Protein encapsulation by electrospinning and electrospraying

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Highlights

- Electrospinning and electrospraying can be used for protein encapsulation.
- Use of biocompatible, FDA-approved materials may facilitate clinical translation.
- Coaxial architectures offer advantages over blend and emulsion techniques.
- Preservation of bioactivity and sustained release profiles can be achieved.
- Potential for scaled-up manufacturing paves the way for product commercialisation.

Abbreviation list

ALP Alkaline phosphatase
BMP Bone morphogenetic protein
bFGF Basic fibroblast growth factor
BSA Bovine serum albumin
CAM Chorioallantoic membrane
CD Circular dichroism
CTGF Connective tissue growth factor
DSDP Dual-source dual-power
EC Endothelial cell
ECM Extracellular matrix
EGF Epidermal growth factor
ELISA Enzyme-linked immunosorbent assay
FDA United States Food and Drug Administration

FITC Fluorescein isothiocyanate

GAG Glycosaminoglycan

GDNF Glial cell line-derived neurotrophic factor

GI Gastrointestinal

HRP Horseradish peroxidase

IGF-1 Insulin-like growth factor

IFN-γ Interferon-γ

LbL Layer-by-layer

LSCM Laser scanning confocal microscopy

MSC Mesenchymal stem cell

NGF Nerve growth factor

OVA Ovalbumin

PDGF Platelet-derived growth factor

SDS-PAGE Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SMC Smooth muscle cell

TE Tissue engineering

TGF-β Transforming growth factor β

TNF-α Tumour necrosis factor α

tPA Tissue plasminogen activator

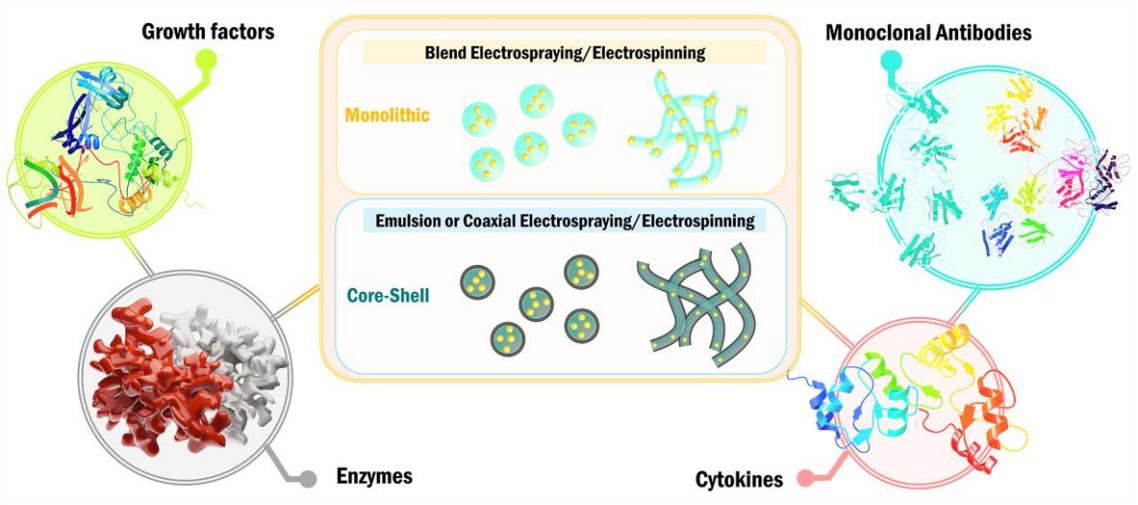
VEGF Vascular endothelial growth factor

Abstract

Given the increasing interest in the use of peptide- and protein-based agents in therapeutic strategies, it is fundamental to develop delivery systems capable of preserving the biological activity of these molecules upon administration, and which can provide tuneable release profiles. Electrohydrodynamic (EHD) techniques, encompassing electrospinning and electrospraying, allow the generation of fibres and particles with high surface area-to-volume ratios, versatile architectures, and highly controllable release profiles. This review is focused on exploring the potential of different EHD methods (including blend, emulsion, and co-/multi-axial electrospinning and electrospraying) for the development of peptide and protein delivery systems. An overview of the principles of each technique is first presented, followed by a survey of the literature on the encapsulation of enzymes, growth factors, antibodies, hormones, and vaccine antigens using EHD approaches. The possibility for localised delivery using stimuli-responsive systems is also explored. Finally, the advantages and challenges with each EHD method are summarised, and the necessary steps for clinical translation and

scaled-up production of electrospun and electrosprayed protein delivery systems are discussed.

Graphical abstract



Keywords: electrospinning, electrospraying, protein encapsulation, drug delivery, tissue engineering

1. Introduction

Peptides and proteins are arguably the most multifunctional biomolecules in the body, participating as catalysts in biochemical reactions, driving inflammatory responses, modulating cell proliferation and differentiation, and regulating metabolic and signalling pathways [1]. A very wide range of pathological conditions, including genetic, metabolic, inflammatory, and oncological diseases, arise from abnormalities related to endogenous protein function. Consequently, interest in peptides and proteins as therapeutic biological agents has dramatically increased over the past few decades. In 1982, recombinant insulin was approved by the United States (US) Food and Drug Administration (FDA) and became the first recombinant protein-based therapeutic agent to be introduced to the market [1–3]; since then, more than 60 FDA-approved protein drugs have been commercialised, and a great number more are currently under evaluation in preclinical and clinical trials [4].

Therapeutic protein-based agents offer several advantages over traditional small molecule drugs. Owing to their larger size and multiple sites of interaction, they act on the intended target with a very high specificity, potentially decreasing the adverse side effects that are inevitably associated with small molecules [1,5]. Further, proteins usually carry out physiological functions that are too complex to be fully reproduced by any other compound [1]. As an example, transforming growth factor β (TGF-β) is a cytokine involved in a multitude of biological responses, including wound healing, inflammation, adult stem cell differentiation, and embryonic development [6]. Thus, the number of protein-based therapeutics is expected to grow exponentially in the future, particularly with rapid evolution of the fields of genomics, transcriptomics, and proteomics, which facilitate the screening and testing of potential targets [5].

However, the clinical application of peptide- and protein-based therapeutic agents has been held back owing to several obstacles associated with their formulation and methods of administration. For instance, oral delivery has the advantage of allowing self-administration and high patient compliance, but it results in low systemic bioavailability of the protein due to degradation in the gastrointestinal (GI) tract. Since proteins have high molecular weights and often present several ionisable groups, overcoming biological membranes like the intestinal epithelium or the blood-brain barrier is often challenging [7,8]. Ergo, most peptide- and protein-based therapeutics are administered *via* parenteral injections [9], even though this method can be highly problematic. Once in the blood stream, these agents have very short half-lives, being susceptible to clearance by immune cells or elimination by the liver or kidneys, depending on their physicochemical and structural characteristics [10,11].

Another problem that is commonly observed with protein drug administration is the occurrence of structural modifications that may compromise protein integrity and the native tertiary structure, causing denaturation, misfolding or aggregation [9,12]. Not only do these structural alterations result in loss of bioactivity, but they can also lead to immunogenicity and higher toxicity [13–15]. These rapid clearance or degradation mechanisms substantially decrease the bioavailability of the therapeutic protein, forcing the administration of higher and more frequent doses, and thereby reducing patient compliance and increasing the possibility for the manifestation of deleterious side effects [9,16]. Finally, proteins have low stability and high susceptibility to degradation during processing, storage, and distribution. The establishment and maintenance of a “cold chain” (temperatures typically near 0 °C) from manufacturer to patient is therefore required [17], representing a significant economic and logistic burden [18,19].

Taking this information into account, it becomes clear that there is a need for new encapsulation and delivery systems capable of preserving protein bioactivity while providing protection against degradation. Sustained release is also required, in order to lower the applied dose and decrease the frequency of administration to overcome the low bioavailability of the protein. Combining biological, pharmacological, and physicochemical principles, it is possible, albeit challenging, to design tailored formulations to achieve desired protein release profiles *via* different routes of administration. Further enhancements may allow for targeted delivery, specific responses to different stimuli (*e.g.* temperature, pH, enzymatic activity, magnetic fields), and easier permeation of biological barriers [20].

Current drug delivery systems exist in a plethora of different forms (such as micro- or nanoparticles and fibrous scaffolds) and their chemical composition can also be very diverse, ranging from inorganic materials to lipid or polymer-based formulations. Frequent protein encapsulation methods include solvent evaporation and extraction, coacervation, polyelectrolyte complexation/ionotropic gelation, and spray drying [21]. Over the last couple of decades, however, there has been an increasing interest in electrohydrodynamic (EHD) techniques for protein drug delivery. EHD processes employ an electric field to solidify a polymer solution for the production of fibres or particles with high surface area-to-volume ratios. EHD techniques encompass electrospinning, in which the final products are nano- or microfibres, and electrospraying, where nano- or microparticles are produced instead [22] (**Figure 1**). The main difference between the two processes is the solution viscosity: electrospinning requires a highly viscous polymeric solution, while at lower viscosities electrospraying occurs.

A wide variety of natural and synthetic polymers can be used in EHD, including biocompatible and biodegradable polymers such as gelatin, hyaluronan, chitosan, poly(ε-caprolactone) (PCL), poly(lactic acid) (PLA), poly(ethylene glycol) (PEG), and poly(D,L-lactide-*co*-glycolide) (PLGA), among many others [23]. The choice of the polymer(s) to be used and the EHD processing conditions will significantly influence the size, structure, morphology, and release behaviour of the fibres or particles generated [24]. This review will first provide an overview of the technical principles of EHD processing, comparing blend, emulsion, and co-/multi-axial electrospinning and electrospraying in the context of protein delivery. Examples of key studies making use of these techniques for the encapsulation of enzymes, growth factors, antibodies, and the fabrication of vaccines will then be presented. Finally, we briefly discuss the scale-up process necessary for industrial production and clinical translation of these protein delivery systems towards the establishment of new biological therapies.

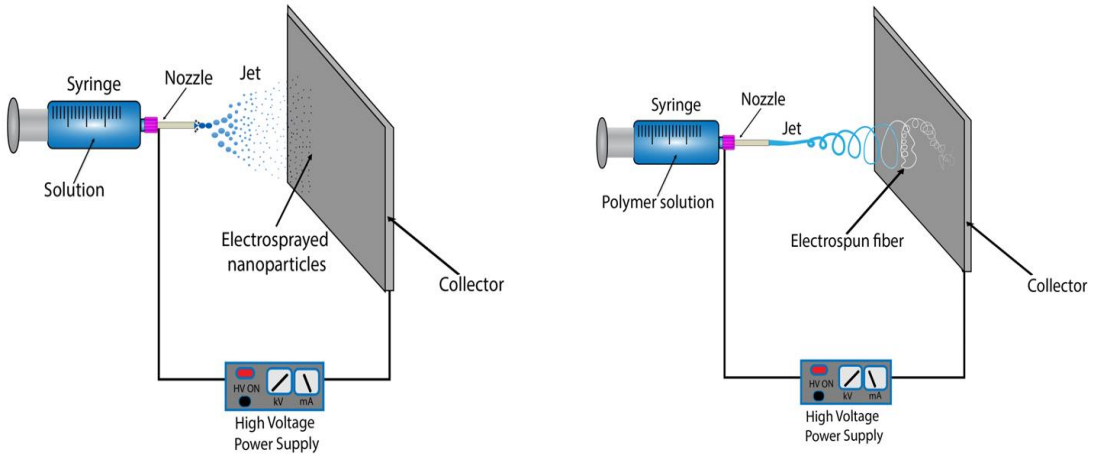


Figure 1. Electrospraying (left) and electrospinning (right) schematics.

2. Fabrication of monolithic and core-shell fibres and particles

Typically, electrospinning refers to the fabrication of continuous fibres from polymer suspensions, solutions, or emulsions extruded through a conductive spinneret under the application of a strong electric field (melts can also be processed). An adjustment of the solution properties and processing parameters allows the production of particles instead of fibres, and this process is called electrospraying [22]. The standard setup for EHD processing (**Figure 1**) includes a high voltage power supply, a syringe pump, a spinneret made from a conductive material, and a collector consisting of a grounded metal plate [25]. The spinneret can comprise a single needle (monoaxial electrospinning; **Figure 2A**), which yields monolithic fibres from a single solution (**Figure 2B**). Alternatively, a coaxial spinneret, with two concentrically nested needles (**Figure 2C**), can be used to process two fluids into core/shell fibres (**Figure 2D**). It is also possible to process three or more fluids (so-called multi-axial electrospinning).

The polymer solution is pumped through the spinneret at a regulated and specified flow rate. Without the application of an electric field, the solution exits the spinneret in the form of droplets, so as to minimise surface tension [22]. Once an electric force is applied to the spinneret, electrostatic charge builds up near the orifice as the polymer solution exits the needle, which, together with the potential difference between the spinneret and the grounded collector, causes the droplet to deform into a conical shape termed the ‘Taylor cone’ (**Figure 2E**) [26]. Further increases in the strength of the electric field cause greater charge accumulation at the droplet surface, generating a repulsive force that will eventually overcome the liquid surface tension and culminate in the formation of a jet [27]. This jet either then accelerates towards the collector, narrowing as it is drawn under the electrical field and yielding fibres, or undergoes Coulombic

explosion. The latter results in the formation of droplets, and ultimately to particles being deposited on the collector. In either case, as the solution travels towards the collector, the solvent is evaporated, resulting in dry structures [28].

EHD techniques thus allow the production of particles and continuous fibres with sizes on the nano- or micro-scale [29,30]. Proteins and other therapeutic agents can be encapsulated within these structures using different variations of these methods, of which the most common are blend, emulsion, and coaxial electrospinning/spraying [31]. Importantly, due to the rapid speed of the drying process, the distribution of an active ingredient within the solution(s) is maintained and propagated to the solid state. This generally results in amorphous solid dispersions of the therapeutic agent within the fibres or particles forming, which usually increases the dissolution rate of the drug. It also means that the presence of two different solutions in the two needles of a coaxial spinneret will generate a core/shell structure if the processing conditions are appropriately set.

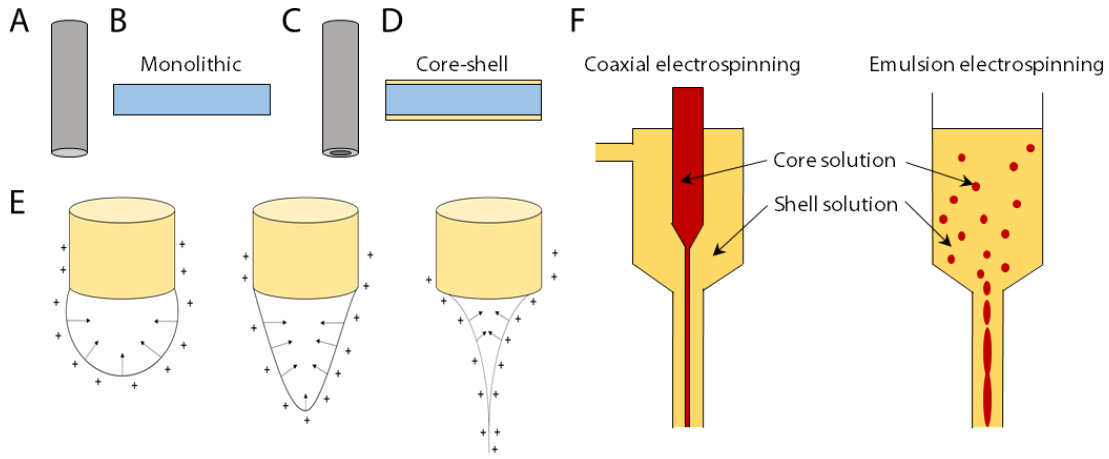


Figure 2. Needle EHD processing. (A) Single-liquid (monoaxial) spinnerets generate (B) monolithic fibres. (C) Coaxial spinnerets result in (D) core-shell fibres. (E) Forces acting on the tip of the spinneret and liquid surface upon application of an electric field. As charge builds up, the liquid droplet progressively loses its spherical shape and the Taylor cone is formed, followed by the ejection of a jet from the surface. (F) Schematic illustration of coaxial vs. emulsion electrospinning: both result in core-shell fibre architectures, but while coaxial electrospinning is performed with a coaxial spinneret emulsion electrospinning makes use of a monoaxial needle.

2.1. Monoaxial EHD processing

2.1.1. Blend electrospinning and electrospraying

The simplest method for the development of a drug delivery system using EHD processing relies on the dissolution or dispersion of the drug of choice with a polymer carrier, usually in a volatile solvent (so called ‘blend’ processing) [31]. A high electrical potential, usually 5-20 kV for monoaxial electrospinning/spraying of a single fluid, is

applied to yield monolithic materials with the drug typically dispersed on the molecular level within the fibre or particle product.

The release profile of a drug from a delivery system can be governed by several different mechanisms. Most commonly, drug release is controlled by the diffusion of the active pharmaceutical ingredient through the carrier matrix, dissolution of the carrier matrix within the release media, or erosion of this matrix in the case of insoluble materials [22]. Other mechanisms such as osmosis and swelling may also regulate the release of the encapsulated active ingredients [32]. Since proteins have a number of ionisable sites, they are often charged during the EHD process, and thus will tend to migrate towards the surface of the jet *via* dielectrophoretic motions [33,34]. Such surface enrichment of the encapsulated drug is common in blend electrospinning/spraying, and is likely to lead to an initial burst of release [35]. Blend fibres have been used successfully for the delivery of antimicrobial peptides and other small molecules [36–38]. However, for labile biomolecules like proteins, issues may arise from the typical use of organic solvents in blend EHD processing. A prolonged exposure to these is likely to cause protein misfolding or aggregation, and consequent loss of activity. This limits the use of blend nanofibres/particles for protein delivery [39].

2.1.2. Emulsion electrospinning and electrospraying

Emulsion EHD processing is a relatively new approach for the fabrication of core-shell nanostructures using a monoaxial nozzle (**Figure 2F**), either through a water-in-oil emulsion (hydrophilic droplet phase and lipophilic continuous phase) or vice-versa [40]. This emulsion is used to directly encapsulate compounds in the core of the nanostructure [41]. More specifically, the process involves the electrospinning/spraying an emulsion of two or more immiscible fluids typically stabilized by surfactants such as Pluronics®, Span® 60, or Span® 80 [42]. The product, unlike a blend fibre, consists of two or more phases that do not mix in the EHD process. Conventional lipophilic polymers include polyesters such as PCL, PLA, PLGA, polyurethanes (PUs), and polystyrene, while classical hydrophilic polymers comprise poly(vinyl alcohol) (PVA), poly(ethylene oxide) (PEO), polyvinylpyrrolidone (PVP), cellulose derivatives, chitosan, and alginates [31]. The former will dissolve freely in organic solvents, while the latter are soluble in water or polar liquids.

For hydrophilic molecules such as proteins, water-in-oil emulsions are used. The active ingredient of interest is dissolved in an aqueous phase, followed by dispersion of these aqueous droplets in an organic solvent. Since organic solvents are more volatile, they evaporate at a faster rate, which increases the viscosity of the lipophilic phase. This

causes the aqueous phase to concentrate in the centre of the jet, thereby resulting in core-shell structures (**Figure 2F**) [43]. Emulsion EHD not only avoids contact of the protein with organic solvents, but also favours their localisation within the centre of the fibres/particles, contributing to a minimisation of burst release effects [44]. Another advantage of this technique is the attainment of a core-shell structure without the use of a complex coaxial spinneret.

The primary disadvantage of emulsion EHD processing is the difficulty in generating stable and uniform core-shell structures using solutions with low surface tension, which characterises most of the solvents used in conventional electrospinning and electrospraying [22]. Emulsion instability is also a common issue. Furthermore, the emulsification process often involves ultrasonication or other mechanical mixing processes to disperse the aqueous phase within the organic solvent, which may affect the structural integrity and functionality of an encapsulated biomolecule.

2.1.3. Experimental considerations

In order for an EHD process to be successful, the optimisation of several solution, operational, and environmental parameters is required, and these will also influence the morphology and structure of the products obtained. A brief summary of the main criteria to be taken into consideration are detailed below, but for more extensive information other recent reviews can be consulted [22,45].

Solution parameters include viscosity, conductivity, and surface tension, which are determined by the chosen solvent and the polymer's molecular weight and concentration [45,46]. The viscosity of the solution reflects the degree of molecular entanglement in the liquid [47]. It should be high enough to allow the formation of the Taylor cone, but sufficiently low to avoid clogging of the spinneret. Lower solution viscosities are obtained by using reduced polymer concentrations and molecular weights. If the viscosity is sufficiently high, then a continuous jet is formed and fibres ultimately generated. However, at low viscosities, the solution viscosity is not enough to overcome the liquid surface tension, causing the jet to break into small charged droplets rather than forming continuous fibres. This is electrospraying, and results in particles. Solvent evaporation during the droplets' trajectory towards the collector decreases droplet size and leads to the accumulation of more charges at their surface, further breaking them into smaller particles (atomisation) [22]. Intermediate viscosity values usually give rise to beaded fibres, halfway between electrospraying and electrospinning (**Figure 3**).

Surface tension is another very important parameter in EHD processing. Since it opposes the jetting effect of the applied electrical force, solutions with increased surface

tension may be difficult to spin, easily giving rise to droplets or beaded fibres. In turn, higher solution conductivity facilitates the EHD process, since it promotes greater charge accumulation at the liquid surface and thereby facilitates Taylor cone and liquid jet formation [48]. Another aspect that must be taken into account is the volatility of the solvent of choice. The solvents used in EHD processing should be sufficiently volatile in order to evaporate completely before reaching the collector, so as to avoid fused fibres or particles and other defects like porous structures. However, if the solvent is excessively volatile, premature evaporation may hamper the generation of products with smaller dimensions, as the time for fibre elongation or particle atomisation decreases [22]. The diameter of the ejected products also increases with increased viscosity and decreased conductivity. Clogging of the spinneret may also occur with highly viscous and low conductivity solutions.

Operational or processing parameters comprise the solution flow rate, the distance from spinneret to collector, and the applied voltage. Increasing the solution flow rate may result in larger particle/fibre diameters, since there is a higher amount of liquid being ejected per unit of time. Very high flow rates may lead to incomplete solvent evaporation and to structural defects (wrinkles, pores, fused fibres/particles). On the contrary, if the flow rate is too low then beaded fibre morphologies or discontinuous processes can result [22,49]. The distance from the spinneret to the collector has a direct influence on the trajectory of the charged polymer jet/droplets: if it is too short, there is insufficient time for the solvent to fully evaporate, and defects may form in the products. If the distance is too long, the yield of the EHD process decreases, since the particles or fibres start depositing on other surfaces as they seek the shortest route to dissipate their charge. Longer distances normally give rise to smaller diameters, as the period of time available for elongation or atomisation is longer. There is interplay between the spinneret-to-collector distance and the applied voltage, since both affect the electrical field gradient. The effect of the applied voltage on product morphology is complex, however, and contradictory results have been reported in the literature [22,50,51]. The applied voltage needs to be high enough to promote the generation of the Taylor cone and ensure stable ejection of the polymer jet (without dripping), but kept under a certain threshold to avert instabilities like multiple jet formation.

Environmental parameters are temperature and humidity, which have a significant influence on solution viscosity, surface tension and conductivity, as well as solvent evaporation rates. High temperatures decrease the viscosity and increase the conductivity of the solution [52], but equally result in faster solvent evaporation rates. Conversely, solvent evaporation is delayed with increased humidity values [22].

The effects of each parameter on the morphology of the obtained products are summarised in **Figure 3**.

