FORMULATION STRATEGIES FOR EXTRACELLULAR VESICLE DELIVERY

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This thesis describes research conducted in the Department of Pharmaceutics at the UCL School of Pharmacy, University College London, from May 2015 to September 2018. I, Ana Rita Marques Pereira Trindade hereby 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 the thesis.

(Signature)

(Date)
To

my amazing parents
for their unconditional and
never-ending support
throughout this journey
Acknowledgements

My PhD journey has been (or was) an interesting one at least. It has been one of the most emotionally and psychological challenging tasks I ever had to face in my life. Having to deal with constant frustration and failure despite the effort you put in - can be quite trialling at times and demotivating to say the least. It was mainly a journey of self-discovery, especially for understanding what I was able to achieve and truly made of. And for that I am thankful.

However, and since I would have never been able to do it without the support of the lovely people in my life some thanks are owed.

Firstly, I would like to express my deepest appreciation and profound gratitude to my supervisor, Dr Gareth Williams. He was there when I needed him and not there when I needed him not to. Especially during the last year when his support and positive thinking never failed to encourage me like a beacon of hope (“there is a light at the end of the tunnel!” he always told me, even though I always thought it was an incoming train…).

Thanks are also due to all the people that at one point or another helped with practical assistance, insightful guidance and helpful advices. You are way too many to possible name (how lucky was I!) and I cherished all our discussions. I extend my acknowledgements to the present and past research group members I had the pleasure of getting to know for the friendly atmosphere in our workgroup. Similarly, a big thank you to all the people in School of Pharmacy, from Pharmaceutics and outside this department, for the amazing time and memories provided.
I also must acknowledge my own Faculty, School of Pharmacy, University College London and the EPSRC - Engineering and Physical Sciences Research Council for providing funding, facilities and equipment so this project could come to life.

A special thanks is due to all my friends that in one way or the other gave me encouragement when I needed the most and for their infinite patience, Dania in particular. Thank you for being there when things got hard, and for continuing to be there when things got really hard, some even despite the distance.

E para a minha família que foi incansável no suporte e amor que me deram ao longo destes quatro anos, mesmo com a distância e com as visitas limitadas, com as vídeo chamadas de fugida só para dar um beijinho, que sempre me fizeram sentir que tinha um lugar para onde voltar - espero tê-los feito orgulhosos.

E após 25 anos desde ter dito “Se a mamã é doutora eu também sou!” e exigir ser tratada como tal (porque títulos não são mais do que isso), espero agora estar muito mais perto de o ser...

...então e agora, já posso ser chamada de doutora também?
**Abstract**

Stem cells therapies have been demonstrating their ability to elicit cell replacement in tissues where there has been a loss of function or damage. However, cell-secreted extracellular vesicles (EVs) have been shown to be responsible for much of these regenerative effects. In the scarred myocardium after an infarct, EVs have shown cardioprotective effects upon injection. However, administration via injection has severe limitations, since much of the administered dose is rapidly cleared from the site of action. Development of implantable EV formulations is then needed.

EVs from foetal bovine serum and from mesenchymal stem cells culture media were isolated and characterised. A differential centrifugation workflow was assessed to identify a robust and reproducible isolation method. Both isolation and protein characterisation were found to be highly dependent on the starting material, indicating that a careful assessment is required for each sample. The effect of EVs on stressed cardiomyocytes in culture was assessed *in vitro* and found to be concentration dependent. FBS EVs were found to be relatively stable over the course of a month at the storage conditions of -80°C, 4°C and 20°C.

The feasibility of formulating EVs into a scaffold by electrospinning was explored, initially using fast-dissolving polymers such as poly(vinyl pyrrolidone). Extensive optimisation of processing conditions was undertaken and no difference in pre and post-processing EVs potency was noted in cell culture assays, confirming electrospinning to be suitable for EV formulation.
Finally, development of sustained-release scaffolds for cardiac applications was investigated, based on poly(lactic-co-glycolic acid) and poly(dioxanone). A range of systems was prepared and morphology, flexibility, shrinkage behaviour and mechanical properties explored. However, scaffolds were found to have a burst release at 24 h with no more release detected over the 4 week period. Overall, the results obtained are encouraging and lay the formulation foundations for potential EV clinical applications.
Regenerative therapies have recently gained significant momentum mainly owed to their potential for replacing tissues which have registered a loss in function or are damaged. These therapies are especially relevant in the cardiac field as an aging population has led to a world-wide increase in heart failure incidence. Mortality rates remain over 50% due to limited efficacy of current treatments, which are palliative rather than therapeutic. The heart is capable of limited self-renewal, but this is insufficient in heart failure. Clinical trials have explored stem cell therapies as potential therapeutic strategies for myocardial regeneration and repair. However, recent evidence suggests that this regenerative effect is indirect and in large part mediated by cell-secreted extracellular vesicles (EVs). EVs are thought to play pivotal roles in intercellular communication and immune response, and in both physiological and pathological conditions. They have been shown to be responsible for the regeneration of tissue in myocardial infarction and involved other conditions such as in Alzheimer, inflammation, among others.

Although EVs have enormous therapeutic potential, all the studies so far employ intravenous or direct injection to the heart. This would be unpleasant for patients, expensive for healthcare providers, and of sub-optimal therapeutic efficacy as the EVs injected will rapidly clear from the injection site. No alternative EV formulation strategies have yet been explored. There is thus a great unmet need to formulate adequate vehicles for local delivery of EVs. In this work, electrospinning was explored as an EV formulation technology. This is a simple technique that has been applied to cells without causing any
genetic, genomic or physiological damage, and hence it was hypothesised to be suitable for the formulation of EVs.

The work developed in this thesis lays the foundation for formulation strategies for the delivery of EVs using electrospinning. A robust method of vesicle isolation was achieved, along with the successful preparation of formulations able to encapsulate and release EVs without compromising their potency. This research shows the feasibility of formulating such complex entities and will lead to impact in the form of implantable EV therapies. It offers a platform technology not only applicable to the cardiac setting but also for a range of other indications that could benefit from the local delivery of EVs, namely in the reconstitution of solids into injectable liquids.
# Table of Contents

Abstract...........................................................................................................................................i

Impact Statement.............................................................................................................................iii

List of Abbreviations and other Units..............................................................................................xi

Chapter 1 – Introduction                                                                

1.1. Preamble ..................................................................................................................................2

1.2. Stem Cell Therapies ...............................................................................................................2

1.2.1. Stem Cell-based Therapies for Heart Failure ..................................................................3

1.3. Extracellular Vesicles ............................................................................................................7

1.3.1. Extracellular Vesicle-based Therapies for Heart Failure ...............................................9

1.4. Stem Cell vs. Extracellular Vesicle-based Therapies ..........................................................12

1.5. Formulation Challenges .........................................................................................................14

1.6. Electrospinning ......................................................................................................................16

1.6.1. Equipment Design and Function .....................................................................................16

1.6.2. Applications in Cardiac Therapeutics .............................................................................21

1.7. Aims and Objectives ..............................................................................................................24

1.8. References ..............................................................................................................................25
Chapter 2 – Materials and Methods

2.1. Materials

2.2. Methods

2.2.1. Extracellular Vesicle Isolation

2.2.1.1. Density and Viscosity Measurements

2.2.1.2. Differential Centrifugation Isolation Protocol

2.2.2. Electrospinning

2.2.2.1. Single Fluid Process

2.2.2.2. Coaxial Fluid Process

2.2.3. Physicochemical Characterisation

2.2.3.1. Nanoparticle Tracking Analysis (NTA)

2.2.3.2. Transmission Electron Microscopy (TEM)

2.2.3.3. Scanning Electron Microscopy (SEM)

2.2.3.4. Optical Microscopy

2.2.3.5. X-ray Diffraction (XRD)

2.2.3.6. Fourier Transform Infrared Spectroscopy (FTIR)

2.2.3.7. Differential Scanning Calorimetry (DSC)

2.2.3.8. Thermogravimetric Analysis (TGA)

2.2.3.9. Shrinkage and Porosity Evaluation

2.2.3.10. Dynamical Mechanical Analysis (DMA)
Chapter 3 – Extracellular Vesicle Isolation and Characterisation

3.1. Introduction.............................................................................................................59

3.1.1. Extracellular Vesicle Isolation.........................................................................59

3.1.2. Extracellular Vesicle Characterisation...............................................................63

3.2. Aims..........................................................................................................................66

3.3. Results and Discussion............................................................................................67

3.3.1. Determination of UC parameters.......................................................................67

Phosphate buffered Saline.........................................................................................68

Foetal Bovine Serum..................................................................................................69
Chapter 4 – Fast-dissolving Electrospun Fibres

4.1. Introduction........................................................................................................122

4.1.1. Electrospinning of Biomolecules................................................................122

4.1.2. Immediate Release Scaffolds......................................................................123

4.2. Aims..................................................................................................................126
Chapter 6 – Conclusions and Future Work

6.1. Summary of Research Findings……………………………………………………………………….209

6.2. Future Work……………………………………………………………………………………………………213
## List of Abbreviations and other Units

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Area</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATR</td>
<td>Attenuated total reflectance</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid assay</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CD</td>
<td>Clusters of differentiation</td>
</tr>
<tr>
<td>cells/cm²</td>
<td>Cells per square centimetre</td>
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<td>CHF</td>
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</tr>
<tr>
<td>cm</td>
<td>Centimetre</td>
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<tr>
<td>cP</td>
<td>Centipoise</td>
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<td>Cardiac progenitor cells</td>
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<tr>
<td>D</td>
<td>Distance to collector</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
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<td>Dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>ES</td>
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<tr>
<td>g</td>
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</tr>
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<td>F</td>
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<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
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<td>-------------</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
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<tr>
<td>HFIP</td>
<td>1,1,1,3,3,3-Hexafluoro-2-isopropanol</td>
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<tr>
<td>HSP</td>
<td>Heat-shock protein</td>
</tr>
<tr>
<td>HUVECs</td>
<td>Human umbilical vein endothelial cells</td>
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<tr>
<td>IC</td>
<td>Intracoronary</td>
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<tr>
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<td>Intramyocardial</td>
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<td>ISEV</td>
<td>International Society for Extracellular Vesicles</td>
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<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>K</td>
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<td>Kilopascal</td>
</tr>
<tr>
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<td>Kilovolt</td>
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<tr>
<td>LVR</td>
<td>Left ventricular remodelling</td>
</tr>
<tr>
<td>mDSC</td>
<td>Modulated differential scanning calorimetry</td>
</tr>
<tr>
<td>mg</td>
<td>Miligram</td>
</tr>
<tr>
<td>mg/μL</td>
<td>Miligram per microlitre</td>
</tr>
<tr>
<td>mg/mL</td>
<td>Miligram per millilitre</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro ribonucleic acid</td>
</tr>
<tr>
<td>mL</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mL/h</td>
<td>Millilitre per hour</td>
</tr>
<tr>
<td>mm</td>
<td>Milimetre</td>
</tr>
<tr>
<td>mN/m</td>
<td>Milinewton per metre</td>
</tr>
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<td>Mega Pascal</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>MVBS</td>
<td>Multivesicular bodies</td>
</tr>
<tr>
<td>Mw</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>N</td>
<td>Newton</td>
</tr>
<tr>
<td>N/min</td>
<td>Newton per minute</td>
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</tr>
<tr>
<td>nm</td>
<td>Nanometre</td>
</tr>
<tr>
<td>NTA</td>
<td>Nanoparticle tracking analysis</td>
</tr>
<tr>
<td>Pa</td>
<td>Pascal</td>
</tr>
<tr>
<td>particles/mL</td>
<td>Particles per mililitre</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCL</td>
<td>Poly(ε-caprolactone)</td>
</tr>
<tr>
<td>PCLA</td>
<td>Poly(L-lactide-co-caprolactone)</td>
</tr>
<tr>
<td>PDO</td>
<td>Poly(dioxanone)</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PEO</td>
<td>Poly(ethylene oxide)</td>
</tr>
<tr>
<td>PES</td>
<td>Polyethersulfone</td>
</tr>
<tr>
<td>PGA</td>
<td>Poly(glycolic acid)</td>
</tr>
<tr>
<td>PLA</td>
<td>Poly(lactic acid)</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>PVP</td>
<td>Poly(vinylpyrrolidone)</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Rotations per minute</td>
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<tr>
<td>s</td>
<td>Second</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SFCA</td>
<td>Surfactant-free cellulose acetate</td>
</tr>
<tr>
<td>SW</td>
<td>Swinging bucket rotor</td>
</tr>
<tr>
<td>T</td>
<td>Temperature</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TFE</td>
<td>2,2,2-Trifluoroethanol</td>
</tr>
<tr>
<td>$T_g$</td>
<td>Glass transition temperature</td>
</tr>
<tr>
<td>TGA</td>
<td>Thermogravimetric analysis</td>
</tr>
<tr>
<td>$T_m$</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>UC</td>
<td>Ultracentrifugation</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
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<tr>
<td>UV</td>
<td>Ultra-violet</td>
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<td>Symbol</td>
<td>Abbreviation</td>
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<tr>
<td>V</td>
<td>Volume</td>
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<tr>
<td>V</td>
<td>Volt</td>
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<tr>
<td>V</td>
<td>Voltage</td>
</tr>
<tr>
<td>V/V</td>
<td>Volume per volume</td>
</tr>
<tr>
<td>w/V</td>
<td>Weight per volume</td>
</tr>
<tr>
<td>WR</td>
<td>Working reagent</td>
</tr>
<tr>
<td>w/w</td>
<td>Weight per weight</td>
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<tr>
<td>XRD</td>
<td>X-ray diffraction</td>
</tr>
<tr>
<td>µL</td>
<td>Microlitre</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometre</td>
</tr>
<tr>
<td>ω</td>
<td>Shrinkage percentage</td>
</tr>
<tr>
<td>ρ</td>
<td>Density</td>
</tr>
<tr>
<td>°</td>
<td>Degree</td>
</tr>
<tr>
<td>°C</td>
<td>Celsius degree</td>
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<tr>
<td>°C/min</td>
<td>Celsius degree per minute</td>
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<td>Percent</td>
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</table>
Chapter 1

Introduction
1.1. Preamble

The development of biopharmaceuticals has been widely explored in the last decades as there is a continuous need for new and more effective therapies. Proteins, genetic material, antibodies and now even cells as active ingredients are either commercially used or being clinically studied.\textsuperscript{1-4} Biopharmaceuticals in general benefit from high specificity and potency compared to small drug molecules.\textsuperscript{5} However, due to their structural complexity biologics can also pose extra formulation and delivery challenges, especially regarding stability.\textsuperscript{5} Therefore, new pharmaceutical delivery systems are called for in order to utilise the new wave of biomedicines that has recently been developed.

1.2. Stem Cell Therapies

Stem cells are primitive unspecialised cells present in all organisms. They possess the capacity to proliferate into additional stem cells or differentiate into more specialised cells. Naturally occurring stem cells can be of embryonic, foetal tissue, amniotic membrane, and umbilical cord origin, or characterised as an adult stem cell.\textsuperscript{6} Embryonic stem cells are pluripotent and are capable of differentiating into any cell of any tissue in the adult body. Adult stem cells, on the other hand, have been identified within specific niches in most human tissues/organs. They possess self-renewal capacity and a multilineage differentiation potential, and as such are regarded as multipotent.\textsuperscript{7}

These stem cells can generate new further differentiated and specialized cells due to their plasticity, and thereby repopulate the tissues in which they reside under homeostatic conditions in addition to regenerating damaged tissue after an injury.\textsuperscript{8} The latter is particularly important in cases of loss or loss of function of specific cell types. In such cases, either direct regeneration or implantation of cells for the repair of damaged or
lost tissues is required. There are numerous diseases that have limited therapeutic options or no cure at all, and here stem cells present their biggest potential. Examples of conditions where stem cells hold promise and have been studied are macular degeneration, spinal cord injury, stroke, burns, diabetes, osteoarthritis, rheumatoid arthritis and heart disease.

Stem cells’ regenerative potential has drawn great interest in the science community: clinical investigations using these products are currently being explored for addressing a wide spectrum of conditions, and are expected to bring substantial benefit to patients. Just in 2017 nearly 200 trials associated with stem cells were collated in the World Health Organization International Clinical Trials Registry Platform, and over 4,000 have been conducted since 1996. However, it was only in 2015 that the first stem cell therapy was approved in Europe, showing this is a field of research still at its infancy.

1.2.1. Stem Cell-based Therapies for Heart Failure

One of the most promising applications of cellular based therapies is in heart failure conditions. Briefly, the heart is constituted of 4 chambers, two atria and two ventricles. Blood is collected in the atrium and pumped away from the heart by the action of the ventricles, through powerful, rhythmic, contractions. The heart’s capacity to contract is attributed to a muscular tissue called the myocardium, which is composed of cardiomyocytes or cardiac muscle cells. Heart failure is a common yet complex clinical syndrome that can result from any structural or functional cardiac disorder that impairs the ventricle function. The most common cause of heart failure is myocardial infarction (MI) as a consequence of coronary artery disease, as depicted in Figure 1.1.
Coronary arteries are responsible for irrigating the heart itself, maintaining the cardiac cells with a continuous supply of blood and oxygen. Cholesterol deposition as plaques, or atherosclerotic plaques, on coronary arteries walls can lead to their occlusion. Blood deprivation initially produces an inflammatory response, followed by extensive ischaemic death of cardiomyocytes within the affected area. This thus results in the partial loss of ventricular function. Over time, a set of complex alterations occurs in the myocardium, a phenomenon known as ventricular remodelling. Remodelling involves an inflammatory reaction followed by the formation of a collagenous, non-contractile fibrotic scar tissue, thinning of the myocardial wall and progressive ventricle enlargement and dilation. These adaptations are an attempt to compensate for ventricular malfunction. However, although interstitial myocardial fibrosis preserves the structural integrity it leads to progressive myocardial stiffening with further impairment of ventricular function. This ultimately contributes to a decrease in ventricular contractile function and output. Progressive deterioration of the cardiac function leads to heart failure, where the heart is
unable to pump enough blood to meet the metabolic demands of the body. The tissue death, or necrosis, is localised and the extent of the damage will depend on the swiftness of treatment.

Heart failure has a steadily growing global incidence due to an aging population and increasing prevalence of cardiovascular risk factors. Furthermore, patients usually have poor prognosis with a 5-year mortality rate of nearly 50%, largely as a result of the limited efficacy of current therapeutic strategies. Currently available therapeutic solutions are fundamentally palliative and do not address the ongoing loss of cardiomyocytes, fibrotic scar formation, and subsequent contractile dysfunction. In fact, apart from heart transplantation, revascularization techniques or left ventricular assist devices that try to prevent further cardiac deterioration and maintain heart function, there are to date no effective therapies to completely replace scar tissue with viable contractile cardiomyocytes.

It was recently discovered that the heart itself has a discrete capacity for cell renewal due to a population of resident cardiac progenitor cells (CPCs), and that adult cardiomyocytes can indeed proliferate. These cells have distinct advantages over other adult stem cell types in the cardiac setting as they are tissue specific and likely pre-committed to a cardiovascular fate. However, the number of CPCs and their cardiomyocyte proliferation rate is grossly insufficient to compensate for the damage inflicted by extensive myocardial infarction or other myocardial diseases. Therefore, there is an urgent need for newer and curative therapeutic options. Cell-based therapies have sparked interest in the scientific community as they offer the unique possibility of a regenerative treatment.

Since the first report of skeleton myoblasts used in heart failure back in 2001, many preclinical and clinical trials currently underway are supporting the benefits of cell therapies in improving patients’ cardiac function. Regardless of the specific details of
stem cell clinical trials, these therapies have been generally regarded as safe, with no major adverse cardiac effects detected when compared to placebo.\textsuperscript{32,33} However, there is clinical heterogeneity in clinical trials design which results in conflicting outcomes.\textsuperscript{34} The lack of standardisation is largely responsible for the heterogeneity of results. Optimisation of therapeutic regimes will need to address the therapeutic mechanism of action, optimal dose, route and frequency of administration, and long-term engraftment effects and consequences, among many others issues that still have not been conclusively clarified.\textsuperscript{18,31}

As previously mentioned, optimisation of the therapy is necessary since engraftment and survival of injected cells seems to be very limited, and transplanted stem cells either do not differentiate or differentiate into only a restricted number of cardiac cells. In fact, a study from Hou and colleagues showed surprisingly low retention rates of stem cells in the heart even when using local injection.\textsuperscript{35} Intramyocardial (IM) and intracoronary (IC) injections involve the direct injection either to the myocardium or to the coronary arteries, respectively. The obtained cardiac cell retention rates in the myocardium were as low as 11\% for IM and 2.6\% for IC. Moreover, the majority of the administered cells exited the heart into the pulmonary circulation, 26\% in IM injection and 47\% in IC.\textsuperscript{35} Of the small population of cells that manage to reach the heart, there is a further failure in cellular engraftment that eventually leads to a high death percentage.\textsuperscript{36,37}

Such evidence should indicate that there is a major problem in feasibility of stem cells to exert regenerative effects. However, quite surprisingly that is not the case and a beneficial effect in the cardiac function is observed regardless of the engraftment percentage.\textsuperscript{37} This suggests that other pathways might be at play in the cardiac function improvement registered after stem cell therapy. Recent studies suggest that the regenerative effect of cardiac stem cells is actually, in large part, indirect and through the so-called paracrine effect. In general, a paracrine effect describes the secretion and release
of biomolecules from one cell that affect the function of nearby cells. Recently various studies have arisen which seem to attribute this paracrine effect to extracellular vesicles (EVs). \textsuperscript{38–40}

\textbf{1.3. Extracellular Vesicles}

Extracellular vesicles (EVs) are anuclear vesicles bound by a lipid bilayer membrane. They are released by many different cells and are thought to play pivotal roles in intercellular communication and immune response, and are involved in both physiological and pathological conditions. \textsuperscript{41,42} Since originally reported as procoagulant platelet-derived particles in normal plasma in 1946, \textsuperscript{43} and referred to as “platelet dust” by Wolf, \textsuperscript{44} significant research has been undertaken in the last decade in the EV area. \textsuperscript{45} The accumulated data have indicated that the contents, size and membrane composition of EVs are highly heterogeneous and dynamic, and depend on a plethora of conditions such as the cellular source, cell health, and environmental conditions. \textsuperscript{46–48}

At present, although there is not a general consensus in EV nomenclature, three main groups are generally accepted: exosomes, microvesicles and apoptotic bodies. \textsuperscript{49} They mainly differ in their size and biogenesis. Apoptotic bodies are particles of relatively large size (1,000 to 4,000 nm) secreted by cells when in a programmed cell death process. \textsuperscript{50} Microvesicles are generally 100 to 1,000 nm in size and are formed by the outward budding of the plasma membrane (\textbf{Figure 1.2}). \textsuperscript{46} It is the third class of naturally occurring extracellular vesicles, exosomes (30 to 200 nm in size), which have been by far the most characterized. Exosomes are currently being explored as therapeutic and diagnostic tools in a myriad of fields. \textsuperscript{51–56} They have consistently been found in most biological fluids including blood, urine, cerebrospinal fluid, bronchial lavage fluid, breast milk and amniotic fluid. \textsuperscript{57} Furthermore, it has been shown that exosomes are released by many different cells and it
is now thought that all mammalian cells and cell lines are competent for exosome production, although the yields may vary. The genesis mechanism of exosomes has been extensively reviewed elsewhere, but in brief they are created through budding of the endosome membrane during its maturation, generating multivesicular bodies (MVBs) as can be seen in Figure 1.2. Endosomes are involved in degradation or recycling pathways of internalized molecules from the plasma membrane and intracellular medium. Later, release of exosomes to the extracellular environment is achieved upon fusion of these MVBs with the plasma membrane.

![Figure 1.2. Simplified diagram of exosome and microvesicle genesis and release from cells. Microvesicles bud directly from the plasma membrane to the extracellular medium. Exosomes on the other hand are primarily formed as intraluminal vesicles by budding into early endosomes and MVBs. Exosome release is achieved by MVBs fusion with the cellular plasma membrane.](image-url)
Regardless of the cell type that they originate from, typical exosomes express major histocompatibility complex (MHC) I and II on their surface as well as other specific membrane markers (CD63, CD9, CD81), and contain heat-shock proteins (HSP-27, HSP-60, HSP-70 and HSP-90), lipids, proteins and nucleic acids such as messenger or micro RNA (mRNA and miRNA, respectively). Although exosomes have been subject of intense study and their structure, composition and biogenesis roughly identified, the extent of their influence in patho-physiological pathways is still not fully understood. Nevertheless, exosomes have been reported to be involved in several cellular mechanisms and substantially contribute to various disease states including cancer, cardiovascular disease, neurodegenerative diseases (such as Alzheimer’s, Parkinson’s, or Prion diseases), inflammation, autoimmune disorders and infectious diseases.

