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Hydrophobic ion pairing as a novel approach to co-axial electrospraying of peptide-PLGA particles

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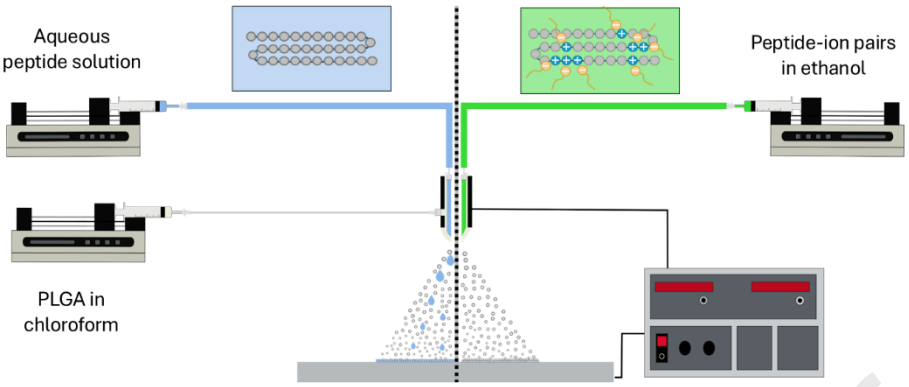
Abstract

Electrospraying is a processing technique that has gained much interest to prepare polymeric particles. The technique operates at ambient temperature, thereby avoiding heat induced degradation of labile therapeutics (e.g. peptides and proteins). Exposure to organic solvents can be minimised by co-axial electrospraying through separation of core (aqueous) and shell (organic) solvents. However, aqueous solutions are often difficult to electrospray due to high surface tension. Immiscibility between the core-shell solvents creates a further process challenge. Herein, we describe for the first time the use of hydrophobic ion pairing (HIP) to encapsulate a polypeptide into polymeric particles prepared by co-axial electrospraying. Peptide ion pairs were prepared to incorporate a model peptide – teriparatide – into an organic solvent, permitting facile electrospraying while also protecting the peptide from denaturation. Teriparatide loaded PLGA particles were generated by electrospraying from aqueous or ethanolic peptide solutions (core). A PLGA solution in chloroform (with and without co-solvents) was employed as the shell solution. The aqueous core solution led to a teriparatide encapsulation efficiency of 79.2 ± 19.8%, which was not significantly different from the ethanolic core (57.1 ± 14.5%). When aqueous solutions were used the process lacked reproducibility, resulting in low process yields (61.3 ± 4.0%). In contrast, when an organic core was used a dry powder bed was achieved with a yield of 102.2 ± 8.8%. The peptide's integrity and biological functionality were retained after electrospraying as ion pairs, as evidenced in a cell-based PTH1R receptor binding assay.

Keywords: teriparatide, peptide, hydrophobic-ion-pairing, electrospraying, PLGA, controlled release, PTH1R



1. Introduction

Advances in the fields of biotechnology, genetic engineering, and analytic technologies have greatly contributed to the increasing importance of therapeutic polypeptides such as peptides and proteins in modern medicine [1–3]. However, several challenges must be addressed to harness the potential of polypeptides. Their physicochemical properties (e.g. hydrophilicity, molecular weight, charge) often limit permeability across biological barriers [4,5]. Polypeptides are sensitive to pH and temperature, and are easily hydrolysed by digestive enzymes, resulting in the loss of biological activity [3]. Most peptide and protein therapeutics require injections [1,5], often reducing patient compliance [6] and require a clinical visit which increases costs [1]. The challenge is magnified because polypeptides generally display short half-lives and thus requiring frequent dosing [4].

The development of prolonged release formulations addresses some of these drawbacks [3] by maintaining drug concentrations within the therapeutic window for days or months [1,7]. Clinically proven strategies are based on biodegradable nano- and micron-sized materials (e.g. polymeric particles and hydrogels), with polymeric microspheres being the most commonly used [8,9]. Polypeptides encapsulated within a microsphere can be gradually released through diffusion and degradation of the polymer matrix [9] and tailored release rates achieved by controlling particle morphology, size, and the polymer matrix [10,11].

Several techniques have been used to fabricate polymeric nano- and microparticles, the most popular being based on emulsion techniques [12]. Hydrophilic compounds are often formulated as a double emulsion – generally a water-oil-water (w/o/w) emulsion. The preparation of polymeric particles is achieved through inclusion of a polymer within the organic phase of the double emulsion and subsequent evaporation of the solvent [13]. This approach has been successfully employed in the preparation of several peptide containing microspheres for controlled and prolonged release, with notable examples being Lupron Depot® and Bydureon BCise® [14]. These peptide-loaded microparticles are clinically approved and offer release profiles of up to 6 months, reducing dosing frequency and thereby significantly improving patient adherence and treatment efficacy [15].

Polypeptide encapsulation efficiency still remains a challenge however [16]. Indeed, double emulsions often lead to dispersed particles and may give low encapsulation efficiencies due to drug diffusion into the continuous phase [2]. The use of organic solvents, high shearing forces and exposure to large interfaces during the emulsification process may result in polypeptide denaturation and aggregate formation [2]. Furthermore, while particle size distribution is generally reproducible, the particles are typically polydisperse [9]. Finally, as hydrophilic molecules, polypeptides tend to display burst release profiles. Often 50% or more of the drug will be released over the initial 24h period leading to challenges with toxicity and maintenance of the required duration of drug release [4,12].

Electrohydrodynamic (EHD) processes have been examined for the formulation of small molecules, polypeptides, DNA and even cells [17,18]. An EHD formulation device is comprised of four principal components: a syringe pump, a high voltage supply, a metallic needle (spinneret) and a collector [19]. An electric field is created

between the spinneret (usually positively charged) and the collector (grounded or negatively charged). This creates repulsive forces at the droplet surface of a solution extruded through the spinneret, and hence the droplet assumes a cone shape (the Taylor cone) [19]. Under the influence of the electric field, the charged liquid is drawn and solidifies at ambient temperature. Distinction is made between electrospinning and electrospraying, where solution properties (e.g. molecular weight and concentration) determine polymer entanglement and thus whether fibres (spinning) or particles (spraying) are produced [18,19]. Particles generated by EHD are nanometre to micrometre in size and present advantages related to drug loading (up to 60%) and encapsulation efficiencies (up to 100%) [11]. Their size, morphology, composition, and release behaviour are highly tuneable by adapting processing parameters and material choice [18,20]. Co-axial electrospraying further adds benefits specifically for biotherapeutics such as polypeptides. The co-axial spinneret, consisting of two concentric but separated needles, provides the possibility of processing two solutions separately. An aqueous peptide solution (core) may be separated from organic solvents (polymeric shell), thereby reducing exposure to organic solvents and the risk of denaturation [18]. The resulting particles have a core-shell structure with the therapeutic localised within the core, thus often reducing the burst release [11,21]. Angkawinitwong *et al.* demonstrated that electrospun core-shell fibres containing bevacizumab presented a zero-order release profile when the protein was processed at its isoelectric point, where it carries a net neutral charge [22].