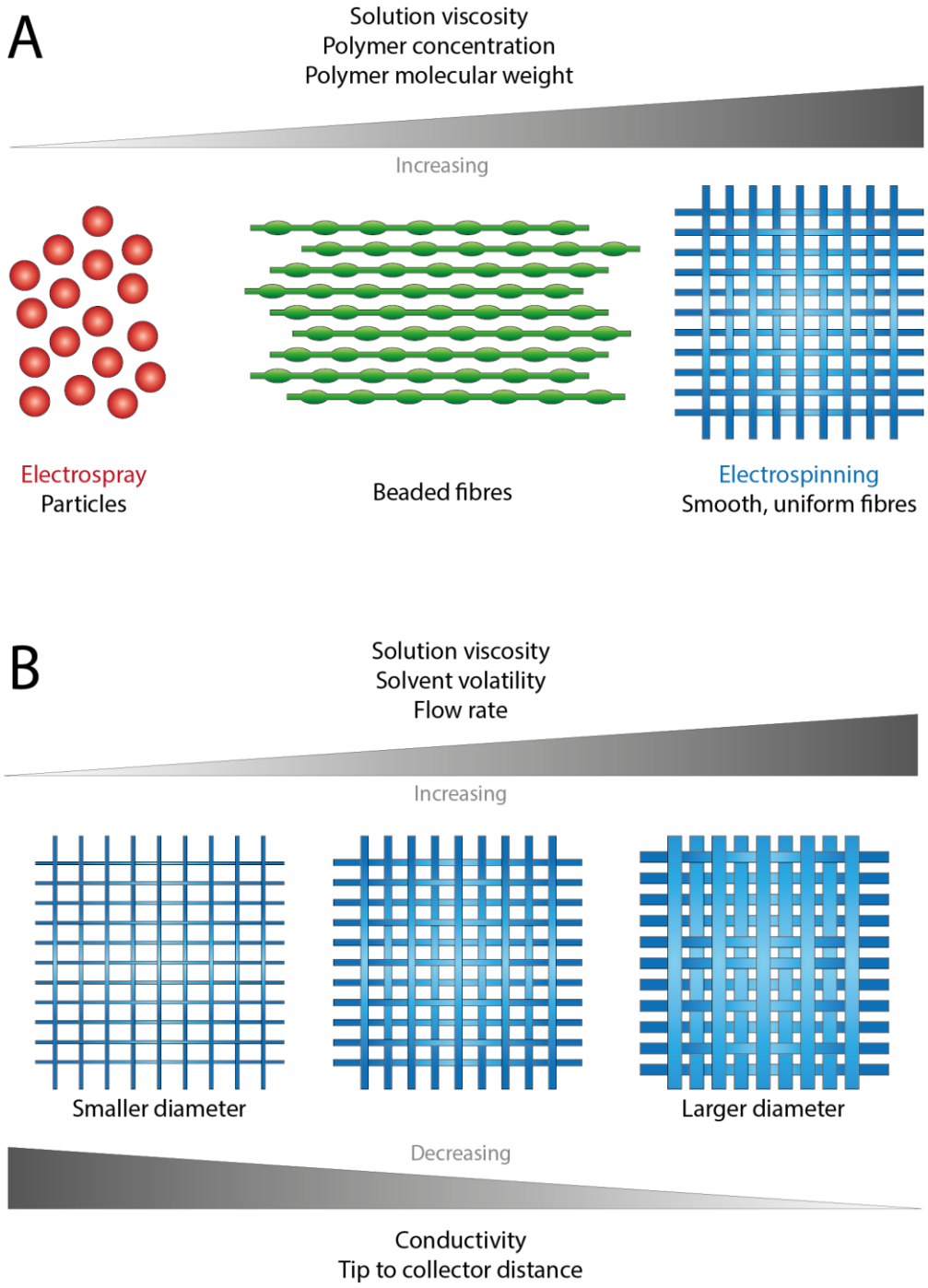


Figure 3. A schematic illustration of the influence of EHD processing parameters on the morphology of the obtained products. A) Effect of solution parameters on the EHD products; B) influence of solution and processing parameters on the diameter of the fibres obtained in electrospinning.

2.2. Coaxial EHD

Coaxial EHD processing uses a spinneret composed of two nested concentric needles [46] (**Figure 2C, F**). This allows the simultaneous dispensing of two solutions:

the core solution is pumped through the inner needle, while the shell solution exits through the outer needle, generating core-shell structures in a single step. As EHD processing is rapid, the core and shell components will have distinct and separate compartments. Coaxial EHD is particularly useful because it can be performed successfully with only one of the fluids independently processable into solid products *via* EHD, expanding the range of polymers that can be worked with [31]. Most frequently, a spinnable shell solution is used to encapsulate an otherwise unspinnable core material. However, the opposite is also possible, where polymeric core solutions can be surrounded by unspinnable sheath materials, such as pure or mixed solvents [53–55] or electrolyte solutions [56,57].

Coaxial processes permit the encapsulation of one or more therapeutic agents within stratified particle carriers or nano/microfibres. This approach can be particularly beneficial with protein active ingredients, since it is possible, for instance, to use an aqueous protein-loaded core solution and a polymer shell solution in an organic solvent. This minimises contact between the protein and the organic solvent, and thus should help to prevent degradation. The use of the coaxial technique can also help to prevent a burst of release and extend drug release [31]. Potential caveats of this method include a limited rate of production and a complex experimental process requiring extensive optimisation. Multi-axial EHD processing (using more than two fluids) can also be performed with multiple concentric needles, generating multi-layered products. Highly tuneable and exquisite architectures can be produced, but this comes at the expense of an increasingly complex experimental process.

2.2.1. Experimental considerations

To achieve successful coaxial electrospinning/spraying, solution, processing, and environmental parameters all need to be considered similarly to monoaxial work (see **Section 2.1.3**). However, there are some additional considerations to be taken into account when performing coaxial EHD, which are listed in **Table 1**.

Table 1. Summary table of key parameters for successful coaxial electrospinning and spraying [22].

Considerations		Impact
Solution parameters	Rate of solidification	• Core and shell solutions should have similar rates of solidification to allow stable co-flow and avoid needle clogging.
	Viscoelasticity	• Core-shell fibres/particles will form if one solution has sufficient viscoelasticity. The other solution may be composed of a non-spinnable solution.
	Miscibility and volatility	• High miscibility of the inner and outer solutions may cause the encapsulated agent to leach from the core to the shell. • Marked volatility differences between the core and shell solvents can result in structural defects (*e.g.* pore formation).
	Flow rate	• The shell flow rate must be greater than that of the core to allow efficient encapsulation. Larger core:shell flow rate ratios result in bigger core components. • The core to shell flow rate ratio is usually 1:3 to 1:10.

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Table 2 summarises the advantages and disadvantages associated with blend, emulsion, and coaxial EHD processing.

Table 2. Summary of the advantages and disadvantages of blend, emulsion, and coaxial EHD processing with regard to protein delivery systems [31,58].

EHD Method	Fibre/Particle Morphology	Advantages	Disadvantages
Blend	Monolithic	• Simplicity	• Direct contact with organic solvents may cause protein denaturation and loss of function
Emulsion	Core-shell	• Protein dissolution within an appropriate solvent and separation from the organic conditions required to dissolve the carrier polymer • Core-shell structures are obtained without the use of complex coaxial spinnerets	• Lack of control over the distribution of the therapeutic agent within the fibres or particles • Unsuitable for polymer solutions with low surface tension • Use of ultrasonication or absence of surfactants during the emulsification process may compromise emulsion stability, protein structure, and bioactivity
Coaxial	Core-shell	• Precise control over the location of the drug within the core or shell of the fibres or particles • High protein loading capacity • Creation of more complex systems and multiphasic release profiles • Non-spinnable polymers can be used in the core or shell of the fibres or particles	• More complex and expensive setup

3. EHD processing for protein delivery

The generation of particles or fibres by EHD techniques has been used to microencapsulate small molecules [59–62], DNA [60,63,64], proteins [65,66], and even cells [67–69] for therapeutic delivery. Electrospinning produces fibres with high interconnected porosity and adjustable pore size, surface functionalisation potential, and structural similarity to the extracellular matrix (ECM), highlighting its applicability in drug delivery, particularly in tissue regeneration [24]. In turn, electrospraying gives rise to nearly monodisperse particles and high encapsulation efficiencies, unlike other frequently used techniques such as spray drying and traditional solvent evaporation (*i.e.*, fabrication of micro- or nanoparticles *via* single or double emulsification) [24,70,71]. Importantly, EHD processing is cost-effective, robust and scalable, which paves the way for its industrial application [22].

It is essential to choose a polymer or polymer mixture that is compatible with the drug selected for delivery [72]. It should be kept in mind that electrospinning and electrospraying usually require the use of organic solvents, which can destabilise the structural integrity and compromise the therapeutic activity of unstable biomolecules, such as proteins [22]. In this case, emulsion or co-/multi-axial EHD methods may be preferred, since these techniques could protect protein drugs from a direct interaction with the organic phase.

As mentioned in **Section 2.1.1.**, the release of drugs encapsulated in polymer delivery systems is controlled by several different processes: the diffusion of the drug through the polymer matrix, the dissolution of the polymer matrix itself, and, in the case of an insoluble polymer template, the degradation of the polymer matrix by erosion [72]. Choosing polymers with distinct density, molecular weight, and hydrophobicity is fundamental to achieve the required release profiles. For example, PEO is a hydrophilic, fast-dissolving, polymer that can be utilised when an immediate drug release is required, while PLGA is largely insoluble in water but degrades slowly by ester hydrolysis, giving more sustained release of the encapsulated drug [22]. A prevailing issue characteristic of most protein delivery systems is the inherent difficulty in controlling the initial release of the incorporated agent(s). The latter often comprises a burst of release, which is usually not desirable for several reasons: i) it is generally not controllable or predictable; ii) elevated drug levels from the burst effect may exceed therapeutic concentrations and induce toxicity; iii) higher drug loading or further administrations are required in order to maintain therapeutic levels over an extended period of time after the initial burst release, which is not only cumbersome to the patient, but also economically inefficient [73,74]. Therefore, the development of new strategies that can avert initial burst effects and promote sustained drug release is now crucial. Co-/multi-axial EHD processes allow for the production of layered structures that may delay protein diffusion and, hence, prevent burst releases.

EHD processing of polymer solutions is a valuable method that can be used for the generation of protein delivery systems, both for local and systemic administration. Appropriate control of the polymer solution, processing, and environmental parameters will result in a highly reproducible and adjustable system that can then be tailored to fulfil specific clinical needs. The key advantages of EHD are summarised in **Table 3**.

Table 3. A summary of the advantages of EHD processing for protein encapsulation and delivery.

EHD processing
• Single-step methodology • Significant encapsulation efficiency (up to 100%) [75]

- High drug loading capacity (up to 60% in weight) [75]
- Particle/fibre uniformity [22]
- Capability of processing over 100 polymers with distinct properties [76]; non-spinnable polymers can be used in coaxial or multi-axial techniques
- Scaffolds with high interconnected porosity resulting in a large surface area, ranging from 10-100 m^2/g [77]
- Adjustable release profile (both hydrophilic and hydrophobic polymers, variations in molecular weight, and different geometries may be used) [78]
- Cost-effective production and reproducibility [79,80]

Early reports demonstrated the potential of EHD processing for the encapsulation of bovine serum albumin (BSA) [81,82], enzymes [82,83], and growth factors [84], which catalysed interest in electrospinning and electrospraying for protein delivery. The next section will provide a comprehensive review of different EHD formulations developed for this purpose, including encapsulation of enzymes, growth factors, antibodies, vaccine antigens, and hormones.

3.1. Bovine serum albumin encapsulation

A lot of studies directed at protein encapsulation by EHD processing are performed using BSA as a model protein, due to its well-known properties, stability, easy accessibility, and low cost. Jiang *et al.* [82] encapsulated BSA in electrospun dextran membranes using water as solvent, proving that the protein could be directly incorporated into an aqueous polymer solution without compromising the stability of the electrospinning process. The structure and morphology of the fibres was preserved for BSA concentrations up to 10 wt.%. Additionally, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and circular dichroism (CD) analyses indicated that there was no protein degradation or denaturation. However, the use of water as the solvent in electrospinning is often challenging, since its low volatility and high surface tension hamper the stability of the EHD process and the production of uniform fibres [22].

Hence, in another report, core-shell nanofibres were produced by monoaxial emulsion electrospinning, in which aqueous solutions of BSA and methyl cellulose (used as a protein stabiliser) were mixed with poly(D,L-lactic acid) (PDLLA) in chloroform solution and ultrasonicated to form emulsions before EHD processing [85]. The authors found that the aqueous phase: organic phase ratios used in the emulsification procedures had a significant influence on the fibre diameter and structure. The fibre diameter was inversely proportional to the aqueous phase fraction (*i.e.*, thinner fibres were obtained for higher aqueous: organic ratios). These observations were expected, since an increase in the aqueous content of the spinning solution will also enhance its

conductivity, leading, as previously mentioned, to smaller diameters. Moreover, fibres with a more defined and complete core-shell structure were obtained with lower volume fractions of the aqueous phase (1.0%). The latter also resulted in a reduced burst effect and more sustained BSA release over time. It was suggested that, because the nanofibres produced from emulsions with higher aqueous: organic volume ratios have smaller diameters, the increased fibre surface area-to-volume ratios and thinner shell components may have led to faster protein release. However, several concerns were raised regarding the effect of ultrasonication on the protein's native structure and conformation. SDS-PAGE analyses did not reveal the occurrence of any aggregation or degradation, but size-exclusion chromatography and Fourier-transform infrared (FTIR) spectroscopy showed that BSA suffered significant modifications in its secondary structure during ultrasonication. The deleterious effects of ultrasonication were also noted after encapsulation of lysozyme in electrospun PDLLA fibres, although to a lesser extent, with a bioactivity loss of ~16% after emulsification [86]. Subsequent studies have replaced ultrasonication with high speed magnetic stirring to minimise mechanical stress, and surfactants like Tween 20® and Span80® were introduced to improve protein stability during emulsification [87,88].

A comparison of the encapsulation of BSA in blend and coaxial electrospinning techniques was performed in two reports, studying fibre morphology and structure, protein distribution, and release profiles [89,90]. Both studies used a shell of PCL in trifluoroethanol (TFE) and an aqueous core of PEG and BSA for coaxial electrospinning, whereas the blend fibres were a composite of both polymers and the protein. The solutions used for blend and coaxial electrospinning had the same PCL, BSA, and PEG concentrations. It was found that, unlike the coaxially electrospun nanofibres, the blend fibres presented an irregular beaded morphology. Furthermore, laser scanning confocal microscopy (LSCM) analyses demonstrated that rhodamine B (in the PCL solution) and fluorescein isothiocyanate (FITC)-conjugated BSA (in the core) were evenly distributed throughout the coaxial nanofibres, while in blend fibres they were mainly concentrated in the bead structures [90] (**Figure 4**). The release profiles were also distinct between the two fibre types: a burst release effect was observed for both blend and core-shell nanofibres, but the latter elicited a more extended protein release profile, over more than 30 days. The work by Ji and co-workers corroborated previous observations [91] showing that the addition of PEG to the nanofibres accelerates protein release, presumably due to its hydrophilic nature facilitating fibre dissolution and the penetration of larger amounts of release medium into the fibres.

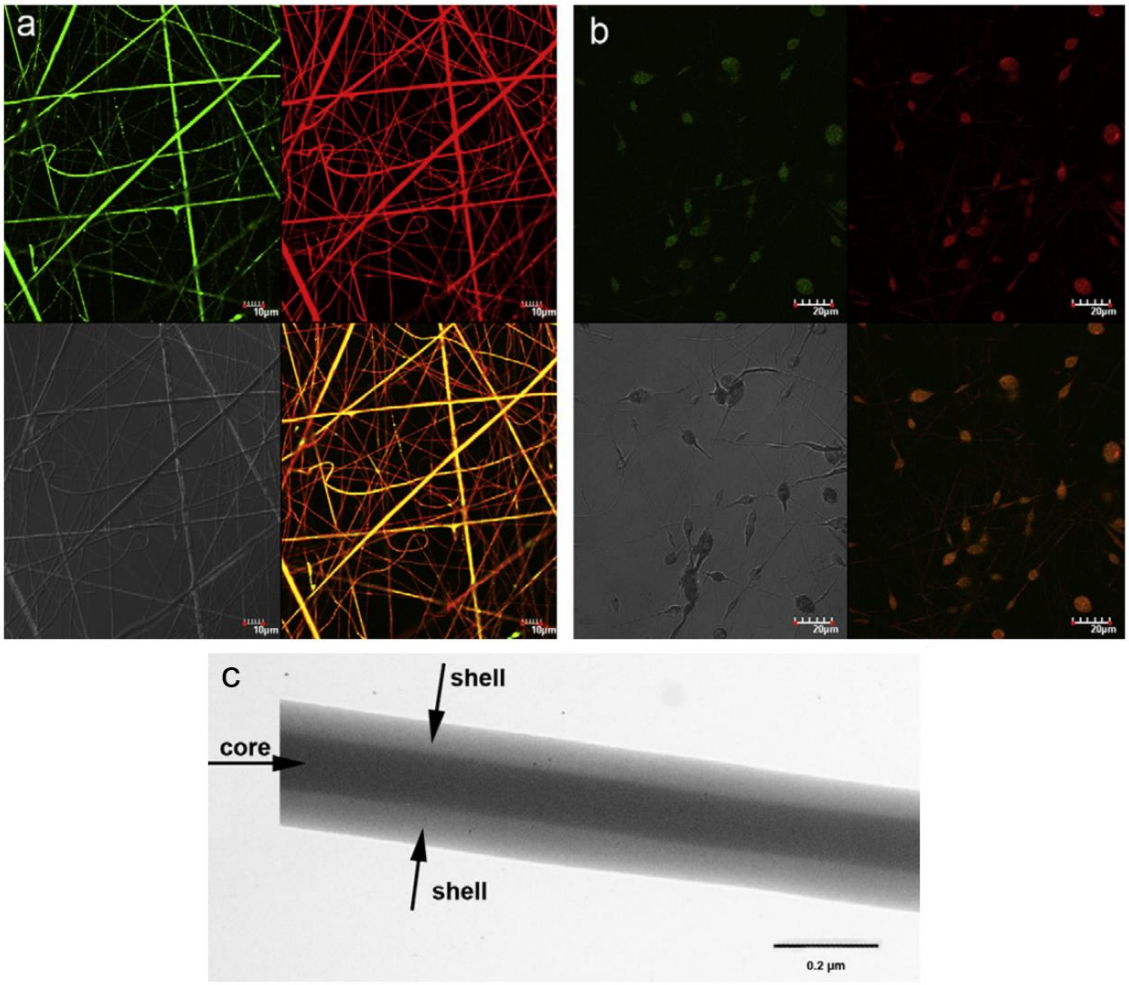


Figure 4. Laser scanning confocal microscopy images demonstrating the distribution of fluorescein isothiocyanate (FITC)-bovine serum albumin (BSA) in (a) coaxial and (b) blend nanofibrous scaffolds. The panels in each individual image correspond to FITC (upper left, green), rhodamine B (upper right, red), natural light (bottom left), and a merge of FITC and rhodamine B channels (bottom right). (c) Transmission electron microscopy (TEM) imaging shows the core-shell structure of the coaxial fibres. Adapted from [90] with permission from Elsevier. Copyright © Elsevier 2010.

A different approach [92] applied triaxial electrospinning for the production of fibres with three distinct layers – a PCL core, an intermediate layer of gelatine, and a PLGA shell – for dual drug delivery. Rhodamine B (RhoB) was incorporated in the PLGA shell, while FITC-conjugated BSA was encapsulated in the gelatine layer of the fibres. A burst release effect followed by a sustained release profile was observed for both molecules, and the release of FITC-BSA was faster when RhoB was present in the shell layer. The authors attributed this phenomenon to the hydrophilic character of RhoB, which promotes the penetration and retention of higher amounts of water in the fibres and, therefore, enhances the diffusion and release of BSA. In addition, the tri-layered scaffolds were associated with higher levels of cell metabolic activity after culture with adipose-derived stem cells than monolithic PLGA and gelatine (core)-PLGA (shell) fibres. PLGA electrospun scaffolds tend to shrink when in contact with cell culture media

[93]. The authors suggest that the addition of the PCL core layer in the triaxial fibres helped reduce the contraction of the fibrous scaffolds more efficiently than the uniaxial and coaxial systems, therefore providing a larger surface area for cell attachment and proliferation.

BSA and lysozyme were successfully encapsulated in PLGA microparticles by emulsion electrospraying in the presence of a surfactant (Pluronic® F127) [94]. In this report, the use of sonication to generate an emulsion and surfactant addition were found to improve the BSA encapsulation efficiency (76.6% was achieved using probe sonication for the emulsification and a Pluronic® F127 concentration of 10%, compared to an encapsulation efficiency of 20% attained by vortex emulsification and absence of surfactant). SDS-PAGE, FTIR, CD, and enzyme-linked immunosorbent assay (ELISA) studies indicate that the secondary structure of the protein was not majorly affected. Likewise, encapsulation in PLGA microparticles preserved the enzymatic activity of lysozyme at 92%. The PLGA concentration and the presence of the surfactant significantly influenced the protein release profiles from the different formulations. For instance, the initial burst was suppressed in the release profile of BSA-loaded microparticles prepared from 10% (w/v) PLGA containing 10% Pluronic® F127, from which BSA was released in a sustained fashion over 35 days. However, protein burst release occurred from particles generated with lower PLGA (6%) and surfactant (0 and 5%) concentrations.

In a later study, Zamani and co-workers [95] performed both emulsion and coaxial electrospraying of PLGA microparticles for the encapsulation of BSA. The protein encapsulation efficiency was superior for coaxially electrosprayed microparticles (approximately 70%) compared to that of emulsion electrospraying (up to 50%), but, interestingly, the latter presented lower burst effects during *in vitro* protein release studies. The authors believed this may be due to a greater extent of adsorption of the protein to the hydrophobic chains of PLGA during the emulsification process, which arises from a large organic-aqueous interface that is not present in coaxial electrospraying. Importantly, it was also emphasised that such protein-polymer interactions had previously been associated with loss of native protein structure and consequent denaturation.

3.2. Enzyme encapsulation

The encapsulation of enzymes in electrospun fibres and electrosprayed particles can be a simple way of evaluating the effects of EHD processing on protein structure and

bioactivity, due to the commercial availability of many standardised enzyme activity assays. One such protein is lysozyme, an anti-bacterial enzyme for which the substrate is peptidoglycan, one of the major components of the cell wall of Gram-positive bacteria [96]. Liu and co-workers created a composite scaffold by combining electrosprayed lysozyme nanoparticles with electrospun PLGA and PEG-PLGA monolithic fibres [97]. The electrosprayed lysozyme solution was prepared by dissolving the enzyme in varying ratios of ethanol (EtOH) and water. Optimal enzymatic activity preservation (approximately 100%) was attained with an EtOH:H_2O ratio of 30:70 v/v, as measured after electrospraying. The electrosprayed lysozyme nanoparticles (with or without PEG) were then dispersed in a PLGA solution for electrospinning. Lysozyme release from the composite mats was low, with only up to 25% of the enzyme loading being detected after 56 days. The authors found that a large fraction of the protein was present within the fibres in the form of insoluble aggregates after the 56 day period, which may explain why such a small amount of the enzyme was detected in the release medium. Of note, lysozyme bioactivity was not assessed after release from the electrospun mats, but it is possible that such structural alterations may have compromised its catalytic activity.

A few strategies have been developed to improve lysozyme stability in suitable solvents for electrospinning and spraying, such as the conjugation of lysozyme with a fatty acid (oleate), which can provide amphiphilicity and, thus, enhance the solubility of the enzyme in organic solvents [91,98]. The enzyme was encapsulated in electrospun PCL-PEG blend fibres, and its solubility in dimethylsulfoxide (DMSO) was improved from 12.6 ± 4.2 mg/mL to 21.1 ± 3.5 mg/mL after ionic conjugation with oleate [91]. Furthermore, the activity of the encapsulated lysozyme released from the fibrous meshes was retained at ca. 90% for over 7 weeks. In a report by Puhl *et al.* [99], electrospinning of lysozyme crystals in a polymer suspension, rather than a solution or emulsion of the protein, was performed (**Figure 5**). The authors proposed that, because protein crystals are more thermodynamically stable and present a smaller solvent-exposed surface area compared to a solution, they could be more suitable for EHD processing in organic solvents. The preparation of lysozyme crystals and suspension in pure PCL and composite PCL-PEG and PCL-PLGA solutions for electrospinning allowed for a relatively homogenous distribution of the protein within the fibres [99]. The bioactivity of the lysozyme released from PCL and PCL-PEG fibres was efficiently preserved; however, the acid degradation products from PLGA in PCL-PLGA fibres and consequent alteration of the release medium pH led to a loss of up to ~30% in bioactivity over a period of 11 weeks. In another report [100], emulsion electrospinning was used for the encapsulation of lysozyme in PCL-PEO composite fibres. After a release period of 24h, the highest

catalytic activity of the enzyme was seen when the emulsification process was performed with a concentration of 0.4% (v/v) of Span80® and a decrease in the sonication amplitude.

