. A similar observation was seen in the PCL/PVP miscible coaxial system of Han and Steckl (2013) and PLGA/PVP coaxial fibres of He et al. (2018). Another explanation to this fast release rate of pirfenidone is owing to the location of the drug molecules on the surface or encapsulated near the surface of the fibres (i.e. less space to travel through the PLGA polymeric matrix), as it will be explained later in section 4.3.5.6.

4.3.5.5. Triaxial Fibres Release using Custom-Made Cages

The purpose of forming a triaxial system was to have a burst release of moxifloxacin, as the outer layer, in order to inhibit the microorganism, and a sustained release of both pirfenidone and the inner core moxifloxacin, to reduce the dose frequency. Nonetheless, a complete release of moxifloxacin was obtained after 2 hours, with no entrapped drug remaining in the core (Figure 4.39), despite the entanglement appearance of the fibre mat after 4 days of release study (Figure 4.40). A complete release of pirfenidone was achieved after 6 hours (Figure 4.39). This was in contrast with Han and Steckl (2013) study on PCL/PCL/PVP triaxial fibres, which demonstrated a complete release of the PCL shell drug and 80% of the PVP core drug after 24 hours release. The unexpected result of the rapid release of moxifloxacin and pirfenidone was probably due to the presence of hydrophilic drugs and PVP in the shell and the core. This allowed the absorption of more PBS into the fibres, which facilitated the diffusion of the loaded drugs via a formed channels. This explanation was also reported in Han and Steckl (2013) study.
Figure 4.39: The release profile of 1% moxifloxacin-loaded into 10% PVP, 2% pirfenidone-loaded into 25% PLGA and 1% moxifloxacin-loaded into 10% PVP from the drug-loaded triaxial fibres using custom made cages. The drug-loaded fibres showed a complete release of moxifloxacin and pirfenidone after 2 hours and 6 hours, respectively. The large graph represents the full release duration (4 days), while the small graph represents the release after 24 hours.
4.3.5.6. Drug Release Study Summary

It was demonstrated that the release of pirfenidone and/or moxifloxacin from the monoaxial and coaxial drug-loaded fibres were controlled by the dialysis bags. This was further confirmed by the complete release of each drug (in powder form) through the bags in a slower rate (i.e. after 24 hours for both drugs) compared to the cages (first time point). This was expected according to the Noyes Whitney equation. One important parameter that can affect the release behaviour across the bags is the rate of stirring, which was constant on 75 rpm throughout the study. In addition, due to the accidental presence of air inside the dialysis bags, an uneven stirring of the buffer within the bags might delayed the release of the drugs. These reasons probably would hinder the release rate of the drugs in powder form through the dialysis bags. Therefore, an alternative release testing method was considered in order to reflect the release of the drugs from the fibres themselves without the influence of the presence of the dialysis bags.

Figure 4.40: SEM image of 1% moxifloxacin-loaded into 10% PVP, 2% pirfenidone-loaded into 25% PLGA and 1% moxifloxacin-loaded into 10% PVP from drug-loaded triaxial fibres after 4 days release. Fibres are entangled and the fibres’ surface are smooth.
This alternative release study showed that the pirfenidone-loaded PLGA monoaxial fibres were able to sustain the release of this drug for at least 15 days, with an initial burst release after 1 hour. However, PVP demonstrated a very rapid release of moxifloxacin from the monoaxial system due to the hydrophilic nature of this polymer.

Loading both drugs into PLGA monoaxial fibres was able to release the majority of the loaded moxifloxacin (87%) and pirfenidone (92%) after 4 hours followed by a complete release in 24 hours. Therefore, loading each drug into separate compartment (layer) was suggested in order to sustain the release of moxifloxacin for a longer period of time (> 4 hours). This will extend the inhibition of bacterial growth allowing the prevention of bacterial regrowth. The drug-loaded coaxial system showed a burst release of pirfenidone (39%) after 30 minutes followed by a complete release after 24 hours while moxifloxacin demonstrated a 60% release after 30 minutes followed by a sustain release for at least 4 days. Consequently, this slight difference was enough to take the drug-loaded coaxial formulation forward, since the intended treatment regimen for ocular abrasion in this thesis is a once daily dose.

Unexpectedly, the addition of a layer of the water soluble polymer PVP with moxifloxacin, as a coat for this coaxial system, resulted a complete release of all the loaded drugs (from each layer) after 6 hours rather than slowing their release. This was considered as a limitation for this triaxial formulation, which will require further optimisation. Despite this release profile, this system was also tested in the next chapter in order to evaluate the performance difference (of each drug) between the coaxial and triaxial systems using microbiological and cell culture studies.

It was reported by Aguilar (2013) that drugs are released rapidly from smaller particles (nanoparticles) compared to larger sizes particles (microparticles). This was explained as due to the large surface area-to-volume ratio, which causes the drug molecules to be released faster due to the easy penetration of the dissolution medium to the matrix and/or the location of the drug at either near or on the surface of the small matrix nanoparticles (Aguilar, 2013). This can further explain the release behaviour of the nanofibres in this chapter.
In addition, the TEM images in Figure 4.8 - formulations 30 and 32 for the drug-loaded coaxial and triaxial fibres, respectively (which were prepared by ACTN), showed that the thickness ratio between the layers of the coaxial and triaxial fibres is almost double. In case of the drug-loaded coaxial fibres, the inner layer was estimated as 167 nm, while the outer layer was 384 nm. On the other hand, the core layer of the drug-loaded triaxial fibres was measured as 66 nm, while the intermediate and the shell layers were 145 and 266 nm, respectively. The thickness of both systems limits the space available for the drug molecules to be near the surface. This could accelerate the release from within the fibres unlike the monoaxial PLGA fibres that showed to have a thickness of 620 nm on TEM image (Figure 4.8 - formulation 10). For this monoaxial fibres, the drug (pirfenidone) molecules had to diffuse, for a longer distance, throughout the hydrophobic polymeric matrix of PLGA allowing the full release of that drug after 15 days compared to 1 day from the coaxial fibres. Therefore, the thickness of the outer layer can be crucial factor for extending the release of the drug in this layer.

This observation was consistent with the behaviour of the coaxial fibres described by Ball et al. (2016) which contained a hydrophobic polymer ethyl cellulose as the outer layer and the water soluble polymer PVP as the inner layer. They reported that by increasing the thickness of the outer layer which was performed by enhancing the shell-to-core volume ratio from 0.5 to 4, the release of maraviroc from the core was extended from 1 to 5 days. Another study by Sriyanti et al. (2018) demonstrated that the release of α-mangostin from PVP nanofibres was faster from the smaller fibre diameter (387 nm) due to higher rate of release medium absorbed compared to the larger diameter fibres (468 nm). This allowed the polymer chain to relax which led to fibres swelling. This can further dissolve the drug rapidly (by the penetrating solvent) allowing its diffusion out of the polymer matrix.

4.3.5.7. Release Kinetic Modelling

The release of a drug from a polymeric system containing a hydrophobic polymer such as PLGA can be through; the drug desorption from the surface
of the system, the diffusion of the drug through the polymer matrix, the erosion of the polymer which facilitate the penetration of the release medium to the polymer matrix, the relaxation of the polymer chain upon contact with the release medium which results swelling of system and the drug being diffused to the outside, the porosity of the system’s surface which allow more release medium penetrates to the inside through these pores, or the combination of more than one of these release mechanisms (Lu et al., 2011, Said et al., 2011, Weldon et al., 2012, Li et al., 2014; Jahangiri et al., 2014). In the case of water soluble polymers such as PVP, the rapid disintegration and dissolving of the polymer upon contact with the release medium is the main release mechanism (Illangakoon et al., 2014). Therefore, selecting a suitable release kinetic model for a hydrophobic polymeric system is essential in order to evaluate the drug release behaviour from this system. Several methods have been used previously to assess the fitting of the drug dissolution profile. These are briefly described in Table 4.8.
Table 4.8: Various models for fitting drug release data. $F$ is the fraction (%) of drug released in time ($t$), $k_0$ is the zero-order release constant, $k_1$ is the first-order release constant, $k_H$ is the Higuchi release constant, $k_{KP}$ is the release constant from polymeric matrices, $n$ is the exponent of the release in function of time $t$ indicating the drug release mechanism, $k_{HB}$ is the Hopfenberg model release constant while the $n$ value is 1, 2, and 3 for film, cylinder or sphere, respectively, $\alpha$ value is the timescale of the process while $\beta$ defines the type of curve ($\beta=1$ for an exponential curve, $\beta >1$ for a sigmoid curve and $\beta <1$ for parabolic curve). Adopted from Siepmann and Siepmann (2008), Dash et al. (2010), Siepmann and Peppas (2012), Ramteke et al. (2014) and Bruschi (2015).

<table>
<thead>
<tr>
<th>Model</th>
<th>Model description</th>
<th>Equation</th>
<th>Parameter(s)</th>
<th>Application(s) example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero-order</td>
<td>Drug is released as a function of time at a constant rate and independent on drug concentration.</td>
<td>$F= k_0^* t$</td>
<td>$k_0$</td>
<td>Drug reservoir that is found in coated (matrix) tablets, transdermal or osmotic systems.</td>
</tr>
<tr>
<td>First-order</td>
<td>Drug release that is dependent on drug concentration (corresponds to a change in drug concentration as a function of time).</td>
<td>$F= 100^* (1- e^{-k_1 t})$</td>
<td>$k_1$</td>
<td>Drugs in porous matrix which the amount of the release drug reduced by time.</td>
</tr>
<tr>
<td>Higuchi</td>
<td>Drug released from homogenous matrices of solid or semi-solid systems by the diffusing medium.</td>
<td>$F= k_H^* t^{0.5}$</td>
<td>$k_H$</td>
<td>Drug particles that are dispersed in an ointment base, a sustained release system (i.e. suspension), matrix tablets or transdermal systems.</td>
</tr>
<tr>
<td><strong>Korsmeyer-Peppas</strong></td>
<td>Drug that is released by desorption, diffusion and/or swelling.</td>
<td>$F = k_{KP} \cdot t^n$</td>
<td>$k_{KP}$, $n$</td>
<td>Drug molecules released from polymeric matrix of a system such as hydrogel, microsphere or microcapsules.</td>
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</tr>
<tr>
<td><strong>Hopfenberg</strong></td>
<td>Drug that is released mainly by polymer erosion, in addition to drug diffusion and polymer chain relaxation from various geometrical dosage forms (which the value of $n$ will be dependent on).</td>
<td>$F = 100 \cdot [1 - (1 - k_{HB} \cdot t)^n]$</td>
<td>$K_{HB}$, $n$</td>
<td>Drug in a dosage form that is a film ($n=1$), a cylinder ($n=2$) or a sphere ($n=3$).</td>
</tr>
<tr>
<td><strong>Weibull</strong></td>
<td>To compare the drug release profiles of matrix systems as a function of a finite time, mainly by diffusion.</td>
<td>$F = 100 \cdot (1 - e^{-\beta t\alpha})$</td>
<td>$\alpha$, $\beta$</td>
<td>Drug that is released from polymeric matrix.</td>
</tr>
</tbody>
</table>
The models with higher $r^2$ values were judged to be the most appropriate models of drug release. As it shows in Table 4.9, the release kinetics of the drug-loaded PLGA and PVP monoaxial fibres followed Weibull model with the $r^2 = 0.9692$ and $r^2 = 0.9996$, respectively, indicating that the release in both systems is due to drug diffusion. This was expected owing to the use of dialysis bags which controlled the rate of drug release. The coaxial system exhibited release profiles for both pirfenidone and moxifloxacin that fit the first-order ($r^2 = 0.9981$ and 0.9749, respectively) and Hopfenberg ($r^2 = 0.9985$ and 0.9751, respectively) models. This suggest that the release of both drugs are governed by the drug concentration in which release can be through drug diffusion across the dialysis bag. In addition, the fibrous system swelling, which results from polymer chain relaxation, and polymer erosion can be further release mechanisms of this coaxial system according to Hopfenberg model. Nevertheless, the elimination of the dialysis bags was preferred in this thesis due to the controlled release that was associated with these bags, as well as, the desire to evaluate the release of the drugs from the fibres themselves.
Table 4.9: Release kinetic modelling showing the coefficient of determination ($r^2$) and the release constant (k) for pirfenidone-loaded PLGA monoaxial fibres, moxifloxacin-loaded PVP monoaxial fibres, pirfenidone- and moxifloxacin-loaded PLGA monoaxial fibres, drug-loaded coaxial fibres and drug-loaded triaxial fibres. Pir: pirfenidone, moxi: moxifloxacin, $k_0$: zero-order release constant, $k_1$: first-order release constant, $k_H$: Higuchi release constant, $k_{HB}$: Hopfenberg model release constant, $\beta$: defines the type of curve, $k_{KP}$: is the release constant from polymeric matrices, $n$: the exponent of the release in function of time $(t)$ indicating the drug release mechanism.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Drug</th>
<th>Zero Order</th>
<th>First Order</th>
<th>Higuchi</th>
<th>Hopfenberg</th>
<th>Weibull</th>
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<td></td>
<td></td>
<td>$r^2$</td>
<td>$k_0$</td>
<td>$r^2$</td>
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<td>Drug Release by Dialysis Bags</td>
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<tr>
<td>25% PLGA fibres</td>
<td>2% pir</td>
<td>0.8608</td>
<td>4.373</td>
<td>0.9310</td>
<td>0.179</td>
<td>0.9455</td>
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<td>10% PVP fibres</td>
<td>1% moxi</td>
<td>0.5694</td>
<td>6.026</td>
<td>0.9966</td>
<td>0.875</td>
<td>0.7267</td>
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<td>Coaxial (25% PLGA/10% PVP)</td>
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<td>0.4864</td>
<td>5.210</td>
<td>0.9981</td>
<td>8.453</td>
<td>0.6394</td>
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<tr>
<td>fibres</td>
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<td>0.5498</td>
<td>3.933</td>
<td>0.9749</td>
<td>4.936</td>
<td>0.6913</td>
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</tr>
<tr>
<td>25% PLGA fibres</td>
<td>1% pir</td>
<td>0.6321</td>
<td>9.391</td>
<td>0.9900</td>
<td>8.723</td>
<td>0.7639</td>
</tr>
<tr>
<td>25% PLGA fibres</td>
<td>2% pir</td>
<td>0.6569</td>
<td>9.055</td>
<td>0.9883</td>
<td>8.887</td>
<td>0.7835</td>
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<tr>
<td>10% PVP fibres</td>
<td>1% moxi</td>
<td>0.3929</td>
<td>142.468</td>
<td>0.9952</td>
<td>129.061</td>
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<td>2% pir</td>
<td>0.5095</td>
<td>35.166</td>
<td>0.9924</td>
<td>12.755</td>
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<td></td>
<td>1% moxi</td>
<td>0.5566</td>
<td>34.575</td>
<td>0.9968</td>
<td>15.075</td>
<td>0.6955</td>
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<td>2% pir</td>
<td>1% moxi</td>
<td>2% moxi</td>
<td>2% pir</td>
<td>1% moxi</td>
<td>2% moxi</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>--------</td>
<td>---------</td>
<td>---------</td>
<td>--------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>Coaxial (25% PLGA/10% PVP)</td>
<td>0.4510</td>
<td>0.3258</td>
<td>0.3605</td>
<td>0.4607</td>
<td>0.9026</td>
<td>0.8759</td>
</tr>
<tr>
<td>Fibres</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>35.629</td>
<td>25.009</td>
<td>35.909</td>
<td>35.825</td>
<td>26.109</td>
<td>331.995</td>
</tr>
<tr>
<td></td>
<td>0.9984</td>
<td>0.9023</td>
<td>0.9029</td>
<td>0.9026</td>
<td>0.9029</td>
<td>0.9029</td>
</tr>
<tr>
<td></td>
<td>1.099</td>
<td>0.6269</td>
<td>0.402</td>
<td></td>
<td>0.009</td>
<td>0.055</td>
</tr>
</tbody>
</table>
The kinetic model of the PLGA monoaxial fibres containing 1% or 2% of pirfenidone showed that the release is governed mainly by drug diffusion, as they follow the Weibull model ($r^2 = 0.9923$ and 0.9910 for 1% and 2% pirfenidone, respectively). This was consistent with Zhang et al. (2012) captopril loaded PLGA fibres release modelling, which also fitted the drug diffusion Weibull model.

PVP monoaxial fibres demonstrated a moxifloxacin release that is concentration dependent (first-order, with $r^2 = 0.9952$) and follows drug diffusion (Weibull, with $r^2 = 0.9948$), polymer chain relaxation and polymer erosion (Hopfenberg, with $r^2 = 0.9952$). This was expected owing to the fast disintegration of the water soluble polymer PVP and the rapid diffusion of the drug from the polymer matrix when the erosion takes place. Kamble et al. (2016) reported a similar release pattern that is a rapid release of irbesartan from PVP fibres which was governed by drug diffusion through the swollen polymer matrix (i.e. following the Korsmeyer-Peppas model) with an $n$ value > 0.5 ($n = 0.5003$). The $n$ value of the Korsmeyer-Peppas model will be discussed later in this section. Another study by Sriyanti et al. (2018) exhibited an accelerated release of α-mangostin loaded into PVP fibres which was dependent on the concentration (i.e. following the first-order model) and the change in the surface area and the diameter of the fibres matrix (i.e. following the Hixson-Crowell model). However, it was also suggested that the release of α-mangostin from the PVP fibres can be due to desorption of the accumulated drug that present on the fibres’ surface, the diffusion of the drug across the matrix, polymer degradation and erosion (Sriyanti et al., 2018). These explanatory mechanisms agree with the above kinetic modelling of moxifloxacin-loaded PVP nanofibres.

Loading both pirfenidone and moxifloxacin into PLGA fibres exhibited a release mechanism that fitted the drug diffusion model (Weibull, with $r^2 = 0.9970$ and 0.9962 for pirfenidone and moxifloxacin, respectively) for both drugs. However, moxifloxacin was also dependent on the concentration (first-order, with $r^2 = 0.9968$) and can also be released after PLGA chain relaxation and/or erosion (Hopfenberg, with $r^2 = 0.9968$). This release kinetic was consistent with the release mechanisms of the PLGA fibres reported by Lu et
al. (2011), Said et al. (2011), Weldon et al. (2012), Li et al. (2014) and Jahangiri et al. (2014), i.e. desorption, diffusion and/or erosion of the polymer.