Nevertheless, electrospraying does present challenges particularly due to the interplay between a large number of processing and formulation factors making optimisation complex [23]. In a co-axial EHD process, additional optimisation of the liquid-liquid interface will be necessary and comparable evaporation rates between core and shell solvents are desirable [11,19]. Finally, while water is often an optimal solvent for pharmaceutical formulations generally and for polypeptides specifically, electrospraying of water is difficult due to its high surface tension, even more so if aqueous buffers are employed (increased electrical conductivity), resulting in an unstable process and a wet powder bed [19].

Here, different strategies to improve co-axial electrospraying of a model peptide (teriparatide) into poly(lactic-co-glycolic acid) (PLGA) microparticles for extended release were explored. Electrospraying was first conducted with an aqueous core solution containing the peptide and a shell solution composed of PLGA in chloroform. Two strategies (co-solvents and hydrophobic ion pairing) were compared with the aim to achieve a stable and reproducible electrospraying process, while maximising peptide encapsulation and maintaining functionality. The release and biological functionality of teriparatide from particles produced in the optimised EHD process set-up were investigated *in-vitro*.

2. Materials and Methods

2.1. Materials

Teriparatide was supplied by Insight Biotechnology (Wembley, UK). Dipalmitoylphosphatidylcholine (DPPC) was provided by Lipoid (Ludwigshafen, Germany). Eudragit EPO was obtained from Evonik (Essen, Germany). Acetic acid, acetonitrile (HPLC grade), trifluoroacetic acid and water (HPLC grade) were obtained

from Fisher (Loughborough, UK). 3-isobutyl-1-methylxanthine, 4-(3-butoxy-4-methoxybenzyl) imidazolidin-2-one, fetal bovine serum, and PLGA (ester terminated, M_w 50,000 – 75,000, lactide:glycolide 85:15) were obtained from Merck Life Sciences (Darmstadt, Germany). Chloroform, ethanol, phosphate buffered saline, sodium dodecyl sulfate, and Trizma were purchased from Sigma Aldrich (Steinheim, Germany). PTH1R encoding plasmid (OHu15045D) was supplied by GenScript (Oxford, UK). Hydrochloric acid was obtained from Honeywell (Seelze, Germany). A cAMP kit was provided by Promega (Madison, WI, USA). Dulbecco's modified essential media, trypsin, and geneticin (G418) were obtained from ThermoFisher (Rockford, IL, USA).

2.2. Electrospraying of PLGA particles

Initial co-axial electrospraying conditions were explored in a design of experiment (DoE) approach based on previously established electrospraying conditions for a monoaxial set-up [24]. A full factorial design was created with Minitab 19 (Minitab®, LLC, State College, PA, USA). The parameters to optimise were the tip-to-collector distance (cm), the flow rate of the shell solution (mL/h) and the flow rate ratio between the core and the shell. For each parameter the levels were predefined and are shown in Table 1.

Table 1: Parameters explored as part of the DoE approach to establish electrospraying conditions for PLGA core-shell particles

Parameter	**Levels**		
Distance [cm]	16	18	20
Flow rate (shell) [mL/h]	0.4	0.6	0.8
Flow rate ratio (core:shell)	1:5	1:10	

The shell solution consisted 62 mg/mL PLGA in chloroform whereas the core solution was 20 mM Tris Buffer (pH 8.3), corresponding to the isoelectric point of teriparatide (pH = 8.3). The core and shell solutions were connected to a co-axial spinneret (Linari Nanotech, Pisa, Italy). The spinneret was comprised of a 15 G external needle (outer diameter: 1.8 mm, internal diameter: 1.35mm) and a 21 G internal needle (outer diameter: 0.8 mm, internal diameter: 0.5 mm). A high voltage DC power supply (HCP 14-20000, FuG Electronik, Schechen, Germany) was attached to the spinneret and the voltage adjusted (14.7 – 20.0 kV) to obtain the most stable electrospraying process. The ground electrode was connected to a Teflon coated collector plate (16 x

20 cm). All experiments were conducted at ambient conditions of 20 – 22 °C and 40 – 50 % RH. The electrospraying process was evaluated for stability (absence of dripping), dryness of powder bed, and particle morphology.

2.3. Teriparatide loaded PLGA particles

2.3.1. Immiscible core-shell solvent system

The optimised electrospraying conditions obtained from the DoE experiment were employed to produce core-shell PLGA particles loaded with teriparatide. The core solution was 1 mg/mL teriparatide in 20 mM Tris buffer (pH 8.3). The shell solution remained 62 mg/mL PLGA in chloroform. The flow rates were set to 0.6 mL/h (shell) and 0.12 mL/h (core) and particles were collected at a tip-to-collector distance of 16 cm. The applied voltage was adjusted to obtain the most stable electrospraying process (14.5 – 19 kV). Experiments were conducted at ambient conditions of 23 – 25 °C and 30 – 35 % RH.

2.3.2. Co-solvents for stabilisation of co-axial electrospraying of PLGA particles

The incorporation of co-solvents (acetonitrile and acetic acid) within the shell solution was investigated. Initially, the strength of the co-solvent was determined. A bi-phasic system of 20 mM Tris-HCl Buffer (pH 8.3) - chloroform at the ratio employed during electrospraying (1:5 *v/v*) was prepared. The volume of each co-solvent required to obtain a single phase was determined through visual inspection. Based on the results, three distinct solutions at different co-solvent – chloroform ratios were prepared containing 62 mg/mL PLGA (see Table 2). These were used as the shell solution for the co-axial electrospraying process and particles were produced with the conditions described in Table 3. The applied voltage was set between 16 – 18 kV. Experiments were conducted at ambient conditions of 21 – 25 °C and 23 – 50 % RH.