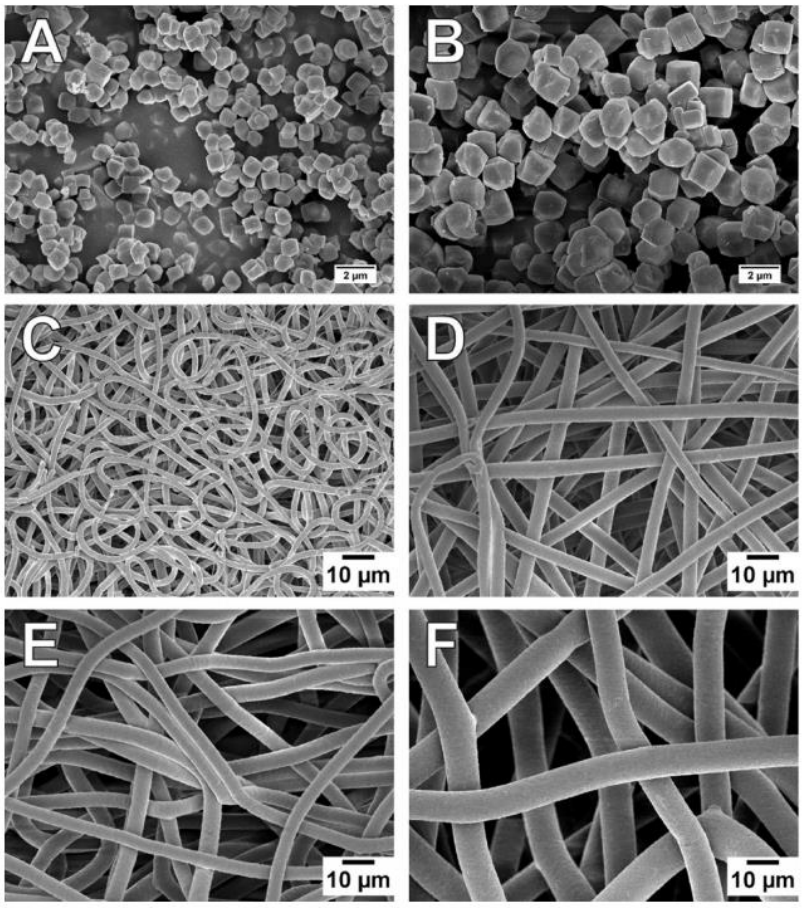


Figure 5. Data from Puhl et al. reporting electrospinning of lysozyme crystals in a polymer suspension. Lysozyme crystals with average diameters of (A) ~0.7 µm and (B) ~2.1 µm were used. (C-F) Non-woven lysozyme crystal-loaded electrospun mats with average diameters of (C) 1.6, (D) 3.6, (E) 5.2, and (F) 10.2 µm were then generated. Reprinted with permission from [99]. Copyright © 2014, American Chemical Society.

Various studies have revealed that the release profiles of lysozyme could be tailored *via* the ratio of hydrophilic (PEO/PEG) to hydrophobic (PCL/PLGA) polymers. Larger amounts of enzyme were released for higher PEO:PCL [100,101] and PEG:PCL [91,99] ratios. It is thus possible to conclude that, at optimised hydrophilic: hydrophobic polymer ratios, both PEO and PEG can act as porogenic agents, since their rapid dissolution in aqueous media increases fibre porosity and enhances protein diffusion and release from the polymer matrix. However, in work by Liu *et al.* [97], this tendency was only verified for a PEG:PLGA ratio of up to 10:100 (w/w), as the lysozyme release was slower at a 20:100 ratio. The authors suggested that this phenomenon resulted from a change in the thermodynamic properties of the scaffold with a higher PEG concentration in the mixture, which facilitated the interaction between PEG and PLGA chains and slowed down

enzyme diffusion and release. It should be noted that the lysozyme encapsulation efficiency was not presented in this study, meaning the lower amount of released lysozyme could also be a result of reduced encapsulation efficiency in the electrospun mats with higher PEG content.

Complex delivery devices may be generated by the combination of various drug carriers in a single construct. For instance, horseradish peroxidase (HRP)-loaded liposomes were prepared and subsequently electrospun into PVA-PCL blend or core-shell fibres, resulting in the incorporation of these vesicles into fibrous scaffolds [39]. The core-shell fibres consistently provided better results, including in terms of improved liposome stability and preservation of HRP activity (average value of 62.3% compared to 9.59% in blend fibres).

In addition to their dissolution in solvents prior to EHD processing, proteins can also be incorporated into particles or scaffolds *after* electrospraying or electrospinning. One example of this was provided by Ma and co-workers, who prepared silica supraparticles (Si-SPs) to be used as an inner ear drug delivery system [102]. Alginate spheres encapsulating several individual silica particles were prepared by electrospraying. The alginate template was then removed, resulting in Si-SPs with an average diameter of ~550 μm. The Si-SPs were incubated with a FITC-lysozyme solution after electrospraying, and it was shown by LSCM that the enzyme not only interacted with the Si-SPs surface, but was also found in the interior of the particles, due to their porous nature. Protein loading capacities of up to 15 μg of lysozyme per particle were achieved. Sustained release profiles over 40 days were obtained for both lysozyme, used as a model protein, and brain-derived neurotrophic factor.

Examples of other enzymes encapsulated in electrospun scaffolds include alkaline phosphatase (ALP) [90] and α-chymotrypsin [103,104]. Importantly, the applications of enzyme-containing electrospun systems are not limited to model studies or drug delivery. In work by Dai and colleagues [105], laccase, an oxidase, was loaded into PDLLA fibres *via* emulsion electrospinning and explored for bioremediation applications. The activity of the encapsulated laccase was reduced to 67% of that of the free enzyme, presumably due to denaturation during the emulsification process. Nevertheless, this catalytic activity was conserved at up to 50% after ten consecutive reactions of the same electrospun mats, highlighting the relatively high operational stability of this system. Similar results were obtained in a separate study [106], where emulsion electrospinning was used for the encapsulation of trypsin into PCL fibres for potential industrial applications. The incorporation of FITC-conjugated trypsin during the electrospinning process enabled the

observation of a relatively uniform distribution of the enzyme within the nanofibres, and catalytic activity after encapsulation was preserved at up to ~66%. The encapsulated enzyme demonstrated higher thermal and storage stability compared to the free enzyme counterpart. Moreover, the operational stability of this system was also satisfactory, with 59% of trypsin's enzymatic activity being conserved after five consecutive reactions.

The studies presented above emphasise the suitability of enzyme-loaded electrospun/electrosprayed systems for both medical and industrial applications. It is worth mentioning, however, that the preservation of catalytic activity during EHD processing remains a challenge in a significant number of these devices, which may limit their practical applications. Because this enzymatic activity is highly dependent on the protein's three-dimensional structure and conformation, the use of harsh processing conditions (*e.g.* contact with organic solvents or extensive organic-aqueous interfaces, ultrasonication) during the electrospinning or electrospraying process should be avoided.

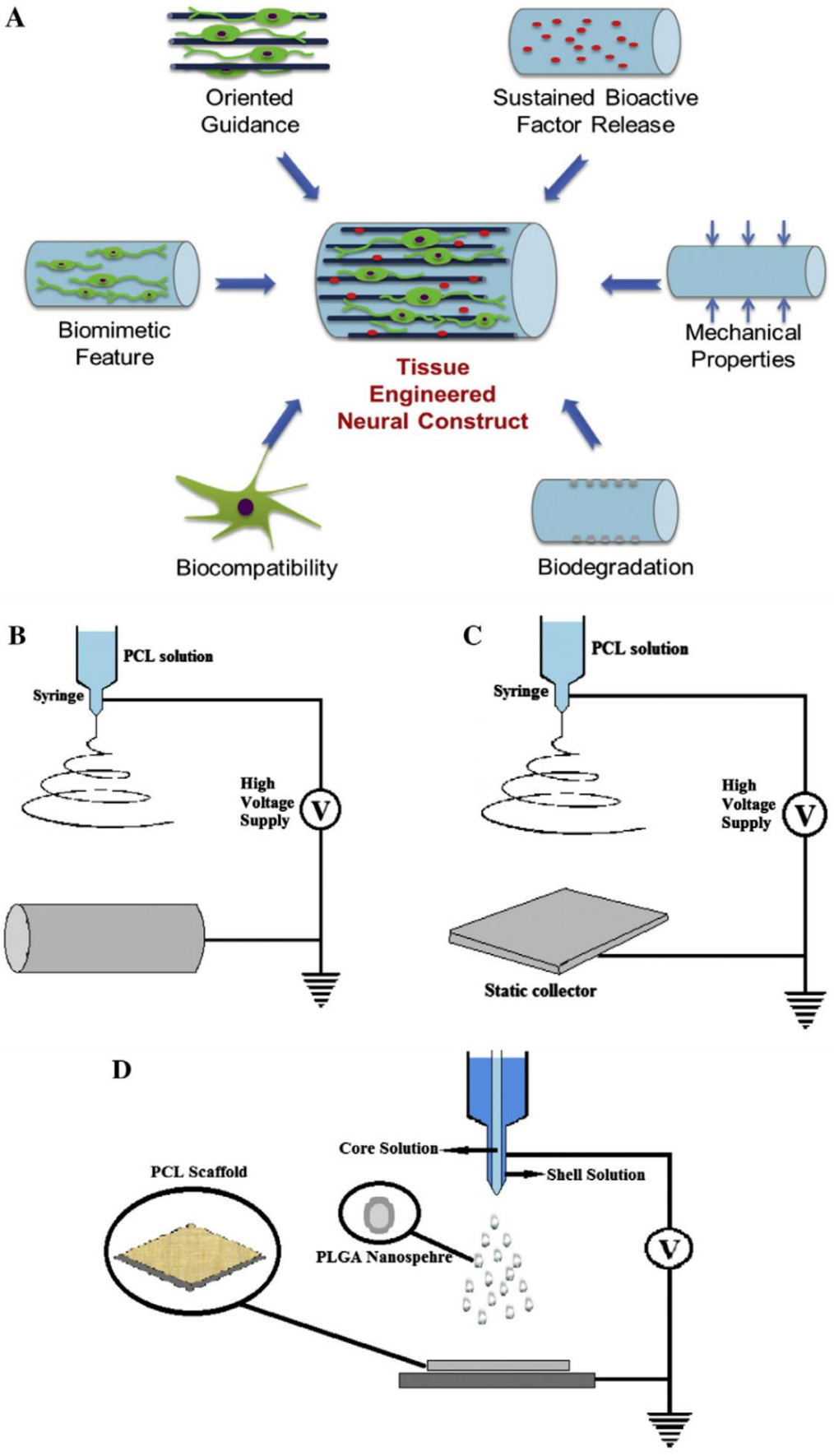
3.3. Growth factor delivery

Growth factor delivery is a strategy widely used in TE to promote cell migration, growth, and differentiation [107]. Nevertheless, the administration of growth factors *in vivo* is highly inefficient, due to their low stability, short half-lives, and rapid inactivation [108,109]. In addition, overdosing with these protein agents is associated with detrimental side effects, including an increased risk of cancer development [110]. As such, the creation of delivery devices capable of preserving the bioactivity of growth factors during processing and storage, and providing sustained release profiles, is pivotal for these molecules to be more extensively used in clinical applications [111].

In the last decade, a lot of effort has been put into fabricating growth factor delivery systems based on scaffolds and microcarriers produced by EHD processing. One of the main areas of focus in these studies is neural TE, in which both mechanical and chemical cues are important determinants for the success of the construct. For example, an aligned electrospun PCL scaffold was combined with BSA-loaded electrosprayed PLGA core-shell spheres in order to provide distinct nano- and micro-scaled topographical cues for the promotion of neural cell growth [112] (**Figures 6** and **7**). In other work, Hu and co-workers developed an aligned nanofibrous scaffold composed of nerve growth factor (NGF)-encapsulating PCL nanofibres [88]. The fibres were produced using emulsion electrospinning, and their alignment was achieved by collection on a cylindrical mandrel rotating at high speed (~3000 rpm). The co-encapsulation of NGF and BSA allowed sustained release of the growth factor over 28 days. Moreover, the protein-loaded scaffolds successfully promoted the adhesion, proliferation and neurite extension of

PC12 cells (a rat adrenal phaeochromocytoma cell line widely used as a neuronal cell model, as their phenotype is similar to that of sympathetic ganglion neurons after NGF-mediated differentiation [113]). A similar approach was used in another study, with the polymer matrix composed of PLGA [114].

Coaxial electrospinning has been used to produce nerve guidance conduits (NGCs) encapsulating NGF and tested in a rat sciatic nerve model (Kuihua et al., 2014; Wang et al., 2012). After excision of a sciatic nerve segment, the NGCs were used as reparative grafts, and shown to improve nerve functional recovery when compared to scaffolds without NGF. In a different report, NGF-loaded PLGA microspheres were incorporated into methacrylated hyaluronic acid fibrous scaffolds [117]. Scaffolds with aligned or random fibres were obtained by collecting them on a rotating mandrel at a high (10 m/s) or low (0.5 m/s) speed, respectively. The microspheres were generated with a water-in-oil-in-water (W/O/W) double emulsion and mixed with the polymer solution before electrospinning. This delivery system successfully stimulated neurite extension in dorsal root ganglion neurons, demonstrating that the encapsulated NGF retained its bioactivity. Importantly, the direction of the neurite growth depended on the orientation of the fibres in the scaffold: in randomly oriented fibres, the neurites grew in several directions, while in aligned scaffolds the neurites tended to grow in the direction of alignment.



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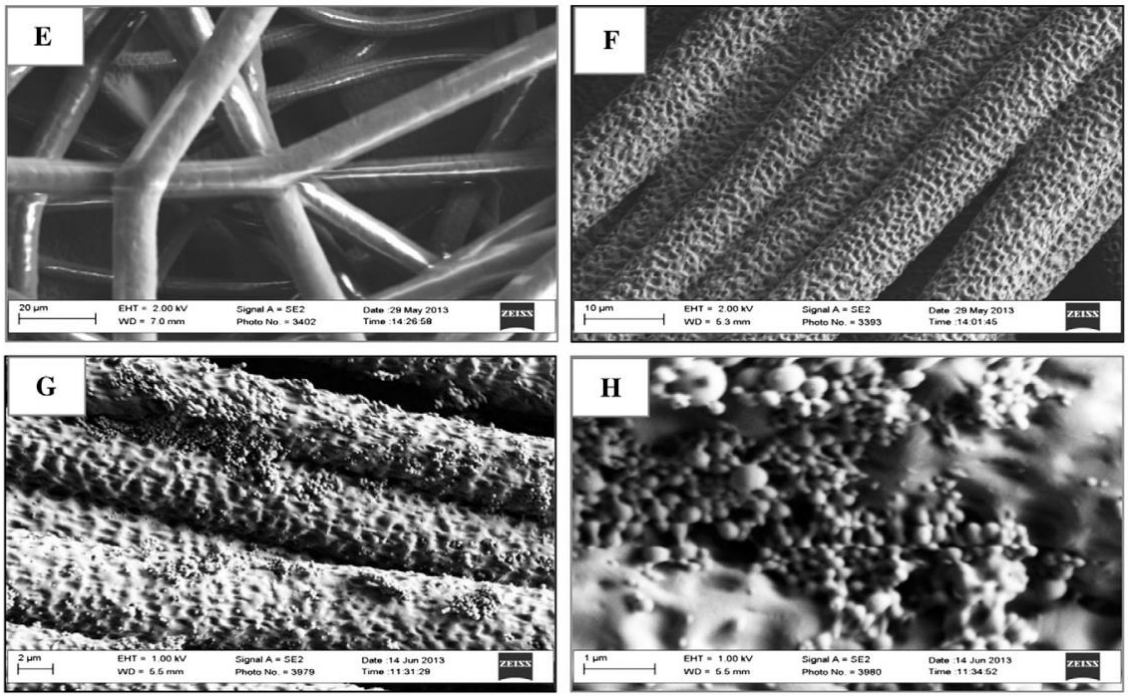

Figure 6. Schematic illustration of (A) the fundamental elements of an ideal scaffold for neural regeneration. These include biochemical and physical cues, such as growth factor delivery and fibre alignment, and appropriate mechanical properties. (B, F) Production of aligned scaffolds by collection of the electrospun fibres on a rotating mandrel. (C, E) Production of random scaffolds by fibre collection on a static collector. (D) Coaxial electrospraying of core-shell PLGA nanospheres on top of the PCL scaffolds. G and H are low and high magnification scanning electron microscopy (SEM) images, respectively, from the aligned composite scaffold. Adapted from [112] with permission from Elsevier. Copyright © 2015, Elsevier Inc.

Simultaneous delivery of multiple growth factors may be more effective in promoting tissue repair, with each performing distinct functions to improve functional recovery or provide a synergistic effect. Dual-source dual-power (DSDP) electrospinning allows the concomitant or sequential extrusion of multiple polymer solutions using separate syringe pumps and power supplies (**Figure 7**), which enables the individual adjustment of parameters such as solution flow rate and applied voltage for each polymer component [118]. This method facilitates the production of hybrid electrospun mats with easily adjustable compositions [118,119]. Liu *et al.* recently published two studies in which the dual delivery of NGF and glial cell line-derived neurotrophic factor (GDNF) was achieved using DSDP emulsion electrospinning, where NGF- and GDNF-loaded PDLLA and PLGA fibres, respectively, were combined into a single hybrid construct [120,121]. Both of these growth factors are involved in neuronal differentiation and regeneration, and their synergistic action in axon branching and elongation has been demonstrated [122]. Encapsulation efficiencies over 80% were achieved for NGF and GDNF, as well as sustained release profiles over 42 days after a relatively small burst release (up to 16.7% and 26.6% for NGF and GDNF, respectively, in the first 24h). *In vitro*, the NGF and GDNF released from the fibrous scaffolds had only a small loss of bioactivity compared to

freshly dissolved growth factors, implying that the electrospinning process does not impair their functionality [121].

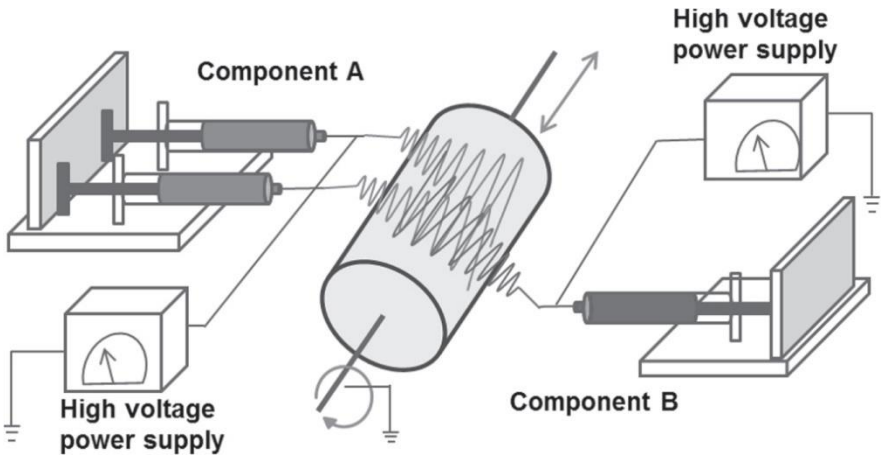

Figure 7. Schematic representation of dual-source dual-power electrospinning for the production of non-woven fibrous mats. Reproduced from [121] under the terms of the Creative Commons CC BY 3.0 license (https://creativecommons.org/licenses/by/3.0/legalcode).

Bone and cartilage TE is another major area of research where EHD methods have been heavily explored. One of the most widely used growth factors in this context is bone morphogenetic protein 2 (BMP-2). BMP-2 is osteoinductive, stimulating progenitor cell proliferation and differentiation into osteogenic phenotypes [123,124], and essential in bone fracture recovery [125]. Furthermore, BMP-2-based therapy has been approved by the FDA since 2002 for clinical use in several pathological conditions [126], and it is currently commercialised under the product names of INFUSE® Bone Graft (Medtronic, US) and InductOS® (Wyeth, Europe) [127]. As such, BMP-2 has been incorporated in a great variety of biomaterials, frequently associated with inorganic, osteoconductive components like hydroxyapatite, for sustained delivery [128–130]. For example, hydroxyapatite nanoparticles incorporated in silk fibroin (SF) and chitosan blend solutions have been used to form the shell of fibres produced by coaxial electrospinning, while the core was composed of an aqueous solution of BMP-2 [131]. These scaffolds demonstrated osteoconductive and osteoinductive properties *in vitro* and *in vivo*. BMP-2 has also been combined with other drugs in several delivery systems for the maximisation of cellular and tissue responses. Coaxial electrospinning was used to encapsulate BMP-2 and dexamethasone in poly(L-lactide-*co*-caprolactone) (PLLACL)-collagen [132] and PLLA-zein [133] based nanofibres. In the first report [132], core-shell (shell: PLLACL-collagen and dexamethasone; core: BMP-2 in PBS stabilised by BSA) and blend nanofibres were compared in terms of the release profiles of BSA over 21 days (however, BMP-2 release was not evaluated). It was shown that the core-shell structured fibres provided a slower release of BSA than blend fibres of the same

composition. In the second study [133], PLLA and BMP-2 composed the core of the fibres, while the shell was constituted of zein, a biocompatible protein extracted from corn, and dexamethasone. The encapsulation of BMP-2 within the fibre core allowed for a more sustained release during 21 days, but also caused the retention of a large amount of protein (45-55%) inside the nanofibres. In both studies, mesenchymal stem cell (MSC) adhesion, growth, and expression of osteogenic markers were promoted more efficiently by the dual drug-loaded scaffolds than blank or single drug-loaded nanofibres.

Recently, Cheng and co-workers successfully developed another dual-release system, where BMP-2-loaded PVA (core)/SF-PCL (shell) nanofibres were produced by coaxial electrospinning and subsequently coated with connective tissue growth factor (CTGF) using a layer-by-layer (LbL) technique (**Figure 8**) [134]. Both BMP-2 and CTGF are important chemical cues in bone regeneration, but while the former is present throughout the whole regenerative process CTGF appears to be more influential in the early stages. Accordingly, the objective was to achieve a fast release of CTGF and a sustained release of BMP-2, promoting both osteogenic and angiogenic responses for bone regeneration. A burst release of approximately 60% of CTGF was achieved over the first 24h, while BMP-2 was released in a controlled fashion over 40 days.