The release model of the drug-loaded coaxial and triaxial fibres were governed mainly by the drug diffusion model (Weibull) which agreed with the above monoaxial PLGA and PVP fibres. In addition, the coaxial fibres swelling and erosion (Hopfenberg) can also play a role in the release of both pirfenidone ($r^2 = 0.9984$) and moxifloxacin ($r^2 = 0.9023$) from this system. Zhu et al. (2015) previously reported that the release of flurbiprofen axetil loaded into the PVP core of the PLGA/PVP coaxial fibres was governed by the diffusion of the dissolution medium into the polymeric network allowing the dissolving of the drug. The dissolved drug will then diffuse out of the polymer matrix that fitted the Fickian diffusion model, as it will be explained next.

Korsmeyer-Peppas mathematical model was utilised in order to further verify the release mechanism of the examined systems. The value of the exponent of the release ($n$) as a function of time in this kinetic model can indicates for the drug release mechanism of a delivery system (Korsmeyer et al., 1983, Siepmann and Siepmann, 2008, Dash et al., 2010, Siepmann and Peppas, 2012, Ramteke et al., 2014; Bruschi, 2015). When $n \leq 0.5$ the release model is known as Fickian (case I) and it describes the drug release by the diffusion process, which the solvent transport rate is larger than the polymer chain relaxation (Deore et al., 2010, Apu et al., 2012). Non-Fickian (case II) release model is when $n = 1$ and the release of the drug corresponds to the relaxation of the polymer chain and the swelling of this system. Anomalous (non-Fickian) transport which the drug release is dependent on diffusion and swelling of the system which occurs when $0.5 < n < 1$. When $n > 1$, the drug release is governed by an extreme form of drug transport that might results from the breaking of the polymer or desorption of the drug from the surface of the delivery system. This latter model is known as Super Case II model (Siepmann and Siepmann, 2008, Dash et al., 2010, Siepmann and Peppas, 2012, Ramteke et al., 2014; Bruschi, 2015).

It was reported by Korsmeyer et al. (1983) and Ritger and Peppas (1987) that the fraction release % ($F\%$) values that are $\leq 60\%$ were only considered. This is due to the constant fractional release profile that can be seen within these
values. Therefore, only the formulations that were examined using dialysis bags and the PLGA loaded with 1% and 2% pirfenidone using the cages were fitted using the Korsmeyer-Peppas kinetic model, as shown in Table 4.10. The use of at least 3 time points was considered in these formulations. The $r^2$ values, which were demonstrated in all the tested systems, except for the moxifloxacin release from the drug-loaded coaxial fibres using the dialysis bags, were low. This low value was probably due to the fast release rates at the early time-points of the dissolution tests. Despite this, the $n$ values were still considered to further verify the release mechanism.

Table 4.10 showed that the $n$ value for PLGA loaded with pirfenidone and PVP loaded with moxifloxacin monoaxial fibres was 0.373 and 0.465, respectively. This indicates that the release kinetic is following the Fickian (i.e. drug diffusion) model ($\leq 0.5$) (Deore et al., 2010, Apu et al., 2012). However, the $n$ values for both pirfenidone and moxifloxacin from the drug-loaded coaxial fibres were $0.5 < n < 1$ which obey the anomalous (non-Fickian) model (i.e. drug diffusion and polymer swelling). All the above results were expected, as the release of all these systems were performed by the use of dialysis bags. On the other hand, PLGA monoaxial fibres which are loaded with 1% and 2% were the only fibrous systems that tested by the custom-made cages and could be fitted with the Korsmeyer-Peppas model. This system exhibited an $n$ values of $>0.5$ and $<1$ for both drug concentrations, which correlate with the anomalous model. The release of these systems were shown above to be following the drug diffusion model (Weibull). However, the $n$ value of this Korsmeyer-Peppas model also indicated that the release of pirfenidone can be governed by swelling. This will be further confirmed in the next result section 4.3.6.
Table 4.10: Korsmeyer-Peppas release kinetic modelling for pirfenidone-loaded PLGA monoaxial fibres, moxifloxacin-loaded PVP monoaxial fibres, pirfenidone- and moxifloxacin-loaded PLGA monoaxial fibres and drug-loaded coaxial fibres. Pir: pirfenidone, moxi: moxifloxacin, \( r^2 \): coefficient of determination, \( k_{KP} \): is the release constant from polymeric matrices, \( n \): the exponent of the release in function of time \((t)\) indicating the drug release mechanism.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Drug</th>
<th>Korsmeyer-Peppas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( r^2 )</td>
</tr>
<tr>
<td><strong>Drug Release by Dialysis Bags</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25% PLGA fibres</td>
<td>2% pir</td>
<td>0.9570</td>
</tr>
<tr>
<td>10% PVP fibres</td>
<td>1% moxi</td>
<td>0.9995</td>
</tr>
<tr>
<td>Coaxial (25% PLGA/10% PVP) fibres</td>
<td>2% pir</td>
<td>0.9909</td>
</tr>
<tr>
<td></td>
<td>1% moxi</td>
<td>0.9875</td>
</tr>
<tr>
<td><strong>Drug Release by Custom-Made Cages</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25% PLGA fibres</td>
<td>1% pir</td>
<td>0.9873</td>
</tr>
<tr>
<td>25% PLGA fibres</td>
<td>2% pir</td>
<td>0.9899</td>
</tr>
</tbody>
</table>
4.3.6. Swelling and Weight Loss Behaviour

The swelling and weight loss behaviour of 2% pirfenidone-loaded into 25% PLGA monoaxial fibres (Formulations 10), 2% pirfenidone-loaded into 25% PLGA and 1% moxifloxacin-loaded into 10% PVP drug-loaded coaxial fibres (Formulations 30) and 1% moxifloxacin-loaded into 10% PVP, pirfenidone-loaded into 25% PLGA and 1% moxifloxacin-loaded into 10% PVP drug-loaded triaxial fibres (Formulations 32) were investigated. These fibres were incubated in the same release media (PBS pH 7.4) at 34.3ºC for 24 hours. The swelling test was performed to assess the penetration of PBS to the polymer matrix which would facilitate the drug dissolution and thus its diffusion out of the fibres, as reported in the Zhang et al. (2012) study on captopril loaded PLGA, PCL or PLA monoaxial fibres. Figure 4.41 showed the highest swelling of the coaxial (324%) and triaxial (347%) fibres after 2 hours contact with PBS, then started to shrink gradually. This might be due to the dissolving of the PVP layer(s) of the coaxial and triaxial fibres. Similarly, the highest swelling of PLGA monoaxial fibres (139%) was after 2 hours, then it shrank slightly compared to the multi-layered systems. After 24 hours, the pirfenidone-loaded PLGA fibres showed a swelling % of 134%, while the drug-loaded coaxial and triaxial fibres exhibited a swelling % of 216% and 232%, respectively, indicating the degradation of the PVP layer(s).

This swelling results suggested that the coaxial and triaxial fibres have a higher swelling ratio than the PLGA monoaxial fibres due to the addition of one or two PVP layers, respectively. This improved the hygroscopic properties of the coaxial and triaxial fibres allowing more buffer uptake. Meng et al. (2011) demonstrated greater buffer uptake by the fenbufen loaded in PLGA blended with gelatine nanofibres compared to the drug-loaded PLGA fibres due to the improvement of the hydrophilicity of the former system by the addition of gelatine, which also accelerated the release of this drug. A similar finding was observed by Sun et al. (2006) in their water uptake study on PLA/PVP coaxial fibres that show more water absorption than the PLA monoaxial fibres due to the presence of the hydrophilic polymer PVP in the coaxial system.

Generally, all fibres swelled which facilitated the release of the loaded drug(s). However, the high swelling difference between the coaxial and triaxial fibres
compared to the PLGA monoaxial fibres correlates well with the accelerated release of both pirfenidone and moxifloxacin from both systems. Zhang et al. (2012) reported that due to the swelling effect of captopril-loaded PLGA, PCL or PLA fibrous systems, more water can penetrate within the matrices which allows the rapid release of captopril from these systems.

Figure 4.41: Swelling behaviour of pirfenidone-loaded PLGA monoaxial fibres, drug-loaded coaxial and triaxial fibres, showing their swelling % over time in the release medium.

The weight loss of the fibres was measured after 24 hours incubation in PBS. Table 4.11 showed that the weight loss of pirfenidone-loaded PLGA monoaxial fibres is lower than the drug-loaded coaxial and triaxial fibres. This contributed to the dissolving of the PVP layer(s) in the multi-layered fibres, which was expected after 24-hour incubation.
Table 4.11: Weight loss behaviour of pirfenidone-loaded PLGA monoaxial fibres, drug-loaded coaxial and triaxial fibres, showing their average weight loss % after 24-hour incubation in the release medium.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Average Weight Loss (%)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pirfenidone-loaded PLGA fibres</td>
<td>5.04</td>
<td>1.77</td>
</tr>
<tr>
<td>Drug-loaded coaxial fibres</td>
<td>38.07</td>
<td>1.85</td>
</tr>
<tr>
<td>Drug-loaded triaxial fibres</td>
<td>47.00</td>
<td>1.73</td>
</tr>
</tbody>
</table>
4.4. Conclusion

Pirfenidone-PLGA/moxifloxacin-PVP coaxial and triaxial fibres were successfully prepared using multi-axial electrospinning techniques. The coaxial fibres were made by using a miscible solvent system of DCM or ACTN to dissolve PLGA and DCM:ethanol or ACTN:ethanol, in a 1:1 ratio, to dissolve PVP. The triaxial fibres were made by dissolving PLGA in ACTN and PVP in ACTN:ethanol (1:1). However, in order to assess these fibres, solvent properties, such as relative viscosity and boiling point, were taken into consideration.

The morphology of prepared fibres were observed using different microscopic imaging. SEM images illustrate the surface characteristics and how the use of high vapour pressure solvents can produce rougher surface fibres (in PLGA fabrication), despite using a lower voltage and tip-to-collector distance. In addition, solvent choice affected the diameter of the fibres. PLGA and PVP fibres that were prepared by using DCM and DCM:ethanol had a diameter of 1900 nm and 925 nm, respectively. Nonetheless, the diameter reduced by the use of ACTN for PLGA and ACTN:ethanol for PVP, to approximately 680 nm for both monoaxial systems. A similar observation was seen when the coaxial fibres were fabricated by using DCM (~ 1200 nm) and ACTN (~ 640 nm). TEM findings have shown the successful coaxial and triaxial fabrication for the drug-loaded fibres. Fluorescence microscopy indicated the encapsulation of moxifloxacin, which was located in the core of the coaxial fibres and in the core and the outer layer of the triaxial fibres. Confocal microscopy was used to confirm the presence of the PLGA layer, in which MB were added instead of pirfenidone, in the outer layer of the coaxial fibres and the middle layer of the triaxial fibres, while it also detected the moxifloxacin presence.

The pirfenidone-loaded PLGA monoaxial fibres indicated that the use of ACTN solvent improved the EE% and Y% when used to prepare PLGA fibres (EE% of 100% and Y% of 82%) compared to DCM (EE% of 89% and Y% of 34%). However, there was no clear advantage, with regard to the EE% and the Y%, of using ACTN or DCM in the preparation of PVP fibres. The coaxial fibres fabricated using DCM showed a higher Y% (83%) compared to the ACTN
(64%), while there was a very slight difference in the EE% for both system (for the DCM, the EE% of moxifloxacin and pirfenidone was 80.1% and 83.5%, respectively, while for the ACTN, the EE% of moxifloxacin and pirfenidone was 87.6% and 79.6%, respectively). Nevertheless, due to the flow rate that was used to prepare the coaxial fibres using DCM (outer layer: 0.2 mL/h and inner layer: 0.8 mL/h), the DL of moxifloxacin (52 µg/mg) was twice that of pirfenidone (27 µg/mg) despite using an initial concentration of 1% w/v for the former drug and 2% w/v for the latter. The coaxial fibres which were prepared by using ACTN had a similar flow rate (0.5 mL/h), hence, the DL of both drugs (42 µg/mg for pirfenidone and 23 µg/mg for moxifloxacin) were accordingly to the initial concentration ratio (2:1). The triaxial fibres exhibited a similar Y% (64%) to the coaxial fibres that were made by using ACTN. Additionally, the DL of moxifloxacin (35 µg/mg) and pirfenidone (39 µg/mg) were almost equivalent, due to the addition of 1% moxifloxacin in the outer PVP layer which equalised the initial concentration of both drugs (at 2%).

The release profile for pirfenidone-loaded PLGA monoaxial fibres showed a sustained release profile (at least for 15 days) compared to a fast release profile of PVP loaded with moxifloxacin (4 hours). This was expected since PLGA is a water insoluble polymer and PVP is a hydrophilic polymer. The drug-loaded coaxial fibres demonstrated a complete release of pirfenidone after 1 day, while 70% of moxifloxacin was release after 4 hours with the remaining 30% entrapped in the core of these fibres for at least 4 days. Unexpectedly, the drug-loaded triaxial fibres exhibited a very rapid complete release of moxifloxacin and pirfenidone after 2 and 6 hours, respectively. DSC and XRD further confirmed the presence of both drugs in the amorphous state and due to their high affinity to PBS, both drugs were released faster than expected. In addition, the large surface area-to-volume ratio offered by the nanofibres and the narrow diameter of these fibres can further explain the rapid release of the multi-layered fibres. Swelling tests exhibited a high degree of swelling for both coaxial (324%) and triaxial (347%) fibres after 2 hours of incubation, which suggested that the absorption of more medium to the fibres matrices compared to the PLGA monoaxial fibres (139%). This allowed the dissolving of
pirfenidone and the PVP layer with its incorporated drug (moxifloxacin), thus accelerating the release of both drugs.

Owing to the proposed treatment of corneal abrasion by a single daily dose of the drug-loaded fibre system, the above release profile of the coaxial system was sufficient to take this formulation forward into *in vitro* studies. This is to evaluate the performance of the loaded drugs (i.e. moxifloxacin and pirfenidone).
Chapter 5

*In vitro* Biological Efficacy Assessment of the Multi-Layered Electrospun Fibres
5.1. Introduction

In order to accelerate the wound healing of corneal abrasion and to prevent or inhibit any associated infection, co-treatment with an antibiotic and an anti-fibrotic is a viable therapeutic strategy. Therefore, dual drug-loaded coaxial and triaxial fibres that contain the antibiotic moxifloxacin and the anti-scarring agent pirfenidone were prepared by electrospinning technique, as outlined in the previous chapter. These fibres were manufactured by loading pirfenidone into the PLGA layer and the antibiotic moxifloxacin in the PVP layer(s). The morphology of the fibres was assessed using different microscopy techniques such as SEM, TEM, light and confocal microscopy, which verified the successful fabrication of the multi-layered fibres. In addition, diffraction (XRD) and thermal analysis (DSC) studies confirmed the molecular dispersion of both drugs within the formulations. Finally, the DL and EE% and the in vitro drug release profile were assessed. The release profile of the coaxial system demonstrated a complete release of pirfenidone after 24 hours while approximately 68.5% moxifloxacin was released after 1 hour. The remaining moxifloxacin was entrapped within the fibres and may be released either after further exposure to the PBS buffer via diffusion or the erosion of the PLGA layer. This release profile was considered favourable owing to the proposed treatment regimen of corneal abrasion, which is a single daily dose. The triaxial fibre system was able to completely release moxifloxacin and pirfenidone after 2 and 6 hours, respectively. These release profiles of both multi-layered fibres were probably due to the penetration of the release medium (PBS) into the fibres’ matrices which was facilitated by the swelling of these fibres.

In this chapter, the in vitro efficacy of the two drugs is assessed, to determine whether incorporation into the fibres had any deleterious effect on their function. The first part of this chapter contains the evaluation of the antimicrobial activity of moxifloxacin. The minimum inhibitory concentration (MIC) for this broad-spectrum antibiotic was initially determined against the Gram positive bacterium *Staphylococcus aureus* (*S. aureus*) and the Gram negative bacterium *Pseudomonas aeruginosa* (*P. aeruginosa*) in bacterial suspension. The zone of inhibition, which is the diameter of clear area of ‘no growth’ on bacterial plates, was measured against both bacteria strains, as an
indication of the bacterial growth inhibition. The drug-loaded fibres were compared with the blank fibres (negative control) and moxifloxacin discs (positive control). Finally, a sterility test for the blank and drug-loaded fibres, which have been used in the in vitro study, has been performed after preparing these fibres under aseptic conditions.

The second part of the chapter involves the assessment of the potential anti-scarring effect of pirfenidone. Dose response curves for pirfenidone, moxifloxacin and the combination of both drugs (in 1:1 combination) have been determined using a rabbit cornea fibroblast cell line (SIRC). This is to demonstrate the toxicity of each drug (alone or in combination) and to obtain the growth inhibitory concentration (IC) at 20% (IC20) and 50% (IC50). Anti-fibrotic drugs are able to reduce the expression of the triggered cytokines that were released as a response to an injury. Therefore, the potential anti-scarring effect of the drug-loaded fibres was assessed by exposing SIRC cell line to TGF-β1 (to mimic the triggering of cytokines) and measuring the expression of α-Smooth Muscle Actin (α-SMA) by the Western blot technique against the untreated group of cells. This protein is up-regulated in response to the accumulation of TGF-β1 cytokine due to the presence of a scar. Beta Actin (β-Actin) protein expression was assessed as an experimental control. Negative and positive controls of blank fibres and single and combined drug solutions were also tested.
5.2. Materials and Methods

5.2.1. Materials

Bacterial strains were purchased from The National Collection of Type Cultures (NCTC, England, UK). The Gram positive bacterium *S. aureus* (NCTC 12981) and Gram negative *P. aeruginosa* (NCTC 10662) were used in this study. Agar powder was obtained from Sigma Aldrich Company Ltd (Dorset, UK). Tryptone soya broth (soybean casein digest medium), Iso-sensitest agar and PBS tablets (pH 7.4) were brought from Oxoid Limited (Hampshire, UK). Fluid thioglycollate medium was purchased from VWR International Ltd (VWR chemicals, Leicestershire, UK). Distilled water was generated by an ELGA Option 4 Water Purifier (Veolia Water Technologies, High Wycombe, UK). Pirfenidone was purchased from Tokyo Chemical Industry UK Ltd (The Magdalen Centre, Oxford, UK), while moxifloxacin hydrochloride was purchased from Cambridge Bioscience Ltd, (Munro House, Cambridge, UK).