1.3.1. Extracellular vesicle-based Therapies for Heart Failure

Extracellular vesicles have been regarded as an exciting new potential therapeutic approach for a range of pathologies. Furthermore, cardiac regenerative therapy has shown specially promising outcomes with several articles published advocating the contribution of EVs and exosomes in particular. For instance, Loyer and colleagues found a transient increase in the generation of EVs both from cardiomyocytes and endothelial cells in the heart of mice after an acute MI. EVs have recently been reported to play key roles in cardiac therapeutics as they protect the heart from ischemia by decreasing cardiomyocyte death, regenerate the scarred myocardium, enhancing the development of new blood vessels, and also have been shown to reduce myocardial infarction size. Chen and co-workers found that in vivo delivery of CPC-derived exosomes in an acute mouse myocardial ischemia/reperfusion model inhibited cardiomyocyte apoptosis by about 53% in comparison with an untreated control.
Very recently, a study from Kervadec and colleagues demonstrated similar cardioprotective effects of CPCs and their secreted EVs.\textsuperscript{40} In this study, mice were treated with either cells or their respective EVs, and both had significant reduction in left ventricular volumes and infarct size 6 weeks after treatment. This very strongly suggests that the outcome of stem cell therapies in the heart is mainly due to EV action. Since usually these EVs are isolated from the culture media of cardiomyocytes, it could be argued that the effects that EVs have on the heart are due to the combination of a range of compounds secreted by cells and not solely to the action of the vesicles themselves. However, in a study by Barile and colleagues, EVs were proven to fully account for the paracrine cardioprotective and proangiogenic activity of human CPCs.\textsuperscript{39} Here, CPC isolation media depleted of exosomes was found to lack the cardiac repair activities inherent to exosome administration, implying a negligible role for soluble factors secreted by these cells. Exosome administration on the other hand promoted the inhibition of cardiomyocyte apoptosis and enhancement of human umbilical vein endothelial cell (HUVEC) tube formation.

Although the effect of EVs in heart failure has been demonstrated by some authors, identification of all the underlying factors and mechanisms of the positive improvements to heart function is yet to be achieved. It has been demonstrated that exosomes or EVs contain a specific cargo dependent on the cellular origin and eliciting stimulus.\textsuperscript{77} Cardiomyocytes are no different, and exosomes could significantly vary in their content when produced under stress conditions (such as hypoxia, inflammation or injury). Hypoxia has been regarded as a potent stimulator of exosome secretion and a 2-fold increase was detected when cardiomyocytes were exposed to moderate hypoxia for 2 hours.\textsuperscript{78} Moreover, hypoxia seems to induce the secretion of vesicles rich in tumour necrosis factor alpha (TNF-\(\alpha\)), a pro-inflammatory cytokine.\textsuperscript{79} Gray and colleagues also
observed that exosomes released by hypoxic CPCs contained higher amounts of proangiogenic miRNAs (such as miR-132 and miR-146a), and were able to enhance tube formation in cultured HUVECs and reduce the expression of profibrotic genes in fibroblasts when compared to normal CPC exosomes. Additionally, the hypoxic exosomes were tested in an ischemia-reperfusion injury model, where improved heart function and inhibition of cardiac fibrosis was achieved.

miRNAs are thought to be involved in many of exosomes’ roles in cardiac repair. It seems that CPC exosomes were found to be enriched in miR-210, miR-132 and miR-146a when compared to fibroblast isolated exosomes, a demonstration of the differences in the EV populations produced by different cell types. Reduced cardiomyocyte apoptosis, enhanced angiogenesis and improved left-ventricle ejection fraction was exhibited when CPC exosomes are injected in infarcted hearts, but such effects are not seen when administering exosomes from fibroblast origins. On the other hand, Khan and colleagues have shown that exosomes from mouse embryonic stem cells augment the endogenous cardiac progenitor cell-based repair programs in the heart, and correlate it with the delivery of miR-294. Furthermore, mesenchymal stem cell (MSC) EVs were shown to significantly improve angiogenesis and cardiac function in the post-MI heart of a mouse model. MSC EVs also increased the proliferation, migration and tube formation capacity of HUVECs. Such effects were correlated with the MSC EVs being enriched with miR-210 as silencing this microRNA led to a loss on the pro-angiogenic effect, both in vitro and in vivo. This thus indicates that the use of EVs from pre-committed or specialised cardiac cell lines or stem cells can exert cardioprotective effects in the infarcted heart.

Overall, the cardioprotective and regenerative properties of EVs derived from cardiac and non-cardiac stem/progenitor cells appear to be promising for the development of therapeutic approaches. It could be argued that further research might lead to the
identification of a precise component on the EV load responsible for the cardiac protection and regenerative effects. Ibrahim and colleagues found that cardiac exosomes are especially enriched with miR-146a, but the administration of this miRNA on its own does not reproduce the cardiac benefits provided by the exosomes. Likewise, miR-146a-depleted exosomes were not able to suppress cardiomyocyte apoptosis at the same level as when miR-146a was present. This leads to the idea that it is the combination of compounds contained in the exosome that exerts an effect, rather than this being attributed to solely a single compound.

While it is relevant to understand the kinetics and the mechanisms of exosome function in cardiac regeneration, it may be counterproductive to deconstruct the vesicles from a therapeutic perspective, even if the exact molecules causing the desired effects could be identified. This is because the administration of free soluble factors would likely be disadvantageous compared to EVs, as with their lipid bilayer the latter are naturally engineered to protect their cargo from degradation, are able to enter and possibly specifically deliver the cargo to target cells.

1.4. Stem Cell vs. Extracellular Vesicle-based Therapies

Despite the enormous therapeutic potential for stem cells to treat a vast array of serious diseases there are still concerns regarding unexpected and potentially dangerous outcomes of such therapies. Issues such as the potential for tumorigenesis and immunogenicity require careful evaluation when translation to clinic is desired. During isolation and expansion of stem cells in vitro, there is an increased risk of contamination and damage to genetic material. Furthermore, there is a risk of spontaneous differentiation of transplanted stem cells in an undesirable direction leading to the formation of tumours. Extracellular vesicle therapies, on the other hand, circumvent
some of the concerns and limitations in using viable cells without compromising the therapeutic advantage of using complex biological agents. Compared to their parent cells, EVs may have a superior safety profile since the lack of a nucleus implies that they are unable to self-replicate and thus do not pose the risk of uncontrolled proliferation upon administration. In fact, nearly 80% of the therapeutic regenerative effect of stem cells has been actually attributed to the release of paracrine substances rather than by direct replication and differentiation. Hence, the administration of EVs would reduce the potential safety risks associated with cellular therapy without markedly compromising the therapeutic outcome.

Moreover, EVs present certain characteristics that are attractive in the development of therapeutic products. Their small size allows the sterilisation of samples by a simple filtration step, and their lipidic membrane is able to protect the biologically active cargo, potentially allowing a longer shelf-life and half-life in patients. However, a critical element in the development of extracellular vesicle therapies, as any other biologic-based therapy, is regulatory compliance so that a manufacturing licence and then a market authorisation can be obtained.

From a regulatory point of view, extracellular vesicles are to be considered a biologic medical product for which manufacturing and clinical trials frameworks already exist, provided by the European Medicines Agency (EMA) and agencies in other continents. To allow these to be marketed and approved, EV products need to have specifications of composition, purity, quantity, potency and sterility perfectly defined and in accordance with the regulations for pharmaceutical manufacturing. Composition and purity are, however, where one of the current biggest challenges lies in the development of EV therapies, given the absence of a standard technology to isolate and characterise EVs. EVs isolated from biological fluids, tissues or in vitro cell cultures are part of the secretome,
which includes soluble molecules like proteins and lipids, extracellular RNA species, and membrane vesicles. Electron microscopy data shows that even highly-purified EV preparations for analytical purposes contain co-contaminants. These are just some of the challenges that need to be overcome, and a recent position paper from the International Society for Extracellular Vesicles (ISEV) assesses the points to consider in translating these therapies to clinical practice in more detail. There is currently no clear composition and therapeutic mechanism of action for each potential EV application, but since this is a relatively new field of study substantial improvements should be expected in the next decade.

Overall, EVs offer a compelling off-the-shelf, cell-free therapeutic option that could be effective, safer, and cheaper than using stem cells themselves.

### 1.5. Formulation Challenges

Findings regarding the enormous therapeutic potential of extracellular vesicles are encouraging, which prompts consideration of possible pharmaceutical formulations. The majority of studies so far use intravenous injection as the administration method. A few other injection administration routes have been explored such as intraperitoneal, intramyocardial, intramuscular, percutaneous, and intranasal, but not extensively. To the best of our knowledge, to date there has been no pharmaceutical processing of extracellular vesicles, which highlights the clear need for developing new and more sophisticated formulations in order to fully maximize their therapeutic potential.

The formulation approach is highly dependent on the application and when considering delivery to the heart, the administration options can be quite narrow. Techniques that have been used for stem cell delivery mainly include intravenous,
intramyocardial and intracoronary injection and can possibly be translated to EVs. Intravenous injection (IV), although it is a simple, safe and fairly non-invasive method of administration, lacks tissue specificity and hence low delivery efficiencies are attained. In fact, one of the biggest concerns is related to the rather short half-life of exosomes in circulation. Takahashi and colleagues found that exosome half-life after IV administration is around 2 minutes, with only a minimal presence in the circulation after 4 hours. In order to avoid such rapid clearance of EVs, local delivery approaches need to be considered.

Intramyocardial administration involves injection directly into the myocardium, most frequently into the left ventricle. It is considered to be the most reliable method of delivery by injection due to the modestly higher cell retention levels within the myocardium. However, it was demonstrated that intramyocardial injections of cells can have some drawbacks, including leakage of the injectate to the pericardium or the vasculature resulting in a rapid cell wash-out with migration to remote organs. This led to the low cell retention rates attained by Hou and colleagues upon IM administration. Moreover, limited cell survival might arise from the stress imposed to cells delivered at high pressure without any protection through a needle.

In a study by Hamdi and co-workers, it was found that intramyocardial delivery of stem cells on a local delivery reservoir leads to superior engraftment functionality when compared to injections in the same area. This thus indicates that the most promising option would be a formulation that involves engraftment directly in the epicardium. In order to achieve that, a formulation that allows production of a solid construct that can then be sutured to the epicardium needs to be developed. Electrospinning has been widely used to produce three-dimensional porous fibrous scaffolds by utilising either natural and synthetic polymers and therefore represents one method which could be used to achieve
such a goal.\textsuperscript{102–104} Moreover, electrospinning has been widely explored in the field of regenerative medicine.\textsuperscript{105–107} In fact, it has also been used for the fabrication of constructs for direct application to the heart.\textsuperscript{102,108,109} Hence, production of such constructs might be of interest for the production of EV cardiac delivery systems.

\section*{1.6. Electrospinning}

Electrospinning has emerged in the last decade as a powerful technique for the design of ultrafine fibrous biomaterials.\textsuperscript{110} Its most common biomedical applications are related to tissue engineering, medical implants and drug release matrixes, but many others have been considered.\textsuperscript{105} Electrospinning has gathered a significant interest owing to several key advantages.\textsuperscript{111} It is a versatile technique that can accommodate a wide variety of compounds while offering the ability to incorporate multiple polymers and bioactive ingredients in one formulation. Moreover, the control available over the structure and morphology of the final construct allows ease of functionalisation and superior mechanical properties.\textsuperscript{112} Due to the small diameter fibres produced (typically on the nanoscale), it provides a very high specific surface area-to-volume ratio, which is desirable for enhancing delivery of active ingredients even at high loadings.\textsuperscript{111} The simplicity of the process (requiring little specialized equipment) and the possibility of large-scale production, already commercially available, makes this technique very attractive for many different applications.\textsuperscript{105}

\subsection*{1.6.1. Equipment Design and Function}

Electrospinning is a fibre producing method from a polymer solution made with a volatile solvent that usually has an active substance dissolved in it. The equipment works
on the principle of applying a high voltage electric field through a conducting needle system (spinneret) with the polymer solution placed above a grounded collector. At a certain critical applied voltage, the repulsive electrical forces overcome the surface tension forces of the polymer solution, and the solution droplet forming at the end of the spinneret deforms into a conical shape, or Taylor cone. This is followed by ejection of a continuous charged jet strand from the tip of the Taylor cone. The ejected jet will then accelerate towards the grounded collector. During the process, the internal and external charge forces cause the unstable and rapid whipping of the liquid jet, with further stretching of the polymer chains within the solution allowing the evaporation of the solvent and therefore fibre deposition. The typical apparatus configuration is depicted in a schematic diagram in Figure 1.3.

![Schematic diagram of the set-up for electrospinning.](image)
However, it should be noted that particles might be produced with this set-up, under a technique named electrospraying. The distinction between electrospinning (the process of producing fibres) and electrospraying (the process of producing particles) depends on something known as the Rayleigh instability. The Rayleigh instability governs the behaviour of the electrified liquid jet. There is a balance between the surface tension of a liquid and the generated electrostatic repulsion. In electrospraying, the molecular entanglement of the feeding solution is insufficient to overcome the Rayleigh instability; thus, surface tension prevails over the electrostatic repulsion and the formed jet breaks up into small charged particles to reduce the surface area. Thus, the ability to produce fibres is highly dependent on solutions molecular entanglements and viscosity, and a minimum critical solution concentration defines the transition from electrospraying to electrospinning.

Furthermore, electrospinning is a simple and straightforward process to operate, involving the adjustment of set parameters determining the quality of the final construct, some already discussed. It thus relies on the balance between the intrinsic solution material properties, process parameters and environmental conditions. Polymer solution parameters include concentration, molecular weight (Mw), viscosity, surface tension, and conductivity. Applied voltage, flow rate and collecting distance are some of the processing parameters worth considering. Environmental conditions such as relative humidity and temperature also have an impact on the technique. Extensive discussion on the relationship of each parameter and their influence in the final product has been reviewed previously.
Polymer solution concentration, molecular weight and viscosity all have an interconnected effect on the electrospinning process. With an increase of polymer concentration or on the molecular weight of the dissolved materials there is a concomitant increase in the number of polymer chains entanglements in a solution, resulting in a rise in fluid viscosity. A sufficiently high polymer viscosity is necessary to generate uniform fibres, whereas solutions with a low viscosity often tend to produce particles.\textsuperscript{114}

The choice of solvent can also have an impact on the fibres produced. The solvent selected needs to be able to firstly dissolve all the solids contained in the feeding solution, and have a boiling point that allows solvent evaporation during jet emission. It should be noted that highly volatile solvents are to be avoided as they can evaporate at the tip of the spinneret and, as the solid materials dry, block the needle and stop the process. Moreover, solvent–polymer interactions critically influence not only the operational conditions but also the viscoelasticity and the critical minimum solution concentration required for electrospinning, thus affecting the final electrospun fibre diameter, crystallinity, tensile strength, aspect ratio, and morphology.\textsuperscript{115}

Electrospinning can be performed in a single-fluid or coaxial mode (Figure 1.4). These differ mainly in the use of either a single needle spinneret or a two-needle system, respectively. In the coaxial system one needle is nested inside another allowing the processing of two solutions at the same time. With the rapid stretching and solvent evaporation, the core-shell structure obtained from the physical separation of solutions in the concentrically arranged needles is propagated into the fibre products. The first report on the application of a coaxial system was in 2003 by Sun and colleagues.\textsuperscript{116} In that work, the authors explored the processability of polymeric systems comprised of poly(ethylene oxide) with and without non-spinnable materials in the core. It was found that it was possible to incorporate non-electrospinnable materials in the core providing
there is a polymer solution in the shell that is amenable to processing by electrospinning. This thus expands the possible applications of electrospinning in the development of drug delivery systems by broadening the range of materials able to be used, this being especially relevant in the case of biomolecules.

![Figure 1.4](image)

Figure 1.4. Schematic diagram of the spinneret set-up for a (a) single-fluid and a (b) coaxial electrospinning system. Next to each diagram is a schematic of the normal morphology of the produced fibres.

The production of coaxial fibres has some advantages over the monolithic analogues from single-fluid processing. These are especially useful in controlled-release formulations, as the production of a core/shell system not only minimizes the initial burst release usually seen in electrospun fibres, but can also allow the creation of complex...
release profiles. Hence, targeting the delivery to specific tissues, cell types and in different parts of the body can be achieved. This is particularly relevant when considering sensitive functional materials such as proteins or even cells, as it provides relative protection even during processing.\textsuperscript{118,119}

### 1.6.2. Applications in Cardiac Therapeutics

In 2006, Townsend-Nicholson and Jayasinghe electrospun cell suspensions for the first time.\textsuperscript{120} They found that cells could be electrospun without any significant impact on their viability, morphology or rate of growth. Since then electrospinning has also been applied to bacteria and virus,\textsuperscript{121} proteins,\textsuperscript{122} and cells,\textsuperscript{119,123,124} to form fibrous scaffolds without causing any genetic, genomic or physiological damage \textit{in vitro} and \textit{in vivo}. These studies laid down the foundations for the application of electrospinning in processing highly sensitive biological entities.

As mentioned above, electrospinning is not a novel technique in tissue engineering and has attracted significant attention as a way to produce organ-specific tissue structure substitutes.\textsuperscript{125–127} Yet, few studies have been applied to cardiac cell therapy with \textit{in vivo} testing of the characteristics and efficiency of the implantation of electrospun formulations. Kitsara and colleagues have demonstrated the biocompatibility of cell-loaded electrospun collagen scaffolds after two weeks of implantation in an ischemic heart.\textsuperscript{128} Processing parameter optimisation was found to be of utmost importance as the scaffolds need to have an adequate capacity to endure the stress that the normal heart contraction might inflict. This reiterates the need to carefully consider all important parameters, such as biocompatibility and mechanical strength, when designing and producing formulations for use in the heart.
Polymer materials, from natural or synthetic origins, are the most widely used materials in cardiac construct preparation.\textsuperscript{129} The advantage of natural polymers is their biocompatibility, but they have variable properties depending on the extraction method and also high associated costs.\textsuperscript{130} Among the natural polymers, the most commonly explored are collagen, elastin, gelatin, fibrinogen, silk fibroin and alginites.\textsuperscript{131} Synthetic polymers, on the other hand, are chemical reproducible and therefore suited for large-scale manufacturing. They usually benefit from better mechanical properties though their biocompatibility can be hampered by their degradation products. The most common synthetic biodegradable polymers suggested for cardiac tissue engineering are polyurethane, poly(\(\varepsilon\)-caprolactone) (PCL), poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and their copolymers.\textsuperscript{125,132}

Regardless of the polymer origin, an understanding of the immune response triggered upon implantation is important in the design of implantable patches.\textsuperscript{133} Castellano and co-workers assessed the effect of different electrospun scaffolds after implantation in the heart, and the consequent immune and functional reaction \textit{in vivo}.\textsuperscript{132} Collagen, poly(\(\varepsilon\)-hydroxybutyrate) (PHB) and PCL were found to have an effect in cardiac remodelling, preventing ventricular dilatation and reducing scar tissue formation in the infarcted heart.\textsuperscript{132} However, only PHB and collagen scaffolds were found to elicit no classical foreign body immune with no encapsulation of polymer fibres or induction of nonspecific immune response, just degrading over time. It should be noted, however, that few studies have been performed to investigate the \textit{in vivo} behaviour of electrospun scaffolds for cardiac therapeutic applications, and there are still several unanswered questions and variables to consider. Nevertheless, electrospinning holds significant promise in the production of scaffolds for cardiac implantation.
To the best of our knowledge, extracellular vesicles have never been electrospun before. Although not extensively investigated, electrospinning has been proposed to be a suitable technique for cardiac tissue engineering and therefore it holds potential to prepare materials able to give sustained local delivery of EVs to the damaged heart.
1.7. Aims and Objectives

This project aims to develop strategies to formulate extracellular vesicles for tissue regeneration applications. Specific objectives were set as follows:

- To develop a robust isolation method to obtain EVs from different biological fluids or cell culture supernatants. To assess a set of characterisation approaches to effectively and reproducibly qualify and quantify the isolated EV samples. To test EV stability under a range of storage conditions.

- To develop fast-release EV-loaded fibres through electrospinning, and assess the scaffolds’ physical properties and the EVs’ biological cues and potency.

- To develop controlled-release electrospun scaffolds for cardiac EV delivery. To assess the scaffolds’ physical and mechanical properties, and explore the active ingredient release profile using a fluorescent liposome as a model vesicle.
1.8. References


Chapter 2

Materials and Methods
2.1. Materials

Chemicals and materials used throughout the experimental work undertaken in this thesis are listed in Table 2.2 and Table 2.1.

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foetal Bovine Serum [FBS, heat inactivated, cell culture reagent]</td>
<td>ThermoFisher Scientific (UK)</td>
</tr>
<tr>
<td>Foetal Bovine Serum [FBS, EV isolation]</td>
<td></td>
</tr>
<tr>
<td>Phosphate Buffered Saline [PBS, pH = 7.4], no calcium, no magnesium</td>
<td></td>
</tr>
<tr>
<td>Dulbecco Minimum Essential Medium [DMEM], high glucose, GlutaMAX</td>
<td></td>
</tr>
<tr>
<td>Trypsin-EDTA (0.05%), phenol red</td>
<td></td>
</tr>
<tr>
<td>Trypan Blue Solution (0.4%)</td>
<td></td>
</tr>
<tr>
<td>PrestoBlue® Cell Viability Reagent</td>
<td></td>
</tr>
<tr>
<td>Micro BCA™ Protein Assay Kit</td>
<td></td>
</tr>
<tr>
<td>Antibiotic Antimycotic Solution (100×), Stabilized</td>
<td>Sigma-Aldrich (UK)</td>
</tr>
<tr>
<td>Gelatin solution [Type B, 2% in H2O, tissue culture grade]</td>
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</tr>
<tr>
<td>Dimethyl sulfoxide [DMSO, cell culture reagent]</td>
<td></td>
</tr>
<tr>
<td>Radioimmunoprecipitation assay buffer [RIPA]</td>
<td></td>
</tr>
<tr>
<td>Sodium dodecyl sulphate [SDS, for molecular biology, purity ≥98.5%]</td>
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</tr>
<tr>
<td>Bradford Protein Assay Reagent</td>
<td>Bio-Rad (USA)</td>
</tr>
<tr>
<td>ExoELISA Complete Kit [CD63, CD9, CD81]</td>
<td>System Biosciences (USA)</td>
</tr>
<tr>
<td>Silica Microspheres, Colloidal (50 and 100 nm)</td>
<td>Polysciences (USA)</td>
</tr>
</tbody>
</table>

Biological samples of mesenchymal stem cells culture media were kindly provided by Professor Mark Lowdell (Royal Free Hospital, UK). Cells were harvested from umbilical cord tissue via a combination of dissection, digestion and mechanical homogenisation. The output was then filtered and cultured. The media of MSCs in culture was then retrieved and processed further for the isolation of extracellular vesicles.
TABLE 2.2. Materials used for formulation experiments

<table>
<thead>
<tr>
<th>Material</th>
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</tr>
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<tr>
<td>H9c2(2-1) cells (ATCC® CRL-1446™)</td>
<td>ATCC (USA)</td>
</tr>
<tr>
<td>Polyethylene oxide [PEO, Mw 400,000]</td>
<td></td>
</tr>
<tr>
<td>Poly(vinyl pyrrolidone) [PVP, Mw 360,000]</td>
<td></td>
</tr>
<tr>
<td>Poly(dioxanone) [PDO, Resomer X 206 S]</td>
<td>Sigma-Aldrich (UK)</td>
</tr>
<tr>
<td>Absolute Ethanol [99.8% purity, analytical grade]</td>
<td></td>
</tr>
<tr>
<td>Acetone [99.5% purity]</td>
<td></td>
</tr>
<tr>
<td>Poly Lactic-co-Glycolic Acid [PLGA, 50:50 Mw 17,000]</td>
<td>Corbion Purac Biomateirals (Netherlands)</td>
</tr>
<tr>
<td>Poly Lactic-co-Glycolic Acid [PLGA, 50:50 Mw 44,000]</td>
<td></td>
</tr>
<tr>
<td>2,2,2-Trifluoroethanol [TFE, purity 99.8%]</td>
<td>Acros Organics (USA)</td>
</tr>
<tr>
<td>1,1,1,3,3,3-Hexafluoro-2-isopropanol [HFIP, purity 99+%]</td>
<td>Alfa Aesar (UK)</td>
</tr>
<tr>
<td>DOPC/CHOL Liposomes labelled with TRITC-DHPE (100 nm)</td>
<td>FormuMax (USA)</td>
</tr>
</tbody>
</table>

2.2. METHODS

2.2.1. Extracellular Vesicle Isolation

2.2.1.1. Density and Viscosity Measurements

Samples of phosphate buffered saline, foetal bovine serum and mesenchymal stem cell (MSC) conditioned media for EV isolation were assessed for their density and viscosity. Sample densities [$\rho$] were determined at room temperature by measuring a known volume [V] using a Discovery Comfort micropipette (HTL, Poland) and dividing it by the weight [m] determined on a CPA 225D Cubis semi-micro balance (Sartorius, Germany) according to Equation 2.1. Samples were measured at least five times.
(Equation 2.1)

\[ \rho = \frac{m}{V} \]

To determine samples densities at a range of different temperatures (4, 10, 12, 15, 20, 25, 30 and 37°C), Equation 2.2 (developed by Schiel and Hage)\(^1\) was used to extrapolate the required values.

(Equation 2.2)

\[ \rho_{PBS} = (-5.0 \times 10^{-6}T^2 + 2.0 \times 10^{-5}T + 1) + [-2.8 \times 10^{-5}T + 0.0097] \frac{[\text{phosphate}]}{0.067} \frac{\text{pH}}{7.4} \]

Viscosity measurements were performed using a rolling-ball Automated Micro Viscometer (Anton Paar, Germany). PBS, FBS and MSC conditioned media samples were assayed along with different ratios of PBS:FBS mixtures, ranging from 1:1 to 1:4 (V/V). Viscosity measurements at temperatures between 10°C and 37°C were performed. Samples were measured at least three times. Dynamic viscosity \([\eta]\) was calculated by multiplying the efflux time in seconds by the viscometer constant (Equation 2.3).