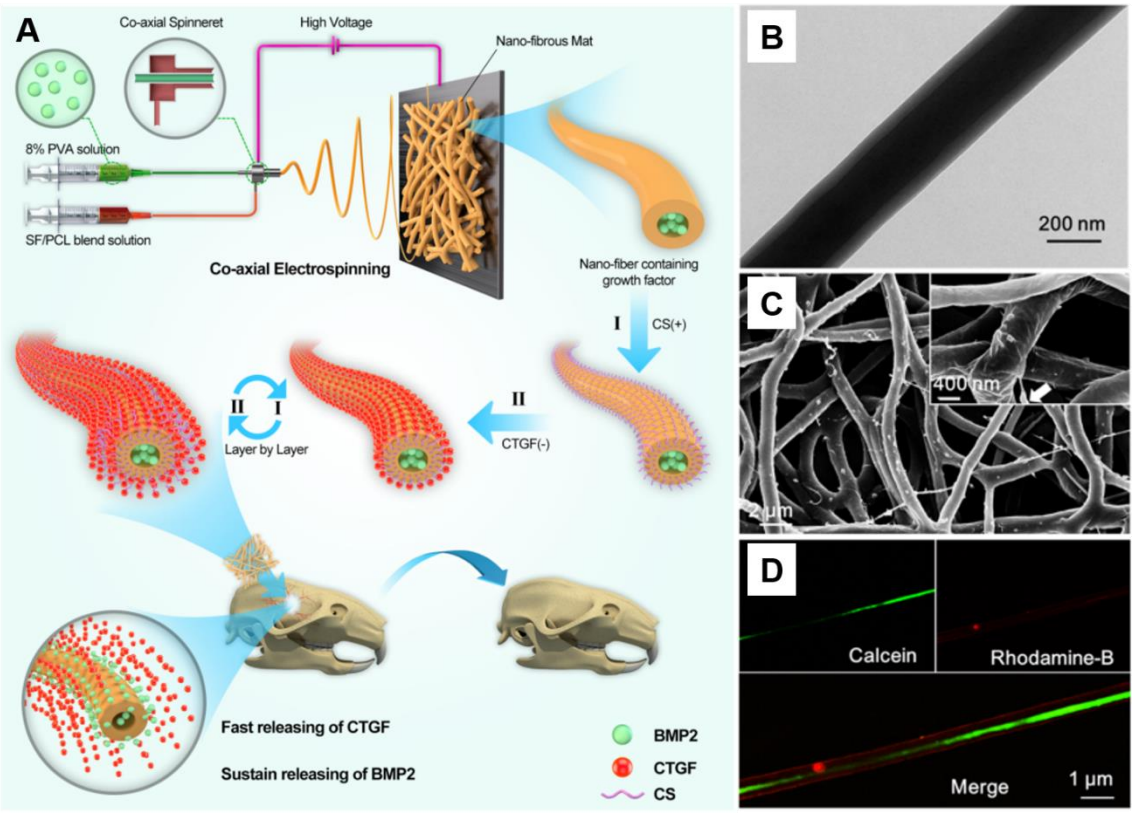

Figure 8. (A) Schematic representation of the coaxial electrospinning process for the production of BMP-2-loaded PVA/SF-PCL core-shell fibres and subsequent layer-by-layer coating with CTGF for *in vivo* implantation. (B) TEM and (C) SEM images of the CTGF-coated core-shell fibres. (D) Incorporation of calcein and rhodamine in the PVA (core) and SF-PCL (shell) solutions, respectively, enabled further confirmation of the fibre core-shell structure using confocal microscopy. Abbreviations: BMP-2 – bone morphogenetic protein 2; CS – chitosan; CTGF – connective tissue growth factor; PCL – poly(ε-caprolactone); PVA – poly(vinyl alcohol); SF – silk fibroin. Adapted with permission from [134]. Copyright © 2019, American Chemical Society.

BMP-7 is another important growth factor widely used in TE for the promotion of bone regeneration [135]. BMP-7 and vascular endothelial growth factor (VEGF), together with human serum albumin, were encapsulated in PLGA-PEG microparticles by blend electrospraying [136]. Protein powders (human serum albumin, BMP-7, and VEGF) were first micronized to reduce particle size and subsequently mixed in an aqueous solution with PEG, followed by lyophilisation. The effect of trehalose as a protein stabiliser in the mixture was also evaluated. A PLGA in chloroform solution was added to the lyophilised particles and the dispersion homogenised by magnetic stirring and sonication. After electrospraying, only trace amounts of BMP-7 and VEGF from the microparticles could be detected by ELISA, indicating denaturation or aggregation. Nevertheless, after critical procedures such as micronisation and vortexing with chloroform, their bioactivity levels were comparable to freshly prepared BMP-7 and VEGF controls in cell-based assays, where they retained *in vitro* efficacy. It is possible that, during extraction or release from the microparticles, reversible modifications occur in the growth factor structure that hinder their ELISA detection, but do not compromise their bioactivity. The addition of

trehalose to the formulation resulted in slower growth factor release, but had no influence on *in vitro* bioactivity.

Cartilage TE is a particularly challenging field, since the naturally low cellular content and the avascularity of this tissue both impair its intrinsic regenerative capacity. In order to promote the homing of chondrocytes to the lesion site and improve graft-host integration, a blend PLGA-PCL mesh was developed for the encapsulation of insulin-like growth factor 1 (IGF-1) [137]. The IGF-1-loaded meshes supported chondrocyte growth and the deposition of cartilage ECM components such as glycosaminoglycans (GAGs) and type II collagen *in vitro*. *In vivo*, IGF-1 seemingly improved graft integration and ECM deposition compared to blank meshes. Another scaffold, composed of TGF-β1-loaded PLGA microspheres incorporated into electrospun PCL nanofibres, was also evaluated for its chondrogenic potential [138]. The authors demonstrated that this fibrous construct was able to support MSC attachment, proliferation, and chondrogenic differentiation, with improved production of GAGs and type II collagen.

VEGF and platelet-derived growth factor (PDGF) have been explored in electrospinning both for bone regeneration [139–141] and cardiovascular TE. Two studies developed dual-delivery systems incorporating both growth factors using modified coaxial and DSDP electrospinning to create double-layered [142] and multi-layered [143] polymer vascular scaffolds (**Figure 9**), respectively. The chemical compositions of these meshes involved both natural (chitosan and gelatin) and synthetic polymers (poly(ethylene glycol)-*b*-poly(lactide-*co*-ε-caprolactone), PLGA, and PCL). In both cases, the encapsulation of VEGF and PDGF was tailored so that the former would be released faster than the latter, leading to differential release profiles. The objectives of this were to promote an early recruitment of vascular endothelial cells (ECs), stimulated by VEGF, and the later homing of vascular smooth muscle cells (SMCs), in which PDGF plays a more determinant role. In addition, the authors intended to avoid PDGF-induced SMC hyperproliferation, which is seemingly inhibited by VEGF [144]. After *in vivo* implantation in a rabbit model, both scaffolds were able to promote the homing of ECs and SMCs, and the combined action of VEGF and PDGF allowed the inhibition of SMC hyperproliferation. It should be noted, however, that the encapsulation efficiencies for both growth factors were quite low in both the double-layered and the multi-layered devices (under 20%).

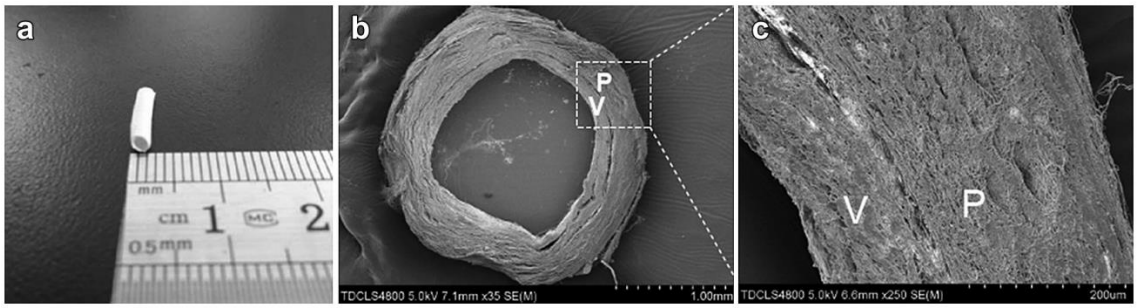

Figure 9. (a) Macroscopic view and (b, c) cross-section SEM imaging of a tri-layered vascular scaffold at low and high magnification. The letters V and P represent the presence of VEGF and PDGF in the inner and middle layers, respectively. The outermost layer was composed of PCL and gelatin, and did not contain any protein. Reprinted from [143] with permission from Elsevier. Copyright © 2013, Elsevier Ltd.

Skin regeneration is another potential application for electrospun TE constructs. PDGF and CTGF have been incorporated in electrospun scaffolds for wound dressing purposes [145,146]. Basic fibroblast growth factor (bFGF)-loaded PEG-PDLLA scaffolds fabricated with an emulsion electrospinning technique have been tested as skin patches in a diabetic mouse model [147]. These constructs markedly accelerated wound closure and healing, promoting skin re-epithelialisation, vascularisation, and the growth of skin appendages like hair and sebaceous glands. The release of bFGF from PLGA-cellulose acetate electrospun fibres was modulated in another study using DSDP and emulsion-coaxial electrospinning [148]. Bi-layered and tri-layered scaffolds were produced by DSDP electrospinning of a bFGF emulsion in PLGA and a cellulose acetate solution (bi-layered scaffolds were composed of a bFGF+PLGA layer and a cellulose acetate layer, whereas in tri-layered scaffolds the bFGF+PLGA stratum was “sandwiched” by two cellulose acetate layers; see **Figure 10a**). Core-shell fibres were generated by coaxial electrospinning, where the core comprised the bFGF and PLGA emulsion and the shell a solution of cellulose acetate (**Figure 10b**). The hybrid scaffolds provided a more sustained release of the protein than that observed for the scaffolds without cellulose acetate. The slowest bFGF release profile was achieved from the tri-layered fibrous scaffold, although the growth factor release was rather incomplete (~40% after approximately one month).

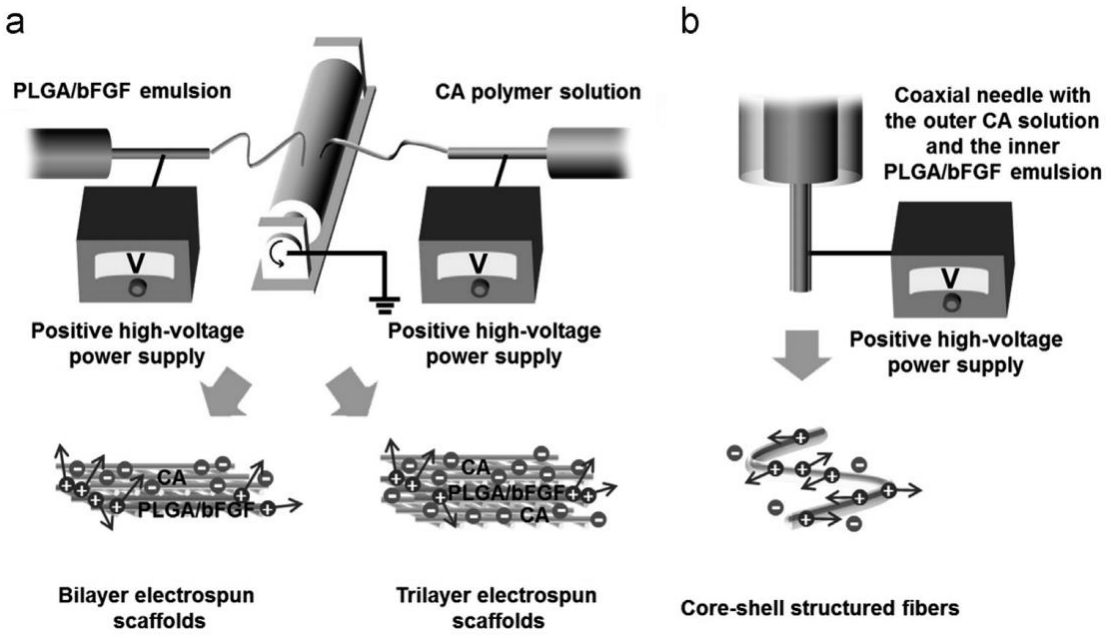

Figure 10. (a) Production of bi-layered and tri-layered scaffolds using dual-source dual-power electrospinning. A bFGF emulsion in PLGA and a CA solution were electrospun sequentially. (b) Generation of PLGA/bFGF (core)-CA (shell) fibres with coaxial electrospraying. Abbreviations: bFGF – basic fibroblast growth factor; CA – cellulose acetate; PLGA – poly(lactide-*co*-glycolide). Reprinted from [148] with permission from Elsevier. Copyright © 2016, Elsevier B.V.

Similarly, epidermal growth factor (EGF) has been encapsulated in electrospun scaffolds for wound healing applications [149,150], and multiple delivery studies of EGF with bFGF [151,152] and VEGF and PDGF [152] were performed. Albright and colleagues designed TGF-β1-loaded PCL-collagen electrospun nanofibres, which were subsequently coated with gentamycin/clindamycin antibiotic-containing micelles using a LbL approach [153]. The objective was to use these fibrous meshes as skin patches for TGF-β1-mediated wound healing, and simultaneous prevention of bacterial growth and lesion infection. Importantly, the encapsulated TGF-β1 seemingly maintained its bioactivity *in vitro*, and the incorporation of both gentamycin and clindamycin in the micelle-coated scaffolds successfully inhibited the growth of a *Staphylococcus aureus* strain.

The existence of such an extensive list of studies applying EHD processing for the generation of tissue engineered constructs demonstrates the potential of this technique for successful outcomes and clinical translation. Although a few reports showed that growth factors may be loaded into emulsion electrospun fibres and still retain a significant portion of their bioactivity, there is a tendency for the use of coaxial electrospinning/spraying instead. Indeed, the incorporation of these molecules in the core of coaxially produced fibres or particles is a more effective way of preventing their

contact with organic solvents or organic-aqueous interfaces than using an emulsion, thereby minimising protein denaturation and loss of functionality.

3.4. Antibody delivery

To the best of our knowledge, the first study reporting the use of EHD processing for antibody encapsulation was performed by Gandhi *et al.* [154]. In this work, anti-$\alpha_v\beta_3$ integrin immunoglobulin G (IgG) antibodies, promising antiangiogenic agents, were incorporated into monoaxial electrospun PCL nanofibres. BSA was encapsulated together with the antibodies, as a potential stabilising excipient. The release profiles of BSA and the antibody from all tested formulations were typically associated with significant burst releases followed by slower release over 30 days. Faster protein release was observed where lower PCL concentrations (11 wt.%) were used in the spinning solutions, and at low antibody: BSA ratios (1:100, w/w). Additionally, the antigen-binding activity of the antibodies to $\alpha_v\beta_3$ integrins expressed by human umbilical vein endothelial cells (hUVECs) was shown to be maintained after release from the nanofibres.

The extensively used protein active ingredient bevacizumab (commercial name Avastin®) is an anti-VEGF antibody used in the treatment of age-related macular degeneration and several types of cancer, including glioblastoma, metastatic colorectal cancer, and non-small cell lung cancer [155]. Bevacizumab was encapsulated in PCL core-shell fibres by Angkawinitwong and co-workers [156]. Using coaxial electrospinning, the authors found that the pH of the core solution in which the antibody was dissolved and electrospun played a crucial role in functional performance. Two distinct pH values were tested: 6.2 (pH of the commercial bevacizumab formulation, Avastin®) and 8.3 in Trizma® buffer (the isoelectric point of the antibody). In general, the core solution with pH 8.3 led to well-defined core-shell structures, zero-order release over two months without any burst effect, and efficient preservation of bevacizumab's structural integrity and VEGF-binding activity. The pH of the core solution affects the antibody charge, which, in turn, influenced its behaviour during the coaxial electrospinning process: at pH 6.2, bevacizumab has a positive charge, and tends to migrate towards the solution surface upon application of an electric field. In contrast, at the isoelectric point of the antibody, it will be neutral, hence remaining in the fibre core. This preferential distribution in the fibre core efficiently protects bevacizumab from contact with the shell solvent (TFE) and losing bioactivity, and promotes a gradual release with no burst observable. Conversely, the localisation of more antibody molecules near the fibre surface results in a more extensive interaction with the organic solvent and a burst of release. Recent work further demonstrated that coaxially electrospun mats composed of (PVA-bevacizumab)-(PCL-gelatin) core-shell nanofibres

were able to decrease the number of blood vessels formed in a chicken embryo chorioallantoic membrane (CAM) model, suggesting the maintenance of antiangiogenic activity [157].

3.5. Vaccines

EHD processing techniques, particularly electrospraying, have been widely studied for the formulation of protein-based vaccines. A few of these reports have focused specifically on the development of oral vaccine formulations. Oral vaccination, compared to the typical subcutaneous immunisation route, can be associated with benefits such as improved vaccine accessibility and distribution (due to the possibility for self-administration), and stimulation of both systemic and mucosal immune responses [158]. The naturally occurring polymer chitosan has attracted much attention in this regard, since it is mucoadhesive [159]. This characteristic favours its retention in the mucosal system for prolonged periods of time [160], maintaining locally high concentrations of the antigen and thus improving immune recognition and response. In a study by Suksamran *et al.*, methylated N-(4-N,N-dimethylaminocinnamyl) chitosan (TM-CM-CS) was used as a coating for ovalbumin (OVA)-loaded calcium alginate and yam starch electrosprayed microparticles [161]. These microparticles were subsequently used for the oral immunisation of mice, and the systemic and mucosal immune responses elicited were evaluated by measuring anti-OVA IgG and secretory IgA (sIgA) antibody levels. The authors showed that the TM-CM-CS coating significantly enhanced the mucoadhesive properties of the delivery system and resulted in elevated levels of both IgG and sIgA. Release of OVA from the microparticles in *in vitro* studies was incomplete, albeit sustained, over the time of the study (24h), with a maximum of ~60% of protein release for uncoated particles and ~40% for chitosan-coated particles.

OVA was also used as a model antigen in a different study [162], where it was encapsulated in coaxially electrosprayed acetalated dextran (Ace-DEX) microparticles and tested *in vitro* and *in vivo* with soluble mirabutide, an adjuvant with an immunomodulatory structure similar to peptidoglycan [163]. Ace-DEX samples with approximately 20, 40, and 60% cyclic acetal coverage (CAC) levels were tested, in order to attain tuneable degradation and protein release kinetics. Using BSA as a model for OVA, 91.8% of the encapsulated protein was released from the Ace-DEX microparticles with 20% CAC after a 48h period, whereas Ace-DEX with higher CAC levels led to much slower protein release. OVA encapsulation efficiencies over 70% were obtained for all microparticle formulations, but the highest value was observed for the 60% CAC Ace-DEX formulation (99.8%). Immunisation studies in a mouse model showed considerably enhanced serum anti-OVA IgG titres compared to mice given soluble OVA, with the most

potent antibody responses seen with the lowest CAC level formulation. Splenocytes from the immunised mice were harvested for *ex vivo* pro-inflammatory cytokine expression analyses upon exposure to OVA. The production of tumour necrosis factor (TNF)-α, interleukin (IL)-2, and interferon (IFN)-γ was also greater in cells isolated from mice vaccinated with encapsulated OVA compared to soluble OVA treatment.

Electrosprayed PVA-coated PLGA nanoparticles were utilised for the encapsulation of subunit antigens in the form of cytomegalovirus peptides, and the properties of this system were compared to particles fabricated by double emulsion methods [164] (**Figure 11**). The production of electrosprayed particles with nanoscale diameters was achieved by decreasing solution flow rate (optimal value of 10 μL/h) and increasing solution conductivity (**Figure 11a, b**). The group found that 80% of the chosen antigen ($pp65_{495-503}$ peptide) was released from the electrosprayed particles over the first 10 days, following a burst release of ca. 20%. The antigen encapsulation efficiency was generally superior for electrosprayed particles than those produced by double emulsion methods. *In vitro* TNF-α and IFN-γ production by $CD8^+$ T cells that were exposed to the antigen-loaded electrosprayed nanoparticles was comparable to those exposed to the soluble antigen counterparts.

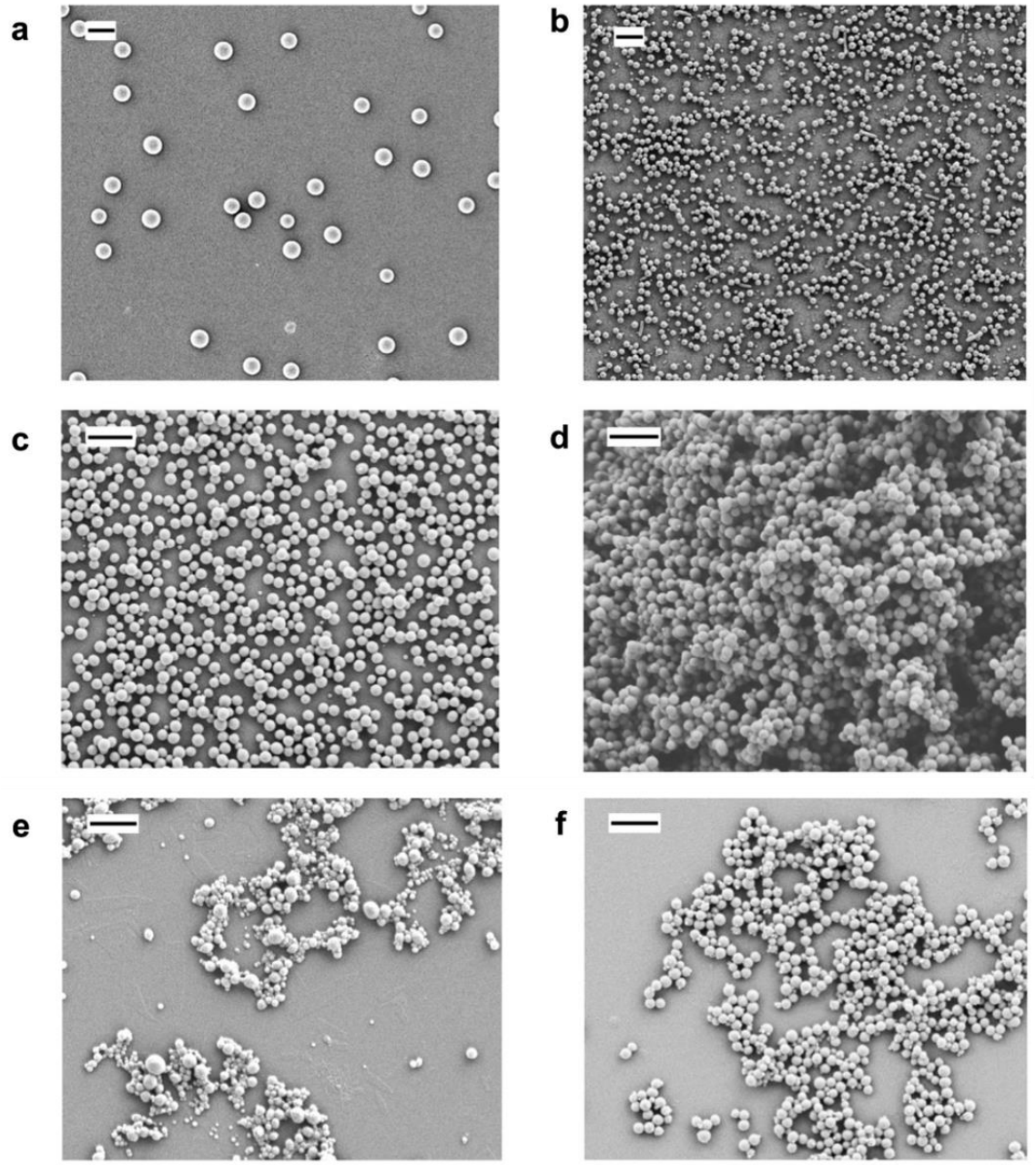

Figure 11. SEM images of PLGA particles electrosprayed from a solution with a conductivity of (a) 4 μS/cm and (b) 115 μS/cm. It is clear that an increase in solution conductivity resulted in a marked reduction of particle diameter. (c) PLGA nanoparticles electrosprayed onto a grounded collector plate. (d) Peptide-loaded PLGA nanoparticles electrosprayed onto a PVA solution for particle coating. (e, f) Comparison between PVA-coated unloaded PLGA particles produced by (e) double (water in oil in water, W/O/W) emulsion and (f) electrospray. Scale-bars represent 1 μm. Reproduced from [164] with permission from Elsevier. Copyright © 2017, American Pharmacists Association®.

3.6. Other examples

Hormone delivery

Insulin is a protein hormone that is secreted by pancreatic β-cells, and is responsible for cellular glucose uptake and carbohydrate, lipid, and protein metabolism [165]. Deficiencies in the production of insulin or the development of insulin resistance are associated with diabetes, a chronic disease that affects millions of people worldwide, with an incidence and prevalence that have been increasing steadily over recent decades (World Health Organization, “Diabetes,” 2020). Exogenous insulin administration, crucial in the management of diabetes, is given mainly subcutaneously. This, coupled with the required high frequency of injection required, can lead to low patient compliance [167]. For this reason, a growing number of studies have explored electrospinning and electrospraying for alternative insulin administration routes.

One such study focused on using fish sarcoplasmic proteins as a polymer carrier to encapsulate insulin into nanofibres *via* blend electrospinning for oral delivery [168,169]. A high encapsulation efficiency of 98.6% was achieved, as well as a loading capacity of 14%. Maximum insulin release was obtained after a period of 3h, and the fibres were capable of protecting the encapsulated protein from α-chymotrypsin degradation. CD studies suggested that, even though the fibres were processed by blend electrospinning and insulin was in direct contact with an organic solvent (hexafluoroisopropanol, HFIP), the protein structure was not affected. The same trend was observed in another report, in which semi-interpenetrating networks of gelatine and insulin were produced by blend electrospinning in HFIP [167]. In this study, it was proven that insulin retained its signalling activity *in vitro* after electrospinning, as it was capable of inducing Akt phosphorylation and adipogenic differentiation of pre-adipocytes. Moreover, the encapsulated hormone was able to better permeate through a porcine buccal mucosa model compared to free insulin. The mucoadhesive properties of chitosan may also be advantageous in this context. Lancina *et al.* [160] produced blend fibres of chitosan, PEO, and insulin for transbuccal delivery, again using HFIP as the solvent. Sustained insulin release was achieved for 24h and its activity *in vitro* was maintained, as assessed by Akt phosphorylation asssays. These studies demonstrate that it is possible to produce insulin delivery systems by blend electrospinning without compromising protein structure or bioactivity.