Rabbit corneal fibroblast (SIRC - Statens Seruminstitut Rabbit Cornea) was purchased from American Type Culture Collection (ATCC® CCL-60™, Manassas, USA). Fetal Bovine Serum (FBS, qualified, heat inactivated, E.U.-approved, South America Origin), Penicillin-Streptomycin (10,000 units/mL for penicillin and 10,000 µg/mL streptomycin), GlutaMAX™ (100X), sodium pyruvate 100 mM (100X), trypan blue solution (0.4%) and trypsin-EDTA (0.25% with phenol red) were obtained from Gibco (Fisher Scientific UK Ltd, Bishop Meadow Road Loughborough, UK). Minimum essential medium eagle, MEM non-essential amino acid solution (100X), Dulbecco’s PBS and thiazolyl blue tetrazolium bromide (MTT powder) were brought from Sigma Aldrich Company Ltd (Dorset, UK). MES SDS running buffer (20X) and SDS-PAGE Bolt™ 4-12% bis-tris plus gels, 15 wells were purchased from Novex® (Life technology, Fisher Scientific UK Ltd, Bishop Meadow Road Loughborough, UK). Trans-Blot® Turbo™ 5X Transfer Buffer and TransBlot® Turbo™ mini-size low fluorescence polyvinylidene fluoride (LF PVDF) membrane were brought from Bio-Rad laboratories (USA). Marvel Original dried skimmed milk powder was purchased from Waitrose (local supermarket, London, UK).
SuperSignal™ west femto maximum sensitivity substrate was obtained from Thermo Scientific (Rockford, US). Recombinant human TGF-beta1 (TGF-β1) cytokine (ab50036), primary antibodies α-SMA (ab7817™) and β-Actin (ab8227™), as well as secondary antibodies Goat Anti-Mouse IgG H&L (HRP) (ab205719™) and Goat Anti-Rabbit IgG H&L (HRP) (ab205718™), were purchased from Abcam (Cambridge, UK).

5.2.2. Methods

5.2.2.1. Determination of the Antimicrobial Efficacy of the Multi-Layered Fibres

5.2.2.1.1. Minimum Inhibitory Concentration (MIC) Determination

To test the MIC of moxifloxacin and to check the antibacterial efficacy of pirfenidone against the Gram positive *S. aureus* and Gram negative *P. aeruginosa*, iso-sensitest broth has been prepared (4.68 gm/200 mL distilled water) and sterilised by autoclave for 15 minutes according to Andrews (2001). Stock solutions of moxifloxacin, pirfenidone and the combination of both drugs (1:1) were prepared by dissolving these drugs in sterile distilled water. A serial dilution of 11 points (64 µg/mL to 0.03 µg/mL) of each drug and their combination (1:1) were added first into a 96-well plate (100 µL in each well), while the 12th point contained no drugs (bacterium only), as experimental control. 100 µL of a 1 x 10⁶ colony-forming-unit/mL (CFU/mL) suspension of each microorganism in sterilised broth was then added into each well. This further diluted the drugs to be in the range of 32 µg/mL to 0.015 µg/mL. The plates were incubated at 37°C for 24 hours, following the method of Andrews (2001). The turbidity of the solution (observed visually) is an indication of microorganism presence. This assay has been performed in duplicate, on 2 separate days, to test the reproducibility of the results.

5.2.2.1.2. Zone of Inhibition Determination

To evaluate the antimicrobial property of the electrospun fibres, the zone of inhibition test has been performed based on the work of Said *et al.* (2011) and
Hilal Algan et al. (2016) in the microbiological assessment of their electrospun fibres. The assessment was performed on the blank and drug-loaded coaxial fibres, made of 25% PLGA and 10% PVP (Section 4.2.2.3. Table 4.3 - formulations 26 and 30, respectively), and triaxial fibres, consist of 10% PVP, 25% PLGA and 10% PVP (Section 4.2.2.4. Table 4.4 - formulations 31 and 32, respectively), that were prepared using ACTN solvent and with the same electrospinning parameters mentioned in Chapter 4. The Gram positive S. aureus and the Gram negative P. aeruginosa were dispersed in sterile PBS (pH 7.4) at a concentration of 1 x 10⁶ CFU/mL. Then approximately 2 mL of this bacterial suspension was inoculated and distributed in iso-sensitest agar petri-dishes. After drying, the tested fibres (in a certain weight that contains 50 µg moxifloxacin) were loaded onto the surface of the agar layer in the petri-dish, being positioned in the middle. This was compared to blank fibres (in a weight that is equivalent to the tested drug-loaded fibres) and a sterile disc that contains 50 µg moxifloxacin, as negative and positive controls, respectively. The petri-dishes were incubated at 37ºC for 24 hours. The diameter of the clear area of ‘no growth’ was measured around the particular formulations in millimetres (mm) as described in Said et al. (2011) and Hilal Algan et al. (2016). This assay was performed in triplicate to test the reproducibility of the results.

5.2.2.1.3. Sterility Testing

Coaxial fibres, blank and drug-loaded (Section 4.2.2.3. Table 4.3 - formulations 26 and 30, respectively) and triaxial fibres, blank and drug-loaded (Section 4.2.2.4. Table 4.4 - formulations 31 and 32, respectively) were prepared with the same electrospinning parameters mentioned but under aseptic conditions. All plastic tubes and emitters (needles) of the Spraybase® electrospinning instrument had been autoclaved prior to their usage. Additionally, the surrounding surfaces had been wiped with 70% ethanol solution, along with the aluminum foil used for collecting the fibres. The yielded fibres were peeled by a pair of tweezers that were disinfected with 70% ethanol, and then kept in 50 mL sterilised centrifuge tubes. These fibres were tested for sterility under aseptic conditions, near the flame of the bunsen burner used in the
microbiology lab, to avoid handling contamination. This is required prior to testing the fibres in cell culture as shown later in section 5.2.2.2.3.

Based on the British Pharmacopoeia (Appendix XVI A. Test for Sterility, 2019), two culture media can be used to test for sterility. Fluid thioglycollate (FTG) medium is used to assess the fibres against mainly anaerobic bacteria (such as Clostridium sporogenes), as well as aerobic bacterial strains (such as S. aureus), to be incubated at a temperature between 30°C and 35°C (Appendix XVI A. Test for Sterility, 2019). Soya-bean casein digest (SBC) medium is used to test fungi (such as Candida albicans) and aerobic bacteria, to be incubated at room temperature (between 20 and 25°C) (Appendix XVI A. Test for Sterility, 2019). After preparing (for FTG, 2.975 g dissolved in 100 mL distilled water, and for SBC, 3 g dissolved in 100 mL distilled water) and autoclaving both media, a volume of 10 to 15 mL was added into airtight sterilised containers. Fibre weights of 1 to 2 mg were added and the systems were incubated for 14 days. A clear solution is an indication of no microorganism growth. This assay has been performed in triplicate to test the reproducibility of the results. In case of contamination, turbidity of the medium would occur in 3 days as maximum for bacterial contamination, while a clear fungus growth would occur in 5 days as maximum for fungus contamination (Appendix XVI A. Test for Sterility, 2019).

5.2.2.2. In Vitro Determination of the Anti-scarring potential of the Multi-Layered Fibres

5.2.2.2.1. Fibroblast Sub-Culturing

Rabbit corneal fibroblasts (SIRC) have been used to assess the anti-fibrotic activity of pirfenidone and moxifloxacin in free, combined and formulated forms. This cell line was propagated and passaged according to ATCC recommendations (ATCC® CCL-60™, 2019). A complete growth medium of minimum essential medium eagle adjusted by 1% penicillin-streptomycin, 1% MEM non-essential amino acid solution, 1% sodium pyruvate (100 mM), 1% GlutaMAX™ and 10% FBS was used. When the cell confluency reached 90%, the consumed medium was aspirated and the 75 cm² flask was rinsed with 5 mL Dulbecco’s PBS to remove all traces of serum, as this contains a trypsin
inhibitor which will interfere with the subsequent experimental procedure. A 3 mL of trypsin-EDTA (0.25%) solution was then added to the flask, to be incubated at 37°C and 5% CO₂ for 5 to 10 minutes. Then, a 5 mL of the complete medium was added to neutralise the trypsin. The solution was then centrifuged at 1500 rpm for 5 minutes at 20°C. The supernatant was removed and a certain volume (5-10 mL) of the complete medium was added to the cell pellet to disperse it (ATCC® CCL-60™, 2019). After complete dispersing of the cells, cell counting was performed by using trypan blue solution (0.4%) to determine the number of cells to be transferred into well plates (for inhibitory concentration determination) or 75 cm² flasks (for sub-culturing).

5.2.2.2. Inhibitory Concentration (IC) Determination

After counting the cells, 40,000 cells per well were transferred to 48-well plates and incubated at 37°C and 5% CO₂. After 24 hours incubation, 50 to 60% confluency had been reached, which was sufficient for the subsequent experimental procedure (Tawfik, et al., 2017). A 0.25 mL of test solution (pirfenidone, moxifloxacin or both drugs, at a serial dilution range of 2.5 and 0.01 mg/mL) was added to each well and the cells were incubated for 24, 48 and 72 hours (37°C and 5% CO₂). Untreated cells were used as controls for this experiment.

The MTT assay is a cell proliferation assay for determining the cell metabolic activity. An MTT solution (at a concentration of 0.5 mg/mL) was prepared by dissolving the MTT yellow powder in the culture media as described in Tawfik et al. (2017). After incubating the cells (exposed to the drugs) at the designated time points (24, 48 and 72 hours), the culture medium was aspirated and the cells were supplemented with 0.25 mL of the MTT solution (in a dark biosafety cabinet) and incubated at 37°C and 5% CO₂ for 4 hours. The yielded purple formazan crystals were solubilised by adding an equal volume (0.25 mL) of isopropanol to each well and gently shaking the plates for 30 minutes. Finally, the colour intensity of each well was measured at 570 nm by a microplate reader and the percentage of cell viability was calculated relative to the untreated group. The results represent the mean (± SD) of at least 3 replicates.
5.2.2.2.3. Cell Treatment with the Multi-Layered Fibres

After counting the cells, 80,000 cells per well were transferred to 24-well plates and incubated at 37°C and 5% CO₂. After 24 hours incubation, 50 to 60% confluency had been reached and the cells were treated for 24 hours with blank and drug-loaded coaxial and triaxial fibres in weights that contain 0.08 mg/mL pirfenidone. This was achieved by aseptically cutting approximately 1.95 mg and 2.05 mg square shaped pieces from the coaxial and triaxial fibre mats, respectively, into each well that contains 1 mL of the culture medium. These fibres weights were measured according to the pirfenidone DL, which was ~ 41 µg/mg (0.041 mg/mg) and ~ 39 µg/mg (0.039 mg/mg) for the coaxial and triaxial fibres, respectively. The assessment was performed on the blank and drug-loaded coaxial fibres, made of 25% PLGA and 10% PVP (Section 4.2.2.3. Table 4.3 - formulations 26 and 30, respectively), and triaxial fibres, consist of 10% PVP, 25% PLGA and 10% PVP (Section 4.2.2.4. Table 4.4 - formulations 31 and 32, respectively), that were prepared using ACTN solvent and with the same electrospinning parameters mentioned in Chapter 4. The results were compared to the untreated control group, as well as, the free and combined drugs that are in similar concentrations (0.08 mg/mL) to the fibres.

After 24 hours incubation (37°C and 5% CO₂), the culture medium was aspirated and the cells were supplemented with 0.5 mL MTT solution (in a concentration of 0.5 mg/mL in a dark biosafety cabinet) and incubated at 37°C and 5% CO₂ for 4 hours. The yielded purple formazan crystals were solubilised by adding an equal volume (0.5 mL) of isopropanol to each well and gently shaking the plates for 30 minutes. Finally, the colour intensity of each well was measured at 570 nm by a microplate reader and the percentage of cell viability was calculated relative to the untreated group. The results represent the mean (± SD) of at least 3 replicates.

In addition, blank and drug-loaded coaxial and triaxial fibres were also assessed using SEM to check the cell proliferation within the fibrous network, using a modified method of Sun et al. (2014) and He et al. (2018). These fibres were incubated (37°C and 5% CO₂) with the cells for 24 hours, then were rinsed with Dulbecco’s PBS then removed from the well and transferred to a new well, with no media. This was kept in the incubator at 37°C and 5% CO₂ for a
maximum of 5 minutes, in order to dry the fibres from the remaining buffer. The dried fibres were then adhered onto an SEM stub, using double sided carbon tabs (Agar Scientific, Stansted, UK). The prepared stub was then given a thin coating of gold (10 nM) in a Quorum Q150T Sputter Coater (Quorum Technologies Ltd. East Sussex, UK) in an argon atmosphere. The coated stub was then transferred and imaged under FEI Quanta 200F (FEI company Ltd, Eindhoven, The Netherlands), at an acceleration voltage of 5 kV.

5.2.2.2.4. Protein Quantification for Western Blot

The anti-scarring effect was evaluated by exposing the SIRC cell lines to TGF-\(\beta\)1, in a concentration of 10 ng/mL, according to the methods of Chowdhury et al. (2013) and Stahnke et al. (2017). This was to mimic the immunological response after an injury, which will trigger the release of cytokines such as TGF-\(\beta\). Therefore, inhibition the expression of this protein is an indication of the anti-scarring effect of pirfenidone. The initial growth and sub-culturing of the cells were similar to that described in section 5.2.2.2.1. After cell counting, 160,000 cells per well were transferred to 6-well plates and incubated at 37\(^{\circ}\)C and 5% CO\(_2\). After 24 hours incubation, confluency had reached 50 to 60%, then the media was aspirated and the attached cells were treated with 2 mL serum free medium (i.e. without FBS) in order to starve the cells, as it will be explained later. After the overnight incubation, the serum free medium was aspirated and a 2 mL complete growth medium was added. Here, the medium will contain TGF-\(\beta\)1 (10 ng/mL), TGF-\(\beta\)1 (10 ng/mL) with pirfenidone (0.04 mg/mL), moxifloxacin (0.04 mg/mL) and the 1:1 drug combination (both in 0.04 mg/mL), as positive controls. These controls were compared with the blank and drug-loaded coaxial (Section 4.2.2.3. Table 4.3 - formulation 26 and 30, respectively) and triaxial (Section 4.2.2.4. Table 4.4 - formulation 31 and 32, respectively) fibres that were placed in a medium contains TGF-\(\beta\)1 (10 ng/mL). All these groups were tested against the untreated cell group, which contains complete growth media with no TGF-\(\beta\)1 for 24 and 48 hours incubation.

After the incubation time point, cells were kept under ice. The medium was aspirated and the cells were rinsed with Dulbecco's PBS, then 0.25 mL of cold lysis buffer was added to each well for 10 minutes. The lysis buffer consists of
NaCl (150 mM), Tris pH 7.5 (50 mM), EDTA (ethylene diamine tetra-acetic acid, 5 mM), NP40 (0.25% v/v), phosphate inhibitor (1 mL/ 100 mL total volume) and protease inhibitor (2 tablets in 100 mL total volume). The lysates were then transferred to 1 mL Eppendorf tubes to be centrifuged at 13,000 rpm and 4°C for 10 minutes. The supernatants were then transferred to new Eppendorf tubes for protein quantification. The bicinchoninic acid (BCA) protein assay was used to quantify the protein concentration in each sample. The protein standards and samples were prepared. Protein standards are known concentrations of proteins which can be used to plot a protein standard curve. Here, each standard concentration (50 µL) was mixed with lysis buffer (5 µL) and the BCA reagent (1 mL). However, the tested samples were prepared by mixing each sample (5 µL) with PBS (50 µL) and the BCA reagent (1 mL). The PBS was used as a diluent while the addition of the 5 µL lysis buffer with the protein standards was used to compensate the addition of 5 µL of the samples that already contain lysis buffer. All standards and samples were heated at 55°C for 30 minutes then transferred to 96-well plates to be measured under a microplate reader at a wave length of 562 nm. The total protein concentration of each sample was determined according to the standard curve.

5.2.2.2.5. Proteins Determination by the Western Blot Technique

After quantifying the protein concentrations, certain volumes of Laemmli sample buffer were added to each sample of the cell lysate protein and boiled (95°C) for 10 minutes, according to the methods of Chowdhury et al. (2013) and Stahnke et al. (2017). Then, equal quantities (25 µL) of each protein sample were loaded in the wells of SDS-PAGE gel and separated at 100 V for 90 minutes using MES SDS running buffer (at a dilution of 1X). This running buffer consists of MES (2-(N-morpholino) ethanesulfonic acid), Tris Base (2-Amino-2-(hydroxymethyl)-1, 3-propanediol), SDS (Sodium dodecyl sulfate, also known as sodium lauryl sulfate) and EDTA at pH 7.4. The gel was transferred to PVDF membranes using transfer buffer (at a dilution of 1X). After transferring of the membrane, it was washed with 1X TBST buffer, which consisted of Tris pH 9.5 (30 g), KCl (2 g), NaCl (80 g) and Tween 20 (1% v/v)
in 1 L total volume, then the pH is adjusted to 7.4. The membrane was blocked with milk (5% w/v in 1X TBST) at room temperature for 2 hours. Finally the membrane was incubated overnight at 4°C with the primary antibodies against α-SMA or β-Actin diluted in 5% milk, as described in Chowdhury et al. (2013) and Stahnke et al. (2017) in the recommended concentrations by Abcam (Cambridge, UK).

After the overnight incubation, the membrane was washed 3 times quickly with TBST, followed by 3 long washes (10 minutes each). Then the membrane was incubated (at room temperature for 1 hour) in the HRP conjugated secondary antibody diluted in 5% milk in the recommended concentration. Secondary antibody, goat anti-mouse or goat anti-rabbit, was used for the membrane which was incubated with α-SMA or β-Actin, respectively. After that, each membrane was washed 3 times quickly with TBST, followed by 3 long washes, as described in Chowdhury et al., 2013 and Stahnke et al., 2017. Finally, specific antigen-antibody complexes were developed by using SuperSignal™ west femto maximum sensitivity substrate and the chemoilluminescence was detected by using Syngene GeneGnome Bio imaging system (Syngene, Cambridge, UK). Optical density of each sample band was normalised to the corresponding β-Actin band, which was further quantified using ImageJ software, following the method of Stahnke et al., 2017).
5.3. Results and Discussion

After preparing coaxial and triaxial fibres loaded with moxifloxacin and pirfenidone, the antimicrobial and anti-scarring assessments of each system have been evaluated. For the antimicrobial assessment, the MIC for both drugs, individually and in combination (1:1) has been measured. This is to evaluate the antibacterial efficiency of moxifloxacin and pirfenidone, and to check whether there is any synergistic or resistance effect due to this combination. Zone of inhibition was the main test to evaluate the effect of drug-loaded fibres compared to the blank fibres (negative control) and moxifloxacin disc (positive control). For the anti-scarring assessment, the IC20 and IC50 were determined for both free form of each drug and the combined (in solution) against rabbit corneal fibroblast cell line (SIRC). These fibroblasts were then treated with TGF-β1 alone or co-treated with TGF-β1 and indicated doses of free drugs solution, blank or drug-loaded fibres, for 24 and 48 hours. The use of TGF-β1 was to mimic the upregulation of this cytokine during scar formation, which increases the secretion of α-SMA and other ECM proteins. The level of α-SMA overexpression has been estimated by using Western blotting against β-Actin, as an experimental control.