(Equation 2.3)

\[ \eta = K(\rho_K - \rho)t \]

Where \(K\) is a calibration constant (mPa.cm\(^3\)/g) of the measuring system, \(t\) the rolling time (s) and \(\Delta\rho\) the density difference between ball [\(\rho_K\)] and sample [\(\rho\)], expressed in g/cm\(^3\). A capillary with 1.6 mm internal diameter was used with a 1.5 mm steel ball and a density of 7.85 g/cm\(^3\). The protocol employed assessed viscosity at an angle of 70°. Viscosity was determined at each measurement temperature and a linear regression fitted to extrapolate the viscosity of all samples at 4°C.
2.2.1.2. Differential Centrifugation Isolation Protocol

Foetal bovine serum (FBS) was used as an initial source of EVs in the optimisation phase due to its wide availability and lower cost. MSC conditioned media from umbilical cord tissue culture was kindly provided by Professor Mark Lowdell (Royal Free Hospital, UK) and used to isolate stem cell secreted EVs. FBS samples were processed according to the diagram in Figure 2.1 whereas MSC samples as described in Figure 2.2. Samples were either filtered through a 200 nm SFCA (surfactant-free cellulose acetate) membrane (ThermoFisher, UK) or subject to a 10k g centrifugation step at 11,600 rpm (rotor T-647.5, Sorvall WX+ Ultra centrifuge, Thermofisher Scientific, UK). All centrifugation steps were performed at 4°C. In both cases, a 100k g ultracentrifugation step at 37,500 rpm followed. The vesicle pellet was resuspended in 200 μL of phosphate buffered saline and stored at -80°C until further use.

Figure 2.1. Schematic representation of the EV isolation ultracentrifugation protocol for foetal bovine serum.
2.2.2. Electrospinning

2.2.2.1. Single Fluid Process

PEO and PVP solutions at different concentrations were initially prepared for optimisation, using either ethanol or TFE as the solvent. The polymer powder and solvent were stirred at room temperature until complete dissolution was observed. PLGA, PDO and PDO:PLGA mixtures were prepared in either acetone or HFIP. All containers used were previously sterilised by autoclaving, and sealed to avoid solvent evaporation. For preparation of EV-loaded polymer solutions, ca. 7.5x10^{11} particles were added from an EV stock solution to 2 mL of the polymeric solution immediately before spinning, amounting to a total of 3.75x10^{12} particles/mass of polymer.

The basic experimental setup used for electrospinning process can be seen in Figure 2.3. An adjustable high voltage power supply (HCP 14-20000, FuG Elektronik GmbH, Germany) was used to supply high DC voltage. A syringe pump (78-9100C, Cole Parmer, UK) was used to control the solution flow. The polymer solution to be electrospun...
was placed in either a 1 mL or 5 mL disposable plastic syringe (Terumo, UK), depending on the volume to be processed. This syringe was attached to a blunt 22G (gauge) stainless steel needle with 0.41 mm internal diameter, to which the positive electrode was clamped directly. The grounded electrode was connected to a 10 x 10 cm metallic stand wrapped with aluminium foil or siliconized paper and fibres were collected at a tip-to-collector distance of 10 to 18 cm. The whole apparatus was disinfected with 70% ethanol prior to any experiments and all materials sterilised, when possible, beforehand. Unless noted otherwise, solutions were electrospun under a fixed flow rate of 1 mL/h, with a controlled voltage of 7 to 20 kV. Experiments were performed under ambient conditions (25 ± 5°C and relative humidity 35 ± 5%). All electrospun fibre scaffolds were stored under vacuum in a desiccator at room temperature, with silica gel beads used as desiccant.

![Figure 2.3. Photograph of a typical single fluid electrospinning setup.](image)
2.2.2.2. Coaxial Fluid Process

Shell solutions were prepared in a similar way to the ones used in the single fluid process. The applicability of the coaxial system developed was tested by using fluorescent liposomes in the core solution. Fluorescent liposomes were used as a model to demonstrate the release profile from the scaffolds. Core solutions were prepared by diluting the fluorescent liposome stock in phosphate buffered saline. The core and shell solutions were then loaded into 1 and 5 mL plastic syringes, respectively. The syringes were mounted separately onto two syringe pumps. The syringe containing the core solution was tipped with a nested feeding channel on a coaxial spinneret (Figure 2.4) with an internal diameter of 0.51 mm (21 G); whereas the shell syringe (internal diameter 1.25 mm, 16 G) was connected via tubing attached to the outer channel of the spinneret. The positive electrode of a high voltage DC power supply was connected to the metal tip of the coaxial spinneret while the grounded electrode was connected to a 10 x 10 cm metal plate collector. The core and shell fluids were then simultaneously fed through the spinneret at fixed flow rates, with the processes performed under ambient conditions (25 ± 5°C and relative humidity 35 ± 5%). All electrospun fibre scaffolds were stored under vacuum in a desiccator at room temperature, with silica gel beads used as desiccant.
Figure 2.4. Photographs and diagram of the used coaxial spinneret, showing it to be comprised of two needles, one nested within the other.

2.2.3. Physicochemical Characterisation

2.2.3.1. Nanoparticle Tracking Analysis (NTA)

Nanoparticle tracking analysis (NTA) was performed using a NanoSight LM14 microscope (Malvern Pananalytical, USA) equipped with a sCMOS camera and 532 nm green laser unless stated otherwise. Some measurements were performed on a NS300 instrument equipped with either a 488 nm blue laser or a 532 nm laser, due to temporary in-house equipment unavailability. Samples were diluted in filtered phosphate buffered saline (220 nm cut-off, PES membrane; BD Biosciences, UK) until the number of particles was in the $1 \times 10^8$ to $5 \times 10^9$ particles/mL range. They were next manually analysed at room temperature in 90 second captures intervals from five separate fields of view per sample, using NanoSight standard analysis routines. Video captures were then analysed with the
NTA software version 3.2 to determine the concentration and size of the measured particles, with corresponding standard error. Videos were recorded with the camera level set at 13 and analysed with detection threshold set at 3. Temperature was monitored throughout the measurements. The NanoSight system was calibrated with 50 and 100 nm silica nanobeads (Polysciences, USA), and the solvent (PBS) was tested to ensure the absence of particles.

2.2.3.2. Transmission Electron Microscopy (TEM)

Transmission electron microscopy of electrospun formulations was performed by collecting a small amount of sample on a copper grid by spinning directly onto it. EV-loaded fibres were dissolved in phosphate buffered saline and EV isolates were analysed as whole-mounted vesicles deposited on EM grids and fixed and contrasted/embedded with uranyl acetate. The samples were then analysed using a FEI CM 120 Bio-Twin FEI-TEM (Philips/FEI, USA).

2.2.3.3. Scanning Electron Microscopy (SEM)

A sample of approximately 0.5 cm per 0.5 cm was cut from each fibre formulation and mounted onto aluminium SEM stubs (TAAB Laboratories, UK) with carbon-coated double-sided tape. Samples were then coated with a 20 nm gold sputter (Quorum Q150T, UK) under an argon atmosphere. A Quanta 200F field emission SEM (FEI, the Netherlands/USA) connected to a secondary electron detector (Everheart-Thomley Detector-ETD) was used to generate SEM images of the materials. Images were taken at a 3,000x, 6,000x, 12,000x and 24,000x magnification. Calculation of fibre or particle diameter was carried out by using v15.0i of the ImageJ software (National Institutes of Health, USA) with a minimum sample size of 75.
2.2.3.4. Optical Microscopy

A digital inverted optical microscope (Evos FL Cell Imaging System, ThermoFisher Scientific, UK) was used for immediate assessment of fibre integrity and mat formation. This allowed optimization of the electrospinning voltage and feed rate for each polymer solution. A glass microscope slide was placed on the aluminium collection plate and removed after circa 1 min. The microscope slide was then viewed under the optical microscope at different magnifications to check for fibre formation and bead presence. Feed rate and voltage were adjusted for each solution until continuous fibres were attained.

2.2.3.5. X-ray Diffraction (XRD)

Samples were mounted on glass sample holders and XRD patterns collected using a MiniFlex 600 (Rigaku, Japan) diffractometer supplied with Cu-Kα radiation (λ= 1.5418 Å). Samples were scanned at 2° per minute over the 2θ range of 3-50°, at 40 kV and 15 mA. Data were analysed using the X’Pert Data Viewer software package (PANAlytical, Netherlands).

2.2.3.6. Fourier Transform Infrared Spectroscopy (FTIR)

Absorption spectra in the infrared region were obtained on a Spectrum 100 FTIR spectrometer (Perkin Elmer, USA) fitted with an attenuated total reflectance (ATR) accessory. Spectra were collected at 4 cm\(^{-1}\) resolution, over the range 650 to 4000 cm\(^{-1}\). 64 scans per sample were obtained and averaged. Samples were analysed at room temperature, with the sampled placed directly onto the measurement window. For EV isolates, 5 µL of sample was mounted on the diamond ATR crystal and a thin dry film was
obtained by slow evaporation of the buffer under ambient conditions, as described elsewhere.\textsuperscript{2} Measurements were performed at room temperature, immediately after drying, which usually took approximately 5 minutes. Data were analysed with the Essential FTIR v3.10.016 software (Operant LLC, USA).

\subsection*{2.2.3.7. Differential Scanning Calorimetry (DSC)}

A differential scanning calorimeter (Q2000 Differential Scanning Calorimeter, TA Instruments, USA) was used to examine the thermal properties of prepared materials. 3 to 4 mg of sample were loaded in aluminium pans (TZero, TA Instruments) and sealed with pin-holed aluminium lids. The pans were assessed at a heating rate of 3°C/min in the modulated mode. Samples were heated from -50 to 300°C in a nitrogen atmosphere. Nitrogen gas was purged through the instrument at a flow rate of 50 mL/min throughout the process. From the DSC thermograms, glass transition temperature [$T_g$] and the melting temperature [$T_m$] were determined for each sample. In order to predict the $T_g$ of polymer blends, the Fox equation was applied (\textbf{Equation 2.4}).\textsuperscript{3}

\textbf{(Equation 2.4)}

\[
\frac{1}{T_g} = \frac{1 - x}{T_g(\text{PLGA})} + \frac{x}{T_g(\text{PDO})}
\]

\subsection*{2.2.3.8. Thermogravimetric Analysis (TGA)}

Thermogravimetric analysis was performed to determine if any residual solvent remained in the scaffolds after electrospinning using a Discovery TGA instrument (TA Instruments, USA). Samples of approximately 5 to 10 mg were mounted in open aluminium pans and heated at 5°C/min from 30 to 450°C. The instrument was purged with nitrogen
gas at a 25 mL/min flow throughout. Data were analysed using the Trios software version 3.3.0.4055 (TA Instruments, USA).

2.2.3.9. Shrinkage and Porosity Evaluation

Scaffolds were cut to a dimension of 1.5 x 1.5 cm, suspended in a sealed glass container with 5 mL of PBS (pH 7.4), and incubated at 37°C for 24 hours. Samples were then rinsed with deionised water and dried overnight at room temperature under vacuum. The area \([A_f]\) of the dried samples was measured and compared to their initial area \([A_i]\), and the shrinkage percentage \([\omega]\) was calculated according to Equation 2.5.

(Equation 2.5)

\[
\omega = \left(1 - \frac{A_f}{A_i}\right) \times 100
\]

Porosity was determined by measuring the experimental scaffolds density \([\rho_{\text{exp}}]\) according to Equation 2.6. To measure samples’ thickness a Mitutoyo Absolute calliper (Mitutoyo, Japan) was used.

(Equation 2.6)

\[
\rho_{\text{exp}} = \frac{m}{A \times \text{thickness}}
\]

Considering the pure polymer density at 20°C \((\rho_{\text{PLGA}} = 1.24 \text{ g/cm}^3; \rho_{\text{PDO}} = 1.38 \text{ g/cm}^3)\) it is possible to calculate the scaffolds porosity (%) using Equation 2.7.

(Equation 2.7)

\[
\text{Porosity} [\%] = 1 - \frac{\rho_{\text{exp}}}{\rho_{\text{pure polymer}}}
\]
2.2.3.10. Dynamical Mechanical Analysis (DMA)

Mechanical properties of scaffolds were determined using a dynamic mechanical analysis instrument (Q800 DMA, TA instruments, USA) equipped with a tension film clamp. Scaffolds were cut in rectangular shape samples of 15 x 6.5 mm dimensions. The initial length and width were determined using a digital calliper (Fisher Scientific, UK). Measurements were carried out at a constant temperature of 37°C, to mimic body temperature, in a controlled force mode with a ramp force of 0.5 N/min to 18 N maximum load under 0.005 N static load. A stress-strain curve was generated for each sample, and the Young’s modulus was determined by calculating the slope in the linear region of the curve using the Universal Analysis software (TA instruments, USA).

2.2.4. Release Studies

Release of fluorescent liposomes from coaxial fibres was performed in vitro by immersing 1 x 1 cm scaffolds in 2 mL of phosphate buffered saline and incubated for 4 weeks at 37°C, safe from light sources with a mixing speed set at 100 rpm. Samples of release medium (200 µL) were withdrawn at different time intervals (1, 7, 14, 21 and 28 days) and measured for their fluorescence at 532 nm with a SpectraMax M2e microplate reader (Molecular Devices, UK). The release experiments were conducted at least in triplicate.
2.2.5. Protein Quantification Assays

2.2.5.1. Bradford Assay

The Bradford protein quantification assay was performed in accordance with the manufacturer’s instructions (BioRad, UK). Briefly, the working reagent (WR) was prepared by mixing one-part Bradford reagent in four parts deionised water (V/V), and 100 µL of WR was added to each well of a 96 well plate. A standard curve was constructed using bovine serum albumin (BSA, Sigma) over the concentration range 0 to 1 mg/mL. Absorbance was read at 590 nm and 450 nm, according to the modified protocol of Zor and Selinger, using a SpectraMax M2e microplate reader (Molecular Devices, UK). Blank absorbance was subtracted from all readings before data analysis. All samples were measured in triplicate.

2.2.5.2. microBCA Assay

Total protein was quantified using the microBCA protein assay kit, a bicinchoninic acid-based quantification assay. The assay was performed in accordance with the manufacturer’s protocol. Briefly, the working reagent (WR) was prepared by mixing microBCA reagents A, B and C in 25:24:1 parts (V/V/V), respectively. 100 µL per well of WR was added to lysed samples in a 96 well plate. Lysis of EV isolates was performed by adding RIPA buffer in the ratio of 2:1 V/V (sample:lysis buffer) and incubating on ice for 15 minutes, with vortexing every 5 minutes. Samples were then sonicated for 10 minutes and added to the plate. Some wells additionally had 2% SDS added to test for the interference of lipids in the samples. The plate was shaken for 30 seconds and then incubated at 37°C for two hours. It was left to cool to room temperature before absorbance was read at 562 nm using a SpectraMax M2e microplate reader (Molecular Devices, UK).
Blank absorbance was subtracted from all readings before data analysis. A bovine serum albumin (BSA) standard curve was prepared for each measurement.

2.2.5.3. Enzyme-Linked Immunosorbent Assay (ELISA)

Specific exosomal proteins were detected by enzyme-linked immunosorbent assay (ELISA), which was used to quantify the tetraspanins CD63, CD9 and CD81. The protocol followed was as recommended by the manufacturer of the purchased kit (EXOELISA, System Biosciences, USA). Briefly, samples were incubated in pre-coated plates at 37°C overnight. Plates were washed with the provided washing buffer before incubation with the primary antibody with shaking at room temperature for one hour to prevent non-specific binding. Another cycle of washing steps, followed by secondary antibody incubation for another hour was done. ELISA substrate added to each well after being washed, before being allowed to stand at room temperature for 40 minutes. Stop buffer was then added to stop the reaction, before the endpoint absorbance was quantified at 450 nm with a SpectraMax M2e microplate reader (Molecular Devices, UK). The blank absorbance reading was subtracted from the measured values.

2.2.6. Cell Culture Assays

2.2.6.1. Cell Culture

The rat cardiomyocyte H9c2 cell line (ATCC, USA) was cultured in DMEM [Dulbecco Minimum Essential Medium] with GlutaMAX (Life Technologies, UK) supplemented with foetal bovine serum (FBS, 10% w/V) and an antibiotic antimycotic solution (5% w/V, Sigma, USA). Cell lines were maintained in a 5% CO₂ atmosphere at 37°C and passaged at sub-confluency every 2 to 4 days. Cell passage was achieved by rising them with phosphate
buffered saline and incubating at 37°C for 5 minutes with trypsin-EDTA (0.05% w/V, Life Technologies, UK) until cells had ceased to adhere to the culture dish. Complete media was used to stop the reaction. The cell concentration was determined using a Scepter™ handheld automated cell counter (Millipore Corporation, USA). A 0.2% gelatin solution (Type B, 2% in H₂O, Sigma-Aldrich, UK) prepared with sterile water was used to coat a fresh flask to increase adherence, and the cells resuspended at 10,000 cells/cm² in complete DMEM.

When cell lines were being established, cryopreserved cells were quickly thawed at 37°C. The thawed cells were diluted 10-fold with pre-warmed complete growth medium and centrifuged at 200 g for 5 to 7 minutes at 21°C. The cell pellet was resuspended in complete medium and incubated at 37°C.

2.2.6.2. Survival Assay

To determine the pro-survival effects of isolated and formulated EVs, stressed H9c2 cardiac myoblasts were incubated with FBS and MSC EVs pre and post-formulation. Cells were stressed by serum deprivation and the number of viable cells with and without treatment at the end of 27 hours was determined, as described elsewhere.⁵ To detect the amount of viable cells a resazurin-based assay that measured the reducing power of viable cells through fluorescence after an incubation of one hour and a half was employed. For that a ratio of 1:10 of reagent and serum-depleted DMEM was used per well. Fluorescence results were normalized against a positive control of non-stressed cells in a complete media (containing serum).
2.2.7. Statistical Analysis

Data were statistically analysed using one-way Analysis of Variance (ANOVA) followed by Tukey’s multiple comparisons test and t-tests. Differences were considered significant when \( p < 0.05 \). GraphPad Prism version 6.00 for Windows (GraphPad Software, USA) was used for statistical analysis.

2.3. References


Chapter 3

Extracellular Vesicle
Isolation and Characterisation
3.1. Introduction

Even though extracellular vesicles (EVs) are increasingly recognized as important biological and therapeutic entities, standardized methods for their isolation and analysis are still lacking.\(^1\) It is however fairly consensual that there is a need to clearly recognize the different experimental systems and sources of EVs that have been reported in literature and chose the most adequate for each application.\(^2\) Here, a brief overview of the explored isolation and characterisation methods is reviewed.

3.1.1. Extracellular Vesicle Isolation

One of the major EV application challenges is associated with their isolation from biological samples. EVs represent only a small fraction of the components present in a culture medium, cytosol or body fluids and although a high yield is desirable, it is also important to obtain samples with as little contaminants as possible. A standardized method for isolation and assessment of EVs is yet to be established.\(^1,3\) Some of the most traditional EV isolation methods utilize vesicles specific properties as size and buoyant density. Such methods count ultracentrifugation,\(^1,4-6\) density-gradient separation,\(^7-9\) size exclusion chromatography,\(^10,11\) and ultrafiltration.\(^11,12\) Other methods utilise the change in EVs solubility and induce the formation of aggregates through precipitation with polyethylene glycol,\(^13\) protamine,\(^14\) or sodium acetate.\(^15\) Newer isolation techniques rely on highly specific interactions of EV surface markers,\(^8,9\) or are based on microfluidic technologies.\(^16\) Several EV isolation methods have been explored in the literature and their advantages and disadvantages considered; the most widely reported are summarised in Table 3.1.\(^1,4,17\)
### Table 3.1. Isolation methods for EV research

<table>
<thead>
<tr>
<th>Separation Principle</th>
<th>Advantages</th>
<th>Limitations</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ultracentrifugation</strong></td>
<td>Sequential separation based on particulate density, size and shape via high speed centrifugation of components</td>
<td>• Reduced cost per run • Absence of contamination risk from separation reagents • Large sample processing capacity • Yields large amounts of EVs</td>
<td>• High equipment cost, labour intensive and long run time • Co-isolation of non-extracellular vesicle material • Might damage vesicles • Efficiency affected by rotor type, g-force and sample viscosity</td>
</tr>
<tr>
<td><strong>Density gradient separation</strong></td>
<td>Isolation separation based on different vesicles densities by using sucrose or synthetically made (such as iodixanol) density gradients.</td>
<td>• High purity of EV isolates</td>
<td>• Requires an ultracentrifugation workflow beforehand • Loss of sample – low yields • Long processing times • Complex</td>
</tr>
<tr>
<td><strong>Size-Exclusion Chromatography</strong></td>
<td>Isolation exploiting the hydrodynamic diameter of vesicles to separate them from larger contaminants, by using a column packed with heteroporous beads</td>
<td>• High sample purity • Gravity flow preserves integrity and biological activity of vesicles • Superior reproducibility • Insensitive to high viscosity samples</td>
<td>• Requires dedicated equipment • Complex and costly • Low sample processing volumes • Not trivial to scale-up • Long run time • Necessary a difference in components Mw &gt; 10%</td>
</tr>
<tr>
<td><strong>Ultrafiltration</strong></td>
<td>Separation by size with the use of filtration membranes, sometimes with the concomitant use of centrifugation</td>
<td>• Fast processing • Does not require specialised equipment • No limitations on sample volume</td>
<td>• Moderate purity of EV preparations • Filter clogging and vesicle trapping • Loss of sample in the filter membrane • Vesicle deformation</td>
</tr>
<tr>
<td><strong>Polymer-based precipitation</strong></td>
<td>Vesicle precipitation with the use of polymers such as polyethylene glycol (PEG) and centrifugation at low g-force</td>
<td>• Low cost and simplicity of process • Preservation of vesicle integrity • No specialised equipment needed</td>
<td>• Contamination and polymer retention</td>
</tr>
<tr>
<td><strong>Immunoaffinity capture</strong></td>
<td>Isolation based on specific interactions between membrane-bound antigens (receptors) of EVs and immobilized antibodies (ligands)</td>
<td>• Highly pure samples • Capacity to retrieve specific EVs subtypes</td>
<td>• High reagent cost, low capacity and yield • Nonspecific binding • Difficulty in detachment of molecules and analysis of intact vesicles • Eluting buffers can damage EV functional activity</td>
</tr>
<tr>
<td><strong>Microfluidic isolation</strong></td>
<td>Isolation on a microscale based on a variety of EV properties such as immunoaffinity, size and density</td>
<td>• Fast, low cost process • High efficiency • Easy to automate</td>
<td>• Low sample capacity • Lack of standardization and method validation • Complexity of devices needed • High cost</td>
</tr>
</tbody>
</table>

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1,4–6
7–9
10,11
11,12
13–15
8,9
16
Most protocols are designed to remove sample contaminant particles in order to isolate small EVs, in particular exosomes. However, the resulting samples can potentially contain a mixture of smaller EVs of heterogeneous subcellular origin. Therefore, from this point onward, we will broadly define the isolates as EVs, or extracellular vesicles, and not specifically as exosomes.

Ultracentrifugation (UC) is considered to be the gold standard and by far the most widely used method of EV isolation.\(^3\) Particle separation is achieved by spinning the samples around an axis at a defined speed at which particles will start sedimenting according to their density and size over time. Larger and heavier particles tend to have faster sedimentation rates and are then easier to pellet at lower speeds. Smaller particles, such as extracellular vesicles, tend to take longer to deposit or require faster centrifugation steps – ultracentrifugation. As the starting biofluid for EV isolation is usually a multicomponent mixture of particles differing in size and density, often a series of consecutive centrifugation cycles of different centrifugal force and duration are applied. A typical ultracentrifugation workflow for extracellular vesicle isolation is presented in Figure 3.1, based on the most commonly used protocol first described by Théry and colleagues.\(^4\) It should be noted however that a number of different protocols can be seen in the literature, ranging from one hour centrifugation steps to overnight steps.\(^4,5,18\) A differential centrifugation protocol is successful when sedimentation rates of discrete populations of particles are significantly different from one another.\(^19\) Larger particles, such as cells and cell debris, are removed from the sample on the first centrifugation steps due to their faster sedimentation rates. Supernatants are then centrifuged in subsequent steps for the isolation of smaller particles. While the methodology is straightforward, the quantity and quality of EVs isolated is sensitive to multiple parameters. Physical separation
depends not only on the centrifugal force applied, but also on the rotor type (fixed angle or swinging bucket), sample viscosity and density.

The centrifuge rotors frequently used for exosome isolation are divided in two categories: swinging bucket (SW) which stand out horizontally from the rotational axis during spinning, and fixed angle (FA) rotors which are held at a constant angle during the whole centrifugation. The type of rotor will affect the sedimentation path length of particles across the tube. In fixed angle rotors, particles have less distance to travel before pelleting against the tube wall, as opposed to the swinging bucket rotor where the sedimentation path length is longer. This can result in a lower pelleting efficiency of the latter if not compensated by prolonged centrifugation duration. It is hence important to assess the instrumentation available before designing a protocol.