Coaxial electrospinning has recently been employed for this same purpose, and core (insulin)-shell (PLGA) fibres were generated for application in wound healing in diabetic patients [170]. The core-shell fibres were compared with blend fibres of the same composition. LSCM studies using recombinant enhanced green fluorescent protein

(reGFP) as a model revealed that protein distribution was more uniform in core-shell fibres than in the blend counterparts. Additionally, in an *in vivo* model, wound closure was faster when the core-shell fibre scaffolds were used than with the blend fibres.

Further examples of protein hormone encapsulation within electrospun fibres include growth hormone (GH)-loaded systems [171]. GH is commonly used to treat children with growth impairment, in order to normalise their height, body composition, and pubertal development [172]. It is also given to adults with GH deficiency [173]. Similarly to insulin, the most frequent method of administration of GH is *via* recurrent subcutaneous injections, owing to its short half-life [174]. A sustained GH release system was created by stabilisation of the hormone within sugar glass nanoparticles, which were subsequently encapsulated in PCL or poly(ester urea) electrospun fibres. GH release was observed for a period of 6 weeks with minimal burst, and the released protein maintained its bioactivity in *in vitro* cellular assays [175].

Chemokine delivery

Shafiq and co-workers [176] developed hybrid PCL-collagen vascular grafts by co-electrospinning from two separate spinnerets, preparing collagen fibres loaded with one of two chemotactic factors: stromal cell-derived factor 1α (SDF-1α), which promotes progenitor cell homing, angiogenesis, and tissue repair [177,178], or substance P (SP), a neuropeptide capable of promoting angiogenesis [179] and modulating MSC migration [180] and cytokine secretion [181]. Both grafts had suitable mechanical and biochemical properties for supporting the growth of ECs and SMCs *in vivo*, allowing for endothelialisation and vascularisation after implantation. Zamani *et al.* focused on the encapsulation of SDF-1α in coaxially electrosprayed PLGA microparticles to develop an injectable protein delivery system for cardiac tissue regeneration [182]. This core-shell system (**Figure 12**) showed sustained release of SDF-1α over 40 days, after a relatively small burst release (26.6% and 16.4% with and without BSA co-encapsulation, respectively). Moreover, in *in vitro* transwell assays, the chemotactic activity of the released SDF-1α towards MSCs was preserved. In a recent study [183], SDF-1α or granulocyte colony-stimulating factor (G-CSF) were separately encapsulated in poly(ethylene oxide terephthalate)/poly(butylene terephthalate) fibres *via* emulsion electrospinning. Even though beaded fibres were observed, rather than uniform, bead-less meshes, these chemokine-loaded scaffolds supported the growth of MSCs.

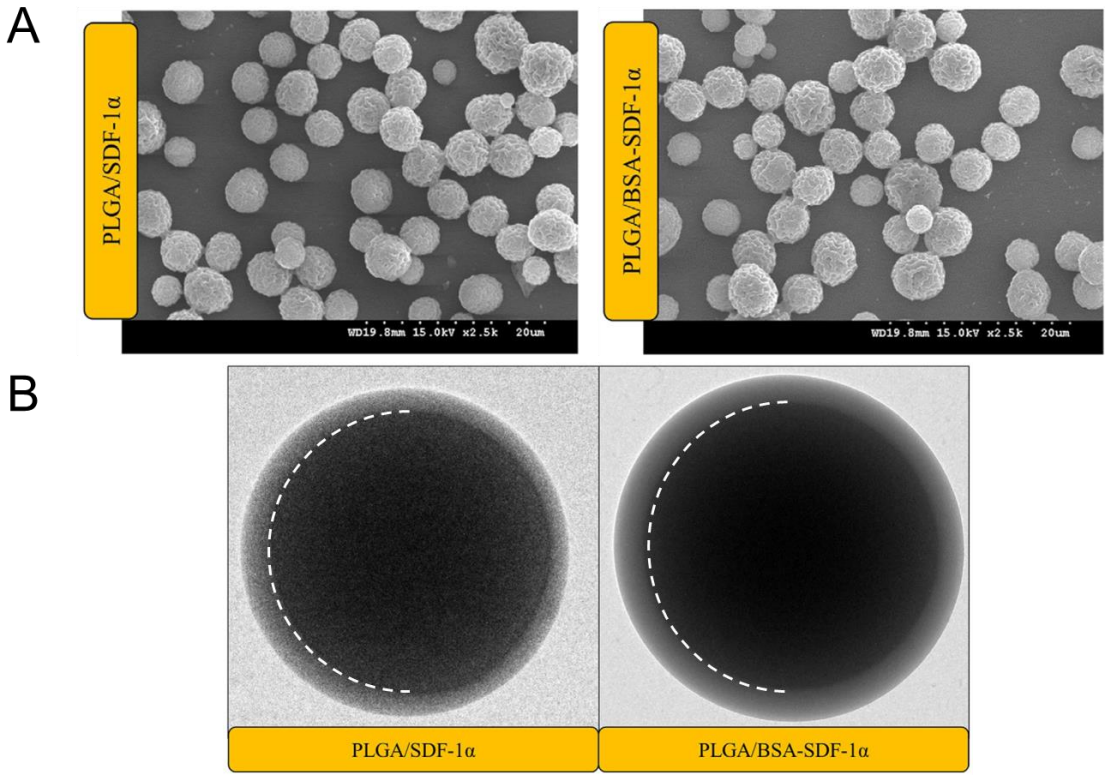

Figure 12. (A) SEM and (B) TEM micrographs of core-shell PLGA microparticles loaded with SDF-1α alone or co-encapsulated with BSA. Abbreviations: BSA – bovine serum albumin; PLGA – poly(lactide-*co*-glycolide); SDF-1α – stromal cell-derived growth factor 1α. Adapted from [182] with permission from Elsevier. Copyright © 2015, Elsevier Inc.

3.7. Stimuli-responsive protein delivery systems

Several strategies have been proposed for localised drug delivery. Active targeting usually relies on the functionalisation of a drug delivery vehicle with specific moieties that are preferentially recognised by a subset of cells or tissues present at the target site [184]. In passive targeting, the therapeutic agents reach the target organ or tissue by passive mechanisms (*e.g.* the enhanced permeation and retention [EPR] effect in tumour tissues) [185]. An effective way of localising delivery is the use of stimuli-responsive systems, in which drug release is triggered by internal or external cues such as changes in local pH or temperature [186].

Sukarto and Amsden developed a temperature-responsive system using electrosprayed P(TMC-CL)$_2$-PEG (poly(1,3-trimethylene carbonate-co-ε-caprolactone)-*b*-poly(ethylene glycol)-*b*-poly(1,3-trimethylene carbonate-co-ε-caprolactone)) microspheres loaded with four different proteins: BSA, lysozyme, BMP-6, and TGF-β3 [187]. The microspheres were then loaded into a *N*-methacrylate glycol chitosan hydrogel to be used as a controlled delivery system for cartilage repair. P(TMC-CL)$_2$-PEG solidified at temperatures lower than 10 °C, allowing its easy dispersion within the hydrogel, but it became a viscous liquid at body temperature, which could then trigger

the release of the encapsulated active factors. Sustained release profiles were attained over up to two months, even though initial burst effects were observed. The isoelectric point of each protein was thought to influence its release. Proteins which were negatively charged at the physiological pH (TGF-β3, BSA) were released faster than those which were positively charged (lysozyme, BMP-6), even though the chitosan hydrogel matrix also had a positive charge. The authors suggested that the positively charged lysozyme and BMP-6 may interact with P(TMC-CL)$_2$-PEG or with its negatively charged degradation products, thus explaining the slower release from the microspheres.

EHD products composed of pH-responsive polymers allow the release of microencapsulated compounds in a pH-dependent manner, which has potential applications for oral protein delivery. The GI tract is characterised by a wide range of pH values throughout its length, with an acidic environment in the stomach and a typically neutral-slightly basic pH in the small and large intestines [188]. Thus, fibres or particles that are insoluble at acidic pH but soluble in neutral conditions can be used for targeted GI delivery. Importantly, such systems are capable of protecting the encapsulated drugs from inactivation or degradation within the acidic milieu of the stomach, allowing their selective and controllable release in the gut [189].

A pH-sensitive device for peroral protein delivery was produced using HRP and ALP as model enzymes [87]. The polymer of choice was Eudragit® L100, an anionic co-polymer of methacrylic acid and methyl methacrylate that is soluble in biological fluids with pH > 6, but not in acidic conditions [190]. Emulsion electrospinning was used for the encapsulation of HRP or ALP into Eudragit® L100 fibres, with very high encapsulation efficiencies for both enzymes (>94%). As expected, protein release was shown to be greatly dependent on the pH of the release medium: only ~5% of the enzymes were detected in a medium at pH 2, but an increase to pH 7 led to release of approximately 100% of the encapsulated proteins in 1h (**Figure 13**). This was attributed to the fast dissolution of the fibres at neutral pH. Among the several processing parameters that were analysed in this study, the authors found that the aqueous: organic phase ratio may have an influence on bioactivity preservation after electrospinning. This influence was only observed for ALP, which had a more accentuated loss of catalytic activity when the aqueous phase was increased from 5 to 20% v/v (80% compared to 50% of bioactivity, respectively). This effect was presumably due to more extensive organic: aqueous interfaces and protein-solvent interactions at higher aqueous phase volumes. Conversely, the enzymatic activity of HRP was preserved at 90% regardless of aqueous phase content. Additionally, it was found that protein stability during storage was improved by fibre encapsulation and lyophilisation, compared to solution formulations.

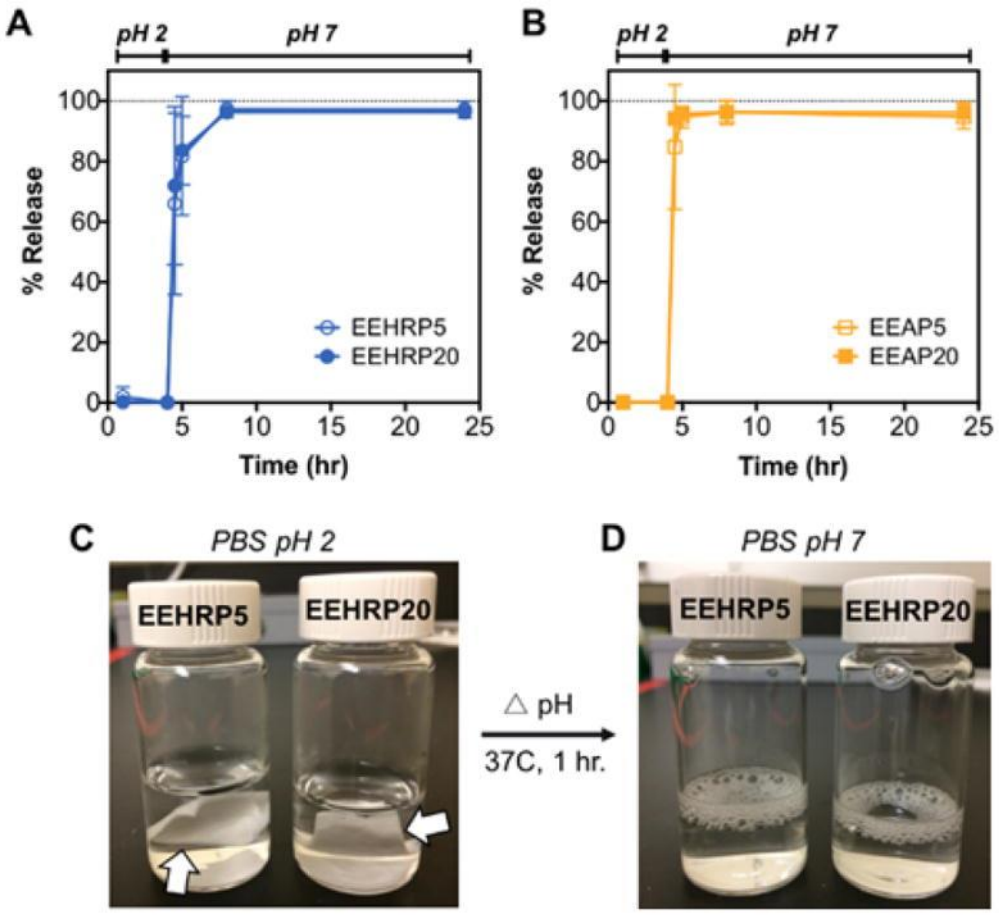

Figure 13. Release profiles of (A) horseradish peroxidase (HRP) and (B) alkaline phosphatase (AP) from pH-responsive Eudragit L100® fibres at pH 2 and 7. (C) and (D) are macroscopic views of the fibrous structure at pH 2 (undissolved) and pH 7 (dissolved). EEHRP5 and EEAP5 are emulsion electrospun HRP and AP formulations with an aqueous phase of 5% (v/v); EEHRP20 and EEAP20 are formulations with an aqueous phase of 20%. Reprinted from [87] with permission from Elsevier. Copyright © 2017, Elsevier B.V.

A different example of a stimulus-responsive system was provided by Yan *et al.*, who fabricated ultrasound-sensitive electrosprayed microbubbles for the local delivery of tissue plasminogen activator (tPA) in ischemic stroke [191,192]. tPA is an enzyme that promotes clot breakdown and prevents thrombosis. Using coaxial electrospraying, microbubbles with a gaseous core (air or sulphur hexafluoride, SF_6) and a shell composed of phosphatidylcholine and PEG were produced. tPA was also incorporated into the microbubble shell (**Figure 14**). The coaxial electrospraying process allowed the generation of microbubbles with minimal aggregation [191]. Furthermore, ca. 80% of the bioactivity of tPA was preserved even at the harshest spraying conditions (voltage of 14 kV). Microbubble bursting and tPA release was dramatically enhanced by the exposure to 2 MHz ultrasonic waves of higher amplitudes, therefore validating the ultrasound-sensitivity of this delivery system [192].

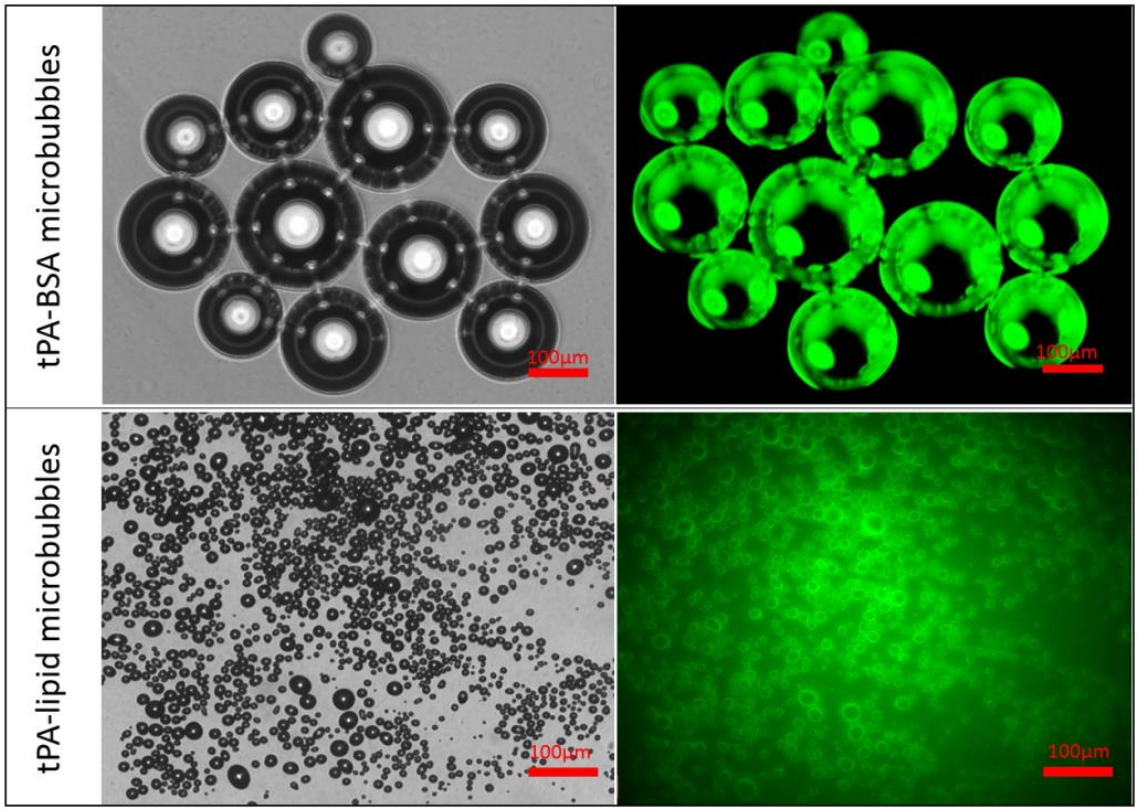

Figure 14. Optical and fluorescent micrographs of FITC-tPA-loaded microbubbles with BSA (top panel) or phospholipids and PEG (bottom panel) as shell components. Abbreviations: BSA – bovine serum albumin; tPA – tissue plasminogen activator. Reprinted from [191] with permission from Elsevier. Copyright © 2016, Elsevier B.V.

The abovementioned examples illustrate how EHD processing can be applied for the development of tailored, stimuli-responsive, materials for targeted delivery and controlled release while maintaining protein bioactivity. These characteristics hold promise for oral protein-based therapies, which are more patient-friendly than parenteral administrations, and for long-acting local delivery, potentially minimising systemic side effects and the need for frequent dosing.

Table 4. Summary table of the compositions and properties of protein delivery systems generated by blend, emulsion, and coaxial electrospinning and electrospraying.

EHD method	Protein(s)	Polymer(s)	Solvent(s)	EE (%)	Protein release	Protein bioactivity after EHD process	Reference
Blend and coaxial electrospinning	BSA ALP	PCL, PEG	TFE	92.1[a]	Burst release (0-4h) Blend fibres (w/ PEG): ~16% Coaxial fibres (w/ PEG): ~15% Coaxial fibres (no PEG): ~13% Cumulative release over 5 weeks Blend fibres (w/ PEG): ~70% Coaxial fibres (w/ PEG): ~60% Coaxial fibres (no PEG): ~50%	ALP: Blend fibres (w/ PEG): 49.3 ± 4.5% Coaxial fibres (w/ PEG): 76.2 ± 8.4% Coaxial fibres (no PEG): 13.4 ± 1.7%	[90]
Blend electrospinning	VEGF	Silk fibroin	Water	n.s.	Burst release in the first 24h followed by a sustained release over 28 days (slower protein release was obtained for scaffolds coated with pure PLGA fibres)	~83% after 28 day release	[140]
Emulsion electrospraying	BSA Lysozyme	PLGA	Proteins: water PLGA: DCM	76.6[b]	No observable burst release[b] Nearly linear release profile over more than 30 days[b]	Lysozyme: ~92%	[94]
Emulsion electrospinning	rhBMP-2 (+ BSA)	PLGA	Proteins: water PLGA: chloroform	~84	Burst release (0-24h): up to 12.5% Cumulative release over 28 days: up to 48%	~67%	[130]
	SDF-1α SP	Type I collagen	Proteins: water Collagen: HFIP	n.s.	Bursts of both SDF-1α and SP releases were observed in the first 24h Cumulative releases over 5 days: ~58% (SP) and ~69% (SDF-1α)	*In vitro* and *in vivo* activity was maintained for both chemokines	[176]
	EGF (+ BSA)	PLGA	Proteins: PBS PLGA: chloroform/acetone (2:1)	n.s.	Burst release on the first 24h was followed by sustained release over 8 days	*In vitro* activity was maintained	[149]
	EGF (+ BSA)	PCL Hyaluronan	Proteins: water PCL: chloroform Hyaluronan: chloroform:formic acid (2:1)	n.s.	Burst release over the first four days (~11%) Cumulative release over 25 days: ~30%	*In vitro* and *in vivo* bioactivity was preserved	[150]

Coaxial electrospraying	OVA BSA	Ace-DEX	Proteins: water (core) Ace-DEX: ethyl acetate:*n*-butanol:ethanol (shell)	20% CAC: 79.4 40% CAC: 70.2 60% CAC: 99.8	BSA release over 48h (pH 7.4) 20% CAC: 91.8% 40% CAC: 17.2% 60% CAC: 5.65%	OVA antigenic potential *in vivo* was maintained	[162]
Blend electrospinning + coaxial electrospraying	BSA	PCL (fibres) PLGA (particles)	PCL: chloroform BSA: water (particle core) PLGA: acetone (particle shell)	80.45%	Burst release in the first 10h (~60%), followed by an extended release over 10 days	*In vitro* activity was superior for PCL composite scaffolds with BSA-loaded PLGA particles compared with scaffolds with bare (non-encapsulated) BSA	[112]
Coaxial electrospinning	CTGF	PVA PLLA	CTGF+PVA: PBS (core) PLLA: DCM:DMF (1:9) (shell)	n.s.	Burst release in the first 24h was followed by an extended release period over 14 days	Bioactivity *in vitro* and in a CAM angiogenesis assay was demonstrated	[146]
Coaxial electrospinning	Bevacizumab	PCL	Core: phosphate buffer (pH 6.2) or Trizma® buffer (pH 8.3) Shell: TFE:deionised water (9:1)	pH 6.2: 72.64% pH 8.3: 63.15%	pH 6.2: biphasic release profile, with a burst over the first 24h and a sustained release over 19 days (cumulative release: 60.6%) pH 8.3: monophasic release profile over 2 months without observable burst release (cumulative release: 55.6%)	Structural and bioactivity preservation over the course of the release study (2 months) was observed for the formulation with a core solution of pH 8.3	[156]

[a] Calculated only for BSA in coaxial fibres without PEG.

[b] Optimised values obtained by using probe sonication (vs. vortex) for the emulsification process and a surfactant (Pluronic® F127) concentration of 10%

Abbreviations

Ace-DEX: acetalated dextran; ALP: alkaline phosphatase; BSA: bovine serum albumin; CAC: cyclic acetal coverage; CAM: chorioallantoic membrane; CTGF: connective tissue growth factor; DCM: dichloromethane; DMF: dimethylformamide; DSDP: dual-source dual power; EE: encapsulation efficiency; EGF: epidermal growth factor; HFIP: hexafluoroisopropanol; n.s.: non-specified; OVA: ovalbumin; PBS: phosphate buffered saline; PCL: poly(ε-caprolactone); PEG: poly(ethylene glycol); PLGA: poly(lactide-*co*-glycolide); PLLA: poly(L-lactic acid); PVA: poly(vinyl alcohol); rhBMP-2: recombinant bone morphogenetic protein 2; SDF-1α: stromal cell-derived factor 1; SP: substance P; TFE: 2,2,2-trifluoroethanol.

4. Discussion and future outlook

The mechanisms governing fibre and particle production by EHD processing and their applicability in various medical and industrial fields have been increasingly explored over the last decade. The relatively simple experimental set-up and low cost of electrospinning and electrospraying have led to a growing number of studies making use of these techniques for TE and drug delivery purposes. Traditional techniques for the production of scaffolds and nano/microparticles include, for instance, freeze-drying (or lyophilisation), solvent casting/particulate leaching, emulsification, and spray drying. However, application of these methods for protein encapsulation is often challenging, due to the requirement for aggressive processing conditions that may result in loss of structure and biological activity. For instance, spray drying typically requires high processing temperatures and may exert shear, interfacial stress, and dehydration stresses on the encapsulated proteins [193]. Similarly, the organic solvents used in solvent casting are unsuitable for protein encapsulation [194]. During lyophilisation, proteins undergo different kinds of stress both while freezing (*e.g.* temperature lowering, generation of extensive aqueous-ice interfaces) and drying (*e.g.* dehydration) [195]. As mentioned previously, emulsification leads to the formation of aqueous-organic interfaces that can also affect protein conformation and bioactivity [196]. Conversely, EHD processing is typically performed under ambient temperature and pressure conditions, thereby avoiding heat-induced denaturation and inactivation of the incorporated proteins. Although during extrusion through the spinneret shear forces may manifest, and a strong electric field is applied, these do not seem to have any deleterious effect on protein stability.