Table 2: Co-solvent blends used for PLGA shell solutions in the co-axial electrospraying process

Co-solvent	**Co-solvent : Chloroform ratio (*v/v*)**		
Acetic acid	1:7	1:3	1:2
Acetonitrile	1:3	1:2	2:1

2.3.3. Hydrophobic ion-pairing of teriparatide

A hydrophobic complex between teriparatide and sodium dodecyl sulfate (SDS) was prepared as described previously [25]. Briefly, teriparatide was dissolved in 1 mM HCl at a concentration of 1.7 mg/mL, to which an equal volume of SDS solution (1.008 mg/mL) was added dropwise. A white precipitate formed which was collected by

centrifugation at 10,000 rpm for 15 min. The supernatant was removed, and the white pellet was redispersed in ethanol (to a theoretical concentration of 1.7 mg/mL). This dispersion of hydrophobic ion pairs (HIPs) was employed as the core solution during the electrospraying process. A 62 mg/mL PLGA solution in chloroform was used as the shell solution. Teriparatide loaded PLGA particles were prepared by electrospraying at a total flow rate of 0.8 mL (core: 0.2 mL/h, shell: 0.6 mL/h), a distance of 16 cm and an applied voltage of 16-18 kV. Experiments were conducted at ambient conditions of 20 – 23 °C and 35 – 55 % RH. An overview of the different electrospraying solutions and conditions used is presented in Table 3.

Table 3: Overview of the different systems employed during optimisation of the teriparatide core-shell electrospraying process

<table>
<tr><th>System</th><th>Core solution</th><th>Shell solution</th><th>Flow rate (core) [mL/h]</th><th>Flow rate (shell) [mL/h]</th><th>Distance [cm]</th></tr>
<tr><td>Immiscible solvent system</td><td rowspan="4">1 mg/mL Teriparatide in 20 mM Tris-HCl Buffer (pH = 8.3)</td><td>62 mg/mL PLGA in chloroform</td><td rowspan="5">0.6</td><td rowspan="4">0.12</td><td rowspan="5">16</td></tr>
<tr><td>Acetic acid (Co-solvent)</td><td>62 mg/mL PLGA in acetic-acid chloroform solutions</td></tr>
<tr><td>Acetonitrile (Co-Solvent)</td><td>62 mg/mL PLGA in acetonitrile chloroform solutions</td></tr>
<tr><td>HIP</td><td>1.7 mg/mL HIPs in ethanol</td><td>62 mg/mL PLGA in chloroform</td><td>0.2</td></tr>
</table>

2.4. Comparison of core compositions for HIP electrospraying

Hydrophobic ion pairs of teriparatide were prepared as described under 2.3.3 with the exception of varying the solvent used for resuspension of the HIP pellet. Three distinct dispersions were prepared containing HIPs in ethanol, HIPs with 10 mg/mL DPPC in ethanol, or HIPs with 37.2 mg/mL Eudragit in 0.1M HCl in ethanol. All other electrospraying parameters were kept as described in Section 2.3.3.

2.5. Characterisation of PLGA particles

2.5.1. Liquid-liquid extraction of teriparatide

A liquid-liquid extraction was employed to separate teriparatide form the PLGA polymer prior to quantification. Approximately 5-10 mg of blank PLGA particles were precisely weighed into a microcentrifuge tube and liquid-liquid extraction was initiated by adding chloroform (150 µL) followed by an equal volume of 0.1% trifluoroacetic acid (TFA) in water. The samples were vigorously shaken for approximately 5 min to ensure full dissolution of the particles in the organic phase and extraction of the peptide. Next, the samples were centrifuged briefly to separate the two layers. 100 µL of the aqueous layer (top layer) was withdrawn and an equal volume of 0.1% TFA in acetonitrile (ACN) added prior to quantification by HPLC. A teriparatide calibration curve was prepared using the same extraction process.

2.5.2. Determination of encapsulation efficiency

Teriparatide concentration was measured with a 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) coupled with an UV-detector. A Supelco Biowide C18 column (4.6 mm × 150 mm × 5 µm) was employed to determine peptide concentrations from a 20 µL injection. The mobile phase consisted of 0.1% TFA in water (mobile phase A) and 0.1% TFA in ACN (mobile phase B). Gradient elution started with 20% B at a flow rate of 1.0 mL/min and was held for 2 min prior to increasing to 60% over the course of 4 min. %B was then increased to 100% over 0.2 min where it was held for 1 min prior to decreasing back to 20% B. The column temperature was set at 25 °C, with UV detection at 210 nm.

For PLGA particles electrosprayed from aqueous teriparatide solutions, liquid-liquid extraction was employed to separate the peptide from the polymer prior to quantification. All other PLGA particles were dissolved in DMSO at a concentration of 4 mg/mL and teriparatide quantified directly.

2.5.3. Morphology and size distribution

For imaging, samples were directly sprayed onto aluminium foil. A small square (~ 1 × 1 cm) was cut from the foil and mounted onto an aluminium stub (TAAB Laboratories, Aldermaston, UK) with carbon-coated double side adhesive tape, prior to sputter coating with gold for 60 s (10 nm gold layer) using a Q150RS sputter coater (Quorum Technologies, Laughton, UK). The coated samples were then analysed using a Phenom Benchtop scanning electron microscope (SEM; ThermoFisher, Eindhoven, Netherlands) with applied voltage of 15 kV. The size of the particles was determined

using the Image J software (National Institutes of Health, Bethesda, MD, USA). For each formulation the size of 100 particles from three different frames each was determined. The size distributions were plotted using Prism (GraphPad Software, San Diego, CA, USA).

2.6. Evaluation of in vitro drug release

Electrosprayed core-shell PGLA particles loaded with peptide ion pairs were dispersed in phosphate buffered saline (PBS, pH = 7.4) at a concentration of 20 mg/mL (0.75 – 1.00 mL). The samples were incubated at 34 °C. At pre-defined time points, samples were shaken for approximately 1 min to obtain a homogenous dispersion. The dispersion was centrifuged at 10,000 rpm for 5 min, following which 50 µL of the supernatant was withdrawn. The samples were then replenished with 50 µL of fresh PBS. Teriparatide content of the individual samples was measured as described in 2.5.2 and cumulative peptide release for each time point was determined using Equation 1:

Equation 1: Determination of teriparatide cumulative release for PLGA particles

$$Cummulative\ release\ (\%) = \frac{C_n \times V + \sum_{i=1}^{n-1} C_i \times V_i}{m_{peptide}} \times 100$$

Where C_n represents the concentration at the time point *n*, V the total volume in the release media. C_i and V_i represent the concentration and the sampled volume at previous time points. The amount of peptide $m_{peptide}$ was based on encapsulation efficiency.