5.3.1. Determination of the Antimicrobial Efficacy of the Multi-Layered Fibres

5.3.1.1. Minimum Inhibitory Concentration Determination

In order to evaluate the effectiveness of moxifloxacin and pirfenidone, individually or combined, against Gram positive S. aureus and Gram negative P. aeruginosa, the MIC for each drug and the combination were measured. A concentration range of 0 to 32 µg/mL was used and the presence of turbidity (grey colour for S. aureus and yellow colour for P. aeruginosa, as shown in Figure 5.1.A and 5.1.B, respectively) indicated bacterial growth. For S. aureus and P. aeruginosa, moxifloxacin exhibited an MIC at 0.125 µg/mL and 4 µg/mL, respectively, as shown in Figures 5.1.A and 5.1.B. This suggested a lower susceptibility of P. aeruginosa to moxifloxacin compared to S. aureus. This finding was in agreement with the Mudgil and Pawar (2013) study, which
suggested a higher susceptibility of *S. aureus* to moxifloxacin loaded into PLGA nanosuspension formulation than *P. aeruginosa* after 12 and 24 hours incubation.

![Figure 5.1: MIC test against S. aureus (A) and P. aeruginosa (B). The minimum concentrating (red circled) which the turbidity (grey or yellow coloured) disappears is the MIC. M: moxifloxacin, P: pirfenidone, MP: moxifloxacin and pirfenidone in 1:1 combination. The combined drugs (MP) was tested twice to verify the absence of any synergistic or inhibitory effects.](image)

According to Andrews (2001), moxifloxacin has an MIC of 0.06 µg/mL and 2 µg/mL against *S. aureus* and *P. aeruginosa*, respectively. In the current test, moxifloxacin showed an insufficient effect at 0.06 µg/mL and 2 µg/mL against *S. aureus* and *P. aeruginosa*, respectively, which was observed as a slight turbidity. However at the concentrations of 0.125 µg/mL and 4 µg/mL, moxifloxacin exhibited MIC values which were within ± one two-fold dilution of
the expected MIC from this previous work (the 0.06 µg/mL and 2 µg/mL). This was considered acceptable according to Andrews (2001). Pirfenidone demonstrated no activity against either bacterial strains at the used concentration range (0-32 µg/mL). The 1:1 moxifloxacin and pirfenidone combination showed similar MIC values to moxifloxacin alone, suggesting that this combination has no synergistic or inhibitory effect against S. aureus and P. aeruginosa (Figures 5.1.A and 5.1.B). Therefore, it was confirmed that moxifloxacin was effective against the used bacterial strains, while the next test (zone of inhibition) will demonstrate the effect of incorporating moxifloxacin within the fibres using electrospinning.

5.3.1.2. Zone of Inhibition Determination

Drug-loaded coaxial and triaxial fibres, which were prepared using ACTN, were assessed against S. aureus and P. aeruginosa in a fibre size equivalent to a dose of 50 µg of moxifloxacin. Blank coaxial and triaxial fibres were used as negative controls, with a weight equivalent to that of the drug-loaded fibres, while a sterile moxifloxacin disc containing 50 µg drug was used as a positive control. After 24 hours incubation, a well-defined zone of inhibition for the drug-loaded coaxial and triaxial fibres was observed, with a similar diameter to that seen with the moxifloxacin disc, against both S. aureus (Figure 5.2.A) and P. aeruginosa (Figure 5.2.B). The zone of inhibition diameter was around 45 mm against S. aureus and about 28 mm for P. aeruginosa, due to the higher effect of moxifloxacin on S. aureus. Table 5.1 summarise the zone of inhibition diameter for S. aureus and P. aeruginosa. Importantly, the blank fibres showed no effect against either strain (Figures 5.2.A. and 5.2.B., respectively). These results suggested that moxifloxacin was successfully released from within the fibre matrix, while retaining its antibiotic potency against the different bacterial strains over the time course of this test. In addition, the presence of pirfenidone and the polymers showed no effect on the antibiotic potency of moxifloxacin, nor any intrinsic antibiotic effect of their own (Figures 5.2.A. and 5.2.B).
Table 5.1: The zone of inhibition diameters of moxifloxacin disc, blank and drug-loaded coaxial and triaxial fibres against S. aureus and P. aeruginosa.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Zones of inhibition (mm) treated by</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Moxifloxacin disc</td>
<td>Blank fibres</td>
<td>Drug-loaded coaxial fibres</td>
<td>Drug-loaded triaxial fibres</td>
</tr>
<tr>
<td>S. aureus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>0</td>
<td>46</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>0</td>
<td>45</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>41</td>
<td>0</td>
<td>45</td>
<td>43</td>
</tr>
<tr>
<td>Average diameter</td>
<td>41.67 ± 0.58</td>
<td>0</td>
<td>45.33 ± 0.58</td>
<td>43.67 ± 0.58</td>
</tr>
<tr>
<td>± SD (n=3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>26</td>
<td>0</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>0</td>
<td>29</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0</td>
<td>28</td>
<td>27</td>
</tr>
<tr>
<td>Average diameter</td>
<td>25.67 ± 0.58</td>
<td>0</td>
<td>28.33 ± 0.58</td>
<td>27.67 ± 0.58</td>
</tr>
<tr>
<td>± SD (n=3)</td>
<td></td>
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</tbody>
</table>
Figure 5.2: Zone of inhibition against *S. aureus* (A) and against *P. aeruginosa* (B). The diameter of the area of no growth is considered as the zone of inhibition. Blank fibre (upper left), moxifloxacin disc (upper right), drug-loaded coaxial fibre (lower left) and drug-loaded triaxial fibre (lower right).
It was reported previously that moxifloxacin-loaded into electrospun fibres has demonstrated an antimicrobial activity against different bacterial strains. Shawki et al. (2010) demonstrated the antibacterial activity of dextran nanofibres loaded with moxifloxacin. This system showed an inhibitory effect against Gram positive \textit{S. aureus} and Gram negative \textit{E. coli} bacteria. A study by Toncheva et al. (2012) was able to show the antibacterial effectiveness of Poly-l-Lactide-Co-d, l-Lactide (coPLA) and a blend of coPLA and PEG containing ciprofloxacin hydrochloride, levofloxacin hemihydrate or moxifloxacin hydrochloride antibiotics against \textit{S. aureus}. Cheng et al. (2015) assessed the antimicrobial activity of ciprofloxacin and moxifloxacin-loaded into a chitosan and PEO blend (9:1) which demonstrated an effective antibacterial results against Gram positive \textit{S. aureus} and Gram negative \textit{E. coli} bacteria. A recent study by Giram et al. (2018) has exhibited an antibacterial activities against Gram positive \textit{S. aureus} and Gram negative \textit{E. coli} moxifloxacin-loaded into Eudragit L-100.

5.3.1.3. Sterility Testing

For the purpose of using the multi-layered fibres in the biological testing (i.e. \textit{in vitro}/\textit{in vivo} studies), these fibres were prepared similarly to the recommended electrospinning parameters (in Section 4.2.2.3. Tables 4.3 and 4.4) but under aseptic conditions. According to the British Pharmacopeia (\textit{Appendix XVI A. Test for Sterility}, 2019), two media were used to test against both aerobic and anaerobic bacteria, as well as fungi. Both media were incubated and monitored (in daily basis) for the full sterility test duration (14 days). Blank and drug-loaded coaxial and triaxial fibres were tested and pictures were captured after 1, 7 and 14 days incubation. Figures 5.3, 5.4 and 5.5 showed the presence of clear solutions for the tested fibres after 1, 7 and 14 days, respectively. In Figure 5.5, contaminated media were demonstrated after intentionally contaminating the inside of the containers in order to check on the suitability of both media. For fluid thioglycollate (FTG) medium, turbidity was the indicator for bacterial growth, while a clear visual fungus growth was the indicator for Soya-bean casein digest (SBC) medium contamination.
Figure 5.3: Sterility test after 1 day incubation in FTG medium (left image) and SBC medium (right image). The absence of a change in the medium colour or a visible turbidity or fungus growth is an indication for the medium sterility.

Figure 5.4: Sterility test after 7 days incubation in FTG medium (left image) and SBC medium (right image). The absence of a change in the medium colour or a visible turbidity or fungus growth is an indication for the medium sterility.
Figure 5.5: Sterility test after 14 days incubation in FTG medium (left image) and SBC medium (right image). The absence of a change in the medium colour or a visible turbidity or fungus growth is an indication for the medium sterility. The FTG contaminated medium (in the left image) showed a change in the medium colour and a visible turbidity. The SBC contaminated medium (in the right image) showed a visible growth of a red coloured fungus.

The absence of turbidity and fungus growth for the blank and drug-loaded fibres after 14 days of incubation was enough to pass the sterility test based on the British Pharmacopeia recommendation (Appendix XVI A. Test for Sterility, 2019). This test was sufficient to ensure that the blank and drug-loaded coaxial and triaxial fibres were sterile (absent from any contamination) and safe to be used in the following cell culture studies, which is a requirement prior to any cell culture assay.

5.3.2. In Vitro Determination of the Anti-scarring Efficacy of the Multi-Layered Fibres

5.3.2.1. Inhibitory Concentration Determination

The SIRC cell line was successfully cultured using ATCC recommendation (ATCC® CCL-60™, 2019). These cells are adherent cells, which means that the cells are attached to the bottom of the growing flask forming a layer, as can be shown in Figure 5.6.
Cell viability was assessed after 24, 48 and 72-hour exposure to solutions of moxifloxacin, pirfenidone or the combination of the two drugs in order to evaluate their toxicity on the cells. The aim of the formulations studied here is to heal the abrasion (scratch) that present on the ocular surface, rather than to kill the native cells, hence a high cell viability (low toxicity) is required. Therefore, 80% cell viability is the lowest that would be acceptable for further studies. This equates to an IC20 value. Figure 5.7 shows the results for the 24-hour exposure of the cells to the drugs. Greater than 80% cell viability was seen with moxifloxacin concentrations of 0.04 mg/mL or less, with higher concentrations leading to increased cell death. Concentrations ranging from 0.08 to 0.31 mg/mL showed a cell viability above 60% but below 80%, with the concentrations above 0.31 mg/mL were toxic to the cells (> IC50). Pirfenidone was less toxic than moxifloxacin, with cell viability of ≥ 80% seen with concentrations up to 0.31 mg/mL. However, the concentrations ranging from 0.62 mg/mL to 2.5 mg/mL showed to be above the IC50, indicating for a lower cell-toxicity compared to moxifloxacin. The effects of the combination followed the pattern of the moxifloxacin alone, indicating that there was no interaction between the two drugs in respect of cell viability.
Figure 5.7: IC20 and IC50 determination after 24-hour exposure to different concentrations of the free drugs showing the dose response curves (top image) and MTT plate pictures (bottom image). The left curve and top MTT plate pictures represent moxifloxacin, the middle curve and MTT plate pictures represent pirfenidone, the right curve and bottom MTT plate pictures represent moxifloxacin and pirfenidone in 1:1 combination.
Figure 5.8 shows the results for the 48-hour exposure of the cells to the drugs. Similar to the 24-hour exposure results, $\geq 80\%$ cell viability was seen with moxifloxacin concentrations of 0.04 mg/mL or less, with higher concentrations leading to increased cell death. However, the effects of these higher concentrations was more noticeable at 48-hour compared to the 24-hour exposure. For instance, the concentration 0.31 mg/mL was considered the IC50 after the 48-hour exposure, while it was above 60% cell viability after 24-hour exposure. In addition, after 48-hour exposure of the cells to pirfenidone, $\geq 80\%$ cell viability was seen with concentrations of $\leq 0.16$ mg/mL, which is lower than seen at 24-hour. Similarly, the combination was marginally more toxic at 48-hour than 24-hour exposure. This indicated that increasing the exposing time of the cells to moxifloxacin and pirfenidone, alone or in combination, can enhance their toxicity.
Figure 5.8: IC20 and IC50 determination after 48-hour exposure to different concentrations of the free drugs showing the dose response curves (top image) and MTT plate pictures (bottom image). The left curve and top MTT plate pictures represent moxifloxacin, the middle curve and MTT plate pictures represent pirfenidone, the right curve and bottom MTT plate pictures represent moxifloxacin and pirfenidone in 1:1 combination.
Figure 5.9 illustrates the results for the 72-hour exposure of the cells to the drugs. The results of moxifloxacin and the combination were similar to those seen at 48-hour. Prolonged exposure to pirfenidone demonstrated an increase in cellular toxicity, with ≥ 80 % cell viability being seen only with concentrations of ≤ 0.08 mg/mL.
Figure 5.9: IC20 and IC50 determination after 72-hour exposure to different concentrations of the free drugs showing the dose response curves (top image) and MTT plate pictures (bottom image). The left curve and top MTT plate pictures represent moxifloxacin, the middle curve and MTT plate pictures represent pirfenidone, the right curve and bottom MTT plate pictures represent moxifloxacin and pirfenidone in 1:1 combination.
This study indicated that moxifloxacin and pirfenidone, individually and in combination, showed dose dependent effects on cell viability, with pirfenidone showing a clear time dependent effect on cell viability compared to moxifloxacin. This study was able to evaluate the toxicity of each drug on the cells through which the concentration of the drugs within the fibres can be measured to avoid the use of toxic concentrations that can inhibit the cells growth. Owing to the 2:1 and 1:1 ratio of pirfenidone and moxifloxacin in the drug-loaded coaxial and triaxial fibres, respectively, drug-loaded coaxial and triaxial fibres with a pirfenidone concentration of 0.04 mg/mL was used. This is to exhibit the effect of these fibrous systems on reducing the expression of α-SMA protein after exposing the cells to TGF-β1 cytokine using the Western blot technique.

5.3.2.2. Cell Treatment with the Multi-Layered Fibres

Despite determining the IC20 of each drug, individually and combined, drug-loaded coaxial and triaxial fibres were intentionally used in a higher concentration of moxifloxacin and pirfenidone. This was equivalent to 0.08 mg/mL of pirfenidone and 0.04 mg/mL of moxifloxacin for the drug-loaded coaxial fibres and 0.08 mg/mL of pirfenidone and moxifloxacin for the drug-loaded triaxial fibres. This higher concentrations were exposed to the cells in order to compare the toxicity effect of the drug-loaded fibres (upon releasing both drugs) to the free drugs in solution form. Blank fibres were used as a negative control, in equivalent weights to the corresponding drug-loaded fibres. Solutions of moxifloxacin, pirfenidone and the 1:1 combination were used, as positive controls, at concentrations of 0.08 mg/mL. Figure 5.10 shows that the drug-loaded fibres reduced the cell viability by about 5 to 9% compared to a reduction of 15 to 20% after 24-hour exposure to the free drugs, while the blank fibres showed no reduction in the cell viability. However, a slightly increase in the reduction of the cell viability % can be observed after 48-hour exposure for all groups (Figure 5.10 and 5.11) compared to the 24-hour exposure. This suggests that the drug-loaded fibres release the drugs at a slower rate than the drug solutions, hence, the effects on the cell viability are lower. These findings demonstrated that despite using drug-loaded coaxial
and triaxial fibres in a slightly toxic concentrations (≥ IC20 and ≤ IC50), these fibrous systems showed a cell viability % more than 90% after 24 and 48-hour exposure compared to the drugs in solution form (~ 80% cell viability). This suggests that both drugs were released at a rate that enabled the cells to survive and proliferate than the cells that were exposed to the drugs in solution forms at a slightly toxic dose (≥ IC20).

Figure 5.10: Cell viability % after 24 and 48 hours cells exposure to free drugs, blank and drug-loaded coaxial and triaxial fibres in equivalent concentrations of 0.08 mg/mL (80 µg/mL).
Figure 5.11: MTT plate pictures after 24 and 48 hours cells exposure to free drugs, blank and drug-loaded coaxial and triaxial fibres in equivalent concentrations of 0.08 mg/mL (80 µg/mL).

Blank and drug-loaded coaxial and triaxial fibres were further examined by SEM, in order to demonstrate the morphology of SIRC cells proliferated on the fibrous network after 24-hour exposure. Figure 5.12 showed that all fibre mats were suitable for cell attachment and spreading, with cells embedded into the fibrous network gaps forming a confluent layer. This could be an indicator for good cell compatibility with the fibre composition and cells have an ingrowth spreading. Sun et al. (2012) demonstrated a similar SEM result with PLGA fibres cultured with human dermal fibroblasts. He et al. (2018) showed a consistent SEM result of PLGA/PVP coaxial fibres cultured with mouse bone fibroblasts.
Figure 5.12: SEM image for cell growth and proliferation on fibrous membranes. Blank coaxial fibre (upper left), blank triaxial fibre (upper right), drug-loaded coaxial fibre (lower left) and drug-loaded triaxial fibre (lower right). Red arrows indicate the presence of cells growth within the fibrous network.
5.3.2.3. α-SMA Expression Determination using Western Blotting

Due to the presence of an injury, the cytokine TGF-β1 will accumulate as a result of the body’s immune system response and will in turn up-regulate the expression of different ECM proteins, such as α-SMA. This high expression can be used as an established marker for the presence of a scar. The healing process will start by down-regulating this protein, and thus suppress the level of TGF-β1 that enabled the α-SMA level to increase. Pirfenidone has been reported to treat corneal scars (Chowdhury et al., 2013; Stahnke et al., 2017), as well as lung (King Jr et al., 2014), heart (Shi et al., 2011), liver (Di Sario et al., 2002) and kidney (Hewitson et al., 2001) scars by reducing the expression of the triggered cytokines that increased the level of ECM proteins such as α-SMA, fibronectin, collagen and platelet-derived growth factor (PDGF).

The SIRC cells were treated with serum free medium and incubated overnight in order to starve them according to the methods of Chowdhury et al. (2013) and Stahnke et al. (2017). Starving will force all the cells to synchronise to the same cell cycle phase and to avoid any interaction of the serum components with the added cytokine or the released proteins during the experimental procedure (Pirkmajer and Chibalin, 2011). Then these cells were exposed to TGF-β1 for 24 and 48 hours, to induce the expression of ECM proteins. In addition to the TGF-β1, cells were exposed to various treatments, including solutions of pirfenidone, moxifloxacin and the combination of the two drugs, as well as, blank and drug-loaded coaxial and triaxial fibres. All these groups were tested relative to the untreated cell group (without the addition of TGF-β1 or any treatment). For the 24-hour exposure, the treatments were added at the same time as the TGF-β1. For the 48-hour exposure, the treatments were added after 24 hours incubation with TGF-β1. The concentration of TGF-β1 that was used is 10 ng/mL to be incubated for 48 hours, which were the optimum conditions to stimulate the expression of ECM proteins according to Narayanan et al. (1989).
Protein quantification was via a BCA protein assay. Figure 5.13 shows the standard curve for this assay. This was used in order to load a similar amount of proteins from each treated group of cells into the wells of the SDS-PAGE gel to be separated.

Figure 5.13: BCA protein standard curve to quantify the amount of proteins from each sample in order to load equivalent amounts of proteins from the tested samples into the wells of the Western blot gel. The curve shows fair linearity ($R^2 \geq 0.996$).

After analysing the samples through the Western blot method, the membranes, in Figures 5.14, demonstrated clearly the enhanced expression of $\alpha$-SMA of the cells after 48-hour exposure to TGF-$\beta1$ but not after 24 hours, which is in agreement with the results of Narayanan et al. (1989). Therefore, only the Western blot results from 48-hour exposure were considered in this study. The resulting band intensity (as shown in Figure 5.14) was quantified using ImageJ software, where the band area of the $\alpha$-SMA for each group was divided by its corresponding band area of the $\beta$-Actin for the same group. For instance, the band area of the $\alpha$-SMA for the TGF-$\beta1$ only treated group was divided by the band area of the $\beta$-Actin for the TGF-$\beta1$ only treated group (i.e. band area of $\alpha$-SMA ÷ band area of its corresponded $\beta$-Actin).
Figure 5.14 illustrates the reduction of $\alpha$-SMA expression after treatment with solutions of pirfenidone, the combination of pirfenidone and moxifloxacin, and interestingly moxifloxacin. In addition, drug-loaded coaxial and triaxial fibres exhibited a similar decrease in the expression of $\alpha$-SMA to the positive controls (free drugs) and a clear down-regulated expression compared to the blank fibres. Surprisingly, both blank coaxial and triaxial fibres were able to reduce the expression of $\alpha$-SMA compared to TGF-β1 only group. This might be due to the presence of the polymers, which will need a further investigation.
Figure 5.14: The top image shows the Western blot band intensities of β-Actin (experimental control) and α-SMA (protein of interest), while the bottom image shows Western blot quantification using the band intensities of both proteins. The bands were obtained after 24 and 48-hour exposure to TGF-β1 alone or in combination with the free drugs, blank or drug-loaded coaxial and triaxial fibres in equivalent concentrations of 40 µg/mL compared to the untreated group. B: blank and DL: drug-loaded.
A similar anti-scarring effect of moxifloxacin had previously reported by Chen et al. (2013) on human corneal fibroblasts. This study showed that the moxifloxacin was able to reduce the expression of α-SMA after TGF-β1 stimulation. In addition, moxifloxacin demonstrated an inhibition of TGF-β-induced fibroblast-to-myofibroblast differentiation (associated with scar formation) by blocking TGF-β receptor type 1, which is considered as a scar healing process (Chen et al., 2013). In addition to the potential anti-scarring effect of moxifloxacin, Weiss et al. (2004) were able to identify an anti-inflammatory effects of this antibiotic via effects on different pro-inflammatory cytokines such as TNF-α, IL-8, and IL-1β.

This Western blot study suggested that the drug-loaded coaxial and triaxial fibres were able to reduce the expression of an up-regulated α-SMA, in a level which is closely similar to the drugs in solution form, resulting in a potential favourable scar healing process. This study also indicated the successful release of pirfenidone and moxifloxacin from the fibres, while retaining their biological activities.
5.4. Conclusions

The microbiological performance of the coaxial and triaxial fibres was successfully assessed by the zone of inhibition study. It was shown that the diameters of the ‘no growth’ area for both drug-loaded coaxial and triaxial fibres were similar to the positive control of the moxifloxacin disc, at the same dose, against both the Gram positive *S. aureus* and Gram negative *P. aeruginosa*. As expected, the blank fibres showed no zone of inhibition against both bacterial strains. Additionally, an MIC test confirmed the susceptibility of these bacterial strains to moxifloxacin, while pirfenidone showed no antimicrobial effect when tested alone, and no synergistic or inhibitory effect when tested in combination with moxifloxacin. Finally, for the purpose of biological assessment, the blank and drug-loaded fibres were prepared under aseptic conditions. The sterility of these fibres was then tested by using two media (FTG and SBC) according to the BP. After 14 days, the blank and drug-loaded coaxial and triaxial fibres showed negative growth, indicating their sterility.

These fibres were also evaluated using Western blot technique on the SIRC cell line. The IC20 was identified for moxifloxacin, pirfenidone and the combination of both drugs. This concentration was proposed to avoid the inhibition of more than 20% of the cell population, which will help in the cell’s proliferation and recovery. Cells were later exposed to blank and drug-loaded coaxial and triaxial fibres (in a concentration above the IC20) for 24 and 48 hours. The intentionally use of a high concentration was to clearly distinguish between exposing the cells directly to the drugs in solution form with the drugs being released from the fibres. The MTT assay suggested that the cell viability was above 90% for the coaxial and triaxial drug-loaded fibres compared to 80 to 85% for the solution form of the drugs in similar concentrations. SEM images of the fibres that were exposed to the cells confirmed the compatibility of this cell line with the fibre components. As expected, the Western blot results demonstrated that the free pirfenidone and the drug-loaded fibres were able to reduce the expression of α-SMA, after the cells were exposed to TGF-β1 for 48 hours. This exposure time was optimum to over-express α-SMA and other ECM proteins. Moxifloxacin was able to decrease the level of α-SMA, which can be an advantage of using this antibiotic for the treatment of both scars and...
infection. In addition, the blank fibres showed a slight reduction in the expression of $\alpha$-SMA, which will need further investigation.

Consequently, the drug-loaded coaxial and triaxial exhibited an antibacterial and anti-scarring efficiency by inhibiting the growth of *S. aureus* and *P. aeruginosa* and reducing the expression of the $\alpha$-SMA, respectively. These results were in similar levels as the solution form of moxifloxacin and pirfenidone, respectively, confirming the successful release of both drugs from these fibrous systems while retaining their biological activities.
Chapter 6

*In Vivo* Assessment of Drug-Loaded Coaxial Electrospun Fibres
6.1. Introduction

The *in vitro* performance of the coaxial and triaxial drug-loaded fibres has been successfully tested in order to assess the ability of the fibres to release both moxifloxacin and pirfenidone while retaining their pharmacological activities. The microbiological assessment for both types of multi-layered fibres were tested using the zone of inhibition test against Gram positive *S. aureus* and Gram negative *P. aeruginosa* bacteria. The findings indicated that these fibres were able to inhibit both bacterial strains in a dose equivalent to a positive control (moxifloxacin disc). The anti-scarring evaluation for the coaxial and triaxial fibres was performed by using the Western blot technique, after exposing rabbit corneal fibroblast (SIRC) cell lines to TGF-β1 cytokine alone or co-treated with each drug individually or combined, the blank and drug-loaded coaxial and triaxial fibres. The TGF-β1 cytokine was applied after starving the cells via overnight incubation in serum free medium to mimic the stress of a scar condition, after which the α-SMA protein expression was measured. This protein expression is usually elevated in response to the accumulation of TGF-β1 cytokine due to the presence of a scar. The results were compared with β-Actin protein as an experimental control. The Western blot findings showed that α-SMA expression was reduced by both pirfenidone and moxifloxacin (in a solution form), as well as, the drug-loaded fibres. This outcome may represent an additional advantage for the use of moxifloxacin in the treatment of corneal abrasion.

After demonstrating the potential delivery capability of the multi-layered fibres, it is reasonable to move on to an animal model, in order to correlate the *in vitro* results to the *in vivo* outcomes. In this chapter, a pharmacokinetic study is performed in New Zealand rabbit eyes, in which samples from the aqueous humour will be withdrawn at certain time points after applying either the drug-loaded coaxial fibres or the free drugs in a solution form. These samples will be evaluated in accordance to a developed standard curve using an ultra performance liquid chromatography (UPLC). Then, an *in vivo* microbiological test is performed to exhibit the infection inhibition ability of the coaxial fibres after infecting one rabbit eye with Gram positive *S. aureus*. Finally, the ocular
irritation for the blank and drug-loaded coaxial fibres was evaluated by the Draize test (Draize et al., 1944). This is to check on the sensitivity of the corneal tissues to the applied fibres.

It must be clearly noted that these in vivo studies were performed after preparing the blank and drug-loaded coaxial fibres, as described in Chapter 4 Section 4.2.2.3. Table 4.3 formulations 26 and 30, respectively, under aseptic conditions. The experimental planning and the sterile fibres were sent to an external collaborator, namely Dr Aws Alshamsan, research group in the College of Pharmacy at King Saud University (Riyadh, Saudi Arabia), who agreed to carry out these animal tests. An ethical approval number KSU-SE-18-25 was awarded by the Research Ethics Committee at King Saud University. The experimental planning of this animal work and the method of analysis using UPLC, as well as, the fabrication of the blank and drug-loaded coaxial fibres were initially performed here at UCL School of Pharmacy. The ethical approval for the work being conducted overseas is going through the appropriate internal approval process, here at UCL, at the time of writing. An animal handling certificate (no. UCL/17/123) has been awarded to Mr Tawfik from the Royal Society of Biology, after successfully achieving the learning outcomes of rat, mouse and rabbit species modules that were organised by the Biological Services at UCL. In addition, the application of the fibres (under the lower eye lid) and the aqueous humour sample withdrawal of one rabbit was demonstrated to Mr Tawfik on the 25th December 2018 in Dr Alshamsan lab at College of Pharmacy at King Saud University.
6.2. Materials and Methods

6.2.1. Materials

Pirfenidone was purchased from Tokyo Chemical Industry UK Ltd (The Magdalen Centre, Oxford, UK), while moxifloxacin hydrochloride eye drops, Vigamox® 0.5% w/v, were obtained from King Khalid University Hospital (Riyadh, Saudi Arabia). *S. aureus* bacterial strain (ATCC® 25923) was bought from ATCC (Manassas, USA). Mannitol Salt Agar (MSA) were purchased from Bacton Dickinson GmbH (Heidelberg, Germany). Acetonitrile HiPerSolv CHROMANORM (for LC-MS) and HPLC grade methanol were brought from BDH Chemical Ltd (Poole, England). Potassium dihydrogen orthophosphate was purchased from WINLAB laboratory chemicals reagents fine chemicals (Leicestershire, UK). Normal saline (0.9% NaCl solution) was obtained from Pharmaceutical Solutions Laboratory (Jeddah, Saudi Arabia). Ketamine hydrochloride (TEKAM®, 50 mg/mL) and xylazine (SETON®, 2% w/v) were brought from Hikma Pharmaceuticals (Amman, Jordan) and Laboratorios Calier (Barcelona, Spain), respectively. Proparacaine hydrochloride ophthalmic solution (Alcaine®, 0.5% w/v) and tropicamide eye drop solution (Mydriacyl, 1% w/v) were purchased from Alcon Laboratories Inc. (Fort Worth, USA) and Alcon Eye Care UK Limited (Camberley, UK), respectively.

6.2.2. Animals

Thirty New Zealand male albino rabbits, weighing 3 to 4 kg each, were obtained from the experimental animal house care centre at College of Pharmacy, King Saud University (Riyadh, Saudi Arabia). They were initially examined and screened for any pre-existing ocular conditions. All animals were maintained according to the guide for the care and use of laboratory animals approved by the centre. All the rabbits were housed in clean cages (i.e. pathogen-free conditions). They were kept under standard laboratory conditions in 12 hours light and dark cycles at ambient temperature (25 ± 2°C), a pellet diet was given with water *ad libitum* and they were fasted overnight before the experiments. All animal studies were approved by the Experimental
Animal Care Centre Review Board (approval no. KSU-SE-18-25). Anaesthetics and artificial restraints were employed in this \textit{in vivo} work.

6.2.3. Methods

6.2.3.1. Pharmacokinetic Study

6.2.3.1.1. Calculation of the Administrated Drug Dose

Drug-loaded coaxial fibres were successfully prepared and the drug loading was estimated as 40 µg/mg for pirfenidone and 21 µg/mg for moxifloxacin. For the purpose of the pharmacokinetic studies, the applied dose was calculated as follows.

For moxifloxacin eye drops (0.5% w/v), 50 µL was used for ocular instillation which is equivalent to 250 µg of moxifloxacin in one dose. For the coaxial fibres, as per loading capacity of moxifloxacin, an equivalent dose to the eye drops was calculated as 11.90 mg of the total fibres weight (contains 250 µg moxifloxacin).

This 11.90 mg of fibres will contain 476 µg (i.e. 0.48 mg) of pirfenidone in one dose of the fibres. Therefore, this pirfenidone amount is added to the 50 µL moxifloxacin eye drop and vortexed.

Consequently, one dose of 50 µL eye drop solution will contain an equivalent dose of moxifloxacin (250 µg) and pirfenidone (476.19 µg) to the drug-loaded coaxial fibres. The fibres were applied under the lower eye lid of the rabbits’ eye.

6.2.3.1.2. UPLC Analysis

In order to analyse the withdrawn samples from the aqueous humour, a UPLC standard curve was developed. Waters Acquity H-Class UPLC system coupled with a Waters UV detector by Acquity UPLC (Waters, Milford, USA) was used. The UPLC system included a quaternary solvent manager, sample manager (Acquity UPLC Waters), with an injection capacity of 10 µL and a column heater. This assay was developed by combining and modifying the parameters.
described in Kalam (2016) and the HPLC developed methods described in Chapter 3, Section 3.2.2.6. The elution of moxifloxacin and pirfenidone was performed on Acquity UPLC BEH™ C₁₈ column (1.7 μm, 2.1 x 50 mm, Waters, USA) maintained at 25°C temperature. An acidic phosphate buffer 20 mM [2.72 g Potassium dihydrogen orthophosphate (MW 136.02) in 1 L of HPLC grade water and adjusted to pH 3.3 by 4-5 drops of phosphoric acid] and ACTN was used as the mobile phase, in a ratio of 65:35, and pumped at a flow rate of 0.12 mL/minute. The injection volume was 5 μL and a total run time of 5 minutes. UV detection was performed at a wavelength of 310 nm. The EMPOWER software was used to control the UPLC-UV system, as well as, to acquire and process the data.

Standard stock solutions of moxifloxacin and pirfenidone were prepared in methanol to get a final concentration of 50 μg/mL. These stock solutions were used for the standard curve and the working standard samples were prepared by modifying the sample preparation methods outlined in Kalam (2016). Working standard solutions of the two drugs, ranging from 250-20,000 ng/mL, were prepared by diluting the stock solution with the mobile phase. Working solutions of both drugs were further prepared by taking 20-25 μL aliquots of each working standard solution, where 50 μL of blank rabbit aqueous humour and 130 μL of ACTN were added. The final volume was adjusted to 500 μL by adding 300 μL of mobile phase and vortexed to yield spiked calibration standards (7 points) ranging from 10 to 500 ng/mL.

The stored aqueous humour samples (at -80°C) were thawed and vortexed for 30 seconds at room temperature before extraction to ensure the homogeneity. To 50 μL of aqueous humour samples, 130 μL of acetonitrile was added and vortexed for 1-2 minutes to precipitate the proteins and centrifuged at 13,500 rpm for 10 minutes at 4°C. After centrifugation, approximately 20 μL of supernatant was transferred into inserts placed in HPLC vials. Now, 5 μL of the supernatant was injected to the column to elute moxifloxacin and pirfenidone by setting the UV-detection wavelength at 310 nm.
6.2.3.1.3. UPLC Method Validation

The developed UPLC assay to quantify moxifloxacin and pirfenidone in the aqueous humor was validated according to the International Conference on Harmonisation (ICH) guideline (ICH, 2005) as follows.

Specificity is the ability of the method to distinguish more than one substance to be analysed among the presence of other components. The specificity of this assay was confirmed by the separation of both drugs in the presence of other potential constituents of the aqueous humour. The linearity of this assay is determined by using 7 concentration points (ranging from 10 to 500 ng/mL). The area under the curve obtained against the corresponding concentration was plotted in order to identify the linear regression equation. The sensitivity of the analytical method can be determined by the LOD and LOQ, which can be calculated by the equations presented in Chapter 3 - Section 3.3.4.4, equations 3.4 and 3.5, respectively. Intra- and inter-day precisions can be evaluated by running each concentration point in triplicate on the same day or in three consecutive days, respectively. Then, the RSD for 3 different concentrations (10, 100, 500 ng/mL) of each drug is measured. According to Parmar et al. (2014) and Dewani et al. (2011) an RSD ≤ 2% is considered as an acceptable precision, which indicates less samples variation. The robustness is the intentional modification of the method of analysis, such as a slight change in the mobile pH or flow rate, which may alter the resulting peak. The absence of any noticeable change on the chromatogram indicates the recovery of this method of analysis.

6.2.3.1.4. Ocular Pharmacokinetics Study

The concentration of drugs in aqueous humour after a single topical ocular dosing of drug-loaded coaxial fibres in rabbits was compared to a single dosing of moxifloxacin (0.5% w/v) and pirfenidone eye drop, as described in Kalam (2016). This is to evaluate the ocular bioavailability of moxifloxacin and pirfenidone released together from the nanofibres and the drug solution. Six rabbits were divided into two groups, each containing three animals (n=3), in which one group (Group 1) will receive the drug-loaded coaxial fibres while the
second group (Group 2) will receive 50 µL of the combined drugs in solution form. Approximately 11.9 mg of the coaxial fibres were inserted under the lower eyelid (conjunctival sac) of the left eyes of all rabbits in Group 1. All the rabbits were placed in restraining boxes which they could freely move their heads throughout the study period. One hour after the dosing, the rabbits were anesthetised by intravenous injection (the ear vain) of a mixture of ketamine HCl (15 mg/kg) and xylazine (3 mg/kg). One drop of proparacaine HCl (0.5% w/v) and one drop of tropicamide (1% w/v) were inserted into the treated eye. Then, 50 µL of aqueous humour was withdrawn using a 29-gauge insulin syringe needle at an angle of 45º through a partial thickness limbal incision at different time points (1, 2, 4, 6, 12 and 24 hours) from the treated eyes of all the rabbits of the respective groups. For each time point, tear fluid sample was collected from the treated eyes of the rabbits (n=3). The collected samples were transferred into centrifuge tubes and analysed by the developed UPLC-UV method in order to quantify the drugs. The pharmacokinetic parameters; i.e. elimination half-life (t₁/₂), time to reach maximum (peak) concentration following drug administration (Tₘₐₓ), maximum (peak) drug concentration (Cₘₐₓ) and area under the concentration verses time curve (AUC₀-time point) were determined by PK-Solver software (Nanjing, China), as described in Zhang et al. (2010). The animals from both groups were sacrificed after the experiment in a humane way, by an intraperitoneal injection of pentobarbitone sodium at concentration of 60 mg/mL and a dose of 60 mg/Kg of body weight.

6.2.3.2. Infection Inhibition Study

Twelve rabbits were divided into four groups (each containing three, n=3). A Gram positive S. aureus was instilled in one eye at the concentration of 1x10⁶ CFU/mL to induce an infection. Before instillation, eye swabs were taken from the eyes to check for any micro-organism growth (i.e. previous infection) on MSA plates. After 24 hours of post S. aureus instillation, eye infections were observed visually (redness). Eye swabs, taken from the infected eyes, were checked for any micro-organism growth on pre-sterilised MSA plates followed
by treatment initiation. To the first group of infected eye rabbits, moxifloxacin eye drops (0.5% w/v) were applied 4 times daily. The infected eyes of the second group were left without further treatment, as the untreated control group. In the third and fourth groups of rabbits, the blank and drug-loaded coaxial fibres were applied under the lower eyelid of the infected eyes. An equivalent weight of the drug-loaded fibres to the moxifloxacin eye drop dose was measured and inserted under the eyelid (group 3). The blank fibres (in a similar weight as the drug-loaded fibres) was also inserted under the eyelid of the rabbits (group 4). Treatment was given daily at the same time and continued for 7 days. The animals from all groups were kept under investigation for 5 days post test then sacrificed on day 12 after the infection (in the humane way described above). Throughout the whole experiment, photographs of the cornea were taken at different time points.

6.2.3.3. Eye Irritation Study

Ocular irritation was measured in rabbit eyes based on the Draize test (Draize et al., 1944, Luechtefeld et al. 2016 and Kalam, 2016). Twelve rabbits were divided into two groups each containing six rabbits (n=6). Equivalent weights of drug-loaded fibres in one group of rabbits and similar weights of blank fibres in the other group were placed under the lower eyelid of the left eyes. The right eyes of both groups were instilled with sterile normal saline to serve as control. The fibres were given once daily for 7 days and the rabbits’ eyes were observed visually throughout the whole study. The eye irritation level was evaluated by the animal discomfort, signs and symptoms in the conjunctiva, cornea, and eyelids according to the scoring guidelines system for ocular irritation testing mentioned in Table 6.1. (Draize et al., 1944, Higuchi et al., 2016 and Kalam, 2016). The maximum total score is the sum of all scores obtained for the cornea, iris and conjunctiva. Scores of 0 are assigned for each parameter if the cornea, iris or conjunctiva is normal. The animals from both groups were kept under investigation for 5 days post-test after the treatment with the blank and drug-loaded fibres, then sacrificed on day 12 (in the humane way described previously).
Table 6.1: Scores for grading the severity of ocular lesions in the ocular irritation studies.

<table>
<thead>
<tr>
<th>Lesions</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>For the cornea</strong></td>
<td></td>
</tr>
<tr>
<td><strong>i. Opacity-degree of density (area which is most dense is taken for reading)</strong></td>
<td></td>
</tr>
<tr>
<td>Scattered or diffuse area - details of iris clearly visible</td>
<td>1</td>
</tr>
<tr>
<td>Easily noticeable translucent areas, details of iris slightly obscured</td>
<td>2</td>
</tr>
<tr>
<td>Opalescent areas, no details of iris visible, size of pupil barely noticeable</td>
<td>3</td>
</tr>
<tr>
<td>Opaque, iris invisible</td>
<td>4</td>
</tr>
<tr>
<td><strong>ii. Area of cornea involved</strong></td>
<td></td>
</tr>
<tr>
<td>One quarter (or less) but not zero</td>
<td>1</td>
</tr>
<tr>
<td>Greater than one quarter but less than one half</td>
<td>2</td>
</tr>
<tr>
<td>Greater than one half but less than three quarters</td>
<td>3</td>
</tr>
<tr>
<td>Greater than three quarters up to whole area</td>
<td>4</td>
</tr>
<tr>
<td><strong>Score equals (i x ii x 5): Total maximum =</strong></td>
<td>80</td>
</tr>
<tr>
<td><strong>For the iris</strong></td>
<td></td>
</tr>
<tr>
<td><strong>i. Values</strong></td>
<td></td>
</tr>
<tr>
<td>Folds above normal, congestion, circumcorneal injection (any one or all of these or combination of any thereof), iris still reacting to light (sluggish reaction is positive)</td>
<td>1</td>
</tr>
<tr>
<td>No reaction to light, haemorrhage, gross destruction (any one/ all of these)</td>
<td>2</td>
</tr>
<tr>
<td><strong>Score equals (i x 5): Total maximum =</strong></td>
<td>10</td>
</tr>
<tr>
<td><strong>For the conjunctiva</strong></td>
<td></td>
</tr>
<tr>
<td><strong>i. Redness (refers to palpebral conjunctiva only)</strong></td>
<td></td>
</tr>
<tr>
<td>Vessels definitely injected above normal</td>
<td>1</td>
</tr>
<tr>
<td>More diffuse, deeper crimson red, individual vessels not easily noticeable</td>
<td>2</td>
</tr>
<tr>
<td>Diffuse beefy red</td>
<td>3</td>
</tr>
<tr>
<td><strong>ii. Chemosis</strong></td>
<td></td>
</tr>
<tr>
<td>Any swelling above normal (includes nictitating membrane)</td>
<td>1</td>
</tr>
<tr>
<td>Obvious swelling with partial eversion of the lids</td>
<td>2</td>
</tr>
<tr>
<td>Swelling with lids about half closed</td>
<td>3</td>
</tr>
<tr>
<td>Swelling with lids about half closed to completely closed</td>
<td>4</td>
</tr>
<tr>
<td><strong>iii. Discharge</strong></td>
<td></td>
</tr>
<tr>
<td>Any amount different from normal (does not include small amount observed in inner canthus of normal animals)</td>
<td>1</td>
</tr>
<tr>
<td>Discharge with moistening of the lids and hairs just adjacent to the lids</td>
<td>2</td>
</tr>
<tr>
<td>Discharge with moistening of the lids and considerable area around the eye</td>
<td>3</td>
</tr>
<tr>
<td><strong>Score equals (i + ii + iii) x 2: Total maximum =</strong></td>
<td>20</td>
</tr>
</tbody>
</table>
6.3. Results and Discussion

After testing the *in vitro* antibacterial and anti-scarring effect of the coaxial fibres, these results have been correlated to an *in vivo* study. A pharmacokinetic test has been performed to evaluate the release of both drugs from the coaxial fibres and to associate the results with the *in vitro* release study that was performed in Chapter 4 (Section 4.3.5.). A UPLC standard curve has been developed to analyse the withdrawn samples from the rabbit aqueous humour. An infected rabbit eye has been treated with a once daily dose of the drug-loaded fibres and 4-time daily doses of moxifloxacin eye drops. Eye swabs have been taken from the infected eyes at certain time points in order to check for bacterial inhibition. The blank and drug-loaded fibres have been tested for eye irritation using the standard Draize test.

6.3.1. UPLC Assay for Moxifloxacin and Pirfenidone Separation

A UPLC method was developed for analysing the presence of both drugs in the aqueous humour. This UPLC method was simple, rapid and very sensitive. In order to validate the method performance, standard curves for moxifloxacin and pirfenidone were generated. Seven spiked standard concentration points ranging from 10 to 500 ng/mL containing each drug were prepared as described previously in Section 6.2.3.1.2. The solutions were injected into the UPLC column in a volume of 5 µL and the peak areas were recorded at 310 nm. The analysis was repeated over three consecutive days.

6.3.1.1. Specificity

A clear analysis of moxifloxacin and pirfenidone in the presence of endogenous constituents of the aqueous humour indicated the selectivity of the method. The chromatograms of the different aqueous humour samples obtained through the developed UPLC method are presented in Figure 6.1. All chromatograms showed no interfering peak in the detection of both drugs during the overall 5 minutes of chromatographic run time which was consistent with Warsi *et al.* (2012), Sushma *et al.* (2015) and Sun *et al.* (2015). There was complete separation between the moxifloxacin and the pirfenidone in the
combined samples, with no overlapping peaks. The individual peaks generally showed a good shape, though a degree of asymmetry with a tailing appearance was evident, which was also seen in Warsi et al. (2012) Parmar et al. (2014) and Dewani et al. (2011). This might be due to injecting high volume of samples into the column, the drugs being adsorbed strongly onto active sites within the column, or poor trapping of these drugs when injected into the column (Watson, 2005). In addition, the retention times for moxifloxacin and pirfenidone were found to be 1.3 minutes and 2.1 minutes, respectively.
Figure 6.1: Representative UPLC chromatograms: (a) blank rabbit aqueous humour, (b) aqueous humour spiked with 100 ng/mL of moxifloxacin and pirfenidone solutions, (c) aqueous humour sample 2 hours after topical application of nanofibres and (d) aqueous humour sample 2 hours after topical application of combined drug solution.
6.3.1.2. Linearity

Calibration curves were found to be linear over the concentration range of 10-500 ng/mL for both moxifloxacin and pirfenidone as shown in Figure 6.2 and 6.3, respectively. The standard calibration curves were constructed by plotting the peak area against the respective concentration. The calibration curves have shown a better linearity for moxifloxacin and pirfenidone with $R^2$ values of 0.9997 and 0.9913, respectively. This was consistent to Warsi et al. (2012), Sushma et al. (2015) and Sun et al. (2015) who obtained $R^2$ values above 0.998 for moxifloxacin and 0.994 for pirfenidone which was slightly less than moxifloxacin. The other parameters related to linearity were listed in Table 6.2. The LOD and the LOQ were also calculated and their values were found to be 0.045 and 0.137 ng/mL for moxifloxacin and 0.054 and 0.166 ng/mL for pirfenidone as mentioned in Table 6.2. Warsi et al. (2012) study demonstrated an LOD and LOQ of 0.04 and 0.12 ng/mL respectively for moxifloxacin detection using UPLC.

![Figure 6.2: UPLC standard curve of moxifloxacin by plotting the area under the curve against the corresponded concentration. The curve shows good linearity ($R^2 \geq 0.9997$).](image-url)
Figure 6.3: UPLC standard curve of pirfenidone by plotting the area under the curve against the corresponded concentration. The curve shows fair linearity ($R^2 \geq 0.991$).

Table 6.2: Linear regression data for the calibration curves and sensitivity (LOD and LOQ) parameters for moxifloxacin and pirfenidone.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Moxifloxacin</th>
<th>Pirfenidone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range (ng/mL)</td>
<td>10 to 500</td>
<td>10 to 500</td>
</tr>
<tr>
<td>Regression equation</td>
<td>$Y = 160.32X + 842.66$</td>
<td>$Y = 306.13X - 1494.37$</td>
</tr>
<tr>
<td>Correlation coefficient ($R^2$)</td>
<td>0.9997</td>
<td>0.9913</td>
</tr>
<tr>
<td>Retention time (Rt)</td>
<td>1.31 minutes</td>
<td>2.10 minutes</td>
</tr>
<tr>
<td>LOD (ng/mL)</td>
<td>0.0453</td>
<td>0.0548</td>
</tr>
<tr>
<td>LOQ (ng/mL)</td>
<td>0.1372</td>
<td>0.1660</td>
</tr>
</tbody>
</table>
6.3.1.3. Intra- and Inter-day Precision

The calculated values of the intraday and interday precision are illustrated in Table 6.3. The developed method was found to be precise, as the percentage of the RSD% values for the intraday and interday precisions were below 2.0%, which was consistent with the recommendations of the International Conference on Harmonization guidelines (ICH, 2005), as well as, Parmar et al. (2014) and Dewani et al. (2011). The percentage recovery values were found in the range of 97.84-99.94% and 98.19-100.02% for moxifloxacin and pirfenidone, respectively, as shown in Table 6.3. This indicated the accuracy of the method for the quantification of moxifloxacin and pirfenidone in aqueous humour samples. This assay has shown precision and accuracy that is in agreement with Dewani et al. (2011), Warsi et al. (2012) and Sushma et al. (2015) for moxifloxacin and Parmar et al. (2014) and Sun et al. (2015) for pirfenidone.
Table 6.3: Intra- and inter-day precision of the developed UPLC method for moxifloxacin and pirfenidone showing the RSD% and recovery% for 3 different concentrations.

| Analyte   | Nominal Concentration (ng/mL) | Intraday precision | Interday precision |  |
|-----------|-------------------------------|--------------------|--------------------|  |
|           |                               | Concentration detected (ng/mL) mean ± SD, n=3 | RSD (%) | Recovery (%) | Concentration detected (ng/mL) mean ± SD, n=3 | RSD (%) | Recovery (%) |
| Moxifloxacin | 10.00                     | 9.79 ± 0.15            | 1.52     | 97.93       | 9.53 ± 0.07            | 0.75     | 95.36       |
|           | 100.00                     | 99.79 ± 0.20           | 0.20     | 99.79       | 98.23 ± 0.19           | 0.20     | 98.24       |
|           | 500.00                     | 499.72 ± 0.18          | 0.04     | 99.94       | 498.65 ± 0.69          | 0.14     | 99.73       |
| Pirfenidone | 10.00                     | 9.99 ± 0.19            | 1.87     | 99.94       | 10.06 ± 0.19           | 1.88     | 100.62      |
|           | 100.00                     | 99.12 ± 0.75           | 0.76     | 99.13       | 99.17 ± 0.86           | 0.87     | 99.17       |
|           | 500.00                     | 499.084 ± 1.86         | 0.37     | 99.82       | 499.03 ± 0.97          | 0.19     | 99.81       |
6.3.1.4. Robustness

The compositions of the used mobile phase were deliberately changed as ± 2 mL each, pH of the mobile phase (± 0.2) and flow rate of the mobile phase (± 0.2 mL/minute) to assess the robustness of this assay. There was no noticeable effect in the chromatograms and the peak areas, which was consistent with Sushma et al. (2015). The values for the robustness of the developed UPLC-UV method are illustrated in Table 6.4.
Table 6.4: Robustness of the UPLC method to analyse 200 ng/mL of moxifloxacin and pirfenidone following changing the mobile phase ratio by ± 2 mL each, pH by ± 0.2 and flow rate by ± 0.2 mL/minute.

<table>
<thead>
<tr>
<th>Optimised conditions</th>
<th>Obtained conc. for the 200 ng/mL of moxifloxacin</th>
<th>Obtained conc. for the 200 ng/mL of pirfenidone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD (n=3)</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>Mobile phase composition; (20 mM KH$_2$PO$_4$ : Acetonitrile; 65:35 ± 2.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(67 : 33, v/v)</td>
<td>199.447 ± 1.974</td>
<td>1.974</td>
</tr>
<tr>
<td>(65 : 35, v/v)</td>
<td>200.836 ± 2.431</td>
<td>1.210</td>
</tr>
<tr>
<td>(63 : 37, v/v)</td>
<td>199.746 ± 2.186</td>
<td>1.094</td>
</tr>
<tr>
<td>Mobile phase flow rate (0.12 ± 0.02)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0.14 mL.min$^{-1}$)</td>
<td>199.808 ± 1.092</td>
<td>0.546</td>
</tr>
<tr>
<td>(0.12 mL.min$^{-1}$)</td>
<td>199.147 ± 3.031</td>
<td>1.522</td>
</tr>
<tr>
<td>(0.10 mL.min$^{-1}$)</td>
<td>199.840 ± 3.222</td>
<td>1.612</td>
</tr>
<tr>
<td>pH of 20 mM KH$_2$PO$_4$ (3.2 ± 0.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(At pH 3.4)</td>
<td>200.995 ± 1.949</td>
<td>0.969</td>
</tr>
<tr>
<td>(At pH 3.2)</td>
<td>200.882 ± 2.474</td>
<td>1.231</td>
</tr>
<tr>
<td>(At pH 3.0)</td>
<td>200.729 ± 2.754</td>
<td>1.372</td>
</tr>
</tbody>
</table>
6.3.2. Ocular Pharmacokinetic Study

The developed UPLC assay was successfully applied for the quantification of moxifloxacin and pirfenidone in rabbit aqueous humour after topical instillation of the dual drug in solution form or in the coaxial fibres. The measured concentrations of both drugs in aqueous humour samples collected at 1, 2, 4, 6, 12 and 24 hours were plotted against the corresponded time points as shown in Figure 6.4. The pharmacokinetic parameters were calculated by PK-Solver software, as described in Zhang et al. (2010), and the results are summarised in Table 6.5.

In the group of animals that were treated with the combined drugs in solution form, the concentrations of moxifloxacin and pirfenidone in the aqueous humour was easily quantified up to 6 hours after ocular instillation. Thereafter, the concentration of both drugs was not measurable, which can be seen in Figure 6.4 (a) and (b), indicating the fast pre-corneal loss of both drugs in the solution form. In contrast, both drugs were easily quantified in the aqueous humour samples up to 24 hours after the topical application of the drug-loaded coaxial fibres in the rabbit eyes, as shown in Figure 6.4 (a) and (b). These extended release results were also demonstrated in Warsi et al. (2012) study on moxifloxacin-loaded nanoplexes and Kalam (2016) study on dexamethasone-loaded into chitosan NPs for ocular delivery. Moreover, Gagandeep (2014) exhibited a longer duration of intra ocular pressure reduction by timolol maleate and dorzolamide hydrochloride-loaded in PVA (up to 16 hours) and PCL (up to 20 hours) nanofibres compared to the conventional eye drop formulation.
Figure 6.4: The aqueous humour concentrations of (a) moxifloxacin and (b) pirfenidone versus time profiles following topical ocular application of both drugs either in solution form (ED-Soln) or coaxial nanofibres (NF) to the rabbits. Each data was represented as mean ± SD (n=3).
Table 6.5: Ocular pharmacokinetic parameters of moxifloxacin and pirfenidone in aqueous humour after topical application of the drug-loaded coaxial fibres and both drugs in solution form. Each data was represented as mean ± SD (n=3) for each time point.