Further, the versatility of the technique has enabled the creation of multiple geometries as a way of regulating protein distribution within the delivery system and the release profile of the active agent(s). Overall, EHD methods have great potential for the encapsulation and delivery of protein active ingredients, but care is needed to ensure that bioactivity is not compromised upon processing. Blend, emulsion, and co-/multi-axial electrospinning and electrospraying are all different variations of EHD processing, and the determination of the most appropriate method for the encapsulation of a certain drug should take several parameters into consideration.

The studies discussed throughout this review, together with those additionally presented in **Table 4**, allow us to draw some conclusions on the most suitable strategies for protein EHD encapsulation. Owing to the poor stability of protein drugs in non-native conditions, particularly when in direct contact with organic solvents, blend

electrospinning and electrospraying are usually not employed for water-soluble protein encapsulation (even though there are exceptions, such as insulin, which is stable even when in contact with HFIP). Instead, these protein agents should be solubilised in aqueous solvents and encapsulated either *via* emulsion or co-/multi-axial EHD methods. Emulsion electrospinning has been broadly used to this end, and promising results were attained in terms of structural and functional protein preservation. However, the emulsification process can be detrimental, decreasing protein stability and resulting in loss of activity of the biological agent. These adverse effects may arise from the contact of the encapsulated proteins with organic-aqueous interfaces or from the ultrasonication procedure that is frequently employed to achieve more uniform emulsions. The use of appropriate surfactants and the replacement of ultrasonication with high speed stirring may improve the stability of the protein drugs throughout the emulsification protocol.

In general, there seems to be an increasing preference for coaxial or multi-axial techniques, as they allow for protein encapsulation in a hydrophilic, possibly non-spinnable, core that is incorporated into a polymer shell. Since the core and shell solutions are kept separated right until they exit the spinneret for fibre or particle formation, the contact between the protein agents and organic solvents used in the shell solution is minimised. Furthermore, even though core-shell structures can also be achieved with emulsion electrospinning/spraying, there is a tendency for the production of more uniform and consistent core-shell geometries with coaxial methods. The obtained release profiles are highly dependent on the distribution of the protein drug within the delivery system, therefore favouring the coaxial approach. In spite of these advantages, the thorough and laborious optimisation steps involved in coaxial and multi-axial methods must be taken into account. The most suitable method for a given application must consider the physicochemical properties of the encapsulated agent, the desired characteristics of the final product, and the complexity of the experimental set-up (**Table 2**).

In order for these protein delivery systems to be used as therapeutic tools, their manufacturing procedure must be upscaled for industrial production. The industrial implementation of electrospinning and electrospraying requires the consideration of several factors. Firstly, it is essential to increase the volume of production from laboratory to industrial scales [197]. A recent review by Vass *et al.* [198] provides a very comprehensive overview of different methods for scaling-up the electrospinning process, including multi-needle, free surface (needleless), melt, and alternating current electrospinning approaches. Methods for the scale-up of electrospraying have received less attention, but some progress has already been made towards this goal. Strategies

like high-throughput nozzles with open-channel architectures [199], multi-nozzle electrospraying [200], and needleless multi-pore electrospraying [201] have been developed to increase production rates. In the latter, solution flow rates of up to 10.5 mL/h were achieved. Recently, a novel high-throughput technology termed electrospraying assisted by pressurized gas (EAPG) was employed for the encapsulation of algae [202] and fish oil [203,204], to be used as nutraceutical supplements. In this method, atomisation of the polymer solution is performed by a pneumatic injector using compressed air, which nebulizes upon application of an electric field. After solvent evaporation, dry particles are then collected in a cyclone as a free-flowing powder [203]. Scaled-up electrospinning and electrospraying production is already performed routinely by a few companies such as InoCure (https://inocure.cz/), which is focused on applying these technologies towards drug delivery, cell culture, and TE.

In addition, it is fundamental that the production can be carried under current good manufacturing practice (cGMP) conditions. The facilities are now available, with companies like Bioinicia focusing on EHD products for biomedical, pharmaceutical, and cosmetic applications and requiring cGMP certification (https://bioinicia.com). Furthermore, it is necessary to guarantee batch-to-batch reproducibility. To this end, tight control of the processing and environmental conditions (temperature and humidity) is fundamental, and is already available in several electrospinning/spraying devices [197].

Another potential obstacle in industrial EHD processing is the difficulty in producing fibres or particles with complex geometries (*e.g.* core-shell) in a high-throughput fashion. This will require specially designed spinnerets or collectors and a very thorough and continuous quality control process [197]. Finally, it should be taken into account that these techniques often involve the use of large quantities of organic solvents, adding environmental and safety concerns. Strict policies for residue treatment and solvent recovery must be applied to ensure environmental safety, bearing in mind that the presence of residual solvents in the products may jeopardise their biomedical application.

Industrial scale EHD fabrication of protein delivery systems has not received much attention to date. However, a few researchers have explored the production of oral formulations (tablets) from protein-loaded electrospun fibres. Vass and co-workers have published a number of reports where β-galactosidase (lactase) was encapsulated within polymer fibres with varying compositions and further processed into tablets with excellent preservation of bioactivity during formulation and storage [205–207]. Feeding rates of up

to 400 mL/h were achieved, corresponding to a productivity of approximately 270 g/h of dry material [205]. The tablets produced were able to preserve the enzymatic activity of lactase at nearly 100% after six months [205] and one year of storage [206,207] at room temperature, a remarkable improvement over liquid formulations of the enzyme. These studies demonstrate the potential of EHD processing for the development of oral solid-state protein active ingredient formulations.

Solid-state protein formulations offer several advantages compared to liquid formulations, since proteins in solution or suspension are usually prone to structural modifications, hydrolytic degradation, and require low-temperature storage and distribution (the abovementioned cold-chain) [208]. Unlike classical drying methods such as lyophilisation or spray drying, EHD processing is a cost-effective technique that is not associated with high processing temperatures or freeze-thawing cycles, therefore contributing to better preservation of the protein conformation and bioactivity [207].

It is hence possible to conclude that EHD processing holds great promise for the production of protein-drug delivery systems, as it provides a gentle encapsulation mechanism with a plethora of possible geometries and allows the development of solid-state formulations, potentially at an industrial scale. Nevertheless, there is still a long way to go before these formulations can be widely used in biomedical scenarios. Clinical trials need to be performed for the evaluation of their safety and efficacy profiles, and regulatory barriers must be overcome before their commercialisation is authorised. Moreover, the industrial implementation of these methods will require further increases in productivity, reproducibility, standardised quality control protocols, and environmental safety policies to ensure regulatory and economic viability.

5. Conclusions

This review has demonstrated that blend, emulsion, and co-/multi-axial electrospinning and electrospraying are suitable for the encapsulation and delivery of a range of protein active ingredients, including enzymes, growth factors, antibodies, and protein-based vaccines. This range of proteins has widely varying structural characteristics, physicochemical properties, and biological roles, but all can be incorporated within electrospun fibres or electrosprayed particles with considerable functionality and structural integrity preservation. EHD methods are exceptionally versatile, since a large range of natural and synthetic polymers can be employed, either individually or in a blend. This results in products with characteristics that can be incrementally varied to provide the desired three-dimensional structures, mechanical properties, degradation rates, and release profiles. In general, co-/multi-axial

electrospinning and electrospraying tend to be the most suitable techniques for the encapsulation of proteins, although promising results have also been achieved by emulsion EHD. Several high-throughput alternatives to the low-throughput methods used in most laboratory research have been proposed over the last few years, enabling increased productivity rates and paving the way for industrial implementation of electrospinning/spraying. Thus, EHD techniques have broad potential in the pharmaceutical and biomedical fields, particularly in drug delivery and tissue engineering, although extensive medical and operational research will be required before widespread clinical application can take place.

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7. References

[1] B. Leader, Q.J. Baca, D.E. Golan, Protein therapeutics: a summary and pharmacological classification, Nat. Rev. Drug Discov. 7 (2008) 21–39. https://doi.org/10.1038/nrd2399.

[2] D. V Goeddel, D.G. Kleid, F. Bolivar, H.L. Heyneker, D.G. Yansura, R. Crea, T. Hirose, A. Kraszewski, K. Itakura, A.D. Riggs, Expression in Escherichia coli of chemically synthesized genes for human insulin, Proc Natl Acad Sci U S A. 76 (1979) 106–110. https://doi.org/10.1073/pnas.76.1.106.

[3] A.J. Clark, R.O. Adeniyi-Jones, G. Knight, J.M. Leiper, P.G. Wiles, R.H. Jones, H. Keen, A.C. MacCuish, J.D. Ward, P.J. Watkins, J.M. Cauldwell, A. Glynne, J.B. Scotton, Biosynthetic human insulin in the treatment of diabetes. A double-blind crossover trial in established diabetic patients, Lancet. 2 (1982) 354–357. https://doi.org/10.1016/s0140-6736(82)90548-7.

[4] A.C.-L. Lee, J.L. Harris, K.K. Khanna, J.-H. Hong, A Comprehensive Review on Current Advances in Peptide Drug Development and Design, Int. J. Mol. Sci. 20 (2019) 2383. https://doi.org/10.3390/ijms20102383.

[5] D.J. Craik, D.P. Fairlie, S. Liras, D. Price, The future of peptide-based drugs, Chem Biol Drug Des. 81 (2013) 136–147. https://doi.org/10.1111/cbdd.12055.

[6] V. Lifshitz, D. Frenkel, TGF-β, in: Handb. Biol. Act. Pept., Elsevier Inc., 2013: pp. 1647–1653. https://doi.org/10.1016/B978-0-12-385095-9.00225-6.

[7] W.M. Pardridge, Blood-Brain Barrier and Delivery of Protein and Gene Therapeutics to Brain, Front. Aging Neurosci. 11 (2020) 373. https://doi.org/10.3389/fnagi.2019.00373.

[8] B.J. Aungst, H. Saitoh, D.L. Burcham, S.-M. Huang, S.A. Mousa, M.A. Hussain, Enhancement of the intestinal absorption of peptides and nonpeptides, J. Control. Release. 41 (1996) 19–31. https://doi.org/https://doi.org/10.1016/0168-3659(96)01353-3.

[9] Z. Antosova, M. Mackova, V. Kral, T. Macek, Therapeutic application of peptides and proteins: parenteral forever?, Trends Biotechnol. 27 (2009) 628–635. https://doi.org/10.1016/j.tibtech.2009.07.009.

[10] A. Jain, A. Jain, A. Gulbake, S. Shilpi, P. Hurkat, S.K. Jain, Peptide and protein delivery using new drug delivery systems, Crit Rev Ther Drug Carr. Syst. 30 (2013) 293–329. https://doi.org/10.1615/critrevtherdrugcarriersyst.2013006955.

[11] A. Patel, K. Cholkar, A.K. Mitra, Recent developments in protein and peptide parenteral delivery approaches, Ther. Deliv. 5 (2014) 337–365. https://doi.org/10.4155/tde.14.5.

[12] J. Shaji, V. Patole, Protein and peptide drug delivery: Oral approaches, Indian J. Pharm. Sci. 70 (2008) 269–277. https://doi.org/10.4103/0250-474X.42967.

[13] M. van de Weert, L. Jorgensen, E. Horn Moeller, S. Frokjaer, Factors of importance for a successful delivery system for proteins, Expert Opin Drug Deliv. 2 (2005) 1029–1037. https://doi.org/10.1517/17425247.2.6.1029.

[14] A.L. Daugherty, R.J. Mrsny, Formulation and delivery issues for monoclonal antibody therapeutics, Adv Drug Deliv Rev. 58 (2006) 686–706. https://doi.org/10.1016/j.addr.2006.03.011.

[15] B. Sharma, Immunogenicity of therapeutic proteins. Part 1: impact of product handling, Biotechnol Adv. 25 (2007) 310–317. https://doi.org/10.1016/j.biotechadv.2007.01.005.

[16] H.A. Schiffter, The Delivery of Drugs - Peptides and Proteins, in: Compr. Biotechnol. Second Ed., Elsevier Inc., 2011: pp. 587–604. https://doi.org/10.1016/B978-0-08-088504-9.00338-X.

[17] J.P. Emond, The cold chain, in: J.R. Williams, S.E. Sarma, S.B. Miles (Eds.), RFID Technol. Appl., Cambridge University Press, Cambridge, 2008: pp. 144–156. https://doi.org/10.1017/CBO9780511541155.012.

[18] L. Wolfson, F. Gasse, S.-P. Lee-Martin, P. Lydon, A. Magan, A. Tibouti, B. Johns, R. Hutubessy, P. Salama, J.-M. Okwo-Bele, Estimating the costs of achieving the WHO-UNICEF Global Immunization Vision and Strategy, 2006-2015, Bull. World Health Organ. 86 (2008) 27–39. https://doi.org/10.2471/BLT.07.045096.

[19] J. Zhang, E. Pritchard, X. Hu, T. Valentin, B. Panilaitis, F.G. Omenetto, D.L. Kaplan, Stabilization of vaccines and antibiotics in silk and eliminating the cold chain, Proc. Natl. Acad. Sci. 109 (2012) 11981–11986. https://doi.org/10.1073/pnas.1206210109.

[20] C. Li, J. Wang, Y. Wang, H. Gao, G. Wei, Y. Huang, H. Yu, Y. Gan, Y. Wang, L. Mei, H. Chen, H. Hu, Z. Zhang, Y. Jin, Recent progress in drug delivery, Acta Pharm. Sin. B. 9 (2019) 1145–1162. https://doi.org/https://doi.org/10.1016/j.apsb.2019.08.003.

[21] Y. Yeo, N. Baek, K. Park, Microencapsulation methods for delivery of protein drugs, Biotechnol. Bioprocess Eng. 6 (2001) 213–230. https://doi.org/10.1007/BF02931982.

[22] G.R. Williams, B.T. Raimi-Abraham, C.J. Luo, Nanofibres in drug delivery, UCL Press, 2018.

[23] D.N. Nguyen, C. Clasen, G. Van den Mooter, Pharmaceutical Applications of Electrospraying, J Pharm Sci. 105 (2016) 2601–2620. https://doi.org/10.1016/j.xphs.2016.04.024.

[24] M. Zamani, M.P. Prabhakaran, S. Ramakrishna, Advances in drug delivery via electrospun and electrosprayed nanomaterials, Int. J. Nanomedicine. 8 (2013) 2997–3017. https://doi.org/10.2147/IJN.S43575.

[25] B.Z. Tang, Electrospinning: principles, practice and possibilities, Royal Society of Chemistry, 2015. https://doi.org/10.1039/9781849735575.

[26] A. Khalf, S. V. Madihally, Recent advances in multiaxial electrospinning for drug delivery, Eur. J. Pharm. Biopharm. 112 (2017) 1–17. https://doi.org/10.1016/j.ejpb.2016.11.010.

[27] C. Huang, S.J. Soenen, J. Rejman, B. Lucas, K. Braeckmans, J. Demeester, S.C. De Smedt, Stimuli-responsive electrospun fibers and their applications, Chem.

Soc. Rev. 40 (2011) 2417–2434. https://doi.org/10.1039/C0CS00181C.

[28] Z. Chen, Z. Chen, A. Zhang, J. Hu, X. Wang, Z. Yang, Electrospun nanofibers for cancer diagnosis and therapy, Biomater. Sci. 4 (2016) 922–932. https://doi.org/10.1039/C6BM00070C.

[29] A. Greiner, J.H. Wendorff, Electrospinning: a fascinating method for the preparation of ultrathin fibers, Angew. Chemie Int. Ed. 46 (2007) 5670–5703. https://doi.org/10.1002/anie.200604646.

[30] Y.-Z. Long, M.-M. Li, C. Gu, M. Wan, J.-L. Duvail, Z. Liu, Z. Fan, Recent advances in synthesis, physical properties and applications of conducting polymer nanotubes and nanofibers, Prog. Polym. Sci. 36 (2011) 1415–1442. https://doi.org/10.1016/j.progpolymsci.2011.04.001.

[31] M. Buzgo, A. Mickova, M. Rampichova, M. Doupnik, Blend electrospinning, coaxial electrospinning, and emulsion electrospinning techniques, in: Core-Shell Nanostructures Drug Deliv. Theranostics, Elsevier, 2018: pp. 325–347. https://doi.org/10.1016/B978-0-08-102198-9.00011-9.

[32] M.L. Bruschi, ed., Main mechanisms to control the drug release, in: Strateg. to Modify Drug Release from Pharm. Syst., Elsevier, 2015: pp. 37–62. https://doi.org/10.1016/b978-0-08-100092-2.00004-7.

[33] X.-Y. Sun, L.R. Nobles, H.G. Börner, R.J. Spontak, Field-Driven Surface Segregation of Biofunctional Species on Electrospun PMMA/PEO Microfibers, Macromol. Rapid Commun. 29 (2008) 1455–1460. https://doi.org/10.1002/marc.200800163.

[34] X.-Y. Sun, R. Shankar, H.G. Börner, T.K. Ghosh, R.J. Spontak, Field-Driven Biofunctionalization of Polymer Fiber Surfaces during Electrospinning, Adv. Mater. 19 (2007) 87–91. https://doi.org/10.1002/adma.200601345.

[35] A. Szentivanyi, T. Chakradeo, H. Zernetsch, B. Glasmacher, Electrospun cellular microenvironments: Understanding controlled release and scaffold structure, Adv. Drug Deliv. Rev. 63 (2011) 209–220. https://doi.org/10.1016/j.addr.2010.12.002.

[36] D.W. Song, S.H. Kim, H.H. Kim, K.H. Lee, C.S. Ki, Y.H. Park, Multi-biofunction of antimicrobial peptide-immobilized silk fibroin nanofiber membrane: Implications for wound healing, Acta Biomater. 39 (2016) 146–155.

[37] X. Wang, T. Yue, T. Lee, Development of Pleurocidin-poly (vinyl alcohol) electrospun antimicrobial nanofibers to retain antimicrobial activity in food system application,

Food Control. 54 (2015) 150–157. https://doi.org/10.1016/j.foodcont.2015.02.001.

[38] G. Zehetmeyer, S.M.M. Meira, J.M. Scheibel, C. de B. da Silva, F.S. Rodembusch, A. Brandelli, R.M.D. Soares, Biodegradable and antimicrobial films based on poly (butylene adipate-co-terephthalate) electrospun fibers, Polym. Bull. 74 (2017) 3243–3268. https://doi.org/10.1007/s00289-016-1896-8.

[39] A. Mickova, M. Buzgo, O. Benada, M. Rampichova, Z. Fisar, E. Filova, M. Tesarova, D. Lukas, E. Amler, Core/shell nanofibers with embedded liposomes as a drug delivery system, Biomacromolecules. 13 (2012) 952–962. https://doi.org/10.1021/bm2018118.

[40] X. Li, Y. Su, S. Liu, L. Tan, X. Mo, S. Ramakrishna, Encapsulation of proteins in poly (l-lactide-co-caprolactone) fibers by emulsion electrospinning, Colloids Surfaces B Biointerfaces. 75 (2010) 418–424. https://doi.org/10.1016/j.colsurfb.2009.09.014.

[41] X. Xu, X. Zhuang, X. Chen, X. Wang, L. Yang, X. Jing, Preparation of core-sheath composite nanofibers by emulsion electrospinning, Macromol. Rapid Commun. 27 (2006) 1637–1642. https://doi.org/10.1002/marc.200600384.

[42] T. Tadros, Principles of emulsion stabilization with special reference to polymeric surfactants, J. Cosmet. Sci. 57 (2006) 153–169.

[43] N. Nikmaram, S. Roohinejad, S. Hashemi, M. Koubaa, F.J. Barba, A. Abbaspourrad, R. Greiner, Emulsion-based systems for fabrication of electrospun nanofibers: Food, pharmaceutical and biomedical applications, RSC Adv. 7 (2017) 28951–28964. https://doi.org/10.1039/c7ra00179g.

[44] X. Xu, L. Yang, X. Xu, X. Wang, X. Chen, Q. Liang, J. Zeng, X. Jing, Ultrafine medicated fibers electrospun from W/O emulsions, J. Control. Release. 108 (2005) 33–42. https://doi.org/10.1016/j.jconrel.2005.07.021.

[45] C. Cleeton, A. Keirouz, X. Chen, N. Radacsi, Electrospun Nanofibers for Drug Delivery and Biosensing, ACS Biomater. Sci. Eng. 5 (2019) 4183–4205.

[46] Y. Lu, J. Huang, G. Yu, R. Cardenas, S. Wei, E.K. Wujcik, Z. Guo, Coaxial electrospun fibers: applications in drug delivery and tissue engineering, Wiley Interdiscip. Rev. Nanomedicine Nanobiotechnology. 8 (2016) 654–677. https://doi.org/10.1002/wnan.1391.

[47] H. Watanabe, Viscoelasticity and dynamics of entangled polymers, Prog. Polym. Sci.

24 (1999) 1253–1403. https://doi.org/10.1016/S0079-6700(99)00029-5.

[48] A. Keirouz, M. Chung, J. Kwon, G. Fortunato, N. Radacsi, 2D and 3D electrospinning technologies for the fabrication of nanofibrous scaffolds for skin tissue engineering: A review, WIREs Nanomedicine and Nanobiotechnology. 12 (2020). https://doi.org/10.1002/wnan.1626.

[49] E. Sapountzi, M. Braiek, J.F. Chateaux, N. Jaffrezic-Renault, F. Lagarde, Recent advances in electrospun nanofiber interfaces for biosensing devices, Sensors (Switzerland). 17 (2017) 1887. https://doi.org/10.3390/s17081887.

[50] S.Y. Gu, J. Ren, G.J. Vancso, Process optimization and empirical modeling for electrospun polyacrylonitrile (PAN) nanofiber precursor of carbon nanofibers, Eur. Polym. J. 41 (2005) 2559–2568. https://doi.org/10.1016/j.eurpolymj.2005.05.008.

[51] S. V. Fridrikh, J.H. Yu, M.P. Brenner, G.C. Rutledge, Controlling the Fiber Diameter during Electrospinning, Phys. Rev. Lett. 90 (2003) 4. https://doi.org/10.1103/PhysRevLett.90.144502.

[52] C. Wang, H.S. Chien, C.H. Hsu, Y.C. Wang, C.T. Wang, H.A. Lu, Electrospinning of polyacrylonitrile solutions at elevated temperatures, Macromolecules. 40 (2007) 7973–7983. https://doi.org/10.1021/ma070508n.

[53] D.G. Yu, C.J. Branford-White, N.P. Chatterton, K. White, L.M. Zhu, X.X. Shen, W. Nie, Electrospinning of concentrated polymer solutions, Macromolecules. 43 (2010) 10743–10746. https://doi.org/10.1021/ma1024363.

[54] D.G. Yu, C. Branford-White, S.W.A. Bligh, K. White, N.P. Chatterton, L.M. Zhu, Improving polymer nanofiber quality using a modified co-axial electrospinning process, Macromol. Rapid Commun. 32 (2011) 744–750. https://doi.org/10.1002/marc.201100049.

[55] H. Zhou, Z. Shi, X. Wan, H. Fang, D.G. Yu, X. Chen, P. Liu, The relationships between process parameters and polymeric nanofibers fabricated using a modified coaxial electrospinning, Nanomaterials. 9 (2019). https://doi.org/10.3390/nano9060843.

[56] D.G. Yu, J. Zhou, N.P. Chatterton, Y. Li, J. Huang, X. Wang, Polyacrylonitrile nanofibers coated with silver nanoparticles using a modified coaxial electrospinning process, Int. J. Nanomedicine. 7 (2012) 5725–5732. https://doi.org/10.2147/IJN.S37455.

[57] M. Wang, T. Hai, Z. Feng, D.G. Yu, Y. Yang, S.W.A. Bligh, The relationships

between the working fluids, process characteristics and products from the modified coaxial electrospinning of zein, Polymers (Basel). 11 (2019). https://doi.org/10.3390/polym11081287.