2.7. Functionality assessment of released teriparatide

2.7.1. HEK293 cell culture

A HEK293 cell line overexpressing PTH1R was previously created by transfecting HEK293 cells with a pcDNA3.1+/C-(K)DYK plasmid vector including the PTH1R gene (GenScript, Oxford, United Kingdom) and overexpressed cells (HEK293-PTH1R) were selected with geneticin [25]. HEK 293-PTH1R cells were grown in DMEM containing 10% *v/v* fetal bovine serum and 600 µg/mL geneticin ("complete medium"). All cells were cultured in humified incubators at 37 °C/5% CO_2 and were passaged at confluency.

2.7.2. Functional assessment of release teriparatide

The functionality of the formulated teriparatide was determined using HEK293-PTH1R cell line, by measuring intracellular cAMP levels after cellular binding of native and processed teriparatide, as described previously [25]. Briefly, release of the formulated peptide was obtained by incubating the PLGA particles in PBS at a concentration of 40 mg/mL overnight (0.35 – 0.55 mL). After incubation, the samples were centrifuged at 10,000 rpm and supernatant (~ 0.20 mL) was withdrawn. Teriparatide concentration within the supernatant was determined by HPLC. A teriparatide stock was prepared in induction buffer. A total of five samples at concentrations in the range of 0.2 pM - 1.8

nM were prepared in induction buffer for all released teriparatide samples, as well as the native peptide.

HEK293-PTH1R cells in complete medium were plated in white clear bottom 96-well plates at a density of 10,000 cells/well and incubated at 37 °C/5% CO_2 overnight. At 70-80% confluency, the media was removed and 20 µL of all teriparatide samples were added to separate wells and incubated at 37 °C/5% CO_2 for 30 minutes, prior to measuring cAMP levels with the Promega cAMP Glo Assay according to the manufacturer's instructions. A cAMP calibration curve between 1.9 nM – 62.5 µM was prepared separately.

2.8. Statistical analysis

Unless otherwise specified, experiments were conducted in triplicates (n=3) and results are presented as mean ± standard deviation. Statistical analysis was performed using ANOVA for comparing more than two variables, or Students t-test for comparing two variables. When ANOVA showed significant differences, a Tukey post-test was employed. Statistical significance was set at $p < 0.05$.

3. Results and discussion

3.1. Electrospraying of core-shell PLGA particles

A DoE approach was employed to identify parameters impacting the electrospraying process. The parameters of interest were the flow rate of the two solutions, the flow rate ratio, and the tip-collector distance. The explored range of these parameters were based on the processing conditions used to electrospray PLGA in a monoaxial set-up [24]. A flow rate of 0.6 mL/h resulted in the most spherical and distinct particles. While particles produced at a 1:10 flow rate ratio appeared more spherical compared to the 1:5 ratio (Supplementary Information, Figure S1.1), a 1:5 ratio theoretically results in particles containing a 5-fold higher teriparatide loading (for the same core solution). For this reason, further experiments were conducted at a flow rate ratio of 1:5. Under these optimised conditions, particles were obtained for all tip-to-collector distances (16 – 22 cm, see Figure S1.2). For greater tip-to-collector distance, a higher voltage is required to generate the same electrical field. Therefore, the tip-to-collector distance was set to 16 cm. Figure 1 shows a representative micrograph of the particles electrosprayed under optimised conditions. The particles displayed a size of 3.5 ± 0.5 µm.

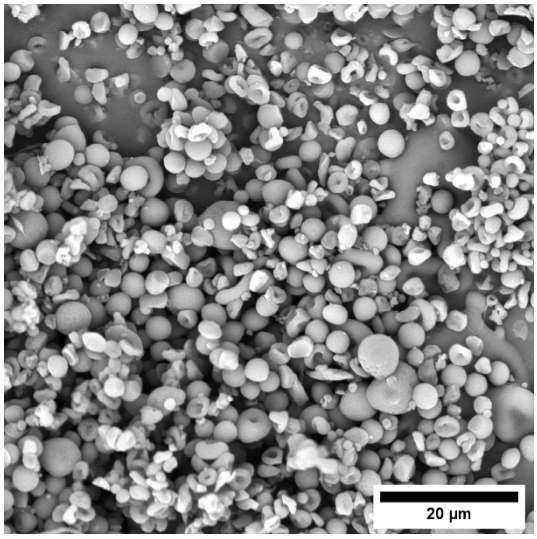


Figure 1: SEM micrograph of the particles prepared under optimised electrospraying conditions. Electrospraying was performed with a shell flow rate of 0.6 mL/h at a 1:5 flow rate ratio between core and shell respectively and a tip-to-collector distance of 16 cm. The scale bar represents 20 µm.

3.2. Teriparatide loading of core-shell PLGA particles

Various approaches were explored to produce teriparatide loaded PLGA particles by electrospraying. Initial attempts to employ an aqueous core comprising the peptide at its isoelectric point and a PLGA in chloroform shell solution did not result in a stable process (Supplementary Information, Section 2). The produced solid presented low and variable teriparatide entrapment efficiencies (28.3 ± 10.4%). The process instability was thought to be due to the high interfacial tension between the core and shell solvents. The introduction of co-solvents (acetonitrile and acetic acid) to the shell solution improved the electrospraying process (Supplementary Information, Section 3). However, improvements in teriparatide loading were only observed when acetic acid was the co-solvent (see Table 4). Further, the impact was strongly dependent on the ratio at which acetic acid was employed. An acetic acid:chloroform system at 1:3 (*v/v*) ratio as the shell solvent produced monodispersed particles with an encapsulation efficiency of 79.1 ± 19.8 %. Nevertheless, none of the solutions led to a stable electrospray process, evidenced by occasional dripping resulting in a wet powder bed (see Figure 2a). The lack of stability and day-to-day reproducibility translates to large variation in the yield (between 50 - 86%).

Table 4: Overview of the characteristics of the electrosprayed particles obtained from co-axial electrospraying of different shell solutions using acetic acid and acetonitrile as co-solvents to chloroform. The tested ratios of co-solvent:chloroform (v/v) were (a) 1:7, (b) 1:3, (c) 1:2, (d) 1:3, (e) 1:2, and (f) 2:1.