**Figure 3.1.** Schematic representation of the EV isolation ultracentrifugation protocol. Cells, dead cells and cell debris are pelleted in the first three centrifugation steps and discarded. Supernatant at each step is recovered and processed through the workflow. At the end, EVs will sediment in a pellet that is then recovered in phosphate buffered saline. The above protocol was developed by Théry and colleagues.
Livshits and colleagues developed a method of predicting the relative amounts of sedimented particles according to the rotor characteristics and duration of the centrifugation step. They took into consideration the viscosity and density of the centrifuged sample, as it will have an impact on particle sedimentation rates. Viscous samples will require longer centrifugation steps to pellet the same sized particles in a liquid carrier. Serum samples are reported to have a higher viscosity when compared to cell culture media. Due to the high viscosity of serum, dilution with PBS is often suggested. Moreover, viscosity increases at lower temperatures. Since isolation of EVs is widely reported to be performed at a temperature of 4°C, this will slow the particle sedimentation rate, which in turn can have a significant impact on the quantity and quality of isolated EVs. It is then critical when designing a centrifugation protocol to assess the parameters that underlie the process. Often, a standard protocol is used, like the one in Figure 3.1, regardless of the equipment available and the starting sample characteristics. Applying the same speed and duration of spin might result in different yields of isolated EVs, depending on the rotor used and the viscosity of the sample in question. This methodology is likely responsible for the heterogeneity of results between research groups.

3.1.2. Extracellular Vesicle Characterisation

Characterisation of EVs has been proven to be technically difficult given their heterogeneity in size and composition, and little consensus has been reached. Establishment of such methods is crucial for the safe application of EVs in a clinical setting. The number of methods used for EV characterization varies widely. In a recent 2016 survey, 9% of respondents reported using only one characterization method, 23% used two methods, 33% three methods, 23% four methods and 12% used five or more characterization methods.
Lötvall and colleagues defined a set of minimal experimental requirements for the characterisation of extracellular vesicles in a position paper from the International Society for Extracellular Vesicles (ISEV).\textsuperscript{2} Here, the main aim is to clearly identify and discriminate EVs from non-EV components.

Characterization of single vesicles within a mixture needs to be performed, according to Lötvall and co-workers requirements, to provide an indication of EV isolates heterogeneity.\textsuperscript{2} At least 2 different technologies should be used to characterize individual EVs. In recent years, several instruments have become available that allow the detection and characterization of individual EVs. These techniques include nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM).\textsuperscript{21} NTA relies on the detection of the light diffraction pattern of particles in suspension in an aqueous solvent. It is a useful technique which provides information regarding the size distribution of particles in a sample and their relative concentration. TEM is another imaging technique that allows the confirmation of membranous vesicles in samples, supporting NTA size distribution results. The combination of both techniques allows the size characterisation of vesicles on the bulk sample (NTA) and on a specific aliquot of the sample (TEM).

Lötvall and colleagues also indicated that a general overview of the protein composition of each EV isolate should be provided.\textsuperscript{2} It is suggested the identification of at least three proteins in at least a semi-quantitative manner in any EV preparation. Often EV preparations are quantitatively expressed in terms of the mass of protein or number of particles present. Assays such as micro BCA or Bradford have been the most commonly implemented to measure EV total protein concentration.\textsuperscript{22} Diverse buffers are known to lyse EVs with different efficiency and as such influence the estimated protein concentration.\textsuperscript{21} Therefore, it is important to know the influence of both the quantification method and the experimental parameters used, and keep a consistent approach
throughout. Measurement of specific protein markers expected to be in EVs is often a chosen approach for vesicle characterisation. Methods such as flow cytometry, western blot, enzyme-linked immunosorbent assay (ELISA) are among some of the selected. ELISAs are convenient assays that allow the detection of EVs by fixing them onto a plate and quantify the exosomal protein present in the sample using an appropriate detection antibody against exosome-associated antigens. Tetrascapins such as CD63, CD9 and CD81 are the most commonly detected proteins by EV ELISA.

Finally, Lötvall and co-workers identify the need to assess the functional activity of EVs. Such assays will vary on a case by case scenario as it is highly dependent on the intended application of the isolated sample. However, they usually comprise of in vitro cell activity studies.

The lack of standardization on isolation and characterisation methods poses many challenges to translation and widespread application of EV-based therapeutics. Moreover, another challenge yet to be extensively studied, involves EV preservation and storage. Stability of vesicles is of utmost importance and must be addressed to enable the use of EVs beyond resource-intensive settings. To date, studies suggest -80°C as the most promising temperature to prevent storage-mediated effects however, the knowledge is still limited. It should be noted that the mechanism of storage-induced changes on EV bioactivity remains unclear. Further understanding of this mechanism would allow the rational design of improved strategies to prevent loss of potency with storage, and would significantly broaden the potential therapeutic applications for EVs.
3.2. Aims

In this chapter, extracellular vesicle isolation and characterisation approaches are investigated for foetal bovine serum and mesenchymal stem cell culture media samples. Both are thought to contain extracellular vesicles. FBS was used as a model fluid for the isolation and formulation of EVs given its reduced cost and ease of availability. MSC derived EVs on the other hand have been shown to elicit therapeutic effects in several pathological conditions, including cardioprotective effects post-myocardial infarction. The aim is to optimise the ultracentrifugation isolation protocol parameters for each EV source, considering sample density and viscosity, and assess different steps in terms of their impact on the final isolate characteristics. Physical characterisation of the EVs obtained in terms of size and morphology, as well as concentration, is reported, along with a quantification of protein content and specific markers. Potency assays of isolated vesicles were performed on serum derived, stressed cardiomyocytes as well as their stability under different storage conditions.
3.3. Results and Discussion

3.3.1. Determination of UC parameters

To design an appropriate isolation protocol, assessment of variables related to the instrumentation available and the specific sample was conducted. According to the model developed by Livshits and colleagues\textsuperscript{18}, in addition to the rotor characteristics it is necessary to know a sample’s density and viscosity, and the specific density of the particles to be isolated. The density of exosomes was taken from the literature, which reports them to have an average density of 1.15 g/mL, varying between around 1.08 and 1.19 g/mL\textsuperscript{4,31}

Phosphate buffered saline, foetal bovine serum and MSC conditioned media samples were experimentally assessed for their density and viscosity. Given that most isolation protocols are performed at 4°C and both viscosity and density decrease with temperature these parameters needed to be adjusted for this. The apparatus available to measure density can only record data at ambient temperature. As we were unable to determine the density of the samples at temperatures other than ambient, it was necessary to extrapolate a value at 4°C. Schiel and Hage (2005) have studied the influence of temperature \([T]\) on phosphate buffer density and derived Equation 3.1.\textsuperscript{32}

(Equation 3.1)

\[
\rho_{PBS} = (-5.0 \times 10^{-6}T^2 + 2.0 \times 10^{-5}T + 1) + [-2.8 \times 10^{-5}T + 0.0097] \frac{[phosphate]}{0.067} \frac{pH}{7.4}
\]

From Equation 3.1, it was possible to calculate the samples’ density at the working temperature of 4°C. However, due to instrumentation limitations, viscosity measurements can only be performed at 10°C or higher. This results in the need to also extrapolate viscosity at 4°C by testing the samples over a wider range of temperatures.
Phosphate Buffered Saline

An initial study on the viscous behaviour of PBS was performed, and the results can be seen in Figure 3.2. As expected, viscosity increased in an inverse relationship to temperature, and the estimated value at 4°C was calculated to be 1.524 ± 0.014 cP.

![Graphs showing experimental values and fitted linear regression for PBS viscosity over a range of temperatures.](image)

**Figure 3.2.** Experimental variation PBS samples viscosity over a range of temperatures from 10 to 37°C. Graphs show (a) the experimental values and (b) the values fitted with a linear regression, with the calculated value at 4°C shown in red. Three independent experiments were performed and mean ± standard deviation values taken for each point.
**Foetal Bovine Serum**

Foetal bovine serum has been reported to have a higher viscosity than other biofluids used for EV isolation. This has prompted researchers to dilute FBS samples with phosphate buffer to compensate for the viscosity increase. Hence, the impact of PBS addition on the viscosity of the overall mixture had then to be assessed. The density of foetal bovine serum:phosphate buffered saline mixtures was measured at different volume ratios. This way, the ultracentrifugation process could be modelled in accordance with actual rather than assumed mixture density. Variation of the PBS content in the mixture did not exert a significant difference in density. The average measured FBS density across all ratios was 0.9868 g/mL, with a standard error of 0.0058 g/mL. Density ranged between 0.9824 and 0.9905 g/mL for all samples. All mixtures were then assessed in terms of their viscosity at temperatures between 10 and 37°C (Figure 3.3), and their density calculated applying Equation 3.1.

![Figure 3.3.](image)

*Figure 3.3.* Experimental variation of viscosity in mixtures of PBS and FBS over a range of temperatures from 10 to 37°C. Three independent experiments were performed and mean ± standard deviation values taken for each point.
Over the temperature range assessed, little variation was observed between replicates of the same sample, with a maximum variation of 0.015 cP at each temperature. The dilution of FBS with PBS results in a decrease in solution viscosity, with the difference being more pronounced as the temperature decreases. This results in a maximum viscosity variation at 10°C of 0.47 cP from phosphate buffer compared to foetal bovine serum. Since it was not possible to measure the viscosity of the samples at 4°C, the usual working temperature, an extrapolation from the obtained data was undertaken and the results are summarised in Table 3.2 and can be visualized in Figure 3.4. There is a difference of around 0.5 cP between the viscosity of phosphate buffer and serum at 4°C.

Table 3.2. Extrapolated viscosities of FBS mixtures at 4°C

<table>
<thead>
<tr>
<th>Mixture [V/V]</th>
<th>Viscosity [cP]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PBS</strong></td>
<td>1.524 ± 0.014</td>
</tr>
<tr>
<td><strong>1:1 PBS:FBS</strong></td>
<td>1.745 ± 0.006</td>
</tr>
<tr>
<td><strong>1:2 PBS:FBS</strong></td>
<td>1.846 ± 0.005</td>
</tr>
<tr>
<td><strong>1:4 PBS:FBS</strong></td>
<td>1.913 ± 0.005</td>
</tr>
<tr>
<td><strong>FBS [0.2µm filtered]</strong></td>
<td>2.035 ± 0.012</td>
</tr>
<tr>
<td><strong>FBS</strong></td>
<td>2.055 ± 0.014</td>
</tr>
</tbody>
</table>
Figure 3.4. Experimental variation of extrapolated viscosity in sample mixtures of PBS and FBS at 4°C. Three independent experiments were performed and mean ± standard deviation values taken for each point.

It is important to assess the influence this difference in viscosity might have on the final centrifugation isolation process. Samples at the extremes of viscosity, in this case PBS and FBS, were used to assess this. Rotor and sample characteristics were inserted in the online calculator developed by Livshits and colleagues and the ideal centrifugation parameters for a 100k g spin for both samples were generated (Table 3.3). In this process a cut-off particle size needs to be established. This is the diameter at which all particles are pelleted. For EV research, there is no consensus in literature regarding the minimum diameter of exosomes as they are usually reported to range between 30 and 50 nm. Hence, a median 40 nm cut-off was chosen for the following isolation experiments.
A 0.5 cP difference in viscosity resulted in an extra 18 minutes of centrifugation being required for FBS to achieve the same isolation conditions as PBS. Had the FBS samples been modelled to PBS parameters (same rotation speed and spin duration) the actual cut-off would be 44 nm and the theoretical amount of 40 nm particles being pelleted would be 91%. This represents a loss of just 9% of the particles intended to be pelleted due to sample viscosity. On top of that, to actually reach a viscosity similar to phosphate buffered saline, serum samples would have to be diluted more than half. This would represent a drastic reduction in the final yield of isolated EVs per processed batch which is not acceptable given the laborious nature of the process. Therefore, given the little impact FBS viscosity might have on EV isolation, it was considered the use of undiluted serum and it is then preferred to adjust the centrifugation parameters to accommodate for a 40 nm cut-off.

Some methods of differential centrifugation for FBS have been suggested in the literature. These often require several centrifugation steps, which usually result in loss of material, impacting negatively the final yield. Here, we sought to minimize the number of processing steps samples underwent. Although there is debate about the desirability of some isolation methods over others, it was decided to focus on two of them: filtration and centrifugation, and explore their impact on isolate concentration, size distribution and protein content. For FBS a two-step approach was chosen with an initial centrifugation or filtration step followed by a 100k g ultracentrifugation. The idea of this initial step is to

| Table 3.3. Parameters for PBS and FBS differential centrifugation at 4°C |
|----------------|----------------|----------------|----------------|----------------|----------------|
| Viscosity [cP] | Density [g/cm³] | Vesicle density [g/cm³] | Centrifugation speed [g] | Cut-off size [nm] | Centrifugation time [min] |
| PBS             | 1.524           | 1.007           | 115             | 100,000        | 40             | 82             |
| FBS             | 2.055           | 0.992           | 115             | 100,000        | 40             | 100            |
remove larger particles from the supernatant. Since exosomes are generally classified as particles sized under 200 nm \(^{38-41}\), it was decided to establish 200 nm as the cut-off for the first step in the isolation process. For the filtration step a 200 nm pore size surfactant-free cellulose acetate (SFCA) filter was used. The choice of this membrane over others has to do with the low binding affinities of the SFCA filters over the more commonly used polyethersulfone (PES). In a study by Vergauwen and colleagues cellulose-based membranes performed at least 2-fold better in particle and EV protein recovery when compared with PES membranes.\(^\text{12}\) Similarly, the 10,000 \(g\) centrifugation step is set to a 200 nm cut-off, eliminating from the sample all components with a size bigger than 200 nm. The calculated parameters for the differential centrifugation of FBS are listed in Table 3.4.

**Table 3.4. Parameters for FBS differential centrifugation at 4°C**

<table>
<thead>
<tr>
<th></th>
<th>FBS viscosity [cP]</th>
<th>FBS density [g/cm(^3)]</th>
<th>Vesicle density [g/cm(^3)]</th>
<th>Centrifugation speed [g]</th>
<th>Cut-off size [nm]</th>
<th>Centrifugation time [min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>10k (g)</td>
<td>2.055</td>
<td>0.992</td>
<td>1.15</td>
<td>10,000</td>
<td>200</td>
<td>42</td>
</tr>
<tr>
<td>100k (g)</td>
<td></td>
<td></td>
<td></td>
<td>100,000</td>
<td>40</td>
<td>100</td>
</tr>
</tbody>
</table>

It is common to add a washing step after the 100k \(g\) step to remove potential contaminants that might be co-isolated.\(^\text{44}\) However, it was found elsewhere that the addition of a washing step at the end of the isolation steps was in general ineffective at removing contaminant proteins.\(^\text{42}\) This step provided as little as a 2-fold increase in particle to protein ratio, a general parameter to assess samples purity. It also led to an incomplete recovery of material, lowering the final yield. Following such findings, the addition of a washing step was no longer considered for any of the samples.
The optimum centrifugation parameters were able to be calculated (Table 3.4) according to the specific sample viscosity, at the experimental temperature used for the ultracentrifuge system available. This approach intends to be more robust than the commonly used approaches that do not consider the above sample and experimental variables.

**MSC conditioned media**

Although culture media has had its viscosity reported elsewhere in the literature, variations in the specific components of the raw media used, combined with the added cell metabolites and debris, might have an impact on the final sample viscosity, which should therefore be assessed. A similar approach was followed for the conditioned media as previously applied for both phosphate buffer and foetal bovine serum samples. The variation in viscosity of undiluted MSC conditioned media with temperature is given in Figure 3.5.

The extrapolated viscosity of MSC conditioned media at 4°C was calculated to be 1.707 ± 0.012 cP. The UC protocol previously reported by Théry and colleagues assumes a differential centrifugation with two initial steps (300 and 2,000 g) to remove remaining suspended cells and debris. These steps do not need to be modelled as the low centrifugation speeds will not be sufficient to pellet EVs. The next steps, however, do need to be modelled. The same workflow approach used for the FBS samples isolation was applied for the MSC conditioned media. A comparison between an intermediate filtration step and a 10k g centrifugation, both with a 200 nm cut-off, was made. Centrifugation parameters were then calculated according to the initial sample characteristics and are summarised in Table 3.5.
Figure 3.5. Experimental variation MSC conditioned media samples viscosity over a range of temperatures from 10 to 37°C. Graph (a) shows the experimental values and (b) the values fitted with a linear regression. The calculated value at 4°C is shown in red. Three independent experiments were performed and mean ± standard deviation values taken for each point.
Table 3.5. Parameters for MSC conditioned media differential centrifugation at 4°C

<table>
<thead>
<tr>
<th></th>
<th>MSC media viscosity [cP]</th>
<th>MSC media density [g/cm³]</th>
<th>Vesicle density [g/cm³]</th>
<th>Centrifugation speed [g]</th>
<th>Cut-off size [nm]</th>
<th>Centrifugation time [min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>10k g</td>
<td>1.707</td>
<td>0.977</td>
<td>1.15</td>
<td>10,000</td>
<td>200</td>
<td>32</td>
</tr>
<tr>
<td>100k g</td>
<td>100,000</td>
<td>40</td>
<td>76</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The viscosity and density values used for the calculation of the centrifugation parameters were determined from the raw MSC conditioned media. However, it could be hypothesized that viscosity changes after each of the intermediate steps due the removal of suspending components in the media. Instead of measuring the viscosity of the sample at each intermediate step it was thought to assess the viscosity of the supernatant after the 100k g centrifugation step of both isolation streamlines (centrifugation and filtration) as this would represent the starkest difference in overall amount of solid components (Figure 3.6).

![Figure 3.6](image_url)

**Figure 3.6.** Experimental variation of viscosity over a range of temperatures from 10 to 37°C for MSC raw conditioned media [○] and 100k g supernatants after EVs isolation: centrifugation [△] and filtration [□] streamline. Three independent experiments were performed and mean ± standard deviation values taken for each point.
No significant difference was seen at any temperature point for processed and unprocessed samples. As a result, the centrifugation parameters calculated in Table 3.5 should be fine to use throughout for all samples.

Similarly to FBS samples, by measuring MSC conditioned media viscosity it is possible to tailor the centrifugation steps to the desired cut-off for a specific centrifuge system.

3.3.2. Extracellular Vesicle Characterisation

Following establishment of the appropriate UC parameters, EV samples were isolated and the products subjected to a detailed characterisation.

3.3.2.1. Size and Concentration

Samples were characterised according to their size and concentration after isolation. For size measurements, nanoparticle tracking analysis was employed for all isolated pellets. As samples are analysed suspended in a solvent it is important to ensure this solvent is particle-free. It was found that phosphate buffer itself, a common solvent in EV research, might present particles in the nanometer range. A careful assessment of the PBS storage and processing conditions was made to obviate this. It was hypothesised that these nanoparticles in suspension could be attributed to phosphate precipitation in solution over time, as older samples tended to present a higher number of particles. This effect was found to be slowed down by storage at 4°C. Moreover, to ensure a particle-free solvent, filtration through a 220 nm cut-off filter was undertaken before every measurement. To ensure there was no particle contribution from external sources, phosphate buffer was always checked before an experiment. The solvent was considered
to not affect measurements if no significant numbers of particles were detected (less than 3 to 5 particles per frame).\textsuperscript{21}

Much has been discussed previously about the use of NTA for the analysis of extracellular vesicles.\textsuperscript{2,21,43,44} It should be noted that although it is a very useful technique, care must be taken in result interpretation and comparison as variability due to operator, software and instrument parameters have been previously reported.\textsuperscript{44,45} One of the parameters that is thought to influence sample concentration and size distribution is the type of laser used. To determine whether this could pose an issue in the present study, two different wavelength lasers were compared on the same instrument.

An experiment with EVs isolated from foetal bovine serum revealed that there was a 60.5 ± 4.2% increase in the detected particle concentration when using a 488 over a 532 nm laser. The capacity to account for a higher concentration of particles in the 488 nm laser might have to do with its higher resolution to detect smaller particles as the wavelength is reduced.\textsuperscript{46} There was an increase of approximately 30% in the number of particles sized under 50 nm detected at 488 nm. In fact, Mcvey and colleagues reported an increase of resolution in EV detection at 405 nm compared to 488 nm.\textsuperscript{46} Simulations based on Mie theory confirmed the increase in sensitivity with the use of a lower wavelength laser. This is particularly relevant in the EV field as the size distribution in these samples is typically left-shifted towards smaller particles. All the work presented in this thesis was performed on an instrument equipped with a 532 nm laser unless stated otherwise, as it was the equipment available. As a result, it should be noted that there might be an underestimation of the final EV content in each sample as exosomes are commonly reported to have a size between 30 and 200 nm.\textsuperscript{33,34,36,38–41,47}
Foetal Bovine Serum

There was no significant difference in the final concentrations of particles below 200 nm between FBS samples initially processed by centrifugation or filtration, as can be seen in Figure 3.7. Samples processed by an initial 10k g centrifugation step had an overall mean particle concentration of $1.03 \times 10^{12} \pm 1.14 \times 10^{11}$ particles/mL whereas filtration processed samples gave $1.04 \times 10^{12} \pm 2.09 \times 10^{11}$ particles/mL, a variation of less than 1%.

Figure 3.7. Particle concentration measured by nanoparticle tracking analysis (NTA) on FBS isolates processed by either an initial 10k g centrifugation step [CENTRIFUGATION] or a 200 nm pore size filtration [FILTRATION], before being isolated by high speed centrifugation (100k g). Isolations were performed in 3 independent experiments, with 3 replicates each.

This demonstrates that the initial step has little to no influence on the particle concentration in the resulting pellet. The overall size distribution of the particles can be seen in Figure 3.8. FBS EV isolates are moderately monodisperse, regardless of the processing method.
Figure 3.8. Particles size distribution and their relative frequency (NTA) on FBS isolates samples processed by either (a) an initial 10k g centrifugation step or (b) a 200 nm pore size filtration before being isolated by high speed centrifugation (100k g). Isolations were performed in at least 3 independent experiments; and the data shown is the mean size distribution per bin of all samples tested.
A breakdown of the raw data in Figure 3.8 into different particle size fractions is given in Figure 3.9. It is fair to assume that exosomes will be pelleted and comprise most of the sample given that more than 90% of the particles are under 200 nm and exosomes are generally regarded as ranging in size between 30 and 200 nm. This shows the suitability of both methods to remove larger particles from the sample. There is a small (just over 5%) percentage of larger particles up to 400 nm present in both samples; these are probably due to the aggregation of EVs of various phenotypes and morphologies, as already reported by Linares and colleagues.48

Figure 3.9. Particle size distribution represented in percentage, measured by NTA on FBS samples processed by either a 10k g centrifugation step [CENTRIFUGATION] or a 200 nm pore size filtration [FILTRATION] before being isolated by high speed centrifugation (100k g). Isolations were performed in at least 3 independent experiments; and the data shown is the mean size distribution per bin of all samples tested.
In terms of particle size, centrifuged samples had a 125.5 ± 3.1 nm hydrodynamic particle mean size, and filtered samples 127.9 ± 5.7 nm. Although there is no statistical difference between both samples, given that particle size distribution lies over a larger range the mean size might not be the most useful information to consider. The modal size should be taken into consideration instead. Little difference can be seen between the modal particle sizes from the two processing methods as well, which are found to be 101.8 ± 4.6 nm for centrifuged samples and 103.1 ± 5.4 nm for the filtered ones (Figure 3.10)

![Graph](attachment:graph.png)

**Figure 3.10.** Particle modal size measured by NTA on FBS samples processed by either a 10k g centrifugation step [CENTRIFUGATION] or a 200 nm pore size filtration [FILTRATION] before being isolated by high speed centrifugation (100k g). Isolations were performed in 3 independent experiments, with 3 replicates each.
Transmission electron microscopy was also employed to more closely visualise the isolated EVs (Figure 3.11).

![Figure 3.11. A representative TEM image of FBS EV isolates, with a demonstrative distribution of different sized particles.](image)

Figure 3.11 depicts cup-shaped, negative stained shrunken vesicles which are characteristic TEM of extracellular vesicles.\(^{49}\) The mean vesicle size is 70.6 ± 44.7 nm. These values are lower than the results obtained from the NTA analysis. The origin of this discrepancy has to do with the way particles are analysed. NTA measures the hydrodynamic diameter as the particles are in suspension, whereas TEM assays the sample on a dry substrate. The hydrodynamic diameter measured by NTA will be necessarily higher than dry TEM. A reduction in vesicle size due to TEM sample preparation has been reported elsewhere.\(^{4,50,51}\) Moreover, TEM measurement detects smaller particles not visualised on the NTA scan, due to an increased imaging resolution.\(^{2}\)
MSC conditioned media

To ensure the integrity and biological functionality of the recovered conditioned media, isolation was carried out either straight away or within a week. Initial low-g centrifugation steps (300 and 2,000 g), to remove cells and cell debris, were performed before storage as recommended elsewhere. Media was then either filtered through a 200 nm pore or centrifuged with a 10k g step. In contrast to the results obtained with FBS, there was a significant difference \((p < 0.02)\) in the final particle concentrations between MSC samples initially processed by centrifugation or filtration, as can be seen in Figure 3.12. Samples processed by an initial 10k g centrifugation step had an overall mean particle concentration of \(2.96 \times 10^{12} \pm 3.22 \times 10^{11}\) particles/mL, whereas filtration processed samples resulted in \(1.51 \times 10^{12} \pm 3.28 \times 10^{11}\) particles/mL, representing a 51% variation in concentration. It should be noted however that these NTA measurements were done on a 488 nm laser unit due to instrument unavailability. This change in instrumentation can have an impact, especially in the final concentration due to variation on detection sensitivity as it was previously mentioned earlier in this Chapter. Thus, values cannot be used for a direct comparison against the results obtained using a 532 nm laser on FBS samples or further isolations from MSC media.