[58] P. McClellan, W.J. Landis, Recent applications of coaxial and emulsion electrospinning methods in the field of tissue engineering, Biores. Open Access. 5 (2016) 212–227. https://doi.org/10.1089/biores.2016.0022.

[59] D. Carson, Y. Jiang, K.A. Woodrow, Tunable Release of Multiclass Anti-HIV Drugs that are Water-Soluble and Loaded at High Drug Content in Polyester Blended Electrospun Fibers, Pharm. Res. 33 (2016) 125–136. https://doi.org/10.1007/s11095-015-1769-0.

[60] P. Davoodi, M.P. Srinivasan, C.H. Wang, Effective co-delivery of nutlin-3a and p53 genes: Via core-shell microparticles for disruption of MDM2-p53 interaction and reactivation of p53 in hepatocellular carcinoma, J. Mater. Chem. B. 5 (2017) 5816–5834. https://doi.org/10.1039/c7tb00481h.

[61] A.R. D'Amato, N.J. Schaub, J.M. Cardenas, A.S. Fiumara, P.M. Troiano, A. Fischetti, R.J. Gilbert, Removal of retained electrospinning solvent prolongs drug release from electrospun PLLA fibers, Polymer (Guildf). 123 (2017) 121–127. https://doi.org/10.1016/j.polymer.2017.07.008.

[62] S. McInnes, T. Macdonald, I. Parkin, T. Nann, N. Voelcker, Electrospun Composites of Polycaprolactone and Porous Silicon Nanoparticles for the Tunable Delivery of Small Therapeutic Molecules, Nanomaterials. 8 (2018) 205. https://doi.org/10.3390/nano8040205.

[63] C. Achille, S. Sundaresh, B. Chu, M. Hadjiargyrou, Cdk2 Silencing via a DNA/PCL Electrospun Scaffold Suppresses Proliferation and Increases Death of Breast Cancer Cells, PLoS One. 7 (2012) e52356. https://doi.org/10.1371/journal.pone.0052356.

[64] Y. Zheng, Y. Wu, Y. Zhou, J. Wu, X. Wang, Y. Qu, Y. Wang, Y. Zhang, Q. Yu, Photothermally Activated Electrospun Nanofiber Mats for High-Efficiency Surface-Mediated Gene Transfection, ACS Appl. Mater. Interfaces. 12 (2020) 7905–7914. https://doi.org/10.1021/acsami.9b20221.

[65] G.N. Salles, F.A. dos S. Pereira, C. Pacheco-Soares, F.R. Marciano, C. Hölscher, T.J. Webster, A.O. Lobo, A Novel Bioresorbable Device as a Controlled Release System for Protecting Cells from Oxidative Stress from Alzheimer's Disease, Mol.

Neurobiol. 54 (2017) 6827–6838. https://doi.org/10.1007/s12035-016-0200-0.

[66] N. Zandi, R. Lotfi, E. Tamjid, M.A. Shokrgozar, A. Simchi, Core-sheath gelatin based electrospun nanofibers for dual delivery release of biomolecules and therapeutics, Mater. Sci. Eng. C. 108 (2020). https://doi.org/10.1016/j.msec.2019.110432.

[67] S.L. Sampson, L. Saraiva, K. Gustafsson, S.N. Jayasinghe, B.D. Robertson, Cell electrospinning: An in vitro and in vivo study, Small. 10 (2014) 78–82. https://doi.org/10.1002/smll.201300804.

[68] D.K. Nguyen, Y.M. Son, N.E. Lee, Hydrogel Encapsulation of Cells in Core-Shell Microcapsules for Cell Delivery, Adv. Healthc. Mater. 4 (2015) 1537–1544. https://doi.org/10.1002/adhm.201500133.

[69] Y. Guo, J. Gilbert-Honick, S.M. Somers, H.Q. Mao, W.L. Grayson, Modified cell-electrospinning for 3D myogenesis of C2C12s in aligned fibrin microfiber bundles, Biochem. Biophys. Res. Commun. 516 (2019) 558–564. https://doi.org/10.1016/j.bbrc.2019.06.082.

[70] K. Songsurang, N. Praphairaksit, K. Siraleartmukul, N. Muangsin, Electrospray fabrication of doxorubicin-chitosan-tripolyphosphate nanoparticles for delivery of doxorubicin, Arch. Pharm. Res. 34 (2011) 583. https://doi.org/10.1007/s12272-011-0408-5.

[71] Y. Xu, M.A. Hanna, Electrospray encapsulation of water-soluble protein with polylactide: Effects of formulations on morphology, encapsulation efficiency and release profile of particles, Int. J. Pharm. 320 (2006) 30–36. https://doi.org/https://doi.org/10.1016/j.ijpharm.2006.03.046.

[72] T. Uyar, E. Kny, Electrospun materials for tissue engineering and biomedical applications: research, design and commercialization, Woodhead Publishing, 2017.

[73] E.P. da Silva, M.R. Guilherme, F.P. Garcia, C. V Nakamura, L. Cardozo-Filho, C.G. Alonso, A.F. Rubira, M.H. Kunita, Drug release profile and reduction in the in vitro burst release from pectin/HEMA hydrogel nanocomposites crosslinked with titania, RSC Adv. 6 (2016) 19060–19068. https://doi.org/10.1039/C5RA27865A.

[74] X. Huang, C.S. Brazel, On the importance and mechanisms of burst release in matrix-controlled drug delivery systems, J. Control. Release. 73 (2001) 121–136.

[75] S.-F. Chou, D. Carson, K.A. Woodrow, Current strategies for sustaining drug release from electrospun nanofibers, J. Control. Release. 220 (2015) 584–591.

https://doi.org/10.1016/j.jconrel.2015.09.008.

[76] N. Hilal, A.F. Ismail, C. Wright, Membrane Fabrication, CRC Press, 2015. https://books.google.pt/books?id=mU23BgAAQBAJ.

[77] G.R. Williams, N.P. Chatterton, T. Nazir, D.-G. Yu, L.-M. Zhu, C.J. Branford-White, Electrospun nanofibers in drug delivery: recent developments and perspectives, Ther. Deliv. 3 (2012) 515–533. https://doi.org/10.4155/tde.12.17.

[78] J. Pelipenko, P. Kocbek, J. Kristl, Critical attributes of nanofibers: Preparation, drug loading, and tissue regeneration, Int. J. Pharm. 484 (2015) 57–74. https://doi.org/10.1016/j.ijpharm.2015.02.043.

[79] E.A. Krogstad, M.J. Rathbone, K.A. Woodrow, Vaginal drug delivery, in: Focal Control. Drug Deliv., Springer, 2014: pp. 607–651. https://doi.org/10.1007/978-1-4614-9434-8_27.

[80] X. Shi, W. Zhou, D. Ma, Q. Ma, D. Bridges, Y. Ma, A. Hu, Electrospinning of Nanofibers and Their Applications for Energy Devices, J. Nanomater. 2015 (2015) 140716. https://doi.org/10.1155/2015/140716.

[81] E.H. Sanders, R. Kloefkorn, G.L. Bowlin, D.G. Simpson, G.E. Wnek, Two-phase electrospinning from a single electrified jet: Microencapsulation of aqueous reservoirs in poly(ethylene-co-vinyl acetate) fibers, Macromolecules. 36 (2003) 3803–3805. https://doi.org/10.1021/ma021771l.

[82] H. Jiang, D. Fang, B.S. Hsiao, B. Chu, W. Chen, Optimization and Characterization of Dextran Membranes Prepared by Electrospinning, Biomacromolecules. 5 (2004) 326–333. https://doi.org/10.1021/bm034345w.

[83] J. Xie, Y. Lo Hsieh, Ultra-high surface fibrous membranes from electrospinning of natural proteins: Casein and lipase enzyme, in: J. Mater. Sci., Springer, 2003: pp. 2125–2133. https://doi.org/10.1023/A:1023763727747.

[84] S.Y. Chew, J. Wen, E.K.F. Yim, K.W. Leong, Sustained release of proteins from electrospun biodegradable fibers, Biomacromolecules. 6 (2005) 2017–2024. https://doi.org/10.1021/bm0501149.

[85] Y. Yang, X. Li, W. Cui, S. Zhou, R. Tan, C. Wang, Structural stability and release profiles of proteins from core-shell poly (DL-lactide) ultrafine fibers prepared by emulsion electrospinning, J. Biomed. Mater. Res. Part A. 86 (2008) 374–385. https://doi.org/10.1002/jbm.a.31595.

[86] Y. Yang, X. Li, M. Qi, S. Zhou, J. Weng, Release pattern and structural integrity of lysozyme encapsulated in core-sheath structured poly(dl-lactide) ultrafine fibers prepared by emulsion electrospinning, Eur. J. Pharm. Biopharm. 69 (2008) 106–116. https://doi.org/10.1016/j.ejpb.2007.10.016.

[87] H. Frizzell, T.J. Ohlsen, K.A. Woodrow, Protein-loaded emulsion electrospun fibers optimized for bioactivity retention and pH-controlled release for peroral delivery of biologic therapeutics, Int. J. Pharm. 533 (2017) 99–110. https://doi.org/10.1016/j.ijpharm.2017.09.043.

[88] J. Hu, L. Tian, M.P. Prabhakaran, X. Ding, S. Ramakrishna, Fabrication of Nerve Growth Factor Encapsulated Aligned Poly(ε-Caprolactone) Nanofibers and Their Assessment as a Potential Neural Tissue Engineering Scaffold, Polymers (Basel). 8 (2016) 54. https://doi.org/10.3390/polym8020054.

[89] Y.Z. Zhang, X. Wang, Y. Feng, J. Li, C.T. Lim, S. Ramakrishna, Coaxial electrospinning of (fluorescein isothiocyanate-conjugated bovine serum albumin)-encapsulated poly (ε-caprolactone) nanofibers for sustained release, Biomacromolecules. 7 (2006) 1049–1057. https://doi.org/10.1021/bm050743i.

[90] W. Ji, F. Yang, J.J.J.P. Van den Beucken, Z. Bian, M. Fan, Z. Chen, J.A. Jansen, Fibrous scaffolds loaded with protein prepared by blend or coaxial electrospinning, Acta Biomater. 6 (2010) 4199–4207. https://doi.org/10.1016/j.actbio.2010.05.025.

[91] Y. Li, H. Jiang, K. Zhu, Encapsulation and controlled release of lysozyme from electrospun poly(epsilon-caprolactone)/poly(ethylene glycol) non-woven membranes by formation of lysozyme-oleate complexes., J. Mater. Sci. Mater. Med. 19 (2008) 827–832. https://doi.org/10.1007/s10856-007-3175-6.

[92] N. Nagiah, C.J. Murdock, M. Bhattacharjee, L. Nair, C.T. Laurencin, Development of Tripolymeric Triaxial Electrospun Fibrous Matrices for Dual Drug Delivery Applications, Sci. Rep. 10 (2020) 609. https://doi.org/10.1038/s41598-020-57412-0.

[93] C. Ru, F. Wang, M. Pang, L. Sun, R. Chen, Y. Sun, Suspended, Shrinkage-Free, Electrospun PLGA Nanofibrous Scaffold for Skin Tissue Engineering, ACS Appl. Mater. Interfaces. 7 (2015) 10872–10877. https://doi.org/10.1021/acsami.5b01953.

[94] J. Xie, C.-H. Wang, Encapsulation of proteins in biodegradable polymeric microparticles using electrospray in the Taylor cone-jet mode., Biotechnol.

Bioeng. 97 (2007) 1278–1290. https://doi.org/10.1002/bit.21334.

[95] M. Zamani, M.P. Prabhakaran, E.S. Thian, S. Ramakrishna, Protein encapsulated core-shell structured particles prepared by coaxial electrospraying: investigation on material and processing variables., Int. J. Pharm. 473 (2014) 134–143. https://doi.org/10.1016/j.ijpharm.2014.07.006.

[96] D.J. Vocadlo, G.J. Davies, R. Laine, S.G. Withers, Catalysis by hen egg-white lysozyme proceeds via a covalent intermediate, Nature. 412 (2001) 835–838. https://doi.org/10.1038/35090602.

[97] X. Liu, L.H. Nielsen, H. Qu, L.P. Christensen, J. Rantanen, M. Yang, Stability of lysozyme incorporated into electrospun fibrous mats for wound healing, Eur. J. Pharm. Biopharm. 136 (2019) 240–249. https://doi.org/10.1016/j.ejpb.2019.01.003.

[98] H. Yoo, H. Choi, T. Park, Protein-fatty Acid Complex for Enhanced Loading and Stability Within Biodegradable Nanoparticles, J. Pharm. Sci. 90 (2001). https://doi.org/10.1002/1520-6017(200102)90:2<194::AID-JPS10>3.0.CO;2-Q.

[99] S. Puhl, L. Li, L. Meinel, O. Germershaus, Controlled protein delivery from electrospun non-wovens: Novel combination of protein crystals and a biodegradable release matrix, Mol. Pharm. 11 (2014) 2372–2380. https://doi.org/10.1021/mp5001026.

[100] T. Briggs, T.L. Arinzeh, Examining the formulation of emulsion electrospinning for improving the release of bioactive proteins from electrospun fibers, J. Biomed. Mater. Res. - Part A. 102 (2014) 674–684. https://doi.org/10.1002/jbm.a.34730.

[101] T.G. Kim, D.S. Lee, T.G. Park, Controlled protein release from electrospun biodegradable fiber mesh composed of poly(ε-caprolactone) and poly(ethylene oxide), Int. J. Pharm. 338 (2007) 276–283. https://doi.org/10.1016/j.ijpharm.2007.01.040.

[102] Y. Ma, M. Björnmalm, A.K. Wise, C. Cortez-Jugo, E. Revalor, Y. Ju, O.M. Feeney, R.T. Richardson, E. Hanssen, R.K. Shepherd, C.J.H. Porter, F. Caruso, Gel-Mediated Electrospray Assembly of Silica Supraparticles for Sustained Drug Delivery, ACS Appl. Mater. Interfaces. 10 (2018) 31019–31031. https://doi.org/10.1021/acsami.8b10415.

[103] T.E. Herricks, S.H. Kim, J. Kim, D. Li, J.H. Kwak, J.W. Grate, S.H. Kim, Y. Xia, Direct fabrication of enzyme-carrying polymer nanofibers by electrospinning, J.

Mater. Chem. 15 (2005) 3241–3245. https://doi.org/10.1039/b503660g.

[104] A. Díaz, L.J. Del Valle, D. Tugushi, R. Katsarava, J. Puiggalí, New poly(ester urea) derived from l-leucine: Electrospun scaffolds loaded with antibacterial drugs and enzymes, Mater. Sci. Eng. C. 46 (2015) 450–462. https://doi.org/10.1016/j.msec.2014.10.055.

[105] Y. Dai, J. Niu, J. Liu, L. Yin, J. Xu, In situ encapsulation of laccase in microfibers by emulsion electrospinning: Preparation, characterization, and application, Bioresour. Technol. 101 (2010) 8942–8947. https://doi.org/10.1016/j.biortech.2010.07.027.

[106] S.C. Pinto, A.R. Rodrigues, J.A. Saraiva, J.A. Lopes-da-Silva, Catalytic activity of trypsin entrapped in electrospun poly(ε-caprolactone) nanofibers, Enzyme Microb. Technol. 79–80 (2015) 8–18. https://doi.org/10.1016/j.enzmictec.2015.07.002.

[107] K. Lee, E.A. Silva, D.J. Mooney, Growth factor delivery-based tissue engineering: General approaches and a review of recent developments, J. R. Soc. Interface. 8 (2011) 153–170. https://doi.org/10.1098/rsif.2010.0223.

[108] Y.R. Yun, J.H. Jang, E. Jeon, W. Kang, S. Lee, J.E. Won, H.W. Kim, I. Wall, Administration of growth factors for bone regeneration, Regen. Med. 7 (2012) 369–385. https://doi.org/10.2217/rme.12.1.

[109] Z. Wang, Z. Wang, W.W. Lu, W. Zhen, D. Yang, S. Peng, Novel biomaterial strategies for controlled growth factor delivery for biomedical applications, NPG Asia Mater. 9 (2017) e435–e435. https://doi.org/10.1038/am.2017.171.

[110] E.J. Carragee, G. Chu, R. Rohatgi, E.L. Hurwitz, B.K. Weiner, S.T. Yoon, G. Comer, B. Kopjar, Cancer risk after use of recombinant bone morphogenetic protein-2 for spinal arthrodesis, J. Bone Jt. Surg. - Ser. A. 95 (2013) 1537–1545. https://doi.org/10.2106/JBJS.L.01483.

[111] W. Ji, Y. Sun, F. Yang, J.J.J.P. van den Beucken, M. Fan, Z. Chen, J.A. Jansen, Bioactive electrospun scaffolds delivering growth factors and genes for tissue engineering applications, Pharm. Res. 28 (2011) 1259–1272. https://doi.org/10.1007/s11095-010-0320-6.

[112] W. Zhu, F. Masood, J. O'Brien, L.G. Zhang, Highly aligned nanocomposite scaffolds by electrospinning and electrospraying for neural tissue regeneration, Nanomedicine Nanotechnology, Biol. Med. 11 (2015) 693–704. https://doi.org/10.1016/j.nano.2014.12.001.

[113] R.H.S. Westerink, A.G. Ewing, The PC12 cell as model for neurosecretion, in: Acta Physiol., NIH Public Access, 2008: pp. 273–285. https://doi.org/10.1111/j.1748-1716.2007.01805.x.

[114] K. Quintiliano, T. Crestani, D. Silveira, V.E. Helfer, A. Rosa, E. Balbueno, D. Steffens, G.P. Jotz, D.A. Pilger, P. Pranke, Neural Differentiation of Mesenchymal Stem Cells on Scaffolds for Nerve Tissue Engineering Applications., Cell. Reprogram. 18 (2016) 369–381. https://doi.org/10.1089/cell.2016.0024.

[115] C.-Y. Wang, J.-J. Liu, C.-Y. Fan, X.-M. Mo, H.-J. Ruan, F.-F. Li, The effect of aligned core–shell nanofibres delivering NGF on the promotion of sciatic nerve regeneration, J. Biomater. Sci. Polym. Ed. 23 (2012) 167–184. https://doi.org/10.1163/092050610X545805.

[116] Z. Kuihua, W. Chunyang, F. Cunyi, M. Xiumei, Aligned SF/P(LLA-CL)-blended nanofibers encapsulating nerve growth factor for peripheral nerve regeneration., J. Biomed. Mater. Res. A. 102 (2014) 2680–2691. https://doi.org/10.1002/jbm.a.34922.

[117] T.J. Whitehead, C.O.C. Avila, H.G. Sundararaghavan, Combining growth factor releasing microspheres within aligned nanofibers enhances neurite outgrowth., J. Biomed. Mater. Res. A. 106 (2018) 17–25. https://doi.org/10.1002/jbm.a.36204.

[118] B. Duan, L. Wu, X. Yuan, Z. Hu, X. Li, Y. Zhang, K. Yao, M. Wang, Hybrid nanofibrous membranes of PLGA/chitosan fabricated via an electrospinning array, J. Biomed. Mater. Res. - Part A. 83 (2007) 868–878. https://doi.org/10.1002/jbm.a.31408.

[119] J. Kang, M. Wang, X. Yuan, An Investigation into Dual-Source and Dual-Power Electrospinning of Nanofibrous Membranes for Medical Applications, Adv. Mater. Res. 47–50 (2008) 1454–1457. https://doi.org/10.4028/www.scientific.net/AMR.47-50.1454.

[120] C. Liu, X. Li, F. Xu, H. Cong, Z. Li, Y. Song, M. Wang, Spatio-temporal release of NGF and GDNF from multi-layered nanofibrous bicomponent electrospun scaffolds., J. Mater. Sci. Mater. Med. 29 (2018) 102. https://doi.org/10.1007/s10856-018-6105-x.

[121] C. Liu, C. Wang, Q. Zhao, X. Li, F. Xu, X. Yao, M. Wang, Incorporation and release of dual growth factors for nerve tissue engineering using nanofibrous bicomponent scaffolds., Biomed. Mater. 13 (2018) 44107.

https://doi.org/10.1088/1748-605X/aab693.

[122] S. Madduri, M. Papaloïzos, B. Gander, Synergistic effect of GDNF and NGF on axonal branching and elongation in vitro, Neurosci. Res. 65 (2009) 88–97. https://doi.org/10.1016/j.neures.2009.06.003.

[123] D. Chen, M.A. Harris, G. Rossini, C.R. Dunstan, S.L. Dallas, J.Q. Feng, G.R. Mundy, S.E. Harris, Bone morphogenetic protein 2 (BMP-2) enhances BMP-3, BMP-4, and bone cell differentiation marker gene expression during the induction of mineralized bone matrix formation in cultures of fetal rat calvarial osteoblasts, Calcif. Tissue Int. 60 (1997) 283–290. https://doi.org/10.1007/s002239900230.

[124] Z. Huang, P.G. Ren, T. Ma, R.L. Smith, S.B. Goodman, Modulating osteogenesis of mesenchymal stem cells by modifying growth factor availability, Cytokine. 51 (2010) 305–310. https://doi.org/10.1016/j.cyto.2010.06.002.

[125] K. Tsuji, A. Bandyopadhyay, B.D. Harfe, K. Cox, S. Kakar, L. Gerstenfeld, T. Einhorn, C.J. Tabin, V. Rosen, BMP2 activity, although dispensable for bone formation, is required for the initiation of fracture healing, Nat. Genet. 38 (2006) 1424–1429. https://doi.org/10.1038/ng1916.

[126] A.W. James, G. LaChaud, J. Shen, G. Asatrian, V. Nguyen, X. Zhang, K. Ting, C. Soo, A Review of the Clinical Side Effects of Bone Morphogenetic Protein-2, Tissue Eng. - Part B Rev. 22 (2016) 284–297. https://doi.org/10.1089/ten.teb.2015.0357.

[127] I. El Bialy, W. Jiskoot, M. Reza Nejadnik, Formulation, Delivery and Stability of Bone Morphogenetic Proteins for Effective Bone Regeneration, Pharm. Res. 34 (2017) 1152–1170. https://doi.org/10.1007/s11095-017-2147-x.

[128] C. Li, C. Vepari, H.-J. Jin, H.J. Kim, D.L. Kaplan, Electrospun silk-BMP-2 scaffolds for bone tissue engineering., Biomaterials. 27 (2006) 3115–3124. https://doi.org/10.1016/j.biomaterials.2006.01.022.

[129] C. Wang, M. Wang, Dual-source dual-power electrospinning and characteristics of multifunctional scaffolds for bone tissue engineering., J. Mater. Sci. Mater. Med. 23 (2012) 2381–2397. https://doi.org/10.1007/s10856-012-4669-4.

[130] C. Wang, W.W. Lu, M. Wang, Bicomponent fibrous scaffolds made through dual-source dual-power electrospinning: Dual delivery of rhBMP-2 and Ca-P nanoparticles and enhanced biological performances., J. Biomed. Mater. Res. A. 105 (2017) 2199–2209. https://doi.org/10.1002/jbm.a.36084.

[131] K.T. Shalumon, G.-J. Lai, C.-H. Chen, J.-P. Chen, Modulation of bone-specific tissue regeneration by incorporating bone morphogenetic protein and controlling the shell thickness of silk fibroin/chitosan/nanohydroxyapatite core–shell nanofibrous membranes, ACS Appl. Mater. Interfaces. 7 (2015) 21170–21181. https://doi.org/10.1021/acsami.5b04962.

[132] Y. Su, Q. Su, W. Liu, M. Lim, J.R. Venugopal, X. Mo, S. Ramakrishna, S.S. Al-Deyab, M. El-Newehy, Controlled release of bone morphogenetic protein 2 and dexamethasone loaded in core–shell PLLACL–collagen fibers for use in bone tissue engineering, Acta Biomater. 8 (2012) 763–771. https://doi.org/10.1016/j.actbio.2011.11.002.

[133] R. Li, Y. Ma, Y. Zhang, M. Zhang, D. Sun, Potential of rhBMP-2 and dexamethasone-loaded Zein/PLLA scaffolds for enhanced in vitro osteogenesis of mesenchymal stem cells., Colloids Surf. B. Biointerfaces. 169 (2018) 384–394. https://doi.org/10.1016/j.colsurfb.2018.05.039.