Co-solvent	Co-solvent : Chloroform ratio	Encapsulation efficiency [%]	Particle size [µm]

	1:7	0.8 ± 1.4	1.4 ± 0.6
Acetic acid	1:3	79.1 ± 19.8	2.9 ± 0.8
	1:2	12.3 ± 10.7	1.4 ± 0.5
	1:3	5.5 ± 9.5	2.2 ± 0.8
Acetonitrile	1:2	5.7 ± 5.5	1.6 ± 0.6
	2:1	7.0 ± 9.8	1.9 ± 0.7

3.3. Hydrophobic ion pairing

Hydrophobic ion pairing can enhance the hydrophobicity of polypeptides through electrostatic interactions of the charged biomolecules with an amphiphilic molecule of opposite charge (e.g. ionic surfactants, fatty acids, phospholipids) [26]. This is a known approach to enhance solubility of polypeptides in organic solvents, thereby improving incorporation of these biomolecules within a variety of drug delivery systems (e.g. polymeric particles, lipid nanoparticles, self-emulsifying drug delivery systems) [12,26–29]. Furthermore, hydrophobic ion pairing confers polypeptides with a greater conformational stability in organic solvents compared to the native molecule. For example, it has been demonstrated that insulin-SDS pairs retain the native structure of insulin, which is likely monomeric, when dissolved in octanol [30]. Formulation processes that were previously limited due to poor solubility and stability in organic solvents have been enabled peptide ion pairing [12,28,29].

The complexation of teriparatide with sodium dodecyl sulfate (SDS) resulted in a hydrophobic complex that may be solubilised in ethanol, and extended stability in this solvent was previously demonstrated [25]. Therefore, encapsulation of teriparatide as an ion-pair within PLGA was examined. The ethanolic solution containing the peptide-surfactant complex constitutes the core solution during the electrospraying process, which is thought to improve the electrospraying process in two ways. Firstly, it avoids the use of aqueous buffers, which often result in an unstable electrospraying process due to high surface tension of aqueous solutions [19]. Additionally, the core and shell solvents are miscible, thereby avoiding interfacial tension [31]. To the best of the authors' knowledge this is the first report of the use of hydrophobic ion pairing to encapsulate a polypeptide into polymeric particles prepared by co-axial electrospraying.

The replacement of the aqueous core solution by an ethanolic solution removed the requirement for co-solvents. Further, it allowed for a slight increase in the flow rate from 0.12 mL to 0.2 mL of the core solution, therefore increasing peptide loading. A

highly stable electrospraying process was obtained, achieving a dry powder bed and as a result yields of ~100% (see Figure 2b).

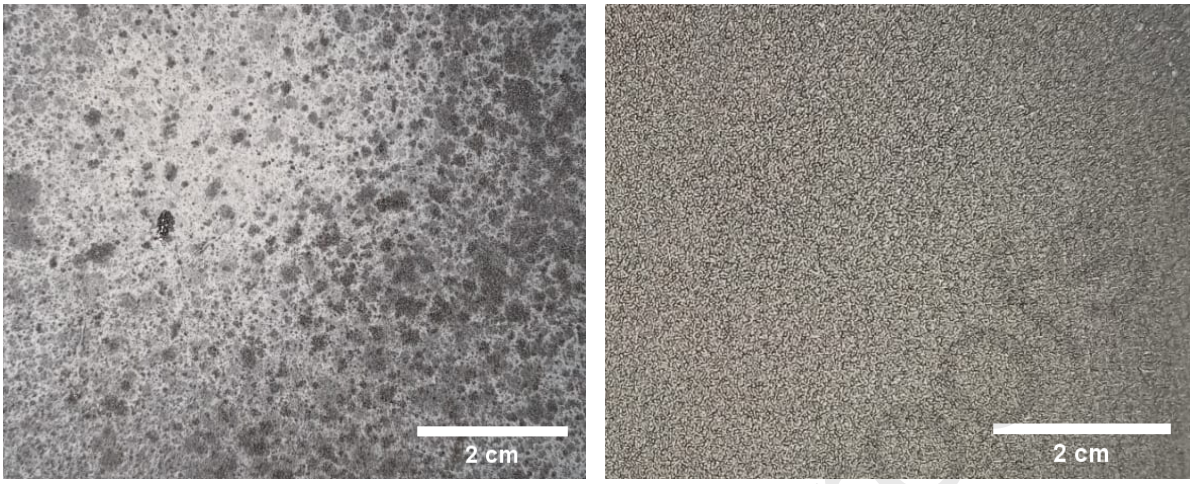
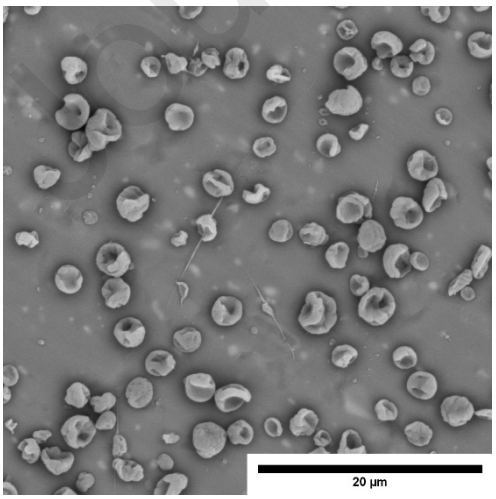


Figure 2: The powder bed observed after electrospraying of teriparatide loaded PLGA core-shell particles using (a) PLGA in an acetic acid:chloroform solvent system (1:3 v/v) as the shell with an aqueous teriparatide core solution and (b) hydrophobic peptide-ion pairs in ethanol as the core solution and PLGA in chloroform in the shell solution. The scale bar represents 2 cm.

The HIP-loaded particles presented an encapsulation efficiency of 51.2 ± 11.7 %, which is comparable to what has previously been achieved by Bussano *et al*. These authors used monoaxial electrospraying set-up to load an insulin-SDS complex into lipid-based microspheres and achieved encapsulation efficiencies of 65% for all of their formulations [32]. Encapsulation efficiencies may be further improved through composition, as observed where the incorporation of 20% palmitic acid increased encapsulation efficiency to > 90% [32].

A representative image of the electrosprayed core-shell PLGA particles loaded with the hydrophobic peptide-surfactant complex is shown in Figure 3a. All particles were in the micrometre range presenting a mean particle diameter of 2.4 ± 0.9 µm (Figure 3b). Their surface is mostly smooth, but the particles appear to be deflated.