<table>
<thead>
<tr>
<th>Pharmacokinetic parameters</th>
<th>Moxifloxacin in solution form (mean ± SD)</th>
<th>Moxifloxacin in the fibres (mean ± SD)</th>
<th>Pirfenidone in solution form (mean ± SD)</th>
<th>Pirfenidone in the fibres (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>1.68 ± 0.23</td>
<td>3.92 ± 0.06</td>
<td>2.52 ± 0.27</td>
<td>4.04 ± 0.27</td>
</tr>
<tr>
<td>$T_{max}$ (h)</td>
<td>2.00 ± 0.00</td>
<td>6.00 ± 0.00</td>
<td>2.00 ± 0.00</td>
<td>6.0 ± 0.00</td>
</tr>
<tr>
<td>$C_{max}$ (ng/mL)</td>
<td>537.05 ± 36.71</td>
<td>463.46 ± 38.51</td>
<td>724.91 ± 54.80</td>
<td>553.03 ± 29.83</td>
</tr>
<tr>
<td>$AUC_{0-24h}$ (ng/mL.h)</td>
<td>2316.29 ± 206.81</td>
<td>4110.69 ± 613.37</td>
<td>2165.75 ± 171.88</td>
<td>5409.51 ± 424.97</td>
</tr>
</tbody>
</table>
The nanofibres provided a higher ocular bioavailability of moxifloxacin and pirfenidone as compared to their solution form. The AUC₀-2₄ₕ of moxifloxacin and pirfenidone from the drug-loaded coaxial fibres was greater than the solution form, with 1.77-times and 2.49-times improved values, respectively. Similarly, the t₁/₂ of moxifloxacin and pirfenidone from the drug-loaded fibres was higher than the solution form, with 2.34-times and 1.43-times increased values, respectively. The C_max of moxifloxacin and pirfenidone from the fibres was 0.86- and 0.76-times improved than that of the solution form, respectively. Although, the calculated values for C_max indicated that the peak aqueous humour concentration of moxifloxacin and pirfenidone was slightly higher from the solution form than the coaxial fibres. Consequently, the drug-loaded coaxial fibres was applied one time a day in an equivalent dose to 4 times a day of moxifloxacin (0.5%) eye drop in the followed infection inhibition test.

6.3.3. Infection Inhibition Study

Before infecting the rabbit eyes with a Gram positive S. aureus bacterium, eye swabs were taken from the eyes to check for any previous infection, as shown in Figure 6.5 and 6.6 (a) pre infection. 24 hours post instillation of S. aureus; the infection in the eyes were observed visually (for eye redness symptom), eye swabs were taken from the infected eyes and the bacterial growth was checked on pre-sterilised MSA plates Figure 6.5 and 6.6 (b) post infection. After confirming the presence of bacterial colonies in all eye swabs, the treatment was initiated.

In the first group of rabbits, marketed moxifloxacin eye drop (0.5% w/v) was applied into the infected eyes, 4 times daily and photographs of the corneal surface were taken at different time points, as shown in Figures 6.5 and 6.6 - Group A. The eye swabs indicated that a complete absence of the bacterium colonies was achieved after 7 days of treatment. A similar outcome was observed in the third group of rabbits, which was treated with a single dose of drug-loaded coaxial fibres (placed under the lower eyelid), as shown in Figure 6.5 and 6.6 - Group C. The use of moxifloxacin antibiotic in the eye drops or the electrospun fibres inhibited the growth of S. aureus similarly after 7 days of treatment. This was expected since moxifloxacin demonstrated a bacterial
growth inhibition using the microbiological tests minimum inhibitory concentration (MIC) and the zone of inhibition, as shown in the previous chapter (Chapter 5 - Section 5.3.1.1. and 5.3.1.2., respectively). It must be noted that treating the eyes for 3 days with both formulations (i.e. eye drops and coaxial fibres) was insufficient to eradicate S. aureus bacterium from the infected eyes, as some bacterial colonies were still detected. However at day 7, the eyes were cleared from any bacterial colonies. Since there was no eye swab performed between days 3 and 7, bacterial inhibition could be achieved in less than 7 days of treatment. Lack of such data can be considered as a limitation of this study.

For the untreated control group (second group), bacterial colonies were still detected after 7 days which was expected owing to the lack of treatment with an antibacterial drug, as it can be seen Figure 6.5 and 6.6 - Group B. The fourth group of rabbits were treated with a once daily dose of blank nanofibres, in an equivalent fibre weights to the drug-loaded ones, and the eye swabs demonstrated the presence of bacterial colonies after 7 days of treatment, as shown in Figure 6.5 and 6.6 - Group D. This was due to the absence of any antimicrobial activity of PVP and PLGA polymers, which was also observed in the zone of inhibition test of the blank fibres in the previous chapter (Chapter 5 - Section 5.3.1.2.). A previous study by Fu et al. (2014) demonstrated the antibacterial inhibition of sodium alginate and PVA blended nanofibres mat loaded with moxifloxacin against S. aureus and P. aeruginosa bacteria by the zone of inhibition test.
Figure 6.5: Eye swabs for the growth of S. aureus on MSA plates; (a) pre infection (no bacterial growth), (b) 24-hour post infection, (c) 3-day post treatment and (d) 7-day post treatment. A: Moxifloxacin eye drops, B: untreated control group, C: drug-loaded coaxial fibres, D: blank coaxial fibres.
Figure 6.6. Photographs of the eyes; (a) (a) pre infection (no bacterial growth), (b) 24-hour post infection, (c) 3-day post treatment and (d) 7-day post treatment. A: Moxifloxacin eye drops, B: untreated control group, C: drug-loaded coaxial fibres, D: blank coaxial fibres.
6.3.5. Eye Irritation Study

The ocular irritation was evaluated by visually observing the cornea, iris and conjunctiva of the rabbits after inserting blank and drug-loaded coaxial fibres under the lower conjunctival sac of one eye once daily for 7 days. However, the other eye was irrigating with a normal saline, as an experimental control. The signs and symptoms were evaluated in accordance to Table 6.1, in which a grade 0 indicates the absence of any signs of discomfort. The scores obtained for the single dose irritation study are illustrated in Tables 6.6 and 6.7 for the blank and drug-loaded coaxial fibres, respectively.

Owing to the frequent ocular application of the nanofibres, a slight irritation was found in a few rabbit eyes. The treated rabbit eyes have shown a slight mucoidal discharge (grade 2) after the application of the fibres, while a few rabbit eyes have shown some watery discharge (different from the normal) which were not mucoidal discharge (grade 1). These obtained scores are illustrated, in details, in Tables 6.6 and 6.7. No corneal opacity was found in any of the treated rabbit eyes during the whole experiment. A combined score for the ocular irritation studies by blank and drug-loaded fibres is shown in Table 6.8. In general, both the blank and drug-loaded fibres demonstrated less distress symptoms to the rabbit eyes. Symptoms of a mild distress such as redness, chemosis (swelling) and presence of discharge were observed with the blank fibres compared to the drug-loaded fibres.
Table 6.6: Weighted individual scores for ocular irritation studies by blank fibres.

<table>
<thead>
<tr>
<th>1</th>
<th>For the cornea</th>
<th>Score for Rabbit 1</th>
<th>Score for Rabbit 2</th>
<th>Score for Rabbit 3</th>
<th>Score for Rabbit 4</th>
<th>Score for Rabbit 5</th>
<th>Score for Rabbit 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lesions</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i. Opacity-degree of density (area which was most dense was considered for the reading)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scattered or diffused area - details of the iris clearly visible</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Easily noticeable translucent areas, details of iris slightly obscured</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Opaescent areas, no details of iris visible, size of the pupil barely noticeable</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Opaque, iris invisible</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ii. Area of the cornea involved</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>One quarter (or less) but not zero</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Greater than one quarter but less than one half</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Greater than one half but less than three quarters</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Greater than three quarters up to whole area</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Total scores obtained = ((i \times ii \times 5)) =</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

2 | For the iris | Score for Rabbit 1 | Score for Rabbit 2 | Score for Rabbit 3 | Score for Rabbit 4 | Score for Rabbit 5 | Score for Rabbit 6 |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Lesions</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i. Values</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Folds above normal, congestion, swelling, circumcorneal injection (any one or all of these or combination of any thereof), iris still reacting to light (sluggish reaction is positive)</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>No reaction to light, hemorrhage, gross destruction (any one/all of these)</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Total scores obtained = ((i \times 5)) =</td>
<td>5</td>
<td>10</td>
<td>5</td>
<td>10</td>
<td>5</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
### For the conjunctiva

<table>
<thead>
<tr>
<th>Lesions</th>
<th>Score for Rabbit 1</th>
<th>Score for Rabbit 2</th>
<th>Score for Rabbit 3</th>
<th>Score for Rabbit 4</th>
<th>Score for Rabbit 5</th>
<th>Score for Rabbit 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>i. Redness [refers to palpebral conjunctiva that coats the inside of eyelids only]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood vessels definitely injected above normal</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>More diffuse, deeper crimson red, individual vessels not easily noticeable</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Diffuse beefy red</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ii. Chemosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any swelling above normal (includes nictitating membrane/ refers to third eye)</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Obvious swelling with partial eversion of the lids</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Swelling with lids about half closed</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Swelling with lids about half closed to completely closed</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>iii. Discharge</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any amount different from normal (does not include small amount observed in inner canthus of normal animals)</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Discharge with moistening of the lids and hairs just adjacent to the lids</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Discharge with moistening of the lids and considerable area around the eye</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total scores obtained = (i + ii + iii) x 2 =</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>10</td>
<td>6</td>
</tr>
</tbody>
</table>
Table 6.7: Weighted individual scores for ocular irritation studies by drug-loaded coaxial fibres.

<table>
<thead>
<tr>
<th></th>
<th>For the cornea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lesions</td>
<td>Score for Rabbit 1</td>
</tr>
<tr>
<td>i. Opacity-degree of density (area which was most dense was considered for the reading)</td>
<td></td>
</tr>
<tr>
<td>Scattered or diffused area - details of the iris clearly visible</td>
<td>0</td>
</tr>
<tr>
<td>Easily noticeable translucent areas, details of iris slightly obscured</td>
<td>0</td>
</tr>
<tr>
<td>Opalescent areas, no details of iris visible, size of the pupil barely noticeable</td>
<td>0</td>
</tr>
<tr>
<td>Opaque, iris invisible</td>
<td>0</td>
</tr>
<tr>
<td>ii. Area of the cornea involved</td>
<td></td>
</tr>
<tr>
<td>One quarter (or less) but not zero</td>
<td>0</td>
</tr>
<tr>
<td>Greater than one quarter but less than one half</td>
<td>0</td>
</tr>
<tr>
<td>Greater than one half but less than three quarters</td>
<td>0</td>
</tr>
<tr>
<td>Greater than three quarters up to whole area</td>
<td>4</td>
</tr>
<tr>
<td>Total scores obtained = (i x ii x 5) =</td>
<td>0</td>
</tr>
</tbody>
</table>

2

<table>
<thead>
<tr>
<th></th>
<th>For the iris</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lesions</td>
<td>Score for Rabbit 1</td>
</tr>
<tr>
<td>i. Values</td>
<td></td>
</tr>
<tr>
<td>Folds above normal, congestion, swelling, circumcorneal injection (any one or all of these or combination of any thereof), iris still reacting to light (sluggish reaction is positive)</td>
<td>1</td>
</tr>
<tr>
<td>No reaction to light, hemorrhage, gross destruction (any one/all of these)</td>
<td>0</td>
</tr>
<tr>
<td>Total scores obtained = (i x 5) =</td>
<td>5</td>
</tr>
</tbody>
</table>
### For the conjunctiva

<table>
<thead>
<tr>
<th>Lesions</th>
<th>Score for Rabbit 1</th>
<th>Score for Rabbit 2</th>
<th>Score for Rabbit 3</th>
<th>Score for Rabbit 4</th>
<th>Score for Rabbit 5</th>
<th>Score for Rabbit 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>i. Redness [refers to palpebral conjunctiva that coats the inside of eyelids only]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood vessels definitely injected above normal</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>More diffuse, deeper crimson red, individual vessels not easily noticeable</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Diffuse beefy red</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ii. Chemosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any swelling above normal (includes nictitating membrane/ refers to third eye)</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Obvious swelling with partial eversion of the lids</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Swelling with lids about half closed</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Swelling with lids about half closed to completely closed</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>iii. Discharge</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any amount different from normal (does not include small amount observed in inner canthus of normal animals)</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Discharge with moistening of the lids and hairs just adjacent to the lids</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Discharge with moistening of the lids and considerable area around the eye</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total scores obtained = (i + ii + iii) x 2 =</td>
<td>8</td>
<td>6</td>
<td>6</td>
<td>10</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>
Table 6.8: Obtained weighted scores for ocular irritation studies by blank and drug-loaded coaxial fibres (combined form of Tables 6.6 and 6.7).

<table>
<thead>
<tr>
<th>Lesions in the eye structure</th>
<th>Individual scores for ocular irritation</th>
<th>Blank nanofibres</th>
<th>Drug-loaded coaxial nanofibres</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Rabbit 1 Rabbit 2 Rabbit 3 Rabbit 4 Rabbit 5 Rabbit 6</td>
<td>Rabbit 1 Rabbit 2 Rabbit 3 Rabbit 4 Rabbit 5 Rabbit 6</td>
</tr>
<tr>
<td>A. Cornea</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i. Opacity</td>
<td></td>
<td>0 1 0 1 0 1</td>
<td>0 0 0 1 0 0</td>
</tr>
<tr>
<td>ii. Area</td>
<td></td>
<td>4 4 4 4 4 4</td>
<td>4 4 4 4 4 4</td>
</tr>
<tr>
<td>Total scores obtained = (i x ii x 5) =</td>
<td>0 20 0 20 0 20</td>
<td>0 0 0 20 0 0</td>
<td></td>
</tr>
<tr>
<td>B. Iris</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i. Values</td>
<td></td>
<td>1 2 1 2 1 0</td>
<td>1 1 0 0 1 0</td>
</tr>
<tr>
<td>Total scores obtained = (i x 5) =</td>
<td>5 10 5 10 5 0</td>
<td>5 5 0 0 5 0</td>
<td></td>
</tr>
<tr>
<td>C. Conjunctiva</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i. Redness</td>
<td></td>
<td>1 2 2 1 2 1</td>
<td>2 1 1 2 0 1</td>
</tr>
<tr>
<td>ii. Chemosis</td>
<td></td>
<td>2 1 2 2 1 1</td>
<td>1 1 2 2 1 1</td>
</tr>
<tr>
<td>iii. Discharge</td>
<td></td>
<td>1 1 0 1 2 1</td>
<td>1 1 0 1 2 1</td>
</tr>
<tr>
<td>Total scores obtained = (i + ii + iii) x 2 =</td>
<td>8 8 8 8 10 6</td>
<td>8 6 6 10 6 6</td>
<td></td>
</tr>
</tbody>
</table>

*Lesions in the eye structure: A. Cornea, B. Iris, C. Conjunctiva.*
Based on the classification of eye irritation score by Kay and Calandra (1962), in Table 6.9, and under the conditions of the above experiments the maximum mean total score (MMTS) obtained for the blank coaxial fibres was 23.83 (> 15.1 but < 25), while for the drug-loaded coaxial fibres it was 12.83 (> 2.6 but < 15).

Table 6.9: Kay and Calandra (1962) classification of the ocular irritation scores.

<table>
<thead>
<tr>
<th>Maximum Mean Total Score (MMTS)</th>
<th>Irritation Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0 - 0.5</td>
<td>None</td>
</tr>
<tr>
<td>0.6 - 2.5</td>
<td>Practically none</td>
</tr>
<tr>
<td>2.6 – 15</td>
<td>Minimally</td>
</tr>
<tr>
<td>15.1 – 25</td>
<td>Mildly</td>
</tr>
<tr>
<td>25.1 – 50</td>
<td>Moderately</td>
</tr>
<tr>
<td>50.1 – 80</td>
<td>Severely</td>
</tr>
<tr>
<td>80.1 – 100</td>
<td>Extremely</td>
</tr>
<tr>
<td>100.1 – 110</td>
<td>Maximally</td>
</tr>
</tbody>
</table>

Table 6.10 demonstrates the MMTS obtained for the blank and drug-loaded coaxial fibres. Therefore, the blank coaxial fibres were categorised as mildly irritating to the rabbits’ eyes, which could be considered as relatively harmless. However, the drug-loaded coaxial fibres were classified as minimally irritating to the rabbits’ eyes. This might be due to the presence of an antibiotic, moxifloxacin, and an anti-scarring and anti-inflammatory drug, pirfenidone, which reduced the level of distress to the front of the eye tissue.
Table 6.10: The MMTS calculations for the blank and drug-loaded coaxial fibres according to the scores from Table 6.9.

<table>
<thead>
<tr>
<th>Blank coaxial fibres</th>
<th>Animal no.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>SUM</th>
<th>Average (SUM/6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornea</td>
<td></td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>20</td>
<td>60</td>
<td>10.0</td>
</tr>
<tr>
<td>Iris</td>
<td></td>
<td>5</td>
<td>10</td>
<td>5</td>
<td>10</td>
<td>5</td>
<td>0</td>
<td>35</td>
<td>5.83</td>
</tr>
<tr>
<td>Conjunctiva</td>
<td></td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>10</td>
<td>6</td>
<td>48</td>
<td>8.00</td>
</tr>
<tr>
<td>SUM total</td>
<td></td>
<td>13</td>
<td>38</td>
<td>13</td>
<td>38</td>
<td>15</td>
<td>26</td>
<td>143</td>
<td>23.83</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug-loaded coaxial fibres</th>
<th>Animal no.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>SUM</th>
<th>Average (SUM/6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornea</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>3.33</td>
</tr>
<tr>
<td>Iris</td>
<td></td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>15</td>
<td>2.50</td>
</tr>
<tr>
<td>Conjunctiva</td>
<td></td>
<td>8</td>
<td>6</td>
<td>6</td>
<td>10</td>
<td>6</td>
<td>6</td>
<td>42</td>
<td>7.00</td>
</tr>
<tr>
<td>SUM total</td>
<td></td>
<td>13</td>
<td>11</td>
<td>6</td>
<td>30</td>
<td>11</td>
<td>6</td>
<td>77</td>
<td>12.83</td>
</tr>
</tbody>
</table>

It is also important to note that all rabbits appeared active and healthy during the study. Apart from the eye irritation that was reported, there were no signs and symptoms of gross toxicity nor any adverse pharmacological effects. Overall, the results of this ocular irritation study suggested the relative safety of the drug-loaded coaxial fibres as compared to normal saline solution. This was consistent with Gagandeep et al. (2014) study, which showed that PVA nanofibres are not irritant to the rabbit eyes. Nevertheless, PCL nanofibres observed a minor irritation, which could be due to the hydrophobicity of this polymer. Another study by Canadas et al. (2016) demonstrated an absence of in vitro cytotoxicity of blank PLGA NPs and pranoprofen-loaded in PLGA NPs against human retinoblastoma cell line (Y-79™). In addition, the drug-loaded NPs exhibited non-irritant characteristics on New Zealand rabbits’ eyes compared to the free drug solution that showed an irritation characteristics to the eyes. This suggested the ocular tolerability of pranoprofen-loaded PLGA NPs and the compatibility of using PLGA polymer for ocular delivery (Canadas et al., 2016).
6.4. Conclusion

The *in vivo* performance of the dual drug-loaded coaxial fibres was successfully evaluated by studying pharmacokinetics, infection inhibition and ocular irritation on New Zealand rabbit eyes. The pharmacokinetics study indicated that the drug-loaded fibres were able to extend the release of moxifloxacin and pirfenidone for 24 hours, compared to 6 hours for the combined drugs in solution form. These findings suggested that a single dose of an equivalent amount of both drugs, can be topically administrated compared to a 4-time daily dose of the eye drop solution. The antibacterial effect of the drug-loaded fibres was tested against *S. aureus* infected rabbits’ eyes. This was achieved by applying a once daily dose of the drug-loaded coaxial fibres, a once daily dose of an equivalent weight of the blank fibres and a 4-time daily dose of the marketed moxifloxacin eye drops for 7 days. The results showed that the drug-loaded coaxial fibres and moxifloxacin eye drops were able to treat the infected eye after 7 days, with a single dose of the fibres compared to 4 applied doses of the eye drops. Moreover, the blank fibres showed no antibacterial efficacy against *S. aureus*, which was similar to the untreated control group after 7 days of post treatment. Finally, the outcome of the ocular irritation test indicated that the drug-loaded fibres were minimally irritating to the rabbit eye tissues compared to the blank fibres, which were mildly irritating. This *in vivo* study suggested that moxifloxacin and pirfenidone dual drug-loaded coaxial fibres can be a potential alternative ocular delivery system to moxifloxacin eye drops for the treatment of corneal infections.
Chapter 7

Conclusions and Future Work
7.1. Conclusions

Corneal abrasion, is a simply a scratch that can be caused by normal daily objects, for instance papers, make-up brushes, tree branches, scissors, etc (Shahid and Harrison, 2013). This condition involves an open wound that can attract microorganisms, such as bacteria and fungi, to cause sight-threatening conditions if not treated. In addition, a scar will generally develop as a response to the natural healing process of the eyes (Cheng et al., 1999, Bourcier et al., 2003, Schaefer et al., 2001, Vajpayee et al., 2000; Wong et al., 2003). Accelerating the treatment after such injury will lower the chance of this condition progressing. The first line of treatment is to prevent any infection developing from this abrasion, by using topical antibiotic eye drops, which will require multiple daily applications and a longer treatment duration. However, by accelerating the healing of the wound (scar), the corneal tissues will recover faster allowing the management of the associated corneal abrasion symptoms and/or infection.

The principal aim of this thesis was to develop a topical dosage form that contains a combination of an antibiotic and an anti-scarring agent in order to inhibit, or even treat, any bacterial infection and to accelerate the wound healing. This was suggested to improve patient compliance by lowering the administered dosage and duration of therapy. A broad spectrum antibiotic, moxifloxacin, was proposed due to its outstanding results for treating corneal infections caused by more harmful bacteria strains, namely *P.* aeruginosa (MacGowan, 1999; Appelbaum and Hunter, 2002). Additionally, this antibiotic is often prescribed for the treatment of corneal abrasion (Robertson et al., 2005, Garcia-Saenz et al., 2001, Callegan et al., 2003, Donnenfeld et al., 2004; Holland et al., 2008). An anti-scarring agent pirfenidone, which was approved for the treatment of idiopathic pulmonary fibrosis (Raghu et al., 2011, du Bois, 2010, Iyer et al., 1999; European Medicines Agency, 2010), was suggested to be combined with moxifloxacin. This anti-fibrotic drug has demonstrated promising outcomes for treating corneal fibrosis (Lin et al., 2009, Zhong et al., 2011, Sun et al., 2011, Lee et al., 2014; Jung et al., 2012). Finally, both drugs were anticipated to be compartmentalised in an electrospun fibre formulation,
owing to the wide application of this type of system in the wound healing and tissue regeneration fields.

Electrospinning is a technique that can form either single or multi-layered fibres, by the application of a high voltage, which will overcome the surface tension of a viscous polymer solution, allowing the solvent to evaporate and thus facilitating the production of a fibrous mat (Williams et al., 2018). Coaxial and triaxial electrospinning can fabricate fibres that contain two and three layers, respectively. Each layer can be used to compartmentalise different drugs, proteins or nucleic acids (Williams et al., 2018). The initial experimental work in Chapter 3 focused on loading moxifloxacin in a hydrophilic polymer, PVA, and pirfenidon in a hydrophobic polymer, PLGA, as single-layered fibres (i.e. monoaxial fibres). After optimising both systems, coaxial electrospun fibres consisting of moxifloxacin-PVA as the core and pirfenidone-PLGA as the shell were considered, in order to sustain the release of pirfenidone and to neither hinder nor slow down the release of moxifloxacin by using a hydrophillic polymer (PVA). Different processing factors, such as polymer MW and concentration, drug concentration, and the applied voltage, were considered and the morphology of the fibres were investigated by using different microscopic imaging techniques. SEM results demonstrated the surface characteristics and how the use of a high evaporation rate solvent (DCM) produced rougher surfaces, in the case of PLGA. TEM findings showed the successful preparation of coaxial fibres, by showing the presence of both inner and outer layers on a single fibre. Fluorescence microscopy indicated the encapsulation of moxifloxacin, which has florescent properties. Furthermore, the EE% and DL of the monoaxial and coaxial drug-loaded fibres were measured. As a result of the low encapsulation of moxifloxacin in the inner core of the coaxial fibres and the low production yield of these fibres, caused by the unstable jet during the preparation, an alternative water soluble polymer was proposed in the next chapter.

Chapter 4 focused on the production and the characterisation of coaxial and triaxial fibres made of the hydrophilic polymer PVP, instead of PVA, and the hydrophobic polymer PLGA. For the coaxial fibres, moxifloxacin-PVP was in the core and pirfenidone-PLGA was in the shell to allow a similar release
performance to the PVA and PLGA coaxial fibres. The triaxial fibres consisted of moxifloxacin-PVP as the core, pirfenidone-PLGA as the intermediate layer and an extra moxifloxacin-PVP layer was introduced as the shell. This additional layer was suggested to enhance the dose of moxifloxacin and to allow its burst release. Different solvents, such as DCM, acetone and ACTN, were considered during the preparation of these fibres and several microscopy techniques were used to assess the yielded fibres. SEM images illustrate the surface characteristics and how the use of high vapour pressure solvents might produce rougher fibre surfaces (in PLGA fabrication), despite using lower voltages and tip-to-collector distances. Solvent choice had an effect on the diameter of the fibres. The diameter of PLGA and PVP monoaxial fibres that are prepared by using DCM and DCM:ethanol were larger than PLGA and PVP fibres prepared by using ACTN and ACTN:ethanol, respectively. Similarly, coaxial fibres that were made by using DCM, had larger diameters than the fibres fabricated by using ACTN. TEM findings have shown the successful coaxial and triaxial fabrication by displaying two and three clear distinctive layers, respectively. Fluorescence microscopy indicated the encapsulation of moxifloxacin, which was located in the core of the coaxial fibres and in the core and the outer layer of the triaxial fibres. Confocal microscopy was used to confirm the presence of the PLGA layer, in which MB was added instead of pirfenidone, in the outer layer of the coaxial fibres and the middle layer of the triaxial fibres. The microscope was able to show clearly the presence of MB and moxifloxacin loaded in coaxial and triaxial fibres compared to the blank fibres.

Solvent choice had a significant effect on PLGA fibre preparation compared to PVP fibres, in which there was no clear effect. PLGA fibres prepared by using ACTN had a greater Y% (82%) than the PLGA fibres made by using DCM (34%). In addition, the DL for the coaxial fibres prepared by using ACTN was more consistent to the initial drug concentration (2:1 pirfenidone:moxifloxacin) than the coaxial fibres made by using DCM. This was due to the similar flow rate (0.5 ml/h) that was used for the core and shell in the coaxial fibres fabricated by using ACTN. Therefore, only the fibres that are made from ACTN were considered for the further tests.
The release profile for pirfenidone-loaded PLGA monoaxial fibres showed a sustained release profile (at least for 15 days) compared to a fast release profile of PVP loaded with moxifloxacin (4 hours). This was expected since PLGA is a water insoluble polymer and PVP is a hydrophilic polymer. The drug-loaded coaxial fibres demonstrated a complete release of pirfenidone after 1 day, while 70% of moxifloxacin was release after 4 hours with the remaining 30% entrapped in the core of these fibres for at least 4 days. Unexpectedly, the drug-loaded triaxial fibres exhibited a very rapid complete release of moxifloxacin and pirfenidone after 2 and 6 hours, respectively. One possible explanation is that the location of the drug molecules near the surface of the fibres, allowing the complete release of the drug. A second probable explanation is due to the high affinity of both drugs to the release media (PBS) and the narrow thickness of the inner layer(s), the release was faster than expected. Another explanation is due to the higher degree of swelling for both coaxial and triaxial fibres compared to the PLGA monoaxial fibres which enabled the absorption of more PBS to the fibre matrix. This will dissolve the pirfenidone and the PVP layer(s) along with the loaded moxifloxacin, allowing the accelerated diffusion of both drugs out of the fibres. Despite the release profile of the coaxial system, this formulation was considered for a single daily dose and was taken forward for microbiological and anti-scarring assessment in the next chapter.

The performance of the prepared coaxial and triaxial formulation was assessed through microbiological and an anti-scarring tests, which were described in Chapter 5. Three experiments were performed for the microbiological assessment. Initially, the MIC was measured and the findings confirmed the susceptibility of both S. aureus and P. aeruginosa bacteria to moxifloxacin. Additionally, there was no evidence of an antimicrobial inhibition of pirfenidone nor any synergistic or inhibitory effects when it was combined with moxifloxacin. The zone of inhibition test was performed to assess the antimicrobial activity of the electrospun fibres against both S. aureus and P. aeruginosa bacteria strains. The results showed that both coaxial and triaxial fibres were able to release moxifloxacin while retaining its antimicrobial efficacy similar to an equivalent dose of moxifloxacin solution. However, the
blank fibres indicated no inhibition zone against both bacterial strains. Finally, a sterility test was performed, in accordance with the BP, and it showed that both systems lack any sign of contamination upon 14 days of incubation in the recommended culture media. This was to evaluate the sterility of the fibres before using them in any biological study.

The anti-scarring assessment was achieved by performing a Western blot technique on the rabbit corneal fibroblasts, SIRC, cell line. The IC20 was measured for moxifloxacin, pirfenidone and the combination of both drugs. This is the concentration at which not more than 20% of the cell population will be inhibited in order to encourage the remaining cells to recover and proliferate. After measuring the IC20, cells were exposed to drug-loaded coaxial and triaxial fibres and their equivalent weights of the blank fibres. The MTT assay suggested that the cell viability was above 90% for the coaxial and triaxial drug-loaded fibres compared to 80-85% for the free drugs in a similar concentration. In addition, the fibres were further investigated by SEM and the images confirmed the compatibility of this cell line with the fibre components. Finally, the Western blot results demonstrated that the free pirfenidone and the drug-loaded fibres were able to reduce the expression of α-SMA protein after the cells were exposed to TGF-β1 for 48 hours. α-SMA is generally over expressed in response to an injury (scar). Interestingly, moxifloxacin was able to decrease the level of α-SMA, which can be an advantage of using this antibiotic for the treatment of both scars and infections.

After expressing the antibacterial and anti-scarring effect of the electrospun fibres, Chapter 6 focused on the in vivo evaluation of these fibres. Three experimental studies were performed on the coaxial fibres on New Zealand Albino rabbit’s eyes. A Draize test was performed and the results indicated that the drug-loaded fibres were minimally irritating to the rabbit’s eyes compared to the blank fibres, which were mildly irritating. A pharmacokinetics study was performed by treating one rabbit eye with either the drug-loaded coaxial fibres or the aqueous solution of moxifloxacin or pirfenidone. The presence of both drugs in rabbits’ aqueous humour were quantified by a developed UPLC method. The findings showed that both drugs in aqueous solution can be easily quantified up to 6 hours compared to 24 hours for the coaxial fibres. The
nanofibres exhibited a significantly higher bioavailability and half-life than the drug solution. Finally, an infection inhibition test was performed after infecting one rabbit eye with *S. aureus* bacterium, in which eye swabs were taken to check on any bacterial growth. The drug-loaded fibres and the marketed moxifloxacin eye drops were able to inhibit the growth of *S. aureus* after 7 days of treatment, with a once daily dose of the fibres compared to 4 daily doses of moxifloxacin eye drops. These *in vivo* results showed the potential application of the drug-loaded coaxial fibres in corneal infections.

Overall, this electrospun dual drug delivery system has successfully demonstrated its ability to load two drugs into two separated layers while sustaining their release compared to the conventional eye drops formulation. This could enhance patient compliance by reducing the frequency of administration. In addition, showing antimicrobial activity against a very harmful bacterium, *P. aeruginosa*, can be another advantage, as it can cause corneal ulcer, a very serious sight-threatening condition. The *in vitro* anti-scarring activity of this system shows a reduction in the expression of a protein (α-SMA) that is responsible for scar formation. This could potentially accelerate the healing of corneal abrasion which involves a presence of a scar tissue on the surface of the eye. The fabrication of a multi-layered system by using miscible solvent systems was able to yield reproducible and reliable fibres, owing to the use of similar flow rates for each polymer solution. This flow rate similarity enabled the production of fibres with the majority of these fibres being multi-layered and not a mixture of single- and multi-layered fibres embedded into each other, which improved the distribution of the loaded drugs within the fibres, hence more precise doses could be obtained.

It must be noted that there were some challenges throughout this study that need to be addressed in taking the approach forward. Optimising the fabrication of a multi-layered fibrous system remains the hardest task. Owing to the different process and polymer solution parameters, such as the flow rate, distance, applied voltage, polymer concentration, solution viscosity and conductivity, can have a profound effect on the resulting fibres and hence, need to be optimised. For the production of bi- or tri-layered fibres, the aforementioned optimisation of the processing parameters becomes even
more relevant, while the miscibility of the spinning solutions needs to be carefully considered to allow the generation of clearly distinctive separated layers. The ease by which defective fibres (e.g. beading) may be produced via relatively small changes to the parameters was also a challenge. Loading one or more active compounds (drug, protein, nucleic acid, etc) can also have an impact on the final product, due to the effect of adding these substances on the spinning solution (i.e. viscosity and conductivity), which would require further optimisation. Despite these challenges, the study was successful in reproducing a sophisticated fibre system that showed considerable promise in terms of the therapeutic applicability and suitability for the purpose.
7.2. Suggested Future Work

Optimising the fabrication of a multi-layered fibrous system has been shown to be challenging owing to the variable electrospinning process and polymer solution parameters that can affect the production of successful fibres, particularly the triaxial system. These fibres were prepared by using a miscible solvent system which successfully created a reproducible fibrous system with high drug loading distribution. However, the dissolution studies have suggested that the drugs were released in an accelerated profile. A future route of research may lie in increasing the diameter of each layer of the multi-layered fibres, as in theory more space may be available which could slow the release. Therefore, more investigation into the effect of the fibres’ diameter on the release of this system is required. In addition, coating the coaxial system with a hydrophilic polymer has negatively impacted the release of the triaxial system. This was due to the high swelling of this system and the attraction of more release medium into the fibres matrix which allows the immediate release of both drugs after a few hours. Hence, changing the outer-layer of this triaxial system to a hydrophobic polymer, such as PLGA or PCL, might be useful in extending the release of these drugs.

The most substantial finding of this thesis is the ability of the multi-layered fibres to release the loaded moxifloxacin while retaining its antibacterial efficacy against a Gram positive S. aureus and Gram negative P. aeruginosa bacteria strains. Similarly, pirfenidone was able to release and down-regulate the \(\alpha\)-SMA protein that is expressed due to a scar. Moxifloxacin also exhibited a reduction in the expression of \(\alpha\)-SMA protein, which can be another benefit of using this antibiotic in the treatment of corneal abrasion. \(\alpha\)-SMA is considered as one of the ECM proteins whose release will be triggered as a response to an injury (i.e. a scratch in this case). Nonetheless, assessing the ability of the drug-loaded fibres and moxifloxacin alone to reduce the expression of other ECM proteins, such as collagen and fibronectin, would be advantageous in understanding the mechanism of scar healing of this fibrous system and antibiotic.
The *in vivo* behaviour of the coaxial system was encouraging, as a single dose of these fibres was equivalent to four doses of the marketed moxifloxacin eye drops to inhibit the corneal infection. Nevertheless, an anti-scarring animal model is required to assess the ability of the drug-loaded fibres to accelerate the healing of an acquired scar. Generally, corneal abrasion is treated for a minimum of 14 days, however, some cases would require a longer duration of therapy. The promising results obtained by the Western blot technique are encouraging to perform an anti-scarring *in vivo* study, especially given that moxifloxacin exhibited an additional pharmacological activity along with its broad spectrum antibiotic activity.
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434


441


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