[134] G. Cheng, C. Yin, H. Tu, S. Jiang, Q. Wang, X. Zhou, X. Xing, C. Xie, X. Shi, Y. Du, H. Deng, Z. Li, Controlled Co-delivery of Growth Factors through Layer-by-Layer Assembly of Core–Shell Nanofibers for Improving Bone Regeneration, ACS Nano. 13 (2019) 6372–6382. https://doi.org/10.1021/acsnano.8b06032.

[135] S. Cecchi, S.J. Bennet, M. Arora, Bone morphogenetic protein-7: Review of signalling and efficacy in fracture healing, J. Orthop. Transl. 4 (2016) 28–34. https://doi.org/10.1016/j.jot.2015.08.001.

[136] N. Bock, T.R. Dargaville, G.T.S. Kirby, D.W. Hutmacher, M.A. Woodruff, Growth Factor-Loaded Microparticles for Tissue Engineering: The Discrepancies of In Vitro Characterization Assays, Tissue Eng. Part C. Methods. 22 (2016) 142–154. https://doi.org/10.1089/ten.TEC.2015.0222.

[137] M.K. Boushell, C.Z. Mosher, G.K. Suri, S.B. Doty, E.J. Strauss, E.B. Hunziker, H.H. Lu, Polymeric mesh and insulin-like growth factor 1 delivery enhance cell homing and graft-cartilage integration., Ann. N. Y. Acad. Sci. 1442 (2019) 138–152. https://doi.org/10.1111/nyas.14054.

[138] W. Zhu, N.J. Castro, X. Cheng, M. Keidar, L.G. Zhang, Cold Atmospheric Plasma Modified Electrospun Scaffolds with Embedded Microspheres for Improved Cartilage Regeneration., PLoS One. 10 (2015) e0134729. https://doi.org/10.1371/journal.pone.0134729.

[139] M. Farokhi, F. Mottaghitalab, J. Ai, M.A. Shokrgozar, Sustained release of platelet-derived growth factor and vascular endothelial growth factor from silk/calcium phosphate/PLGA based nanocomposite scaffold., Int. J. Pharm. 454 (2013) 216–225. https://doi.org/10.1016/j.ijpharm.2013.06.080.

[140] M. Farokhi, F. Mottaghitalab, M.A. Shokrgozar, J. Ai, J. Hadjati, M. Azami, Bio-hybrid silk fibroin/calcium phosphate/PLGA nanocomposite scaffold to control the delivery of vascular endothelial growth factor., Mater. Sci. Eng. C. Mater. Biol. Appl. 35 (2014) 401–410. https://doi.org/10.1016/j.msec.2013.11.023.

[141] T. Briggs, J. Matos, G. Collins, T.L. Arinzeh, Evaluating protein incorporation and release in electrospun composite scaffolds for bone tissue engineering applications, J. Biomed. Mater. Res. - Part A. 103 (2015) 3117–3127. https://doi.org/10.1002/jbm.a.35444.

[142] H. Zhang, X. Jia, F. Han, J. Zhao, Y. Zhao, Y. Fan, X. Yuan, Dual-delivery of VEGF and PDGF by double-layered electrospun membranes for blood vessel regeneration., Biomaterials. 34 (2013) 2202–2212. https://doi.org/10.1016/j.biomaterials.2012.12.005.

[143] F. Han, X. Jia, D. Dai, X. Yang, J. Zhao, Y. Zhao, Y. Fan, X. Yuan, Performance of a multilayered small-diameter vascular scaffold dual-loaded with VEGF and PDGF., Biomaterials. 34 (2013) 7302–7313. https://doi.org/10.1016/j.biomaterials.2013.06.006.

[144] A.H. Dorafshar, N. Angle, M. Bryer-Ash, D. Huang, M.M. Farooq, H.A. Gelabert, J.A. Freischlag, Vascular endothelial growth factor inhibits mitogen-induced vascular smooth muscle cell proliferation, in: J. Surg. Res., Academic Press Inc., 2003: pp. 179–186. https://doi.org/10.1016/S0022-4804(03)00254-3.

[145] M. Piran, S. Vakilian, M. Piran, A. Mohammadi-Sangcheshmeh, S. Hosseinzadeh, A. Ardeshirylajimi, In vitro fibroblast migration by sustained release of PDGF-BB loaded in chitosan nanoparticles incorporated in electrospun nanofibers for wound dressing applications., Artif. Cells, Nanomedicine, Biotechnol. 46 (2018) 511–520. https://doi.org/10.1080/21691401.2018.1430698.

[146] R. Augustine, A.A. Zahid, A. Hasan, M. Wang, T.J. Webster, CTGF Loaded Electrospun Dual Porous Core-Shell Membrane For Diabetic Wound Healing., Int. J. Nanomedicine. 14 (2019) 8573–8588. https://doi.org/10.2147/IJN.S224047.

[147] Y. Yang, T. Xia, W. Zhi, L. Wei, J. Weng, C. Zhang, X. Li, Promotion of skin

regeneration in diabetic rats by electrospun core-sheath fibers loaded with basic fibroblast growth factor., Biomaterials. 32 (2011) 4243–4254. https://doi.org/10.1016/j.biomaterials.2011.02.042.

[148] Q. Zhao, M. Wang, Strategies to incorporate polyelectrolyte in emulsion electrospun nanofibrous tissue engineering scaffolds for modulating growth factor release from the scaffolds, Mater. Lett. 162 (2016) 48–52. https://doi.org/10.1016/j.matlet.2015.09.120.

[149] M. Norouzi, I. Shabani, H.H. Ahvaz, M. Soleimani, PLGA/gelatin hybrid nanofibrous scaffolds encapsulating EGF for skin regeneration., J. Biomed. Mater. Res. A. 103 (2015) 2225–2235. https://doi.org/10.1002/jbm.a.35355.

[150] Z. Wang, Y. Qian, L. Li, L. Pan, L.W. Njunge, L. Dong, L. Yang, Evaluation of emulsion electrospun polycaprolactone/hyaluronan/epidermal growth factor nanofibrous scaffolds for wound healing., J. Biomater. Appl. 30 (2016) 686–698. https://doi.org/10.1177/0885328215586907.

[151] O. Mirdailami, M. Soleimani, R. Dinarvand, M.R. Khoshayand, M. Norouzi, A. Hajarizadeh, M. Dodel, F. Atyabi, Controlled release of rhEGF and rhbFGF from electrospun scaffolds for skin regeneration., J. Biomed. Mater. Res. A. 103 (2015) 3374–3385. https://doi.org/10.1002/jbm.a.35479.

[152] H.J. Lai, C.H. Kuan, H.C. Wu, J.C. Tsai, T.M. Chen, D.J. Hsieh, T.W. Wang, Tailored design of electrospun composite nanofibers with staged release of multiple angiogenic growth factors for chronic wound healing, Acta Biomater. 10 (2014) 4156–4166. https://doi.org/10.1016/j.actbio.2014.05.001.

[153] V. Albright, M. Xu, A. Palanisamy, J. Cheng, M. Stack, B. Zhang, A. Jayaraman, S.A. Sukhishvili, H. Wang, Micelle-Coated, Hierarchically Structured Nanofibers with Dual-Release Capability for Accelerated Wound Healing and Infection Control, Adv. Healthc. Mater. 7 (2018). https://doi.org/10.1002/adhm.201800132.

[154] M. Gandhi, R. Srikar, A.L. Yarin, C.M. Megaridis, R.A. Gemeinhart, Mechanistic examination of protein release from polymer nanofibers, Mol. Pharm. 6 (2009) 641–647. https://doi.org/10.1021/mp800160p.

[155] V. Gerriets, A. Kasi, Bevacizumab, (2020). https://www.ncbi.nlm.nih.gov/books/NBK482126/ (accessed June 15, 2020).

[156] U. Angkawinitwong, S. Awwad, P.T. Khaw, S. Brocchini, G.R. Williams, Electrospun formulations of bevacizumab for sustained release in the eye, Acta

Biomater. 64 (2017) 126–136. https://doi.org/10.1016/j.actbio.2017.10.015.

[157] S.O.L. de Souza, M.C.A. Guerra, L.G.D. Heneine, C.R. de Oliveira, A. da S. Cunha Junior, S.L. Fialho, R.L. Oréfice, Biodegradable core-shell electrospun nanofibers containing bevacizumab to treat age-related macular degeneration, J. Mater. Sci. Mater. Med. 29 (2018) 173. https://doi.org/10.1007/s10856-018-6187-5.

[158] J.E. V. Ramirez, L.A. Sharpe, N.A. Peppas, Current state and challenges in developing oral vaccines, Adv. Drug Deliv. Rev. 114 (2017) 116–131. https://doi.org/10.1016/j.addr.2017.04.008.

[159] I.A. Sogias, A.C. Williams, V. V. Khutoryanskiy, Why is chitosan mucoadhesive?, Biomacromolecules. 9 (2008) 1837–1842. https://doi.org/10.1021/bm800276d.

[160] M.G. Lancina, R.K. Shankar, H. Yang, Chitosan nanofibers for transbuccal insulin delivery, J. Biomed. Mater. Res. - Part A. 105 (2017) 1252–1259. https://doi.org/10.1002/jbm.a.35984.

[161] T. Suksamran, T. Ngawhirunpat, T. Rojanarata, W. Sajomsang, T. Pitaksuteepong, P. Opanasopit, Methylated N-(4-N,N-dimethylaminocinnamyl) chitosan-coated electrospray OVA-loaded microparticles for oral vaccination, Int. J. Pharm. 448 (2013) 19–27. https://doi.org/10.1016/j.ijpharm.2013.03.015.

[162] N. Chen, M.M. Johnson, M.A. Collier, M.D. Gallovic, E.M. Bachelder, K.M. Ainslie, Tunable degradation of acetalated dextran microparticles enables controlled vaccine adjuvant and antigen delivery to modulate adaptive immune responses, J. Control. Release. 273 (2018) 147–159. https://doi.org/10.1016/j.jconrel.2018.01.027.

[163] Z. Jakopin, Murabutide Revisited: A Review of its Pleiotropic Biological Effects, Curr. Med. Chem. 20 (2013) 2068–2079. https://doi.org/10.2174/0929867311320160002.

[164] B. Furtmann, J. Tang, S. Kramer, T. Eickner, F. Luderer, G. Fricker, A. Gomez, B. Heemskerk, P.S. Jähn, Electrospray Synthesis of Poly(lactide-co-glycolide) Nanoparticles Encapsulating Peptides to Enhance Proliferation of Antigen-Specific CD8+ T Cells, J. Pharm. Sci. 106 (2017) 3316–3327. https://doi.org/10.1016/j.xphs.2017.06.013.

[165] G. Wilcox, Insulin and Insulin Resistance, Clin. Biochem. Rev. 26 (2005) 19–39. https://doi.org/10.1016/S0025-7125(03)00128-7.

[166] Diabetes, (n.d.). https://www.who.int/news-room/fact-sheets/detail/diabetes (accessed July 15, 2020).

[167] L. Xu, N. Sheybani, S. Ren, G.L. Bowlin, W.A. Yeudall, H. Yang, Semi-interpenetrating network (sIPN) co-electrospun gelatin/insulin fiber formulation for transbuccal insulin delivery, Pharm. Res. 32 (2015) 275–285. https://doi.org/10.1007/s11095-014-1461-9.

[168] K. Stephansen, M. García-Díaz, F. Jessen, I.S. Chronakis, H.M. Nielsen, Bioactive protein-based nanofibers interact with intestinal biological components resulting in transepithelial permeation of a therapeutic protein, Int. J. Pharm. 495 (2015) 58–66. https://doi.org/10.1016/j.ijpharm.2015.08.076.

[169] K. Stephansen, M. García-Díaz, F. Jessen, I.S. Chronakis, H.M. Nielsen, Interactions between Surfactants in Solution and Electrospun Protein Fibers: Effects on Release Behavior and Fiber Properties, Mol. Pharm. 13 (2016) 748–755. https://doi.org/10.1021/acs.molpharmaceut.5b00614.

[170] C.H. Lee, K.C. Hung, M.J. Hsieh, S.H. Chang, J.H. Juang, I.C. Hsieh, M.S. Wen, S.J. Liu, Core-shell insulin-loaded nanofibrous scaffolds for repairing diabetic wounds, Nanomedicine Nanotechnology, Biol. Med. 24 (2020). https://doi.org/10.1016/j.nano.2019.102123.

[171] J.S. Choi, S.H. Han, C. Hyun, H.S. Yoo, Buccal adhesive nanofibers containing human growth hormone for oral mucositis, J. Biomed. Mater. Res. - Part B Appl. Biomater. 104 (2016) 1396–1406. https://doi.org/10.1002/jbm.b.33487.

[172] M.B. Ranke, J.M. Wit, Growth hormone - past, present and future, Nat. Rev. Endocrinol. 14 (2018) 285–300. https://doi.org/10.1038/nrendo.2018.22.

[173] J.J. Díez, S. Sangiao-Alvarellos, F. Cordido, Treatment with growth hormone for adults with growth hormone deficiency syndrome: Benefits and risks, Int. J. Mol. Sci. 19 (2018). https://doi.org/10.3390/ijms19030893.

[174] H.H. Kwak, W.S. Shim, M.K. Choi, M.K. Son, Y.J. Kim, H.C. Yang, T.H. Kim, G.I. Lee, B.M. Kim, S.H. Kang, C.K. Shim, Development of a sustained-release recombinant human growth hormone formulation, J. Control. Release. 137 (2009) 160–165. https://doi.org/10.1016/j.jconrel.2009.03.014.

[175] Y. Gao, Y. Xu, A. Land, J. Harris, G.M. Policastro, E.P. Childers, T. Ritzman, J. Bundy, M.L. Becker, Sustained release of recombinant human growth hormone from bioresorbable poly(ester urea) nanofibers, ACS Macro Lett. 6 (2017) 875–

880. https://doi.org/10.1021/acsmacrolett.7b00334.

[176] M. Shafiq, Q. Zhang, D. Zhi, K. Wang, D. Kong, D.H. Kim, S.H. Kim, In Situ Blood Vessel Regeneration Using SP (Substance P) and SDF (Stromal Cell-Derived Factor)-1α Peptide Eluting Vascular Grafts, Arterioscler. Thromb. Vasc. Biol. 38 (2018) E117–E134. https://doi.org/10.1161/ATVBAHA.118.310934.

[177] M. Xaymardan, M. Cimini, R.D. Weisel, R.K. Li, Bone Marrow Stem Cells. Properties and Pluripotency, in: Princ. Regen. Med., Elsevier Inc., 2008: pp. 268–283. https://doi.org/10.1016/B978-012369410-2.50018-8.

[178] E. Yaniz-Galende, R.J. Hajjar, Stem cell and gene therapy for cardiac regeneration, in: Card. Regen. Repair, Elsevier Ltd., 2014: pp. 347–379. https://doi.org/10.1533/9780857096708.4.347.

[179] H.C. Seegers, V.C. Hood, B.L. Kidd, S.C. Cruwys, D.A. Walsh, Enhancement of angiogenesis by endogenous substance P release and neurokinin-1 receptors during neurogenic inflammation, J. Pharmacol. Exp. Ther. 306 (2003) 8–12. https://doi.org/10.1124/jpet.103.050013.

[180] H.S. Hong, J. Lee, E. Lee, Y.S. Kwon, E. Lee, W. Ahn, M.H. Jiang, J.C. Kim, Y. Son, A new role of substance P as an injury-inducible messenger for mobilization of CD29+ stromal-like cells, Nat. Med. 15 (2009) 425–435. https://doi.org/10.1038/nm.1909.

[181] Y. Jin, H.S. Hong, Y. Son, Substance P enhances mesenchymal stem cells-mediated immune modulation, Cytokine. 71 (2015) 145–153. https://doi.org/10.1016/j.cyto.2014.10.003.

[182] M. Zamani, M.P. Prabhakaran, E.S. Thian, S. Ramakrishna, Controlled delivery of stromal derived factor-1α from poly lactic-co-glycolic acid core-shell particles to recruit mesenchymal stem cells for cardiac regeneration, J. Colloid Interface Sci. 451 (2015) 144–152. https://doi.org/10.1016/j.jcis.2015.04.005.

[183] F.F.R. Damanik, G. Spadolini, J. Rotmans, S. Farè, L. Moroni, Biological activity of human mesenchymal stromal cells on polymeric electrospun scaffolds, Biomater. Sci. 7 (2019) 1088–1100. https://doi.org/10.1039/c8bm00693h.

[184] B.S. Pattni, V.P. Torchilin, Targeted Drug Delivery Systems: Strategies and Challenges, in: Springer, Cham, 2015: pp. 3–38. https://doi.org/10.1007/978-3-319-11355-5_1.

[185] Z.P. Aguilar, Chapter 5 - Targeted Drug Delivery, in: Z.P.B.T.-N. for M.A. Aguilar

(Ed.), Elsevier, 2013: pp. 181–234. https://doi.org/https://doi.org/10.1016/B978-0-12-385089-8.00005-4.

[186] A. Bhardwaj, L. Kumar, S. Mehta, A. Mehta, Stimuli-sensitive Systems - an emerging delivery system for drugs, Artif. Cells, Nanomedicine, Biotechnol. 43 (2015) 299–310. https://doi.org/10.3109/21691401.2013.856016.

[187] A. Sukarto, B.G. Amsden, Low melting point amphiphilic microspheres for delivery of bone morphogenetic protein-6 and transforming growth factor-β3 in a hydrogel matrix, J. Control. Release. 158 (2012) 53–62. https://doi.org/10.1016/j.jconrel.2011.10.015.

[188] A.K. Philip, B. Philip, Colon targeted drug delivery systems: A review on primary and novel approaches, Oman Med. J. 25 (2010) 70–78. https://doi.org/10.5001/omj.2010.24.

[189] L. Liu, W. Yao, Y. Rao, X. Lu, J. Gao, pH-Responsive carriers for oral drug delivery: challenges and opportunities of current platforms, Drug Deliv. 24 (2017) 569–581. https://doi.org/10.1080/10717544.2017.1279238.

[190] S. Thakral, N.K. Thakral, D.K. Majumdar, Eudragit®: A technology evaluation, Expert Opin. Drug Deliv. 10 (2013) 131–149. https://doi.org/10.1517/17425247.2013.736962.

[191] W.C. Yan, X.J. Ong, K.T. Pun, D.Y. Tan, V.K. Sharma, Y.W. Tong, C.H. Wang, Preparation of tPA-loaded microbubbles as potential theranostic agents: A novel one-step method via coaxial electrohydrodynamic atomization technique, Chem. Eng. J. 307 (2017) 168–180. https://doi.org/10.1016/j.cej.2016.08.081.

[192] W.C. Yan, Q.W. Chua, X.J. Ong, V.K. Sharma, Y.W. Tong, C.H. Wang, Fabrication of ultrasound-responsive microbubbles via coaxial electrohydrodynamic atomization for triggered release of tPA, J. Colloid Interface Sci. 501 (2017) 282–293. https://doi.org/10.1016/j.jcis.2017.04.073.

[193] N. Grasmeijer, V. Tiraboschi, H.J. Woerdenbag, H.W. Frijlink, W.L.J. Hinrichs, Identifying critical process steps to protein stability during spray drying using a vibrating mesh or a two-fluid nozzle, Eur. J. Pharm. Sci. 128 (2019) 152–157. https://doi.org/10.1016/j.ejps.2018.11.027.

[194] D. Shi, Introduction to Biomaterials, Tsinghua University Press, 2006.

[195] S. Luthra, J.P. Obert, D.S. Kalonia, M.J. Pikal, Investigation of drying stresses on proteins during lyophilization: Differentiation between primary and secondary-

drying stresses on lactate dehydrogenase using a humidity controlled mini freeze-dryer, J. Pharm. Sci. 96 (2007) 61–70. https://doi.org/10.1002/jps.20758.

[196] M. Van De Weert, J. Hoechstetter, W.E. Hennink, D.J.A. Crommelin, The effect of a water/organic solvent interface on the structural stability of lysozyme, J. Control. Release. 68 (2000) 351–359. https://doi.org/10.1016/S0168-3659(00)00277-7.

[197] J. Xue, T. Wu, Y. Dai, Y. Xia, Electrospinning and electrospun nanofibers: Methods, materials, and applications, Chem. Rev. 119 (2019) 5298–5415. https://doi.org/10.1021/acs.chemrev.8b00593.

[198] P. Vass, E. Szabó, A. Domokos, E. Hirsch, D. Galata, B. Farkas, B. Démuth, S.K. Andersen, T. Vigh, G. Verreck, G. Marosi, Z.K. Nagy, Scale-up of electrospinning technology: Applications in the pharmaceutical industry, Wiley Interdiscip. Rev. Nanomedicine Nanobiotechnology. (2019). https://doi.org/10.1002/wnan.1611.

[199] H. Fu, R.A. Hoerr, P.J. Ryan, A High-throughput Electrospray Nozzle for Nanoparticle Production, TechConnect Briefs. 1 (2014) 101–104. https://briefs.techconnect.org/papers/a-high-throughput-electrospray-nozzle-for-nanoparticle-production/ (accessed June 17, 2020).

[200] M. Parhizkar, P.J.T. Reardon, J.C. Knowles, R.J. Browning, E. Stride, R.B. Pedley, T. Grego, M. Edirisinghe, Performance of novel high throughput multi electrospray systems for forming of polymeric micro/nanoparticles, Mater. Des. 126 (2017) 73–84. https://doi.org/10.1016/j.matdes.2017.04.029.

[201] C. Zhang, M.W. Chang, Z. Ahmad, W. Hu, D. Zhao, J.S. Li, Stable single device multi-pore electrospraying of polymeric microparticles via controlled electrostatic interactions, RSC Adv. 5 (2015) 87919–87923. https://doi.org/10.1039/c5ra18482g.

[202] C. Prieto, J.M. Lagaron, Nanodroplets of docosahexaenoic acid-enriched algae oil encapsulated within microparticles of hydrocolloids by emulsion electrospraying assisted by pressurized gas, Nanomaterials. 10 (2020). https://doi.org/10.3390/nano10020270.

[203] M.A. Busolo, S. Torres-Giner, C. Prieto, J.M. Lagaron, Electrospraying assisted by pressurized gas as an innovative high-throughput process for the microencapsulation and stabilization of docosahexaenoic acid-enriched fish oil in zein prolamine, Innov. Food Sci. Emerg. Technol. 51 (2019) 12–19. https://doi.org/10.1016/j.ifset.2018.04.007.

[204] P.J. García-Moreno, A. Pelayo, S. Yu, M. Busolo, J.M. Lagaron, I.S. Chronakis, C. Jacobsen, Physicochemical characterization and oxidative stability of fish oil-loaded electrosprayed capsules: Combined use of whey protein and carbohydrates as wall materials, J. Food Eng. 231 (2018) 42–53. https://doi.org/10.1016/j.jfoodeng.2018.03.005.

[205] P. Vass, Z.K. Nagy, R. Kóczián, C. Fehér, B. Démuth, E. Szabó, S.K. Andersen, T. Vigh, G. Verreck, I. Csontos, G. Marosi, E. Hirsch, Continuous drying of a protein-type drug using scaled-up fiber formation with HP-β-CD matrix resulting in a directly compressible powder for tableting, Eur. J. Pharm. Sci. 141 (2020). https://doi.org/10.1016/j.ejps.2019.105089.

[206] P. Vass, E. Hirsch, R. Kóczián, B. Démuth, A. Farkas, C. Fehér, E. Szabó, Á. Németh, S.K. Andersen, T. Vigh, G. Verreck, I. Csontos, G. Marosi, Z.K. Nagy, Scaled-Up production and tableting of grindable electrospun fibers containing a protein-type drug, Pharmaceutics. 11 (2019). https://doi.org/10.3390/pharmaceutics11070329.

[207] I. Wagner, Z.K. Nagy, P. Vass, C. Fehér, Z. Barta, T. Vigh, P.L. Sóti, A.H. Harasztos, H. Pataki, A. Balogh, G. Verreck, I. Van Assche, G. Marosi, Stable formulation of protein-type drug in electrospun polymeric fiber followed by tableting and scaling-up experiments, Polym. Adv. Technol. 26 (2015) 1461–1467. https://doi.org/10.1002/pat.3569.

[208] U. Angkawinitwong, G. Sharma, P.T. Khaw, S. Brocchini, G.R. Williams, Solid-state protein formulations, Ther. Deliv. 6 (2015) 59–82. https://doi.org/10.4155/tde.14.98.