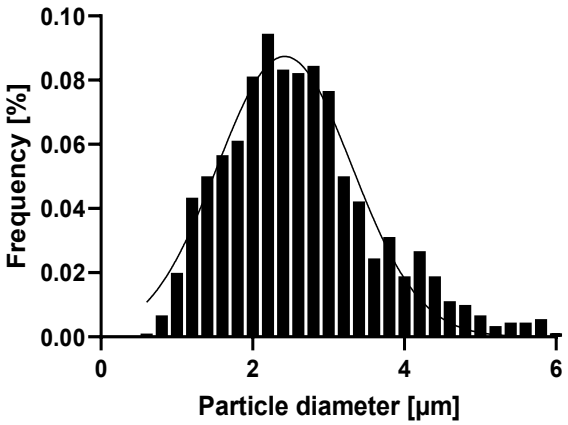


Figure 3: (a) SEM image of the electrosprayed core-shell particles from an ethanolic core solution containing hydrophobic peptide ion pairs and the respective (b) particle size distribution. The scale bar represents 20 µm.

3.4. Comparison of core composition for HIP electrospraying

Non-reducing disaccharides such as trehalose are commonly used cryoprotectants for peptides and proteins and have been shown to play an important role maintaining the stability of protein in electrosprayed particles [33]. However, given their limited solubility in pure ethanol these disaccharides would not be suitable to act as cryoprotectants for the peptide ion pairs. Thus, an alternative use of a phospholipid (DPPC) to impact stability and thereby potentially improve encapsulation efficiency was investigated. HIP complexes of teriparatide and SDS were previously shown to remain stable during thin-film production (drying) in the presence of phospholipids [25]. In addition, our group has shown that the nature of the polymer impacts stability of proteins during electrospraying, with polyvinylpyrrolidone (PVP) outperforming dextran and polysucrose [33]. As PVP is a hydrophilic polymer and may act as a porogen resulting in increased burst release, incorporation of Eudragit EPO was investigated [29]. Eudragit EPO was incorporated within the core as the polymer is only soluble in aqueous media (pH < 5.0) and was expected to modulate release profiles. Figure 4 compares the encapsulation efficiencies obtained from PLGA particles electrosprayed using an ethanolic HIP dispersion with and without the addition of DPPC or Eudragit EPO. The presence of DPPC, while presenting slightly higher mean encapsulation efficiency, shows no significant difference (one-way ANOVA, $p>0.05$) in encapsulation efficiency compared to particles prepared without DPPC. The presence of Eudragit negatively impacted the electrospraying process (dripping and wet powder bed), which may have led to greater variations in encapsulation efficiency observed.

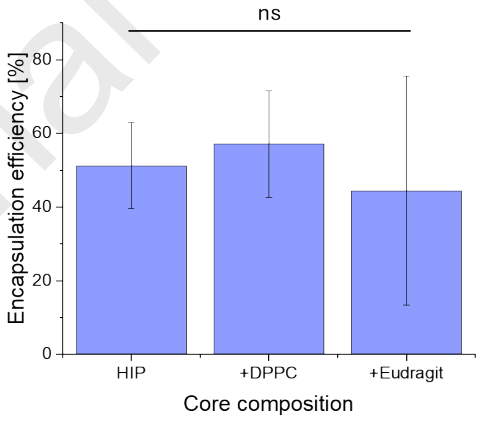
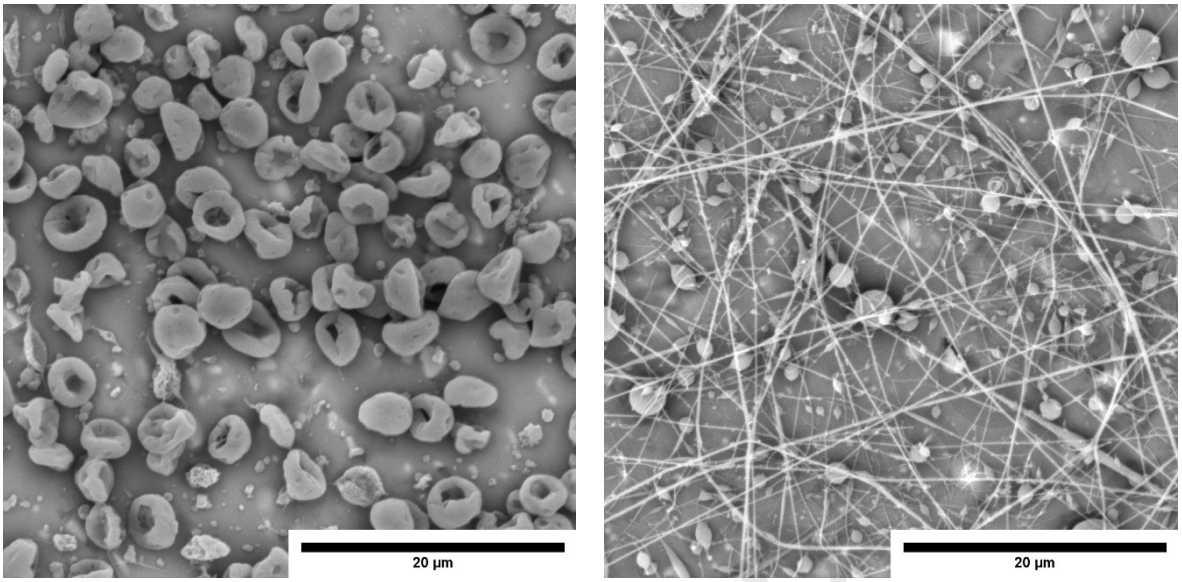


Figure 4: Encapsulation efficiency of teriparatide in PLGA particles with the addition of DPPC or Eudragit within the ethanolic HIP dispersion. Data is shown as mean ± s.d., n=3.

SEM images of the electrosprayed core-shell PLGA particles containing HIP and either DPPC or Eudragit EPO are given in Figure 5a and b respectively. The mean diameter of the DPPC containing particles is 2.7 ± 1.0 µm (Figure S4.1a). As above, the particles appear to be deflated. Ethanol is a miscible but poor solvent, thus likely to cause polymer chains to coil up and extend the distance between them. Higher PLGA

concentrations may be required for inter-polymer chain entanglement and production of smooth and spherical particles [34].



(a) (b)

Figure 5: SEM images of core-shell particles electrosprayed with an ethanolic core solution containing hydrophobic peptide ion pairs and (a) DPPC, (b) Eudragit EPO. The scale bar represents 20 µm.

In contrast, Eudragit EPO containing samples were composed of both particles and fibres. The incorporation of the polymer likely increased entanglement resulting in the presence of fibres [23]. The Eudragit EPO particles present a mean diameter of 1.3 ± 0.5 µm (Figure S4.1b), but a greater extent of polydispersity was observed, further suggesting an unstable cone-jet mode (Table 5) [23].

Table 5: Size distribution of the electrosprayed core-shell particles from an ethanolic core solution containing hydrophobic peptide ion pairs. The size range was determined as the difference between the largest and smallest particle measured.