The size distribution in the MSC pellet samples is depicted in Figure 3.13. There is a 3.9% difference in the isolated amounts of particles under 200 nm, with the filtration-processed samples being slightly more enriched in these particles. Over 80% of sample is sized under 200 nm, with the remaining percentage being mainly larger particles (around 15%).
Figure 3.12. Particle concentration measured by NTA (488 nm laser) on MSC conditioned media samples processed by either a 10k g centrifugation step [CENTRIFUGATION] or a 200 nm pore size filtration [FILTRATION] before being isolated by high speed centrifugation (100k g). Replicates were performed, and mean values taken for each condition. There is a significant difference between groups *p < 0.05.

Figure 3.13. Particle size distribution represented in percentage, measured by NTA (488 nm laser) for MSC conditioned media samples processed by either a 10k g centrifugation step [CENTRIFUGATION] or a 200 nm pore size filtration [FILTRATION] before being isolated by high speed centrifugation (100k g). Three replicates were performed, and mean values taken for each condition; and the data shown is the mean size distribution per bin of all samples tested.
Although a significant difference was found in the amount of particles isolated it did not seem to affect considerably the average size of particles obtained. The centrifuged samples had a mean $158.2 \pm 7.1$ nm hydrodynamic particle mean size and the filtered samples $150.2 \pm 9.8$ nm. The modal particle size did not differ much too, being $133.2 \pm 9.9$ nm for the centrifuged samples and $135.8 \pm 21.6$ nm for filtered ones (Figure 3.14).

**Figure 3.14.** Particle modal size measured by NTA (488 nm laser) on MSC samples processed by either a 10k g centrifugation step [CENTRIFUGATION] or a 200 nm pore size filtration [FILTRATION] before being isolated by high speed centrifugation (100k g). Three replicates were performed, and mean values taken for each condition.

Overall, the size of particles isolated by both methods is similar but there seems to be a higher number of vesicles isolated through having an intermediate centrifugation step. This leads to conclude that isolation with differential centrifugations is the preferred method for MSC conditioned media samples. It should also be noted that isolation of EVs via an intermediate filtration step was not easily attained. The filter used was easily clogged (with volumes of just 10 mL), which made the process of sample processing tiresome and led to a lot of sample being wasted. As a result, it was determined that the
The centrifugation approach was the most appropriate for MSC EVs and the reason why not more independent experiments were performed on the filtration processing route. This highlights the importance of assessing different isolation protocols and make a case by case assessment before choosing how to process samples. Here, it is evident with the difference in processing protocols chosen for FBS and MSC conditioned media samples.

Isolation of EVs was then assessed for the centrifugation streamline and a comparison between the initial batch and the following measured by nanoparticle tracking analysis with different lasers is in Figure 3.15.

![Figure 3.15](image_url)

**Figure 3.15.** Particle concentration measured by NTA with a 488 nm and a 532 nm laser on different batches of MSC conditioned media samples processed by a 10k g centrifugation step before being isolated by high speed centrifugation (100k g). Three replicates were performed, and mean values taken for each condition. Three independent experiments, with three replicates each, were done for samples measured by a 532 nm laser. There is a significant difference between groups (****p < 0.0001).

There is a significant difference on isolates particle concentration detected with a 488 nm or a 532 nm NTA laser (p < 0.0001), resulting in a 42% difference between samples. This reinforces the need to keep a consistent analytical approach to avoid incorrect assumptions.
When analysed with a 532 nm NTA instrument, MSC-derived EV isolates had a $1.25 \times 10^{12} \pm 3.90 \times 10^{11}$ particles/mL concentration, a mean particle size of $128.8 \pm 4.1$ nm and a modal size of $108.3 \pm 4.3$ nm.

It was impossible to compare the 10k and 100k g pellets as the former was not sufficiently compacted and was therefore difficult to retrieve it completely at the end of the step. This is an issue already known for fixed angle rotors, as particles reach the tube wall and continue gliding down.\textsuperscript{18} This loose pellet required samples to be filtered through a higher pore size filter (450 nm) to remove possible resuspended pellet pieces, before proceeding to the high speed centrifugation step.

Morphological analysis via TEM was also performed on MSC conditioned media samples, and an exemplar image can be seen in Figure \textbf{3.16}. TEM images of MSC cell culture media isolates, similarly to FBS EV samples, show cup-shaped, negative stained shrunken vesicles characteristic of EVs.\textsuperscript{49} Vesicles processed by an intermediate centrifugation step had a $119.3 \pm 56.7$ nm average diameter and through a filtration workflow $87.9 \pm 43.7$ nm. There was no significant difference between groups. It should be noted that the definition of what is and is not extracellular vesicle particles in TEM analysis is an extremely subjective idea. There is then, some possible user error in selecting the particles to measure on the TEM images and care must be taken when using the size values provided this way. There seems to be a certain background amount of amorphous particles in TEM images of MSC EVs samples, likely due to the presence of pelleted proteins and/or ribonucleoprotein aggregates.
Figure 3.16. A representative TEM image of MSC EV isolates processed by either a (a) 10k g centrifugation step or a (b) 200 nm pore size filtration before being isolated by high speed centrifugation (100k g), with a demonstrative distribution of different sized particles.
3.3.2.2. Protein Content

Total Protein Assay Selection

Protein characterisation methods such as Bradford and BCA have been widely employed in the EV field. The reasoning to choose between one and another is not exactly clear in most studies, and a careful approach needs to be taken to decide which assay might suit the application better.

The Bradford assay is widely used to determine protein content because of its rapid and convenient protocol. However, it has been reported to underestimate protein concentration in membrane containing samples and more recently in EV isolates. This can be linked to the relatively low sensitivity of common protocols, and the use of an optimised protocol might improve the method characteristics. The working concentration range for Bradford at which there is linearity is narrow and usually at high protein contents, from 2 to 10 mg/mL. This requires unknown samples to fall within the limited working range. This nonlinearity might be the reason why Bradford is not sometimes chosen by researchers in their EV research, as samples are usually scarce and dilute. Zor and Selinger (1996) proposed an alternative Bradford methodology that increases the assay sensitivity and is thus able to be applied to more diluted samples. Briefly, they suggest measuring absorbance at two wavelengths 590 and 450 nm instead of the usual 590 nm. The protein-dye complex has an absorbance maximum at 590 nm whereas the free dye at 450 nm. As increasing quantities of protein are added to the system, reduction of the 590 nm background can be calculated by measuring the change of absorbance at 450 nm, where the protein-dye complex does not absorb. This results in a linear relationship between protein concentration and the ratio of absorbance measurements, 590 nm over 450 nm.
Furthermore, a comparison between the standard Bradford method and Zor’s revised protocol for lower concentrations of a control protein, bovine serum albumin (BSA), can be seen in Figure 3.17.

![Absorbance measurements of BSA up to a concentration of 10 µg/mL with (a) the standard Bradford method and (b) Zor and Selinger’s modified method.](image)

Three replicates were performed and mean ± standard deviation values taken for each point.
Zor’s modification allowed the fitting of a linear curve for concentrations between 0.5 and 10 µg/mL of protein. This shows that accurate detection of lower protein contents is possible with the modified Bradford method, and this can thus be used for determination of the total protein content of EV samples.

As an alternative to Bradford, the bicinchoninic acid (BCA) assay has been widely used. This allows the use of buffers and detergents without interfering with the detected protein content, which is a significant advantage over other protein assay procedures. BCA thus allows the measurement of the protein content inside vesicles. However, BCA has been reported to show overestimation of protein content in the presence of lipids and lipoproteins. This poses issues when assaying EVs as they include lipids in their composition. Morton and Evans suggested the addition of 2% sodium dodecyl sulphate (SDS) to overcome the lipid interference.\textsuperscript{54} In the literature, there does not seem to be a clear consensus as to whether the Bradford or BCA assays is more appropriate, so a comparison of both assays was done for both FBS and MSC samples (Figure 3.18).

There was no significant difference in the detected protein content between any of the experimental conditions tested for FBS EV isolates (Figure 3.18a). There was however significant differences in MSC EV isolate protein content under the tested experimental conditions ($p < 0.05$; Figure 3.18b). Regardless of the samples being lysed or not, the addition of SDS does not seem to affect the detected protein content. This either can be related to the lack of lipidic component interference with the assay (due to a low lipid concentration, for example), or the addition of SDS being incapable of reducing the interference of lipids in the first place in these samples. Surprisingly, lysed samples presented lower readings than non-lysed ones. This might possibly be due to the presence of proteases in solution, which could digest the proteins in the sample. Bradford readings were generally lower than the ones obtained using the BCA method, corroborating what
has been found previously in the literature.\textsuperscript{12} Since no conclusive trend was observed in the different conditions tested with the BCA method, and that the same results were not found on both isolates, it is hard to select an approach that would work for all samples.

It was decided to use the Bradford method for all samples for characterisation purposes even though there might be an underestimation of the concentration of total protein present. It has been the recommendation of ISEV that consistency in the analysis is key and as long as the protocol is completely defined comparison between samples can be reached.\textsuperscript{2}

Electing the Bradford method as the assay of choice for isolated EV characterisation is not without further optimisation. It has been suggested that the isolation of proteins from samples can be used as a way to reduce the influence of contaminants in protein quantification assays.\textsuperscript{55} One such method is through precipitation using acetone. Some experiments were performed to explore this however with no success, so such methodologies were abandoned.
Figure 3.18. Protein content of (a) FBS and (b) MSC EV isolates measured by BCA and Bradford assays. Different experimental conditions were tested for the BCA assay: with or without a lysis step, and in the presence or absence of 2% SDS. Three replicates were performed and mean ± standard deviation values taken for each sample.
**Foetal Bovine Serum**

The total protein content on isolated samples does not vary significantly between the two processing methods (Figure 3.19), with $8,032 \pm 2,070 \mu g$ of protein per mL obtained for the 10k $g$ centrifuged samples and $7,916 \pm 2,161 \mu g/mL$ for the filtered isolates. FBS EVs samples mean protein content amounted to less than 1.5% variation between the two processing methods (see Figure 3.20). Thus, the final protein content does not seem to be dependent on the pre-processing stages.

**Figure 3.19.** Protein content measured by Bradford assay on FBS isolates processed by either an initial 10k $g$ centrifugation step [CENTRIFUGATION] or a 200 nm pore size filtration [FILTRATION] before being isolated by high speed centrifugation (100k $g$). Isolations were performed in 3 independent experiments, with 3 replicates each.
It is important to characterise samples appropriately as functional assays are not only confounded by particle concentration but also by the specific amount of protein. Differences on the quantitative results between detection methods have previously been noted in the literature. It is hence important to clearly specify the protein quantification method used to ensure reproducibility between research groups can be achieved. There is to date little consensus in the most appropriate protocols for EV characterisation, especially when considering isolate purity. One of the proposed indicators in use is the particle to protein ratio, which gives a measure of any protein contamination of samples. As described by Webber and Clayton, isolates with a ratio of $3 \times 10^{10}$ particles per µg of protein or greater are considered to be of high purity. The calculated particle to protein ratio for FBS isolates is represented in Figure 3.21.
Figure 3.21. Particle to protein ratio calculated from particle concentration and protein content measured by nanoparticle tracking analysis and Bradford assay of FBS isolates processed by either an initial 10k g centrifugation step [CENTRIFUGATION] or a 200 nm pore size filtration [FILTRATION], before being isolated by high speed centrifugation (100k g). Isolations were performed in 3 independent experiments, with 3 replicates each.

Samples processed by either workflow do not show any significant difference ($p > 0.05$) in their particle to protein ratio. Both have values below the suggested limit defined by Webber and Clayton.\textsuperscript{42} Centrifuged EV isolates demonstrated a particle to protein ratio of $1.37 \times 10^8 \pm 3.92 \times 10^7$ particles/µg of protein and samples processed with a filtration step have $1.41 \times 10^8 \pm 3.84 \times 10^7$ particles/µg of protein. It should be noted that this approach has its own shortcomings as it assumes that all particles detected by nanoparticle tracking analysis are EVs which is often not accurate. Protein aggregates, large salt crystals and other components might be present and provide an overestimation of the true EV number. Another of this measurement caveat is linked to the assumption that each vesicle contains the same amount of protein, which is also unlikely. Nevertheless, and bearing these issues in mind, particle to protein ratio is still a useful quantitative approach to establish the relative purity of vesicles preparations. It is thus clear that the particle to protein ratio of samples is below the high purity threshold defined by
Webber and Clayton. It should also be noted that differential centrifugation protocols often produce samples with a higher content of co-isolated non-vesicular macromolecule contaminants. It is then not surprising that a lower particle to protein ratio is obtained in samples processed by UC.

In addition to measurement of total protein content, the presence of specific proteins characteristic of exosomes were assayed. Tetraspanins such as CD63, CD9 and CD81 are often found on EVs and were tested on FBS isolates. FBS isolates were found to possess all three tetraspanins.

**MSC conditioned media**

Similarly to FBS isolates, MSC-derived EVs were also assessed for their total protein content. No significant variation on protein content was registered on samples isolated by either one of the two processing methods as depicted in Figure 3.22. Samples with a 10k g centrifugation step had a $1,380 \pm 524 \, \mu g$ of protein per mL whereas filtered samples demonstrated a $1,044 \pm 169 \, \mu g/mL$. Hence, the final total protein content, as seen already with the FBS samples, does not seem to be dependent on the pre-processing stages.

An ELISA assay allowed the identification of specific extracellular vesicles markers such as the tetraspanins CD63, CD9 and CD81. MSC-derived EVs were particularly concentrated in CD81. This is a marker that is exclusively detected in small EVs. It was detected some CD81 in the supernatant from the isolation procedure, possibly from small particles still in suspension that were not able to be isolated. In fact, in a study from Cvjetkovic and colleagues it was found that after a 70 minute UC protocol, supernatants still contained significant quantities of remaining EVs in suspension. However, it should be noted that in the MSC EVs samples this amount was vestigial as there was a 71% difference.
in the detected protein from the isolate to the supernatant control. Samples also expressed CD63 and CD9. In fact, it is important to note that the single presence of CD81 without CD63 it is not enough to qualify the existence of exosomes in the sample.\textsuperscript{58} CD63 was found to be 51% more present in the isolate when compared to the supernatant control. The higher presence of this marker in the supernatant can be attributed to the presence of other vesicles.\textsuperscript{58} MSC isolates were found to express all markers specific to extracellular vesicles hence, providing the confidence that these vesicles were isolated from the culture media.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{protein_content.png}
\caption{Protein content measured by Bradford assay on MSC-derived isolates processed by either initial 10k g centrifugation step [CENTRIFUGATION] or a 200 nm pore size filtration [FILTRATION] before being isolated by high speed centrifugation (100k g). Isolations were performed in 3 independent experiments, with 3 replicates except for filtration samples due to processing constrains.}
\end{figure}
To assess the relative purity of isolated samples and to compare between FBS and MSC isolated EVs, the particle to protein ratio of samples was determined and compared (Figure 3.23). A significant difference ($p < 0.0001$, t-test) was found between the isolates. MSC-derived EV samples were found to have a higher ratio of $1.25 \times 10^9 \pm 2.23 \times 10^8$ particles/µg of protein when compared to the $1.41 \times 10^8 \pm 3.85 \times 10^7$ particles/µg of FBS samples. This 89% increase in particle to protein ratio reflects the presence of less contaminants in the MSC-derived EV samples after isolation.

![Figure 3.23](image.png)

**Figure 3.23.** Particle to protein ratio calculated from particle concentration and protein content measured by nanoparticle tracking analysis and Bradford assay of FBS and MSCs-derived EVs isolated by the preferred UC protocol. FBS samples were processed with an initial 200 nm pore size filtration whereas MSC ones via a 10k $g$ centrifugation step, before both being isolated by high speed centrifugation (100k $g$). Isolations were performed in 3 independent experiments. There is a significant difference between groups (****$p < 0.0001$).
3.3.2.3. Potency Assay

Extracellular vesicles, as discussed previously, have been shown play a major role on the activation of pro-survival mechanisms and reduction of cardiomyocyte apoptosis in cardiovascular disease models.59,60 Here, FBS (Figure 3.24) and MSC-derived (Figure 3.25) isolated EVs were incubated with stressed cardiomyocytes to assess their pro-survival effects.

![Figure 3.24](image)

**Figure 3.24.** Cell viability of rat cardiomyocytes after incubation with FBS EVs. Fluorescence results were normalized against a positive control of non-stressed cells in a complete media (containing serum). Cardiomyocytes were serum deprived and incubated for 27 hours before cell viability was determined. At least three independent experiments were performed, and individual values are presented with the mean effect calculated across all samples. Statistically significant differences are indicated with p-values, which are * unless otherwise detailed: **p < 0.01; ****p < 0.0001.
As shown in Figure 3.24, serum deprivation of H9c2 rat cardiac cells led to a dramatic decrease in more than 50% in the number of viable cells. It was found that FBS EV isolates promoted survival of these stressed cardiac cells. With the addition of EVs a dose-dependent cellular viability was registered reaching approximately 90% viable cells after $1 \times 10^{11}$ particles were added in a total media volume of 400 µL.

Analogous experiments were performed with MSC-derived EVs, and again a dose-dependent response to the addition of EVs to stressed cardiomyocytes was noted (Figure 3.25).

**Figure 3.25.** Cell viability of rat cardiomyocytes after incubation with MSC EVs. Fluorescence results were normalized against a positive control of non-stressed cells in a complete media (containing serum). Cardiomyocytes were serum deprived and incubated for 27 hours before cell viability was determined. At least three independent experiments were performed, and individual values are presented with the mean effect calculated across all samples. Statistically significant differences are indicated with p-values, which are * unless otherwise detailed: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. 
There are up to 86% viable cells when $5 \times 10^{10}$ particles of MSC conditioned media are added to stressed cardiomyocytes. The data also shows a nearly 15% mean increased survival effect upon administration of MSC-derived EVs when compared to the same number of FBS EVs added. Thus, showing that MSC EVs can be discretely more efficient in promoting survival upon stressing conditions than FBS EVs. Such result might have to do with the specific cargo inside the isolated vesicles.

3.3.2.4. Stability under Storage

There is no consensus on whether storage has an impact on the quality and yield of isolated EVs. To understand whether isolated EVs can be formulated, their stability under different storage conditions needed to be assessed. Three different temperature conditions (-80°C, 4°C and 20°C) that are likely to be used in the formulation workflow for EVs were investigated. This study was carried out on FBS EVs for one month. The effect of aging was assessed by nanoparticle tracking analysis for particle size (Figure 3.26) and total particle concentration (Figure 3.27), and Bradford assay to determine total protein content (Figure 3.29).

The modal particle size was shown to be stable over the course of the 4 weeks, regardless of the storage conditions with no significant difference between the different time points (Figure 3.26). Storage at -80°C rendered at most a 3.6% variation on modal particle size while 7.3% and 6.0% were attained for 4°C and room temperature, respectively. Higher variations were detected for 4°C and 20°C however a shift inferior to 10% can hardly be significant indicating that EVs appear to be stable at all tested storage conditions for at least one month.
Figure 3.26. Modal particle size measured by nanoparticle tracking analysis on FBS EVs over the course of one month under different storage conditions: (a) -80°C, (b) 4°C and (c) room temperature (20°C).
Figure 3.27. Particle concentration measured by nanoparticle tracking analysis on FBS EVs over the course of one month under different storage conditions: (a) -80°C, (b) 4°C and (c) room temperature (20°C). Statistically significant differences are indicated with p-values, which are * unless otherwise detailed: *p < 0.05; **p < 0.01; ***p < 0.001.
There is a general trend for the particle concentration to decrease upon storage (Figure 3.27). Storage at -80°C seems to produce the least change in concentration over time, with variation being significant only after 3 weeks. In contrast, at 4 and 20°C there is a significant change in particle concentration after one week’s storage. Given that the modal size was the same throughout, and the particle size distribution is also roughly the same throughout (Figure 3.28), the decrease in concentration can only be attributed to the loss of particles of smaller sizes or, in the case of room temperature storage, to some aggregation. The NTA system used has some limitations in detecting particles at the lower end of the size range; hence, if the EVs shrink or break down into smaller particles the overall concentration of the samples stored is reduced.

The protein content was also assessed for all samples across the whole four week storage period, with the Bradford assay (Figure 3.29). This method was applied as it measures total protein content in the sample with no lysis of vesicles required prior to the assay. This allows the detection of any possible increase in protein content in the event of vesicle disruption with storage.

There was no significant difference in the total protein content of the EV samples regardless of the storage conditions applied. This indicates that major degradation of the proteins in the sample did not occur at tested temperatures. Also, it means there was no disruption of the vesicles in the samples. This reiterates the hypothesis that changes in particle concentration are not due to the breakdown of the EVs, but rather to their reducing in size below the NTA tracking limit.
Figure 3.28. Particle size distribution measured by nanoparticle tracking analysis on FBS EVs over the course of one month under different storage conditions: (a) -80°C, (b) 4°C and (c) room temperature (20°C).
Figure 3.29. Total protein content measured by Bradford assay on FBS EVs over the course of one month under different storage conditions: (a) -80°C, (b) 4°C and (c) room temperature (20°C).
EV isolates were also stored at a higher temperature condition (37°C) to assess their stability under physiological conditions. This is particularly relevant to understand the feasibility of developing formulations that stay implanted for longer periods of time. In our study, the particle concentration increased by almost 65% after 2 weeks of storage. This is expected due to evaporation of the suspension solvent, and hence concentration of the vesicles in the sample. Sokolova and colleagues previously reported a vesicle size reduction of roughly 50% after 48 h at 37°C.\textsuperscript{61} However, no significant difference on particle modal size was seen over the course of two weeks storage. There is however a 25% increase in the total protein content detected after one week of storage. The increase in protein content is tightly related with the increase in sample concentration and does not necessarily represent the leak of proteins from vesicles.

The effect of storage conditions on EV morphology and potency has been widely explored. However, the literature is not in agreement as to the effect of temperature and storage time. A study by Kalra and colleagues investigated the storage of plasma EVs at -80°C, -20°C and 4°C and confirmed their stability up to 90 days across all storage conditions.\textsuperscript{62} A decrease in EV number was noted at 4°C, but not at lower temperatures. On the other hand, Lőrincz and co-workers found that storage at 4°C and 20°C (room temperature) resulted in a significant decrease of EV numbers and vesicles potency after just 1 day.\textsuperscript{63} There is a general consensus that storage at lower temperatures will have a protective effect on vesicles, and -80°C storage is generally regarded as most appropriate since it preserves EV integrity and function.\textsuperscript{30,63,64} One of the concerns of storing samples at lower temperatures however is the effect that repeated freeze-thaw cycles might have on EV integrity. A recent study showed that multiple freezing and thawing had no effect on the biophysical properties of exosomes stored at -20°C and -80°C.\textsuperscript{61} It should be noted however, that some disagreement on EV stability across the literature have been
reported.\textsuperscript{29} Translation of stability data for EVs from different sources might also not be possible, as the isolated vesicles will vary in surface lipid and protein composition as well as their internal content. Such potential variability has been investigated previously for liposomes, where the specific surface composition was found to have a critical influence on sample stability.\textsuperscript{65}

Overall, there was not much difference between the storage conditions tested on vesicle morphology and protein content. It can be concluded that -80°C is adequate to store our samples and that processing at room temperature does not exert a significant deleterious effect on samples. This allows the handling of EVs while formulating without the fear of losing their potency.
3.4. Conclusions

One of the challenges in extracellular vesicle research lies in the absence of a standardised isolation and characterisation protocol. A tailored ultracentrifugation protocol was developed and validated for foetal bovine serum (FBS) and mesenchymal stem cell (MSC) EVs. In this study, the processing parameters were adapted to the samples tested by measurement of their specific density and viscosity. This allows the tuning of processing conditions to ensure the deposition of the appropriate sized particles at each step. Reproducible isolations were attained as a result of such optimisation. While a filtration step was found to be adequate for FBS samples, a pure differential centrifugation protocol had to be applied to MSC culture media samples due to membrane clogging.

Isolated EVs from both FBS and MSC conditioned media were characterised in terms of size, concentration, total protein content, presence of specific protein markers and assessed for their functional performance in vitro after isolation. The isolates were found to be in the expected size range for exosomes, with consistently more than 80% of the sample sized up to 200 nm; their concentration and protein contents were also determined. All samples were found to express the tetraspanins CD63, CD9 and CD81, indicative of the presence of extracellular vesicles. All isolates were found to have a positive effect on cardiomyocyte survival after stress, but MSC samples were found to be discretely more potent than FBS at the same concentration. This indicates the superior influence of MSC extracellular vesicles in the regeneration of cardiac cells, and their applicability in cardiac therapeutics.
Moreover, isolated FBS EVs were shown to be relatively stable at a range of temperatures (-80°C, 4°C and 20°C) over one month period. This revealed little effect of the storage temperature over time, with the protein content and size being maintained over the course of a month. The fact that the EVs are stable, and especially at room temperature, provides confidence that EVs can be formulated without degradation due to detrimental environmental conditions.
3.5. References


Chapter 4

Fast-dissolving Electrospun Fibres
4.1. Introduction

As a first step to formulate extracellular vesicles (EVs) it is necessary to understand that these are a combination of biological components ranging from lipids and proteins to nucleic acids. Such biomolecules are frequently fragile and easily degraded. The individual components of EVs have been the subject of extensive research to determine the best formulation strategies. It is then vital to carefully assess the formulation approach considered in order to ensure it does not affect the morphology or functionality of the active components.

4.1.1. Electrospinning of Biomolecules

Biological materials have previously been successfully encapsulated in electrospun fibres. For example, DNA has been encapsulated to be applied as a gene therapy. DNA was found to be released from the electrospun scaffolds intact, capable of cellular transfection and encode the production of the desired protein after processing. Proteins have also been successfully electrospun, with β-nerve growth factor being released from a copolymer of poly(ε-caprolactone) and ethyl ethylene phosphate scaffolds in a sustained manner over at least 3 months, and with its bioactivity retained.

Moreover, living biological material such as viruses, bacteria and mammal cells have recently been encapsulated via electrospinning. Virus dispersions have successfully been electrospun with the water soluble poly(vinylpyrrolidone). The resulting fibres maintained their ability to infect bacterial hosts after being resuspended in a buffer solution. In fact, the first report of cell electrospinning was in 2006 by Townsend-Nicholson & Jayasinghe. The authors described for the first time the use of a coaxial setup to process an highly concentrated cellular suspension (10⁶/mL) of human 1321N1 astrocytoma (brain
tumour) cells in culture medium as the core solution. It was found that cells remained viable post-electrospinning and no statistical differences were observed between the viability of post-electrospun and that of control cells passed through a needle but without any potential difference applied. A follow-up study further demonstrated the ability of cells at even higher concentrations ($10^7$/mL), to be processed by electrospinning with no loss of viability.\(^{10}\) Moreover, cell-loaded fibres have successfully been grafted into mice, and cells ability to proliferate \textit{in vivo} was found to be unaffected by the electrospinning process.\(^{11}\) A range of other cell types has been electrospun, with a number of resultant applications. For instance, Ang and colleagues have entrapped human umbilical vein endothelial cells in polyethylene oxide and polycaprolactone electrospun fibres, and showed that cells were viable for over two weeks post-processing.\(^{9}\) Likewise, cardiac myocytes have been electrospun into fibres with potential applications in cardiac tissue engineering.\(^{8}\)

Such findings show that polymer-based electrospun fibres and complex biological components can be formulated without total loss of biological functionality. Here we will explore whether this extends to the use of electrospinning for extracellular vesicles formulation.

### 4.1.2. Immediate Release Scaffolds

Fast-dissolving systems have become increasingly important as formulation strategies for drug delivery.\(^{12,13}\) Traditional immediate release formulations possess a highly porous matrix, which proves to be of utmost importance when fast dissolution is required as they allow the increase in surface contact area with the solvent. Several technologies have been developed to achieve this highly porous network, such as spray drying, freeze-drying, 3D printing and others.\(^{14,15}\) Electrospinning counts itself as one of the techniques now widely applied to achieve immediate release formulations.\(^{16}\) Drugs amorphously
dispersed in the polymer matrix have been found to possess long term stability when formulated by electrospinning, due to polymer chains been found to provide steric hindrance to drug recrystallization. This is especially interesting if the aim is to formulate scaffolds that can be used as an off-the-shelf product. In fact, Lopez and colleagues found that poly(vinyl pyrrolidone) scaffolds loaded with griseofulvin, an antifungal agent that is known to readily recrystallise, were stable up to 8 months stored in a desiccator at room temperature.\(^{17}\) The potential of using these formulations to produce scaffolds that can be later reconstituted and used when needed is enormous. Moreover, solid formulations are a good alternative to overcome the instability of liquid-based products. It should be noted that to our knowledge there is no information regarding the use of such approach for biomolecules.

Hence, more than 40 different types of polymers have been used to fabricate nanofibres through electrospinning.\(^{18}\) However, the process of choosing a polymer as a formulation vehicle needs to be carefully considered. Properties such as biocompatibility are essential when scaffolds have a therapeutic application. Thus, electrospun biodegradable polymer fibres have received a lot of interest due to potential applications in biomedical fields.\(^{19}\) Two such polymers are poly(ethylene oxide) and poly(vinyl pyrrolidone).

Poly(ethylene oxide) (PEO) is a well-known synthetic polymer with potential applications including soft tissue engineering and drug delivery due to its biocompatible properties and ability to be easily electrospun.\(^{20,21}\) Moreover, it is amphiphilic and therefore soluble in water, as well as in some other organic solvents such as ethanol and acetone. High solubility in aqueous solutions is a desirable characteristic for immediate release fibres.\(^{16}\) Fibres produced from water soluble fast-dissolving polymers will experience an immediate release when in an aqueous medium due to the high surface-area-to-volume
ratio of electrospun fibre scaffolds, allied to their high porosity. The high porosity allows water to infiltrate rapidly and the high surface area accelerates the dissolution process.

Poly(vinyl pyrrolidone) (PVP) is a synthetic amorphous polymer with high solubility both in water and in many organic solvents. It presents low chemical toxicity, high hydrophilicity, and excellent physiological compatibility and biocompatibility. These characteristics make it a suitable biomaterial to use in numerous medical and non-medical applications ranging from the pharmaceutical industry and medicine to optical and electrical applications, among others. PVP has been previously electrospun into fibres numerous times. Moreover, a range of PVP-based fast-dissolving drug delivery systems have been developed. These have been reported to include active ingredients such as vitamin D, ketoprofen, irbesartan, and curcumin, among others.

The hypothesis for understanding the feasibility of applying electrospinning as a formulation technique for extracellular vesicles is yet to be determined and this chapter intends to prove it. Not only the use of fast-dissolving formulations allows the analysis of EVs characteristics and potency post-electrospinning more easily it could have a potential application in extending the stability of EV-formulations in the solid state.
4.2. Aims

This chapter is a proof-of-concept study aiming to demonstrate the feasibility of using electrospinning as an extracellular vesicle formulation approach. Electrospun fibres of fast-dissolving materials were formulated and prepared to allow the rapid evaluation of EV stability and potency post-processing. A detailed characterisation of the formulations was undertaken, including evaluation of morphology, physicochemical properties and biological activity.
4.3. Results and Discussion

4.3.1. Initial Formulation Attempts

Polyethylene oxide was initially chosen as the polymer to formulate fast-dissolving extracellular vesicle-loaded scaffolds. A preliminary study was first performed to assess whether fibres could be produced, and vesicles loaded easily. A representative image of fibres obtained can be seen in Figure 4.1 along with the diameter distribution of samples.

![Figure 4.1. Scanning electron microscopy images of (a) electrospun PEO fibres and (b) analogous FBS EV-loaded fibres. Histograms of fibre diameter distribution are depicted next to the respective formulation. Diameter size measurements were performed using Image J [(a) n = 75 and (b) n = 100].](image-url)
Blank PEO fibres were found to have an average diameter of $905 \pm 352$ nm, whereas $791 \pm 152$ nm was obtained for the EV-loaded ones. It is possible to detect a significant reduction ($p < 0.0041$, t test) on the fibre diameter when EVs are added to the formulation. Such a reduction in size could be due to a conductivity difference in the electrospinning solutions. Charge repulsion at the surface of the electrospinning jet causes the solution to stretch more and hence reduce the final diameter of the fibres. A reduction in fibre diameter has previously been reported for PEO with increasing conductivity of the spinning solution. Addition of vesicles to the polymer solution is expected to have increased its conductivity due to the negatively charged lipidic membranes of EVs. DNA for instance has been previously reported to significantly increase the conductivity of polymer solutions more than 20-fold. No extracellular vesicles could be observed on the SEM images, probably due to their size being at the limit of detection of the instrument used and full encapsulation within fibres.

It proved impossible to assess the produced PEO formulations for EV-loading or potency since the polymer did not dissolve completely in aqueous media or phosphate buffered saline. One of the parameters at play here could be the relatively high molecular weight polymer used as it is known that molar mass affects solubility of materials. The high molecular weight results in polymer molecules being very long, hence extensive entanglements between molecules in the fibre will take time to unravel. An incompletely dissolved polymer could potentially skew the final loading and potency results as there might be EVs still entrapped in the polymer matrix. These observations led us to conclude that although the used PEO is considered to be soluble in aqueous media, it was not the ideal model polymer to use for this study.
The use of other hydrophilic polymers, such as poly(vinyl pyrrolidone), was therefore considered for the production of extracellular vesicle-loaded scaffolds. Blank PVP fibres were first produced and attempts made to dissolve them in phosphate buffered saline. The scaffolds fully dissolved within a few seconds even in small volumes of PBS, and thus PVP was chosen for the preparation of fast-dissolving scaffolds.

4.3.2. Poly(vinyl pyrrolidone) Electrospinning Optimisation

Before loading EVs into the scaffolds it was necessary to assess which parameters would produce the best spinning conditions. Several parameters are crucial to the process success, including the polymer concentration, voltage and distance to collector. It has been reported elsewhere that PVP concentrations ranging from 6 to 12% (w/V) in ethanol are regarded as optimal for 1,300 kDa polymers. And although the Mw of polymer actually used is lower, those parameters functioned as good starting points. Polymer solutions in ethanol were prepared initially, but addition of EVs led to precipitation. Ethanol is used as part of a number of nucleic acid and protein precipitation protocols, which might explain the precipitation observed when EVs are added. As such, there is a need to choose a different solvent system. 2,2,2 trifluoroethanol (TFE) has been successfully used to electrospin gelatin and has been noted for its biocompatibility, conductivity and high dielectric constant. In fact, Roccatano and colleagues found that addition of TFE to peptide mixtures in water promoted stability rather than inducing denaturation.

Therefore, several PVP concentrations ranging from 2 to 12% (w/V) in TFE were prepared and electrospun with different processing parameters (voltage and distance to collector) to assess which combination produced the most homogeneous fibres. The flow rate was not varied here in order to simplify the optimisation procedure. Lower flow rates are usually recommended in electrospinning to allow enough time for polarization of the
polymer solution, while high rates will result in beaded fibres with thick diameters (mainly due to the reduced drying time prior to reaching the collector and low stretching forces). It was decided to keep the flow at a constant rate of 1 mL/h throughout the optimisation stage. Such rate was already reported in different publications on PVP electrospinning as being optimal.40,41 In Figure 4.2 and Figure 4.3 representative images for each set of conditions tested are depicted.

Only at lower polymer concentrations of 2% (w/V) the formation of beaded fibres was observed due to low solution viscosity, which was not enough to prevent Coulombic-repulsion and caused the partial breakup of the charged jet (Figure 4.4 (a)). All other conditions tested produced fibres with different diameters and size homogeneity; summary plots depicting the effect of different parameters on fibre diameter and relative standard deviation are presented in Figure 4.4.

It is possible to infer from the plots that fibre diameter is largely independent of voltage, while both polymer concentration and distance to collector have a greater influence on the final diameter. At higher polymer concentrations and lower distances to collector the thickest fibres from the conditions tested can be found, with mean values of around 1,500 nm. Similar findings have been previously found elsewhere.26,42 With an increase in polymer concentration not only more material is being expelled per unit of time, more chain entanglements and a concomitant increase in viscosity is registered.33 The augmented viscoelastic force as the polymer concentration increases prevents the jet segment from being stretched by the constant Coulombic force, resulting in the obtained fibres having larger widths.
Figure 4.2. Scanning electron microscopy images of PVP fibres produced at different processing conditions with variation of the voltage (V, kV), polymer concentration (C, % w/V) and distance to collector (D, cm) in TFE. Flow rate was kept constant at 1 mL/h for all samples. Images correspond to fibres produced from 2, 5 and 8% w/V PVP solutions in TFE at the following processing conditions: (a) V9C2D13.5, (b) V8C5D12, (c) V8C5D18, (d) V10C5D15 (e) V12C5D12, (f) V9C8D13.5 and (g) V10C8D15.
Figure 4.3. Scanning electron microscopy images of PVP fibres produced at different processing conditions with variation of the voltage (V, kV), polymer concentration (C, % w/V) and distance to collector (D, cm) in TFE. Flow rate was kept constant at 1 mL/h for all samples. Images correspond to fibres produced from 10 and 12% w/V PVP solutions in TFE at the following processing conditions: (a) V8C10D15, (b) V10C10D15, (c) V8C12D12 and (d) V12C12D18.
Figure 4.4. Ternary plot depicting the effect that variation of polymer concentration, distance to collector and voltage have on the (a) fibre diameter, and the (b) relative standard deviation of PVP fibres prepared from TFE. Polymer concentration varied from 2 to 12% (w/V), distance to collector from 12 to 18 cm and voltage from 8 to 12 kV. Flow rate was kept constant at 1 mL/h.
However, it should be noted that the average fibre diameter does not provide any information on the homogeneity of the sample and the variation of thicknesses: relative standard deviation, RSD, should be looked at instead. Looking at Figure 4.4 (b) there is a clear region situated in the middle of the overall set of parameters tested that produces the smallest variation in diameter. Figure 4.5 shows the variation of fibre diameter with the polymer concentration, regardless of distance to collector or voltage applied.

![Variation of the mean diameter of PVP electrospun fibres produced with polymer concentrations ranging from 2 to 12% w/V in TFE. Diameters were measured from SEM images using ImageJ (n=100).](image)

The concentration of polymer is the main parameter influencing the fibre diameter, as different combinations of potential and distance to collector do not seem to change this trend one way or another. At a concentration of 5% (w/V) the smallest relative variation is attained, and smooth uniform fibres are produced. This was thus considered to be the optimal concentration to work with.

The variation of fibre diameter with distance to collector (Figure 4.6) and voltage applied (Figure 4.7) were also plotted.
CHAPTER 4 – Fast-dissolving Electrospun Fibres

Figure 4.6. Variation of the mean diameter of PVP electrospun fibres with the distance to collector, ranging from 12 to 18 cm. Diameters were measured from SEM images using ImageJ (n=100).

Fibre diameter appears to be independent of distance to collector. Generally increasing the distance allows the fibres sufficient time to elongate before reaching the collector, however distances either too close or too far will incur in fibres with a non-uniform size. Often this parameter is tightly dependent on the polymer concentration and applied potential.

Figure 4.7. Variation of the mean diameter of PVP electrospun fibres with the applied voltage ranging from 8 to 12 kV. Diameters were measured from SEM images using ImageJ (n=100).
Similarly, there is no clear variation in fibre size with the applied potential. The literature reports that increasing voltage can result in both narrower fibres or thicker ones, much dependent on the other variables at play.\textsuperscript{33,44}

The optimisation studies revealed fibre diameter and relative standard variation to be dependent on the feeding solution concentration with 5\% (w/V) being the condition that provided the best results. And although fibre morphology is not directly dependent on distance to collector and applied voltage these were defined at 18 cm and 8 kV, respectively.

### 4.3.3. Extracellular Vesicle-loaded Fibres

As electrospinning with a 5\% w/V PVP solution rendered the most uniform fibres, FBS EVs were initially loaded in this polymer concentration. However, it was impossible to spin as the addition of the EVs suspension in phosphate buffered saline diluted out the polymer, reducing its concentration, and the resultant solution became unspinnable. The failure to successfully electrospin at these concentrations may be attributed to the reduction of the overall solution viscosity. It was then decided to increase the polymer concentration to 10\% w/V for EV loading experiments. Fibres were initially produced and tested with FBS EVs to assess the feasibility of the process, and later generated with more therapeutically relevant vesicles derived from MSCs. The feeding solution of 10\% (w/V) PVP was loaded with $3.75 \times 10^{11}$ EV particles/mL in both cases and their morphology can be seen in the representative SEM images in Figure 4.8.

Smooth fibres were obtained for both blank and EV-loaded PVP samples. There was a reduction in fibre diameter with the addition of EVs, from $1,396 \pm 235$ nm to $455 \pm 188$ nm for FBS-loaded samples and to $400 \pm 121$ nm for MSC-loaded ones. This
This reduction could be due to a conductivity difference in the electrospun solutions. Thus, and as already discussed for the PEO fibres, the addition of EVs to the polymeric solution most likely increased solutions conductivity. A reduction in fibre diameter has been found for PVP elsewhere when the electrical conductivity is increased. Regardless, it is important to have a homogeneous system, which in this case translates as a uniform size distribution in order to get reproducible performance.

Other characterisation techniques were then employed to assess the formulated fibres. FTIR was employed to explore the presence of extracellular vesicles in the fibres. From the IR spectra it is possible to gather general information concerning the molecular constituents and their structures in a mixture or formulation. It was hypothesised that EVs might be detected within the formulation since FTIR has previously been used to determine the protein-to-lipid ratio in biological samples by Navarro and colleagues, exploiting the distinctive absorption bands that lipids and proteins show in IR spectra. The spectra obtained for the fibres are shown in Figure 4.9.

Bulk PVP and the blank electrospun fibres present identical spectra, from which it can be inferred that there are no molecular changes induced by dint of processing through electrospinning. The most characteristic peaks from the PVP FTIR spectrum are a broad peak at around 1,650 cm\(^{-1}\) corresponding to C=O carbonyl stretching, and bands at 1,290 cm\(^{-1}\) (C-H bending), 1,420 cm\(^{-1}\) (C-N bending) and at around 2,950 cm\(^{-1}\) (asymmetric CH\(_2\) stretching). The broad absorption band at around 3,400 cm\(^{-1}\) is due to O-H stretching vibrations of adsorbed water on the fibre surface, and possibly to the presence of some residual water within the fibres. Such results are in line with previous reports.
Figure 4.8. SEM images of fibres prepared from 10% w/V PVP in TFE showing (a) blank, (b) FBS EV-loaded, and (c) MSC EV-loaded systems. Histograms of size distribution are depicted next to the respective formulation. Size measurements were performed using Image J (n = 100).
Figure 4.9. Representative ATR-FTIR spectra of PVP bulk polymer [PVP bulk], blank PVP [ES PVP] electrospun fibres, foetal bovine serum EVs [FBS EVs], mesenchymal stem cell derived EVs [MSC EVs], FBS EV-loaded fibres [ES FBS EV-loaded PVP] and MSC EV-loaded fibres [ES MSC EV-loaded PVP].
An analysis of the FTIR spectrum for the FBS EVs shows two amide absorptions, one at around 1,650 and the other at 1,540 cm\(^{-1}\). These have been reported previously as the main features for all biological samples.\(^{47}\) The first originates mainly from the C=O stretching vibrations of the protein peptide backbone, and the second from the N-H bending vibrations of the peptide groups. The strong band at around 3,290 cm\(^{-1}\) (superposed with the broad, overlapped OH stretching vibrations) belongs to the NH stretching vibrations of the peptide groups of proteins. The dominant antisymmetric and symmetric stretching vibrations of the lipid acyl CH\(_2\) groups correspond to the bands at 2,960 and 2,870 cm\(^{-1}\), respectively. The absorption band at around 1,450 cm\(^{-1}\) belongs to the bending (scissoring) vibration of lipid acyl CH\(_2\) groups, while the band at 1,400 cm\(^{-1}\) is attributed to bending vibrations of both lipid and protein CH\(_3\) groups. Bands in the spectral region of 1,200 to 950 cm\(^{-1}\) are generally attributed to the stretching vibrations of the phosphodiester groups of phospholipids and to the C-O-C stretching vibrations of phospholipids, triglycerides and cholesterol esters. However, it should be noted that this spectral region could be masked by the broad phosphate vibrations bands of the isotonic PBS buffer, as noted in the literature.\(^{47}\) The lower concentration of the MSC EV samples led to less intense features in the spectrum, but comparable peaks to those noted in the FBS samples were observed.

It is not possible to distinguish any of the characteristic peaks of the biological samples in the FTIR spectra of the EV-loaded fibres. As can be seen from Figure 4.9, all EV samples exhibited a sharp peak near 1,650 cm\(^{-1}\), which may be obscured by the PVP carbonyl vibration peak in the fibres. Other smaller characteristic peaks of the EVs overlap with the major features of PVP. The high ratio of polymer to added EV in the formulation might be associated with the difficulty in differentiating peaks from the different constituents.
Electrospun fibres were also assessed for physical form, initially using X-ray diffraction (Figure 4.10).

The pattern for raw PVP shows a characteristic broad halo between 7 and 30° (2θ), consistent with its well-known amorphous nature. After processing through electrospinning, PVP remains amorphous as has been reported elsewhere. The EV-loaded formulations' patterns are shown to be amorphous as well.

**Figure 4.10.** Representative XRD patterns of EV-loaded [ES FBS/MSC EV-loaded PVP] and blank [ES PVP] electrospun fibres, and the bulk polymer [PVP bulk].
The standard fibre characterisation techniques are unable to confirm the presence of extracellular vesicles in the formulation. It is important to assess whether the EVs are able to maintain their structure and remain unaltered after processing. To have an idea of the extent of EV encapsulation in the polymer matrix, nanoparticle tracking analysis was performed on fibres dissolved in phosphate buffered saline. The results are depicted in Figure 4.11. Contrary to what was expected, it was not possible to directly detect the formulated EVs by NTA due to the noise provided by the polymer: an equally polydisperse profile was attained for the non-loaded (blank) formulation.

Although we cannot clearly identify the EVs through size measurements there is a distinct difference in the overall concentration of particles detected between the two formulations. The diluted NTA prepared sample for blank PVP fibres had a concentration of $1.03 \times 10^9 \pm 6.36 \times 10^7$ particles/mL, a $4.0 \times 10^8$ difference from the $1.43 \times 10^9 \pm 5.19 \times 10^7$ particles/mL obtained with the FBS EV-loaded formulation. More particles were detected from the EV-loaded formulations, which can be attributed to the presence of vesicles in the dissolved mixture. Moreover, the obtained difference is roughly in line with the theoretical EV concentration of $3.75 \times 10^8$ particles/mL, expected for the diluted NTA sample. Analysis of the MSC EVs-loaded scaffolds was not undertaken due to a lack of confidence in the quantitative loading information that NTA could provide. This analysis, however, was able to confirm the successful loading of the scaffolds with extracellular vesicles.
Figure 4.11. Nanoparticle tracking analysis data from dissolved (a) FBS EV-loaded PVP fibres and (b) blank PVP fibres.
To confirm the integrity and presence of vesicles post-formulation, fibres were then dissolved and assayed by TEM imaging, as shown in Figure 4.12.

![Figure 4.12](image)

**Figure 4.12.** Representative transmission electron microscopy image of dissolved FBS EV-loaded PVP fibres, with a demonstrative distribution of different sized particles. Scale bar represents 200 nm.

It was possible to recognise rounded structures with a stained lipid bilayer in the dissolved samples. These are consistent with previous literature data on EVs. These structures have a mean diameter of 50 ± 10 nm, smaller than the sizes attained previously by nanoparticle tracking analysis (see Section 3.3.2.1). This is expected: NTA relies on the hydrodynamic diameter of samples in an aqueous medium while TEM involves a drying step which usually leads to shrinkage. There is some background in the TEM images, likely due to the presence of polymer. Overall, it is evident that EVs are able to be electrospun and maintain their structure after being formulated.
Another way to assess the presence of vesicles in the scaffolds is by quantifying the protein content post-dissolution: the results of such assays can be seen in Figure 4.13. The presence of proteins in the final dissolved formulation can be correlated with the initial theoretical loading. As expected, the total protein content was lower for the formulated samples. There is an estimated encapsulation yield of 76.8 ± 1.6% for FBS EVs electrospun fibres and 70.6 ± 8.5% for the MSC EVs-loaded ones, based on the theoretical loading. It is unclear what might have happened to the non-encapsulated EVs, but it should be noted that the calculation assumes a homogeneous distribution of EVs in the scaffold and no losses in sample transfer. These might well be contributors to the reduction in the total amount of protein detected for the formulated materials. Blank PVP fibres were also assayed to rule out any possible contribution of the polymer to the overall absorbance. The polymer provided a vestigial contribution to the absorbance (at most 3%) and can therefore be ignored.

Although it is relevant to prove the presence of EVs, it is crucial to understand whether the formulated vesicles are still potent post-processing. To determine their potency, a survival stress assay was performed using rat cardiomyocytes (Figure 4.14). Cells were cultured in stressing conditions with serum deprivation for 27 hours and the effect of the EV-loaded fibres on cell survival assessed.
Figure 4.13. Total protein content measured by Bradford assay for: (a) FBS EVs and FBS EV-loaded PVP scaffolds; and (b) MSC EVs and MSC EV-loaded PVP scaffolds. A theoretical loading of 3.4x10^9 particles for the FBS samples and 1.14x10^9 particles for the MSC ones were assayed corresponding at 1 µL of the initial suspension. There is a significant difference between the groups in both cases **p < 0.01 and ***p < 0.001.
There is a positive effect on cell survival after the addition of EVs, regardless of whether the vesicles were formulated or not. Surprisingly, as the amount of EV-loaded fibres added increased there is a decrease in cell survival. This could be explained by the increasing amount of polymer added: this is detrimental to cardiomyocyte survival, as is clear from the blank polymer fibres leading to viability values as low as 11.9%. As PVP is regarded as a biocompatible polymer such an effect was not initially expected. However, it should be noted that the ratio of polymer mass per surface area of the well (1.9 cm$^2$) and in comparison to the total volume of media added (400 µL) is quite high. The addition of 5 mg of loaded fibres produced a survival after stress of 73 ± 21%, which is in line with the effect from the addition of the same number of unprocessed EVs. At this concentration, the
polymer added does not seem to influence viability when compared to the control. The apparent reduction of potency from 5 mg to 10 mg and then to 15 mg is of the same order of magnitude as with the blank PVP fibres. There is a 12 to 15% variation registered from 5 mg to 10 mg added and 18 to 22% from 10 mg to 15 mg. Overall, it can be taken from the data that FBS EVs can be electrospun and still remain as potent as before processing.

Data showing the effect of EVs from mesenchymal stem cells are presented in Figure 4.15. Given what was found and discussed previously for the FBS EV formulations, here only 5 mg of fibres were assayed.

**Figure 4.15.** Cell viability of rat cardiomyocytes after incubation with increasing concentrations of MSC isolated EVs and 5 mg of PVP electrospun fibres (loaded and blank). This fibre mass corresponds to a theoretical EV number of $1.88 \times 10^{10}$. Three independent experiments were performed with at least two replicates each and individual values are plotted. There is a significant difference between the blank and the EV-loaded fibres $**** p < 0.0001$. 
The addition of MSC EV-loaded fibres produced a survival after stress of 87 ± 9%, the same value as the unprocessed EVs. Mesenchymal stem cell derived extracellular vesicles are clearly able to be electrospun with no loss in potency. These results highlight the feasibility of using electrospinning as an effective formulation strategy for extracellular vesicles, opening up the possibilities for solid state approaches for EV storage and delivery. It could prove itself particularly useful, as an example, for injection reconstitution or for direct topical application but further studies need to be done to establish those.
4.4. Conclusions

Fast-dissolving scaffolds were developed to allow a rapid evaluation of extracellular vesicles loading. Moreover, it was found that the choice of solvent is especially relevant when processing biological material in single fluid electrospinning. Ethanol was deemed unsuitable as it prompted sample precipitation. Trifluoroethanol was used instead since it is a gentler solvent on biological samples. Poly(ethylene oxide)-based vesicle-loaded fibres were first prepared but problems were encountered due to incomplete dissolution of scaffolds in aqueous media. Poly(vinyl pyrrolidone) was hence employed instead to fabricate fast-dissolving fibre matrices. Optimisation of the processing parameters of voltage, distance to collector and polymer concentration for fibre production were undertaken. For PVP, the polymer concentration was found to be the most important parameter to control to ensure successful production of smooth homogeneous fibres.

PVP was then loaded with either FBS and MSC extracellular vesicles and the resultant fibres assessed for their physical and biological characteristics. The fibres were found to be smooth and cylindrical, and to comprise amorphous materials (determined by x-ray diffraction). The addition of EV isolates produced fibres with a smaller diameter than the PVP blank control due to a change in the conductivity of the feeding solution. IR spectroscopy did not allow the presence of EVs in the fibres to be confirmed mainly due to the high mass ratio of polymer to EVs. Nanoparticle tracking analysis provided evidence of intact EV incorporation into the fibre matrix, despite the polymer forming interferent aggregates in the nanoscale range. A stark difference between the number of particles detected upon dissolution of the EV-loaded and blank fibres was observed, with this difference being roughly equal to the initially loaded amount of vesicles, indicating their continued presence after electrospinning. This was further confirmed by TEM. An analysis
of protein content indicated an estimated encapsulation efficiency of 70-75%. Furthermore, EVs showed no loss in potency after electrospinning. When both fresh and electrospun EVs were added to serum deprived, stressed, cardiomyocytes both were equally potent in restoring cell viability. EVs hence can be electrospun and retain their potency. This offers the potential to use electrospun EV formulations in fast-release applications or as a platform for more complex controlled-release systems.
4.5. References


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Chapter 5

Controlled-release
Electrospun Formulations
5.1. Introduction

The delivery of active substances loaded in electrospun scaffolds for tissue regeneration has been widely researched in the last decade.\textsuperscript{1,2} A breath of materials has been used, which arises from the multiplicity of diseases, administration route, dosage range and special requirements that may apply.

5.1.1. Scaffolds for Cardiac Repair

One promising approach in myocardial infarction (MI) therapy is the design and implantation on the damaged heart surface scaffolds with regenerative cues. Recently, cellularized scaffolds to promote heart recovery and improve cardiac function have been thoroughly investigated.\textsuperscript{3–6} Liu and colleagues developed a poly(ε-caprolactone) (PCL), elastin and collagen scaffold that not only increased the proliferative capacity of anchored cells but also improved cardiac function, reduced infarction area and restricted left ventricular remodelling (LVR) 4 weeks after implantation.\textsuperscript{3} Another study demonstrated similar improvements with radio-frequency plasma surface functionalized electrospun PCL fibres as a matrix loaded with MSCs.\textsuperscript{5} The scaffolds led to an improvement in cardiac function and attenuated dilatation in a rat model of chronic MI 4 weeks after implantation. This thus paves the way to use polymer constructs to promote improvement in cardiac function.

In all cardiac scaffolds developed, certain properties and characteristics are essential to ensure their suitability for heart implantation. Their design requires the selection of biocompatible, biodegradable materials that possess mechanical and physicochemical properties suitable for cardiac application.
Flexible scaffolds strong enough to allow surgical handling and with adequate mechanical resistance post-implantation are key. The best constructs should have a Young’s modulus between the tens of kPa and 1 MPa. Depending on the function and the positioning of the scaffold in the heart, different mechanical properties are required. The native heart is reported to have a Young’s modulus 3-fold lower than that of ischemic tissue. This increase in stiffness can be explained by the scaring and fibrosis the ischemic heart goes through during remodelling. It is expected then that a formulation to be applied in an ischemic heart will have to possess different mechanical properties than that of native heart tissue.

Biocompatibility and biodegradable properties are universal requirements for a scaffold material, regardless of the tissue type in which the system is to be applied to. Suitable materials need to be compatible with the natural tissue eliciting a low inflammatory response after implantation, thus allowing healing and avoiding scaffold rejection by the body. This is especially important in scaffolds that are expected to remain in the body for extended periods of time. That said, it is important to note that biocompatibility is not an intrinsic property of a material, as it depends on the biological environment and the tolerability that exists with respect to specific drug-polymer-tissue interactions. Biodegradable materials degrade in vivo either enzymatically or non-enzymatically (or both), to produce biocompatible, toxicologically safe by-products which are further eliminated by normal metabolic pathways. Over the past decade, the use of such materials in or as adjuvants in controlled drug delivery has increased dramatically.
5.1.2. Polymeric Materials

Biomaterials used in drug delivery and tissue regeneration scaffold development can be broadly classified as synthetic and naturally occurring polymers. Polymers such as poly(lactic acid) (PLA), poly(ε-caprolactone) (PCL), poly(ethylene oxide) (PEO), and copolymers such as poly(L-lactide-co-caprolactone) (PCLA) and poly(lactic-co-glycolic acid) (PLGA) are synthetic materials extensively investigated for fibre fabrication with appropriate properties for tissue engineering and drug delivery applications.\textsuperscript{1,12,13} Polysaccharides such as alginate, cellulose, chitin, chitosan, hyaluronic acid, starch, dextran, and heparin, or proteins such as collagen, gelatin, elastin, silk, among others comprise some of the most common natural polymers used in drug delivery, and more specifically in electrospinning.\textsuperscript{6,14}

Natural polymers hold a set of advantages due of their inherent biocompatibility and non-toxic degradation products, as well as providing intrinsic biochemical cues. This results in scaffolds that induce low immune responses. However, most of this category of polymers lack the adequate physical and chemical characteristics to produce an acceptable scaffold for drug delivery, or are difficult to electrospin on their own.\textsuperscript{15} Usually, an extra crosslinking step or the addition of synthetic polymers is necessary to make them insoluble in aqueous environments and mechanically resistant.\textsuperscript{16} This has led to the development of hybrid materials, which consist of a blend of both synthetic and natural polymers. However, such blends may results on altered fibres’ 3D structure and porosity, but also their biocompatibility, due to the introduction of non-biocompatible chemical agents.\textsuperscript{16} Moreover, appropriate solvent selection for the electrospinning process is crucial to avoid or reduce biomolecule denaturation.
Synthetic polymers, on the other hand, are chemical reproducible and therefore suited for large-scale manufacturing and simple quality control processes. They usually benefit from better mechanical properties than natural materials, though their biocompatibility can be hampered by their degradation by-products. Moreover, their cost is much lower in comparison to natural polymers, and because there is the possibility to tune the synthesis process their mechanical properties can be adapted effectively. Additionally, the rate of degradation can be controlled to some extent by altering parameters such as the polymer blend composition and ratio of amorphous to crystalline segments. Kim and colleagues demonstrated that the degradation rate (via hydrolysis) of blends containing different ratios of PLA, PLGA, PLA-b-polyethylene glycol-b-PLA, and free lactide could be tuned by varying their composition (thus altering the hydrophobicity/hydrophilicity). On the negative side, synthetic polymers are missing key biochemical cues for tissue regeneration. These should be introduced either via adding an extra step of functionalization or via combining synthetic materials with natural polymers. This flexibility in key parameters makes scaffolds based on synthetic materials more amenable for use in scaffolds over natural polymers.

As one of the main characteristics required of a scaffold is biocompatibility and biodegradability, biodegradable aliphatic polyesters are commonly used. Poly(lactide), poly(lactide-co-glycolide) and poly(ε-caprolactone) have attracted much interest for applications ranging from medical implants, bone fixation parts, scaffold fabrication and controlled drug release devices to sustained release systems for pesticides and fertilizers.
PLGA is Food and Drug Administration (FDA) approved for multiple therapeutics, and has been used to construct delivery vehicles for drugs, proteins and various other macromolecules such as DNA, RNA and peptides.\textsuperscript{10} PLGA is biodegradable and widely biocompatible. It undergoes degradation through hydrolysis in aqueous environments, providing a consistent degradation profile.\textsuperscript{20} Polyester PLGA is a copolymer of poly(lactic acid) (PLA) and poly(glycolic acid) (PGA). When crystalline PGA is co-polymerized with amorphous PLA the overall degree of crystallinity of the resulting PLGA is reduced, and an increase in hydration and hydrolysis rate is observed. Thus, a higher content of PGA leads to faster degradation rates, except for 50:50 PLA/PGA, which exhibits the fastest degradation of all copolymer ratios. Generally, the copolymer PLGA is preferred over its constituent homopolymers for drug delivery, mainly because PLGA offers superior control of degradation properties by varying the lactic and glycolic acid monomer ratio. The possibility of controlling the degradation rate in the copolymer also allows matching with the tissue regeneration rate required for tissue engineering applications, and control of drug release kinetics for drug delivery. PLGA is also advantageous because its degradation products can be removed through natural metabolic pathways. Electrospun PLGA has been formulated either on its own or in blends with other polymers to improve the adhesion and proliferation of cells on scaffolds.\textsuperscript{21}

Poly(dioxanone) (PDO) is a biodegradable semi-crystalline polymer with excellent bioabsorbability and biocompatibility characteristics. It is widely used as a tissue repair and scaffold material in the medical field, especially as an FDA approved absorbable wound closure suture in clinical practice.\textsuperscript{22,23} The degradation products of PDO have been proven to have low-toxicity, and cause lower inflammatory response rates than some PLGAs.\textsuperscript{24} Due to the high crystallinity and hydrophobicity of the polymer, it can be considered a slow to moderately fast degrading polymer. Poly(dioxanone) electrospun scaffolds have been
previously investigated for cardiac tissue engineering. For instance, a bioabsorbable valved PDO patch was developed with the aim of reconstructing the right ventricular outflow tract (RVOT), frequently necessary during congenital heart surgery.\textsuperscript{4} Scaffolds seeded with MSCs were implanted in the RVOT of growing lambs and shown to be completely degraded after 8 months, and replaced by viable fully functional tissue similar to the native tissue. The growth potential of the engineered scaffold was evident, with less fibrosis, less calcifications and no thrombus, stenosis or aneurisms compared with the unseeded control. Furthermore, the same authors investigated further a set of different polymer scaffolds from polyurethane, polyhydroxyalkanoate or polydioxanone. PDO was shown to produce the best \textit{in vitro} properties performing better in a rat model of partial inferior vena cava replacement.\textsuperscript{25}
5.2. Aims

After proving that extracellular vesicles can be successfully processed by electrospinning in the previous chapter, the next challenge is to develop formulations for EV delivery that might be applicable in the ischemic heart. To achieve that, biocompatible polymers were assessed for their capacity to produce scaffolds with appropriate morphology, mechanical strength, flexibility, residual solvent content, among other properties that make them adequate for implantation in the heart. Once that is established, formulations with a controlled release profile were produced and tested for their suitability in delivering EVs in a sustained manner over time.
5.3. Results and Discussion

5.3.1. Poly Lactic-co-Glycolic Acid Scaffolds

PLGA was chosen as a delivery vehicle since it is already FDA approved for several therapeutic applications, as well for its biodegradability, biocompatibility and sustained release properties.\textsuperscript{20} The 50:50 ratio material was specifically elected for presenting degradation rates of under a month. This is relevant as it would provide delivery of EVs over the course of the most critical time after an MI, hindering remodelling and prompting the regenerative potential of the tissue. A polymer with a relatively low molecular weight was first explored, as it would have a theoretical faster degradation and release than other 50:50 PLGAs. According to the manufacturer, 17k Mw 50:50 PLGA has an estimated time to complete mass loss of 2 to 4 weeks, as compared to 44k Mw at 3 to 4 weeks. Solutions of the lower molecular weight polymer were prepared, and attempts made to electrospin them.

Campos and colleagues have electrospun 50:50 PLGA with a molecular weight between 30k and 60k at a 20\% (w/V) concentration.\textsuperscript{26} Given that the molecular weight of the polymer used here is lower, higher starting concentrations of 30\% (w/V) were used to assess the spinning conditions. The conditions tested with varied applied potential and distance to collector resulted in the images depicted in Figure 5.1. Acetone was chosen as the solvent as it is a sustainable and non-toxic material, although it has received limited attention for the electrospinning of biocompatible polymers.\textsuperscript{27} It is important that the solvent system chosen is not classed as toxic or hazardous, since any residual solvent in the scaffold could have potential long-term impacts on the environment and user’s health. Regardless of the processing parameters, beads or beaded fibres were produced from this
polymer. Such morphologies have been previously attributed to low solution viscosities in polymer electrospinning experiments. However it seems impractical to increase the polymer concentration much more to attain an appropriate viscosity for electrospinning, mainly due to the cost of the polymer.

PLGA with a 44k molecular weight was then considered. A higher Mw will lead to more chain entanglements, which results in a more viscous solution even at a lower polymer concentration. In order to survey the optimal conditions for electrospinning 44 kDa PLGA, a range of concentrations from 10 to 30% (w/V) were investigated. The processing of 10% (w/V) solutions led to electrospraying with particles being produced (Figure 5.2). Increasing the concentration to 20% (w/V) produced a mixture of particles and fibres, indicating the need to increase further the polymer concentration to ensure uniform fibres are produced. This, once again, is tightly related to the solution viscosity and polymer concentration. In order for electrospinning to occur a minimum concentration of the polymer solution is required. Therefore, 30% (w/V) PLGA solutions were prepared and electrospun at different conditions of voltage and distance to collector, and the resulting fibres can be seen in Figure 5.3. Fibre diameter seems to increase with both applied voltage and distance to collector. There is however a smaller relative standard deviation in samples produced at higher voltages and distances, indicating that the use of elevated applied potentials and increased distance of tip to collect might be preferable to obtain more uniform samples (Figure 5.4 (b)).
Figure 5.1. Scanning electron microscopy images of 17kDa 50:50 PLGA electrospun at different processing conditions of voltage (V, kV), polymer concentration (C, % w/V) and distance to collector (D, cm) in acetone. Images correspond to fibres produced at the following processing conditions: (a) V20C30D20, (b) V10C30D10, (c) V10C30D20 and (d) V20C30D10.
Figure 5.2. Scanning electron microscopy images of 44kDa 50:50 PLGA electrospun at different processing conditions of voltage (V, kV), polymer concentration (C, % w/V) and distance to collector (D, cm) in acetone. Images correspond to fibres produced with the following processing conditions: (a) V10C10D10, (b) V20C10D10, (c) V20C10D20 and (d) V15C20D15.
Figure 5.3. Scanning electron microscopy images of 44k Da 50:50 PLGA electrospun at different voltage (V, kV) and distance to collector (D, cm) in acetone. Images correspond to fibres produced from 30% (w/V) PLGA solutions at the following processing conditions: (a) V10C30D10, (b) V10C30D20, (c) V20C30D10, (d) V20C30D20 and (e) V15C30D15.
Figure 5.4. Ternary plot depicting the effect that variation of flow rate, distance to collector and voltage have on the (a) fibre diameter, and the (b) relative standard deviation of PLGA fibres prepared from acetone. Distance to collector varied from 10 to 20 cm and voltage from 10 to 20 kV.
Although uniform smooth fibres were obtained, another solvent was tested to assess its capacity to produce homogeneous PLGA fibres, as acetone being a very volatile solvent has the tendency to evaporate right at the spinneret tip blocking it. HFIP, or 1,1,1,3,3,3-Hexafluoro-2-isopropanol, is a solvent that has been used in the formulation of PLGA scaffolds in the past. A concentration of 30% (w/V) was used, based on successful findings with acetone. Bead-free fibres could be obtained at any condition of distance to collector, flow rate or applied voltage (Figure 5.5).

A combination of all three parameters is relevant to assess produced fibres characteristics. At higher voltages and flow rates but lower distances to collector fibres of smaller diameter were produced. However, the relative standard deviation was shown to be more dependent on distance and flow rate (Figure 5.6). At higher distances and lower flow rates the smallest deviations are obtained. In fact, a faster flow rate will usually lead to thicker fibres as more material is being ejected from the spinneret at a given unit of time. Therefore, the conditions that demonstrated the smallest relative standard deviation (8.7%) in fibre diameter was at higher voltages (20 kV), a flow rate of 0.5 mL/h and a 15 cm distance from spinneret to collector and thus are considered optimal. This are then the conditions taken forward for fibre production and processing.
Figure 5.5. Scanning electron microscopy images of 44kDa 50:50 PLGA electrospun with different voltage (V, kV), flow rate (F, mL/h) and distance to collector (D, cm) in HFIP. Images correspond to fibres produced from 30% (w/V) PLGA solutions at the following processing conditions: (a) V15F0.5D10, (b) V15F0.5D15, (c) V20F0.5D20, (d) V20F0.5D15, (e) V15F1D15, (f) V15F1.5D15 and (g) V20F1.5D10.
Figure 5.6. Ternary plot depicting the effect that variation of flow rate, distance to collector and voltage have on the (a) fibre diameter, and the (b) relative standard deviation of PLGA fibres prepared from HFIP. Flow rate varied from 0.5 to 1.5 mL/h, distance to collector from 10 to 20 cm and voltage from 15 to 20 kV.
The optimised conditions for consistent, smooth fibre scaffold formulations were found. The scaffolds being generated in this work will need to deliver extracellular vesicles in a controlled manner to the heart epicardium, it is then also important to ensure their adequability for \textit{in vivo} delivery. One key property is the scaffold behaviour when immersed in an aqueous environment at physiological temperature (37°C). Its behaviour at the aforementioned conditions is relevant as it will mimic the environment that scaffolds would be subjected to \textit{in vivo}. After immersion in phosphate buffered saline, the PLGA scaffolds not only became quite rigid to the touch, there was also a $55 \pm 7\%$ reduction in size after just 24 hours (\textit{Figure 5.7}).

\textit{Figure 5.7.} Photograph of PLGA electrospun scaffolds before (on the left) and after (on the right) incubation in phosphate buffered saline at 37°C for 24h.

Such characteristics are not desirable for cardiac suturing and implantation. As the heart is a muscle, scaffolds implanted on it need to be flexible enough to withstand the normal rhythmic contractions. Moreover, it is not known what effect this shrinkage would have on EV release from the scaffold, but as the surface area to volume would be significantly reduced an altered release profile from the fresh scaffold could be expected.
Shrinkage of PLGA electrospun constructs has been previously reported. Ru and colleagues reported a 75% shrinkage of 50:50 PLGA electrospun scaffolds following a 24 hour incubation at 37°C in phosphate buffered saline. This shrinkage behaviour is closely related to the polymer’s glass transition temperature ($T_g$). $T_g$ is the temperature at which a material changes from a hard and brittle glass-like state to a more flexible, rubbery state. During electrospinning, the polymer solution is subjected to a high electrical potential that results in a high degree of alignment of polymer chains within the fibres. Also, the solution drawing in electrospinning results in the polymer chains having a more extended structure than the molecules in raw PLGA particles would have. When at temperatures above the $T_g$, the main chain of the aligned polymer will gradually become randomly coiled due to thermally induced relaxation resulting in the overall shrinkage of the scaffold.

The glass transition temperature of PLGA was then determined by differential scanning calorimetry (DSC). The $T_g$ for bulk PLGA was determined to be 49.5°C, and that of the electrospun scaffolds was 39.6°C (Figure 5.8). These values are in accordance with the literature. The $T_g$ of the electrospun samples is lower than that of the raw PLGA: the electrospinning process thus exerts a plasticizing influence on the polymer. This results in the scaffold transitioning from brittle to ductile at a lower temperature. There could be some added plasticizing effect through the inclusion of residual amounts of solvent or water from the surrounding air. Furthermore, and when in an aqueous environment water acts as a plasticizer and thus reduces further the $T_g$ of wet scaffolds. For 50:50 PLGA electrospun materials, the $T_g$ of wet scaffolds has been reported to be around 31°C. This is below the temperature used for the shrinkage assay in this work, resulting in the relaxation of the polymer chains (Figure 5.8) and undesirable shrinkage of the scaffolds.
Figure 5.8. Representative DSC thermogram of PLGA fibres by modulated DSC with a scan from 0 to 250°C at 3°C/min. The glass transition temperature at around 40°C is quickly followed by an endothermic relaxation.

This shrinkage behaviour hinders the development of PLGA-based formulations, since the scaffolds would not possess the necessary characteristics for the intended therapeutic application. To resolve this, some methods to reduce the shrinkage of scaffolds were considered. Jiang and colleagues reported a reduction in the dimensional shrinking of scaffolds by preparing blends of poly(ethylene glycol) (PEG) and chitosan with PLGA. Pure PLGA scaffolds shrank to 76% of their original size at similar incubation conditions as used in this work, whereas the blended fibres registered a 3% reduction only. The PEG and chitosan present in the scaffolds will swell in an aqueous environment and thus prevent shrinkage, even when the PLGA chains relax. This occurs despite the fact that the $T_g$ of the electrospun blends was found be lower than pure PLGA fibres, due to mixing with PEG (which has a transition temperature of -54°C). In another work, scaffolds were fabricated with PLGA blended with PCL. PCL is flexible, so can overcome the brittle nature.
of PLGA. The results showed that the PLGA/PCL composite scaffold offered improved flexibility.

Therefore, the blending of polymers appeared as an appropriate strategy to improve the properties of the controlled-release formulations being developed here. The addition of another synthetic polymer was thus considered. Poly(dioxanone) is a bioabsorbable polymer that has been electrospun previously and shown to allow MSC differentiation onto their scaffolds.23,38,39 This polymer is semi-crystalline and has a glass transition temperature between -10°C and 0°C and no relaxation. The incorporation of PDO in the polymer matrix with PLGA will potentially restrict PLGA chains movement at physiological temperature, thus preventing its relaxation and concomitant coiling of molecular chains.

5.3.2. Poly(dioxanone) Scaffolds

Assessment of the optimal spinning conditions for poly(dioxanone) was undertaken, before blending PDO with PLGA for scaffold production. PDO has been previously electrospun at a concentration of around 9% (w/V) in HFIP elsewhere.38,39 Hence a range of different polymeric solutions ranging from 5 to 15% (w/V) in HFIP were assessed here, under different processing conditions. A concentration of 5% (w/V) revealed to be insufficient, resulting in either electrospaying or the formation of beaded fibres, regardless of the applied voltage, flow rate or distance to collector. Higher polymer concentrations of 10% (w/V) were then explored. A range of flow rates were tested, from 1 to 10 mL/h, a higher range due to what was reported in literature.38 However, at higher flow rates it was not possible to spin due to jet instability, particularly at closer distances to the collector (10 cm). Fibres were attained at 20 cm but presented significant amounts of
beading. At even higher polymer contents (15% (w/V)), most of conditions experimented did not produce fibres successfully due to jet instability.

Those conditions where electrospinning was successful and that appeared to have produced smooth fibres under the optical microscope were probed by SEM, and the resultant images are presented in Figure 5.9. All fibres are approximately cylindrical in shape. However, fibres prepared at higher flow rates appear to be increasingly irregular. As the flow rate increases, solvent from the feeding solution is not allowed to evaporate fast enough to produce uniform fibres. Therefore, only slower feeding rates should be considered. Similar findings were already reported elsewhere.38

Similarly to previous optimisation assays, a ternary plot with the variation of fibre diameter with different conditions of voltage, distance and flow rate was drawn in Figure 5.10. Diameter of fibres seems to be directly dependent on applied potential, regardless of applied flow rate or distance. However, it is at lower voltages, higher distances and slower flow rates that the minimum diameter variation is attained.

However, it should be considered that PDO will be blended with PLGA at an expected ratio in which the amount of the later is still be significantly higher than the former. Thus, the PLGA optimised processing conditions should take precedent, or a set of very approximate operating conditions. Nevertheless, the scaffolds produced at a 1 mL/h of flow rate, at a distance to collector of 15 cm and an applied voltage of 20 kV seem to have produced fibres with the best characteristics.
Figure 5.9. SEM images of fibres prepared from 10% (w/V) PDO in HFIP at different conditions of voltage (V, kV), distance to collector (D, cm) and flow rate (F, mL/h). Images correspond to fibres produced at the following processing conditions: (a) V10F1D10, (b) V10F1D20, (c) V15F5D15, (d) V20F1D10, (e) V20F1D20, (f) V20F1D20 and (g) V20F1D15.
Figure 5.10. Ternary plot depicting the effect that variation of flow rate, distance to collector and voltage have on the (a) fibre diameter, and the (b) relative standard deviation of PDO fibres prepared from HFIP. Flow rate varied from 1 to 10 mL/h, distance to collector from 10 to 20 cm and voltage from 10 to 20 kV.
5.3.3. Poly(dioxanone)/Poly Lactic-co-Glycolic Acid Scaffolds

Given the need to produce scaffolds with a different mechanical behaviour than pure PLGA fibres in an aqueous environment, an initial investigation based on the glass transition of blends of PDO and PLGA was performed to assess the most appropriate blend to use. The Fox equation was used to calculate the blends’ theoretical glass transition temperatures (Equation 5.1). This equation takes into consideration the relative mass contribution of each component and the \( T_g \) of the pure materials.

\[
\frac{1}{T_g} = \frac{1 - x}{T_{g(PLGA)}} + \frac{x}{T_{g(PDO)}}
\]  

(Equation 5.1)

The \( T_g \) of each electrospun polymer was then determined by modulated temperature DSC (mDSC). Electrospun PDO showed a \(-6.7^\circ\text{C}\) (Figure 5.11) transition and PLGA \(39.6^\circ\text{C}\). An extrapolation of the \( T_g \) values of blends based on the Fox equation was then undertaken and can be seen in Figure 5.12. According to these calculations, a 1:100 (w/w) ratio of PDO:PLGA should have a dry glass transition temperature below \(37^\circ\text{C}\). Scaffolds were then produced at a range of PDO:PLGA ratios, and their experimental \( T_g \) measured; the results are depicted in Figure 5.12.
**Figure 5.11.** Representative DSC thermogram of PDO fibres by modulated DSC with a scan from -50 to 250°C at 3°C/min. Glass transition temperature at around -7°C with a melting temperature at about 106°C.

The equation did not describe the experimental $T_g$ variation behaviour as it can be seen in the graphic representation on Figure 5.12. The Fox equation is often regarded as being simplistic, as it bases its predictions on the pure components and assumes perfect system miscibility. Brostow and colleagues have shown that the Fox equation fell short in predicting the $T_g$ variation of PEO and epoxy resin blends. It is possible that the complexity of the scaffold system cannot be described fully by the equation. The experimental data showed that only the material with a 1:1 (w/w) ratio of PDO and PLGA had a dry glass transition temperature below 37°C.
Figure 5.12. Variation of glass transition temperature ($T_g$) with the relative mass amount of PLGA ($x$) present in the blend with PDO. The Fox equation was used to model the theoretical variation of $T_g$ ($\bullet$). Experimental values ($\square$) were measured on samples by mDSC.

The scaffolds were produced at the same processing conditions of applied potential, flow rate and distance to collector, and a representative image of the fibre morphology at each blend ratio is depicted in Figure 5.13.

The cylindrical and smooth fibres produced with different contents of PDO and PLGA were assessed for their shrinkage behaviour in similar conditions as reported previously (24 hours incubation at 37°C in phosphate buffered saline). The results are given in Figure 5.14.
Figure 5.13. Scanning electron microscopy images of fibres produced from PDO/PLGA mixtures under a 20 kV applied voltage, at 15 cm distance to collector and 0.5 mL/h flow rate in HFIP. Images correspond to the following produced electrospun fibres: (a) PLGA [30% (w/V)], (b) 1:100 (w/w) PDO:PLGA, (c) 1:20 (w/w) PDO:PLGA, (d) 1:10 (w/w) PDO:PLGA, (e) 1:5 (w/w) PDO:PLGA, (f) 1:2 (w/w) PDO:PLGA, (g) 1:1 (w/w) PDO:PLGA and (h) PDO [10% (w/V)].
**Figure 5.14.** Shrinkage percentages of fibres made from PLGA, PDO and blends at different polymer ratios, after incubation for 24h with phosphate buffered saline at 37°C. At least three independent experiments were performed and mean ± standard deviation values taken for each condition. There is a significant difference between groups (****p < 0.0001).

Unsurprisingly, pure PLGA electrospun scaffolds showed the highest reduction in size after the 24 h period. Only after reducing by 10-fold the amount of PLGA in the scaffold is there a significantly different shrinkage behaviour, to a value of 39 ± 7% (p < 0.0001, t-test). There is then a progressive decrease in the overall change in size of the scaffolds after immersion as the amount of PDO in the blend increases. At a 1:1 (w/w) ratio of PDO:PLGA, incubation at 37°C in an aqueous environment appears to have no impact on the scaffold size and flexibility (**Figure 5.15**). PDO scaffolds also did not show any signs of shrinkage. Flexibility was assessed manually by folding the scaffold and by an experienced surgeon for their expert opinion. Photographs of handling PLGA and 1:1 (w/w) PDO/PLGA scaffolds post-incubation is represented in **Figure 5.15**.
In addition to containing uniform sized fibres and having adequate flexibility, scaffolds need to present some degree of porosity to ensure an adequate surface area for delivery. When developing scaffolds for tissue regeneration, an adequate porosity will allow cells to infiltrate and proliferate and contents to be released, while ensuring gas and nutrient exchange for therapeutical application. Scaffold porosity suitable for cellular penetration is typically in the range of 60 to 90%. Pure PLGA scaffolds present a 66.2 ± 5.4% porosity which is in line with what was attained previously. Blends porosity varied between 42 and 68%. Although here we are not producing a scaffold for cellular infiltration or delivery it is always beneficial to assess the material capacity to perform under certain circumstances representing a superior scaffold able to be used in different contexts.

A battery of characterisation assays was performed on the produced scaffolds to understand their physical properties. Infrared spectroscopy has been frequently used to
investigate the conformational changes of polymers. FTIR spectra for bulk and electrospun polymers are shown in Figure 5.16.

![FTIR Spectra](image)

**Figure 5.16.** Representative ATR-FTIR spectra of PLGA and PDO bulk polymer, PLGA and PDO electrospun fibres, and the different electrospun PDO/PLGA (w/w) ratios (1:100, 1:20, 1:10, 1:5, 1:2 and 1:1).
For pure PLGA fibres, the spectrum shows a strong characteristic absorption band at about 1,745 cm\(^{-1}\) attributed to the stretching vibration of carbonyl (C=O) bond and bands in the 1,084 to 1,452 cm\(^{-1}\) region denoting the presence of the ester group. Other major peaks observed were at around 1,270 cm\(^{-1}\), 1,170 cm\(^{-1}\) and 1,080 cm\(^{-1}\) (asymmetric and symmetric C-O vibrations). Similar spectra have been recorded elsewhere.\(^{43,44}\) Comparison of the spectra obtained for bulk PLGA and its electrospun fibres showed no major differences, confirming electrospinning to be a process that does not impart any changes to the PLGA structure.

The PDO spectrum on the other hand showed peaks at 1,732 cm\(^{-1}\) (ester carbonyl group), 1,125 cm\(^{-1}\) (C–O–C of the ester, with shoulder broadening at 1,100 cm\(^{-1}\) due to the ether group), 1,066 and 1,055 cm\(^{-1}\) (C–O bands of ester) and for aliphatic CH\(_2\) groups at 2,925 cm\(^{-1}\). The peaks in the fingerprint region, 929, 873, 840, 723, 580, 507, and 452 cm\(^{-1}\) are of interest as they denote the amorphous and crystalline phases of polyesters. Full agreement with spectra reported elsewhere was found.\(^{45}\) Similarly to PLGA, electrospinning did not induce any conformational changes in the PDO polymer structure.

The spectra of the blend fibres represent a composite of the single-component systems. As the amount of PDO present in the scaffold increases, PDO contribution can be more clearly seen. At a ratio of 1:1 (w/w) there is a band at 1,733 cm\(^{-1}\) with a shoulder at 1,745 cm\(^{-1}\), characteristic of PDO and PLGA respectively. A greater amount of PLGA in the fibres results in the masking of the characteristic bands of PDO by PLGA, and at ratios lower than 1:2 (w/w) PDO/PLGA it is not possible to identify any PDO specific bands. This demonstrates the contribution of both polymers to the spectra but little can be said about the nature of mixing in PLGA and PDO blends.
In order to assess the physical form of the polymer scaffolds, the materials were assessed by X-ray diffraction (Figure 5.17).

**Figure 5.17.** Representative XRD patterns of PLGA and PDO bulk polymer, PLGA and PDO electrospun fibres, and the different electrospun PDO/PLGA (w/w) ratios (1:100, 1:20, 1:10, 1:5, 1:2 and 1:1).
PDO electrospun scaffolds are semi-crystalline, as is the pure polymer, with two distinct Bragg reflections visible between 21° and 23°. These Bragg reflections are visible in the diffraction patterns scaffolds comprising up to 1:5 (w/w) PDO/PLGA. At lower PDO contents, crystalline material cannot be seen, and the patterns display only an amorphous halo. This is identical to the pattern of pure PLGA, indicative of the amorphous nature of the materials. Such findings are in accordance with previous reports in literature.38,46

Having selected the 1:1 (w/w) PDO/PLGA as the best performing scaffold of the set, further characterisation was undertaken. Another important issue to take into consideration in producing implantable scaffolds is the possible presence of residual solvent in the scaffolds. Organic solvents, such as HFIP, used in the production of nanofiber matrices, can be harmful to cells.47,48 It is hence relevant to quantify whether there is any residual solvent. A thermogravimetric analysis (Figure 5.18) was then performed on the 1:1 (w/w) PDO/PLGA scaffolds (n = 3), and very little mass loss below 150°C was registered (0.41 ± 0.09 %). This thus indicates that very little solvent is still present in the scaffolds after drying, and they should be safe for implantation. It is difficult to differentiate between HFIP and residual water in the scaffold, so it is possible that the amount of this organic solvent is even lower in the system. Rosa and colleagues analysed the effect of increasing concentrations of HFIP on human adipose stem cells, considering concentrations up to 500 ppm.31 No nefarious effect on cell viability was recorded by these authors across the whole range of concentrations, demonstrating that even the higher concentrations of HFIP did not result in cellular toxicity. Concentrations of less than 1% in the scaffold were deemed acceptable for in vivo implantation.31
It is then necessary to assess the mechanical properties of the scaffold. Mechanical testing was performed on scaffolds with a 1:1 (w/w) ratio of PDO/PLGA. A Young’s modulus of $8.83 \pm 0.65 \text{ MPa} (n = 3)$ was attained for these samples. This Young modulus is higher than that which would be ideal for cardiac scaffolds. Electrospun scaffolds made of PLGA and PCL blends have shown an increase in Young modulus with an increase in PLGA content, and Rosa and colleagues found that PLGA fibre constructs presented Young modulus values around 15 MPa. Previous reports on the mechanical properties of electrospun PDO and PLGA scaffolds reveal that PDO is less stiff, hence more elastic, than PLGA, and that electrospun PDO possesses comparable mechanical properties to collagen and elastin, which are natural materials. It should be noted that the measurements were performed in dry conditions, which can be not fully representative of what might happen once the scaffold is implanted. In fact, differences in the ultimate tensile strength were previously found on electrospun PLGA:collagen scaffolds when measured in dry and
wet conditions.\textsuperscript{51} Tensile strength, which is the maximum stress the material can withstand before breaking under tension, was found to be 1.55 MPa in dry conditions and decreased approximately 40\% to 0.65 MPa in wet mode. This also has an impact on the calculated Young modulus. Although Sadeghi-Avalshahr and colleagues did not provide the value of Young’s modulus, the stress-strain plot shows a clear difference in the slope, and hence in the Young’s modulus, under wet and dry conditions, with the value being higher for dry measurements.

5.3.4. Poly(dioxanone)/Poly Lactic-co-Glycolic Acid Coaxial Scaffolds

The 1:1 (w/w) PDO/PLGA system was then selected for the production of controlled-released formulations, due to its improved flexibility over the other scaffolds. To achieve that, a coaxial system will ultimately be prepared with the EV suspension in the core and a shell comprising the PDO/PLGA blend. It was anticipated that detection of EVs in the scaffold or being released from it would be extremely challenging, and thus a model vesicle was employed. Liposomes were used to mimic EVs. They are usually a good choice of a biological reference material as they are produced from a bulk material, their composition is well characterized, and their size distribution tends to be quite narrow.\textsuperscript{52} The used liposomes are fluorescently labelled, permitting them to be quantified in a relatively straightforward manner. The concentration of fluorescent particles in suspension in phosphate buffered saline was determined by NTA in scatter and fluorescent mode (Figure 5.19).
CHAPTER 5 - Controlled-release Electrospun Formulations

Nanotracking particle analysis revealed that the liposomes had concentrations of 2.18x10^{14} particles/mL in phosphate buffered saline with 8.33x10^{13} fluorescent particles per millilitre. This means that roughly 40% of the sample exhibits fluorescence detectable at 532 nm in the NTA. This can be used to determine the loading in the formulated fibres.

Figure 5.19. Representative size distribution of a sample of fluorescent liposomes assessed by nanoparticle tracking analysis in (a) scatter and (b) fluorescent mode.
For the fabrication of fibres, some of the parameters need to be carefully evaluated. Moreover, having the coloured liposome solution in the core aided in the visualisation of the Taylor cone hence easing the assessment of the spinning solutions behaviour and determine the best processing conditions. One of them being the relative flow rates of the core and the shell solutions. The shell is most commonly processed at a higher rate when compared to the core, a flow rate ratio between around 1:3 and 1:10 is generally regarded as adequate for successful fibre formation. A relative ratio of core-to-shell flow rate of 1:5 was found to be optimal for our system and was therefore employed in the scaffold production. Also, a voltage of 15 kV and a distance to collector of 12 cm were employed. Fibres were fabricated, and their morphology can be seen in Figure 5.20. The fibres obtained were cylindrical and had a mean diameter of 1,035 ± 296 nm, giving a relative standard deviation of 29%.

Figure 5.20. An SEM image of coaxial fibres prepared from 1:1 (w/w) PDO/PLGA in HFIP, with a core suspension of fluorescent liposomes, together with a histogram of size distribution. Size measurements were performed using Image J (n = 100).

Fibres produced were confirmed to be coaxial, with clear core and shell layers as confirmed by transmission electron microscopy (Figure 5.21).
Figure 5.21. TEM image of coaxial fibres prepared from 1:1 (w/w) PDO/PLGA in HFIP with loaded fluorescent liposomes.

The liposome-loaded scaffolds were then assessed for their release. Samples were immersed in 2 mL of phosphate buffered saline at physiological temperature (37°C) under constant mixing at 100 rpm. The quantitative fluorescence was detected in aliquots taken at periodic time points to assess the number of fluorescent liposomal particles released from the scaffolds. The cumulative release is represented in Figure 5.22.

Figure 5.22. Cumulative release profiles of fluorescent liposomes from coaxial 1:1 (w/w) PDO/PLGA electrospun scaffolds.
A burst release after 24 hours of just over 10% of the whole loaded amount of liposomes can be seen. However, over the course of the 4 weeks assayed, no more particles were apparently released from the polymer matrix. It could be hypothesised that liposomes in such incubation conditions somehow lose their fluorescence over time leading to an underestimation of the detected fluorescence. However, no loss in fluorescence from control samples of liposomes suspended in PBS is seen over the 4 week period. It is then difficult to attribute a clear reason for such phenomenon. It could be hypothesised that degradation of the polymer is too slow for the 4 week timeframe. It would have then been necessary to assess the degradation behaviour of such fibres once incubated and their pore formation. The formation of pores allows vesicles to escape and be released from the polymer matrix. Therefore, another factor might be here at play with vesicles being somehow bound to the polymer chains and getting entrapped in the matrix. It was not possible to troubleshoot the issue in the timeframe given for this project and the release mechanism of liposomes from coaxial 1:1 (w/w) PDO/PLGA fibres is yet to be determined.
5.4. Conclusions

Coaxial formulations were prepared as scaffolds for the extended delivery of extracellular vesicles in this chapter. The proposed application is for a cardiac patch to implant in the heart epicardium and allow a sustained release profile of vesicles, to confer cardioprotective effects post-myocardial infarction.

On a first stage, poly(lactide-co-glycolide) of different molecular weights electrospinning conditions were optimised. Successful electrospinning was found to be highly dependent on polymer molecular weight and concentration. Fibres were produced and their behaviour in an aqueous environment at physiological temperature assessed (37°C) after 24 hours. However, the scaffolds produced shrink considerably and increase their stiffness upon incubation in such conditions. This is directly related to PLGA’s intrinsic polymer relaxation, which arises due to the PLGA glass transition temperature being very close to the test temperature. By relaxing, PLGA chains tend to coil, hence significantly shrinking. One strategy to overcome such stiffness after an aqueous incubation, is the inclusion of a different polymer in a blend. Poly(dioxanone) was selected for this role owing to its applicability in the clinic and particularly in the cardiac setting. Moreover, it does not relax with temperature.

A range of polymer blends with different ratios of PLGA and PDO were fabricated and assessed in terms of their physical characteristics. FTIR showed that no molecular changes occurred after electrospinning, and the semi-crystallinity associated with PDO was only observable in blends with high ratios of this polymer. Only at a 1:1 (w/w) PDO/PLGA ratio could no shrinking be detected; this was also the only formulation that retained adequate flexibility for implantation, and was therefore selected for further development. Very minimal solvent content was detected in the scaffolds post-production, deeming them
safe to be implanted. This was then taken forward and used to formulate model coaxial formulations loaded with fluorescent liposomes in the core as model vesicles. Fibres produced were uniform in size and demonstrated to be core-shell. Release studies over four weeks demonstrated a burst release after 24 hours and not much being released afterwards. Such hinderance in vesicle release could be intrinsically connected to the specific delivery mechanism from this fibre system which needs to be clarified. The scaffolds produced in this work demonstrated some of the properties required for the development of implantable cardiac patches but there is still room for improvement in terms of their mechanical and release properties.
5.5. References


Chapter 6

Conclusions and Future Work


### 6.1. Summary of Research Findings

The work described on this thesis delivers the fundamental knowledge necessary to further develop extracellular vesicle formulations for tissue regeneration applications using electrospinning.

One of the challenges in extracellular vesicle research lies in the absence of a standardised isolation and characterisation protocol. In *Chapter 3*, different ultracentrifugation protocols were explored and validated on foetal bovine serum (FBS) and mesenchymal stem cell (MSC) samples. The protocol was designed according to sample viscosity, allowing the sedimentation of the appropriate sized particles at each step. While an intermediate filtration step was found to be adequate for FBS samples, a pure differential centrifugation protocol had to be applied to MSC culture media samples due to membrane clogging. Isolates were found to be in the expected size range for exosomes, with consistently more than 80% of the sample sized up to 200 nm; their concentration and protein contents were also determined.

All isolates were found to have a positive effect on cardiomyocyte survival after stress, but MSC samples were found to be discretely more potent than FBS at the same concentration. This indicates the superior influence of MSC extracellular vesicles in the regeneration of cardiac cells, and their applicability in cardiac therapeutics. A study on different storage conditions influence on FBS EV stability was also performed. This revealed the little effect storage temperature (-80°C, 4°C or 20°C) had over time, with protein content and size remaining constant over the course of a month. The fact that EVs are stable, especially at room temperature, provides the confidence necessary to allow them to be formulated without the fear of degradation due to detrimental environmental conditions.
After extracellular vesicles isolation and characterisation, development of fast-release formulations was achieved in *Chapter 4*. Optimisation of the electrospinning processing conditions for selected polymers (poly(ethylene oxide) [PEO] and poly(vinyl pyrrolidone) [PVP]) was first performed. It was found that the choice of solvent is especially relevant when processing biological material in single fluid electrospinning. Ethanol was deemed unsuitable as it prompted sample precipitation. Trifluoroethanol was used instead due to being a gentler solvent on biological samples. Although PEO was able to be electrospun, it was abandoned as a fast-release polymer since it did not allow the full dissolution of scaffolds in aqueous media. PVP was able to produce scaffolds and polymer concentration was found to be the most important parameter to control to ensure the successful production of smooth homogeneous fibres.

The addition of EV isolates produced fibres with a smaller diameter than the PVP blank control due to a change in conductivity of the feeding solution. Physical characterisation of fibres by FTIR did not allow the identification of EV within the fibres, mainly due to the high mass ratio of polymer-to-EVs. X-ray diffraction showed that the fibres, both EV-loaded and blank, were amorphous post-electrospinning. Difficulties were encountered when using nanoparticle tracking analysis to check the vesicle loading, as the PVP scaffold itself forms aggregates in the nanoscale range, interfering with the concentration detected by the instrument. Nevertheless, a stark difference between number of particles detected upon dissolution of the EV-loaded and blank fibres was observed, with this difference being roughly equal to the initially loaded amount of vesicles, indicating their continued presence after electrospinning. This was further confirmed by TEM.
Isolated EVs showed no loss in potency after electrospinning. When fresh and electrospun EVs were added to serum-deprived, stressed cardiomyocytes both were equally potent in restoring cell viability. This offers the potential to use electrospun EV formulations in fast-release applications (such as formulations for reconstitution for injectable administration) or as a platform for more complex controlled-release systems.

Proof-of-concept that EVs can be electrospun and retain their potency encouraged the development of more complex scaffolds in Chapter 5. The proposed application is for an implantable patch to be placed in the hearts’ epicardium to confer cardioprotective effects post-myocardial infarction by allowing the sustained release of extracellular vesicles. Poly(lactide-co-glycolide) (PLGA) of different molecular weights was first processed under different conditions in an extensive electrospinning optimisation process. Successful electrospinning was found to be highly dependent on polymer molecular weight and concentration. Fibres were produced and their behaviour in an aqueous environment at physiological temperature assessed after 24 hours. PLGA fibres were found to shrink considerably due to polymer relaxation, attributed to PLGA $T_g$ being very close to the test temperature.

To solve this problem, PDO was introduced to the system. A range of polymer blends with different ratios of PLGA and PDO were fabricated and assessed in terms of their physical characteristics. Only at a 1:1 (w/w) PDO:PLGA ratio no shrinking could be detected; this was also the only formulation that retained adequate flexibility for implantation, and was therefore selected for further development. FTIR showed that no molecular changes occurred after electrospinning, and the semi-crystallinity associated with PDO was only observable in blends with high ratios of this polymer. Only very minimal solvent content was detected in the scaffolds post-production, deeming them safe to be implanted. However, scaffolds were found to not possess the right mechanical properties,
with higher Young modulus than that of the native/ischemic heart, but the measurements undertaken could have been affected by the experimental conditions (dry measurements instead of wet).

Coaxial fibres were then produced with a PLGA:PDO shell and a core loaded with fluorescent liposomes as model vesicles. Fibres were confirmed to be coaxial by transmission electron microscopy. Unfortunately, the release profile of the scaffolds does not align with the necessary release desired for the cardiac implant. Scaffolds seem to demonstrate just a burst release with no more particles being released over the 4 week period. Hence, there are still improvements to be made for the formulation before it can be tested with extracellular vesicles.
6.2. Future Work

The work described on this thesis provides the fundamental knowledge necessary to further develop extracellular vesicle formulations for tissue regeneration applications using electrospinning. However, further investigation on the subject is necessary to develop scaffolds able to be assessed in vivo.

In Chapter 3, additional work could be undertaken to further characterise the isolates. It would potentially be beneficial to add another purification step such as ultrafiltration or size-exclusion chromatography to the isolation protocol, and assess the obtained yield and particle to protein ratio. On the characterisation side, a Western blot assessment would give an added benefit to confirm the ELISA results. Western blot was in fact tried in this work but without success, indicating the need to further develop and optimise the assay method. The use of Western blotting would allow the detection of more extracellular vesicle markers, with less sample needed and with a higher sensitivity and accuracy. An additional potency assay with a different cell line such as human umbilical vein endothelial cells (HUVECs) would allow the assessment of the angiogenic properties of vesicles, which are relevant in the context of post-myocardial infarction therapy. The assessment of the ideal storage and handling conditions of MSC derived vesicles could be explored in more detail, similarly to the work undertaken for FBS isolates. Also, an investigation of the EVs potency and the presence of specific markers after aging, as well as size, concentration and protein content variation, would provide a more complete picture on the appropriate storage and processing conditions.
Chapter 4 described the formulation of fast-release constructs and the proof of concept that EVs can be processed by electrospinning with no detrimental effects on their potency. However, some additional assays could help clarify the therapeutic potential of the fast-release scaffolds produced. Assessment of how stable and potent vesicles are within the polymer matrix over time and under different storage conditions would give valuable information on the possible use of the formulation as an off-the-shelf product. If scaffolds are to be considered for potential clinical applications methods to sterilise them need to be addressed and the possible impact in their structure and release profile assessed.

The development of controlled-release formulations for cardiac implantation was discussed in Chapter 5. It should be noted that a final scaffold ready for in vivo work was not attained so the future work is closely related to the caveats of this chapter. Testing of mechanical properties should be performed in an aqueous environment and iterative formulation development undertaken to match these to the native tissue. A degradation study on blank fibres to assess the hydrolysis process and pore formation over time could be performed to try to establish a possible release mechanism of large lipidic compounds from the core of the fibres. This should be complemented with another release study with increased liposome loadings, to assess whether the release is concentration dependent. If successful, loading with extracellular vesicles should be undertaken and their release and potency tested; favourable results in these assays could then be followed by an in vivo study in a myocardial infarction rat model.