Core composition	HIP	+DPPC	+Eudragit EPO
Mean ± s.d [µm]	2.4 ± 0.9	2.7 ± 1.0	1.3 ± 0.5
Size range [µm]	5.4	4.3	7.2

3.5. Evaluation of in-vitro drug release

The release of peptides from PLGA particles is typically characterised as biphasic with an initial burst release followed by a second phase of continuous release [35]. The initial burst release is generally associated with accumulation of the drug near the

surface of the particles or presence of pores in the polymeric shell [11]. The pore network creates a diffusion pathway to the surface and is thought to be formed by the incorporated peptide. The extent of the network will depend on the loading and size of the biomolecule [36]. In water, PLGA degrades through hydrolysis into lactic acid and glycolic acid, creating additional pores through which the drug can be released [35]. In some cases, a dormant phase is described where little to no release is observed. If present, this is observed between burst and continuous release [36]. Core-shell particles have a less water-accessible core compared to particles prepared by double emulsion, thus often presenting a reduced burst release and overall slower drug release [21,26]. The release of teriparatide from each of the formulations was measured over 21 days (Figure 6).

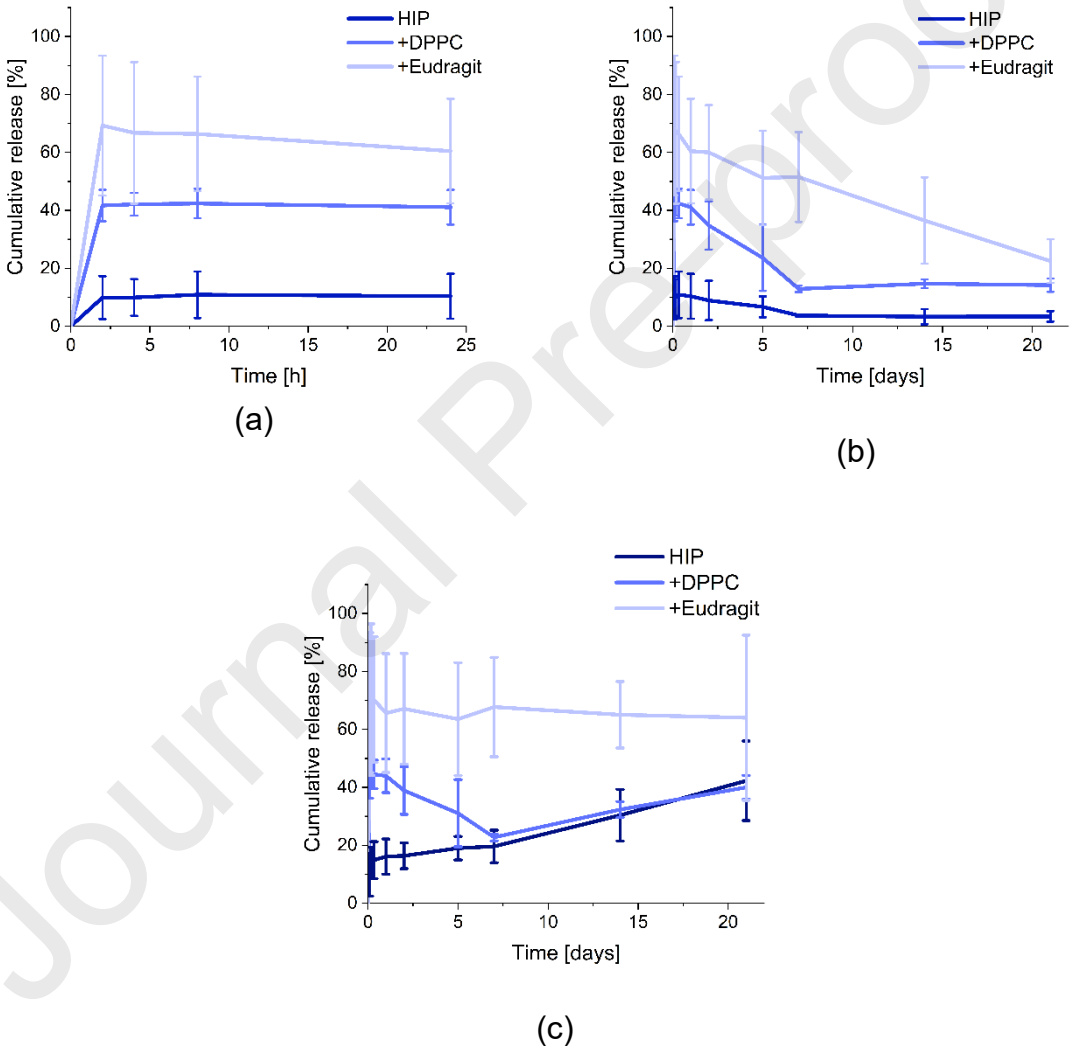


Figure 6: Release of teriparatide from electrosprayed PLGA particles comparing the impact of core composition and shown as cumulative release profile over (a) 24 h, (b) 21 days, (c) corrected for teriparatide degradation. PLGA particles were prepared in triplicates and values are presented as mean ± s.d., n=3.

Here, the core composition appears to have a strong influence on the initial burst release (Figure 6a). The particles obtained from the HIP dispersion without any other components presented the lowest burst release with (10.9 ± 8.1 % within the first 2 h),

whereas when Eudragit EPO was incorporated the highest burst release (69.2 ± 24.1 % within 2 h) was observed. Eudragit EPO is an acid soluble polymer requiring a pH < 5.0 to dissolve. It was initially anticipated that incorporation of Eudragit EPO within the core of the particles may delay the release of teriparatide, first requiring sufficient degradation of PLGA to locally decrease pH to < 5.0 [37]. However, the core-solution was comprised of 0.1M HCl in ethanol, required to solubilise the polymer. The presence of HCl during electrospraying is likely to have resulted in premature solubilisation of Eudragit EPO thereby forming pores in the polymeric network and resulting in a rapid release of teriparatide. The incorporation of DPPC within the core solution resulted in an increased burst release (41.7 ± 5.4 % within 2 h). As the only difference in these particles was the presence of DPPC, it is hypothesised that DPPC was subjected to greater dielectrophoretic movement, ultimately leading to greater pore network within the particles [38].

Following the initial burst release a decrease in cumulative release was observed for all particles (Figure 6b). It was found that that prolonged incubation at 34 °C negatively affected peptide stability (see Supporting Information, Section S5). The stability of biomolecules throughout their lifecycle is a known and major challenge [9]. The cumulative release of teriparatide was corrected by the degradation rate of the peptide (Figure 6c). The degradation rate was estimated from the AUC of secondary peaks (Supplementary Information, Section S6), but may underestimate teriparatide degradation especially for the DPPC containing particles. It is thought that the presence of DPPC may alter degradation routes and thus products. Nevertheless, from Figure 6c it can be observed that Eudragit EPO containing particles released all the peptide within the first 2h. On the other hand, particles containing only HIPs or HIP and DPPC in the core demonstrate continuous release over a 21-day period with 42.3 ± 13.7 % and 40.0 ± 4.1 % released respectively.

Overall, the results show that a very low burst release can be achieved from peptide ion-pair electrospraying. Moreover, the amount of burst release is dependent on core composition. Exploration of alternative counter-ions for hydrophobic ion pairing could further reduce or even eliminate the burst release effect. Indeed, Lu *et al.* demonstrate the impact of counter-ion on release kinetics for polycaprolactone-polyethylene glycol nanocarriers containing polymyxin ion pairs. The ratio between peptide and counter-ion further impacts release kinetics [28]. Moreover, a prolonged and close-to-linear peptide release from the PLGA microparticles is achieved from day 2 and day 7 for the HIP and HIP-DPPC particles respectively. While peptide stability was a challenge this is unlikely to be problematic *in vivo* as peptides are generally cleared from the blood stream in matter of minutes. Teriparatide has an elimination half-life of 1 h after subcutaneous administration of immediate release formulations which means >90%is cleared in 4 h, which is considerably shorter than the observed peptide degradation rates [36,39].

3.6. Functionality assessment of released teriparatide

The preservation of teriparatide's biological activity was assessed in a cell-based intracellular cAMP assay. Teriparatide binds to parathyroid hormone 1 receptor (PTH1R), and upon teriparatide binding, intracellular cAMP levels increase [40]. In this assay, cAMP levels in a PTH1R overexpressing HEK293 cell line were measured after exposure to teriparatide [25]. The cells were exposed to identical concentrations of

teriparatide released from HIP-PLGA particles and native (unprocessed) teriparatide as control. Figure 7 shows cAMP levels measured after a 30-minute exposure to 0.14 ng/mL of the teriparatide samples. Cells treated with the native peptide and teriparatide released from the electrosprayed particles showed no significant difference in cAMP levels (0.024 µM (native), 0.032 µM (HIP-PLGA), and 0.038 µM (HIP-DPPC-PLGA)), thus demonstrating functionality of teriparatide.

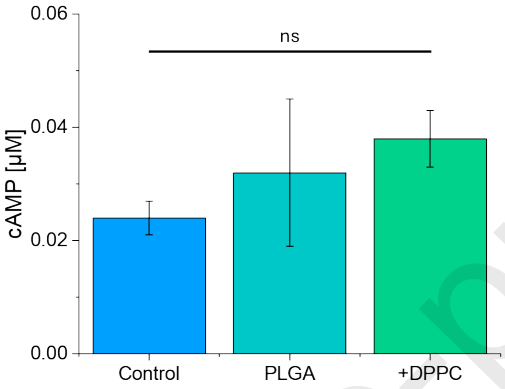


Figure 7: cAMP levels measured in a PTH1R overexpressing HEK293 cell line after exposure to identical concentrations of native (unprocessed) teriparatide (=control) and teriparatide released from the HIP-PLGA particles (mean ± s.d., n= 3).

4. Conclusion

In this work teriparatide loaded core-shell PLGA particles were produced by electrospraying. After initial optimisation of the conditions, electrospraying of the peptide in the form of hydrophobic ion pairs (HIP) was explored for the first time. Utilising a HIP approach, the peptide could be solubilised in an ethanolic solution without loss of stability and biological activity. Comparing the electrospraying of teriparatide in the form of a HIP with the native peptide in an aqueous solution demonstrated clear advantages of the HIP approach. The HIP-loaded ethanolic core led to improved stability of the electrospraying process with increased day-to-day reproducibility, evidenced by a particle yield of 102.2 ± 8.8% (ethanol) compared to 61.3 ± 4.0% (aqueous). Electrospraying of teriparatide in HIPs led to an encapsulation efficiency of 51.2 ± 11.7 % which was not significantly different to aqueous teriparatide solutions (79.2 ± 19.8%). The effect of adding two excipients – DPPC and Eudragit EPO – to the ethanolic HIP dispersion during electrospraying was explored, and it was determined that the extent of burst release may be modulated via the core composition. Continuous and near linear release of the peptide (from day 2) over at least 21 days (end of study) was observed for HIP-PLGA particles. Finally, peptide integrity and its biological functionality was retained, as evidenced by the results of a cell-based assay utilising the PTH1R receptor. This suggests the potential of the HIP approach to generate stable prolonged release delivery systems for peptide active ingredients.

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CRediT authorship contribution statement

Conceptualization, C.S.S., C.J.M., S.B. and G.R.W; methodology, C.S.S.; formal analysis, C.S.S.; investigation, C.S.S.; resources, C.J.M., S.B., and G.R.W.; writing – original draft preparation, C.S.S.; writing – review and editing, C.S.S., C.J.M, S.B., G.R.W; visualisation, C.S.S.; supervision, S.B. and G.R.W.; funding acquisition, G.R.W.

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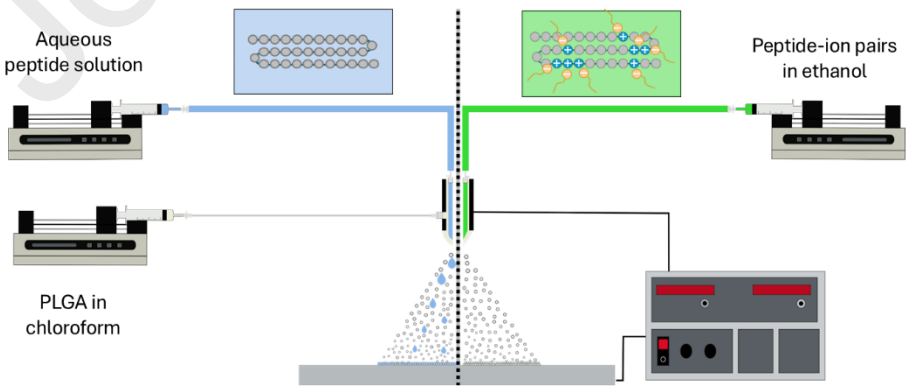

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: