Hollow-layered Nanoparticles for Therapeutic Delivery of Peptide Prepared Using Electrospraying

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Abstract

The viability of single and coaxial electrospray techniques to encapsulate model peptide - angiotensin II into near mono-dispersed spherical, nanocarriers comprising *N*-octyl-*O*-sulphate chitosan and tristearin, respectively, was explored. The stability of peptide under controlled electric fields (during particle generation) was evaluated. Resulting nanocarriers were analysed using dynamic light scattering and electron microscopy. Cell toxicity assays were used to determine optimal peptide loading concentration (~1 mg/ml). A trout model was used to assess particle behaviour *in vivo*. A processing limit of 20 kV was determined. A range of electrosprayed nanoparticles were formed (between 100-300 nm) and these demonstrated encapsulation efficiencies of $\sim 92 \pm 1.8$ %. For the single needle process, particles were in matrix form and for the coaxial format particles demonstrated a clear core-shell encapsulation of peptide. The outcomes of *in vitro* experiments demonstrated triphasic activity. This included an initial slow activity period, followed by a rapid and finally a conventional diffusive phase. This was in contrast to results from *in vivo* cardiovascular activity in the trout model. The results are indicative of the substantial potential for single/coaxial electrospray techniques. The results also clearly indicate the need to investigate both *in vitro* and *in vivo* models for emerging drug delivery systems.

Keywords: Nanoparticles, Electrospraying, Encapsulation, Tristearin, Angiotensin II

1 Introduction

Nanoparticles (NPs) can be synthesised in a wide range of sizes, shapes, and depending on the molecular basis of the structure, can be termed soft or hard. Nanotechnologies are used to achieve targeted delivery of drugs into cells or tissue in a specific manner, enhanced delivery of poorly water-soluble drugs, co-delivery of two or more drugs for combination therapy and visualization of drug delivery by combining therapeutic agents with imaging modalities [1].

The preparation of effective drug delivery systems still represents a timely ongoing challenge in medicine. However, there is a rapidly growing interest in NPs as drug delivery systems [2,3]. Both microparticles and NPs are used therapeutically to transport therapeutic agents through blood vessels and release the active at a desired target site [4]. Thus, the design of ideal drug carriers to obtain sustained and controlled release have attracted much attention over the last few decades. These can also decrease the instability, variation of drug dosage and the concentration constant over the designated treatment period to maximise the efficacy of the therapeutic agent [5,6].

Peptides have also gained interest as pharmaceutical actives but their delivery to target cells and stability (both during manufacturing and following administration) are obstacles in their deployment. To circumvent these problems, encapsulation of peptides and proteins has become the focus of an alternative approach for developing novel drug delivery systems. Generating nano-scale particles with the ability to control surface and morphological features has been shown to have a distinct impact on active protection and controlled release [1]. Several methods have been developed during the last two decades for preparation of NPs, where the active is dissolved, entrapped, encapsulated or attached to a NP. Commonly used methods for protein/drug encapsulation include solvent evaporation [7,8], nanoprecipitation

or solvent displacement [9,10], emulsification (which is a modified version of the solvent evaporation) [11], dialysis [12] and supercritical fluid technology [13]. However, these methods expose peptides to various factors affecting their stability such as organic solvents or high temperatures. These cause polymer degradation, which may promote drug deactivation [14]. Another critical concern related to engineering and mixing methods, includes the risk of drug inactivation due to structural breakdown or deformation arising from exposure to high temperatures or elevated shear stress during particle preparation. Shear stress at the aqueous/organic interface in emulsion methods is mainly responsible for protein denaturation and aggregation, and is generally difficult to avoid [15]. Moreover, low encapsulation efficiency and loading capacity, high initial burst release and broad size distribution of the particles are other negative attributes of emulsification processes [16,15]. These limits provide platforms for the development of new techniques with the ability to yield more promising peptide-protein based drug delivery systems.

Electrospraying (ES) is an ambient temperature platform technology that is currently being used for the preparation of a host of nanoscaled drug delivery morphologies [17,18,19].

ES involves the application of an electric field to a liquid (formulation) flowing through a conducting capillary. The applied electrical charges compete with the inherent surface tension of the formulation (liquid) to deform this liquid droplet into a meniscus. Build-up of the electrical field at the capillary outlet eventually leads to a greater electrostatic force compared to relative surface tension. This causes elongation at the meniscus and subsequent disintegration of the initial liquid meniscus into fine droplets (micro- to nano-size). The charged droplets are collected in the direction of a grounded collector, whereas the solvent (vehicle) evaporates and solidifies resulting in particles [20].

The coaxial mode is a modified form of ES, where two or three capillary needles can be concentrically aligned to enable compartmentalisation within NPs. ES has also been used to generate bio-responsive polymer-peptides with key emphasis on particle size variations (100-600 nm) [21]. ES allows sufficient control of characteristic shapes and sizes on the micro- to sub-micron scale integrating biopolymers and active molecules (e.g. proteins or growth factors) for controlled release [22]. Angiotensin II (Ang II) is an octapeptide hormone, which plays a vital role in cardiovascular homeostasis. Until recently, Ang II was only seen as a regulatory hormone controlling blood pressure and aldosterone release. It is now widely accepted that locally formed Ang II can activate the expression of many substances, including growth factors, cytokines, chemokines, and adhesion molecules, which are involved in diverse cellular processes, from cell growth/apoptosis, to fibrosis and inflammation [23].

Tristearin is a solid lipid (triglyceride) that has been used in the development of drug delivery carriers [24,25]. Matrix systems prepared using triglycerides have shown to protect labile or altered agents from degradation and allowing controlled drug release [26]. Specifically, tristearin has been utilised to demonstrate efficient immobilisation of drugs through high pressure homogenisation which suggests the material is viable and applicable to more aggressive processing routes (e.g. when compared to emulsion methods). Tristearin based drug delivery systems are valuable in the development of drug carriers for improving their bioavailability by better diffusion through biological membranes [27,28]. Amphiphilic materials have also gained appreciable interest in recent times due to their adaptability within polar and non-polar environments. One such amphiphilic polymeric material is *N*-octyl-*O*-sulphate chitosan (NOSC) which has demonstrated good potential biocompatibility, tissue distribution and good *in vivo* and *in vitro* outcomes (no acute toxicity) [29]. In this study, Ang II was used as a model peptide for encapsulation optimisation using the ES process. It also

has wide-ranging physiological roles throughout the body, acting via different subtypes of specific G protein-coupled receptors [30]. In this report, the preparation of core-shell peptide encapsulated nano structures *via* ES technique is described. The resulting structures were evaluated for cell toxicity, *in vivo* and *in vitro* peptide release.

2 MATERIALS AND METHODS

2.1 Materials

N-octyl-*O*-sulphate chitosan (NOSC) and tristearin (MW891.48 g/mol) were used in this study. NOSC was synthesised as previously described [31]. Tristearin, solvents including dichloromethane (DCM), dimethyl sulfoxide (DMSO, > 99%), Heptane, human Ang II (MW 1046.18 Da) were all purchased from Sigma-Aldrich, Dorset, UK. Water (HPLC grade) was purchased from Fisher Scientific, Loughborough, UK. Centrifugal filters (Amicon Ultra-4-ultra cell membrane 3 kDa MWCO) were purchased from Merck Millipore Ltd, Cork, Ireland. Float-A-Lyzer G2 dialysis filters (8-10 KD MWCO) were obtained from Spectrum Laboratories Inc, CA, US. MTT tetrazolium reduction assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was purchased from Sigma-Aldrich, Dorset, UK.

2.2 Solution preparation

A series of solutions were prepared for the electrospraying process. For the first solution, NOSC was added to DMSO (0.1w/v %) and was mechanically stirred until complete dissolution. Ang II (10 mg) was added to 10ml of this solution and was mechanically stirred for a further 2 h ensuring complete dissolution. The second solution comprised NOSC, Ang II and Water (HPLC grade). For this, Ang II was dissolved in water (0.1 w/v %) through mechanical stirring. Once dissolved, NOSC (also 0.1 w/v %) was added to this solution and stirred for a further 2 h. For the final solution, tristearin was dissolved in DCM (1 w/v %)

through mechanical stirring at the ambient temperature (23 °C). The solutions are summarised in Table 1.

2.3 Characterisation of solutions

The solutions were characterised for surface tension, electrical conductivity and viscosity.

Solution viscosity was determined using an Ostwald viscometer (size E) and applying equation (1) :

$$\eta = kt$$

Where η is the apparent viscosity, k the viscosity constant for size E (0.362) and t is the experimental time of transit of the liquid in the capillary. Surface tension was measured using a Kruss Easydyne V2-05 tensiometer (KRÜSS GmbH, Hamburg, Germany) using the plate method. Electrical conductivity was measured using a Hanna instrument HI-9033 (Hanna Instruments Inc., Woonsochet, US) conductivity probe. In all cases, the mean value of five consecutive readings was taken.

2.4 Electrospaying process

Ang II was encapsulated into NOSC and tristearin by the ES process based on a method used previously [17,18]. Tristearin is a triglyceride and is utilised in the food industry as an emulsifying agent. However, it is also used in other products as a hardening agent. For this study, tristearin was the ideal material to generate a core-shell which solidified rapidly after the ES process. It was therefore deployed in the outer needle during coaxial electrospaying to enable a rapid wax shell layer. The ES device (Profector Life Sciences Ltd, Ireland) consists of specifically designed stainless steel capillary nozzles (Rame-hart Instrument Co, NJ, US). The diameter of the single needle processing head was 508 μm . For the coaxial

needle setup, the inner and outer diameter were $\sim 900\ \mu\text{m}$ and $1900\ \mu\text{m}$ respectively. In both instances, the capillary nozzle is connected to a high-voltage supply (capable of generating an electrical field of up to $\sim 30\ \text{kV}$). Solutions were perfused into the nozzle using a programmable syringe pump (World Precision Instruments, Florida, US). Once optimised through applied voltage and flow rate (to enable the stable ‘cone-jet’ mode) [32] a more uniform particle production process was achieved [33]. For particle preparation, the deposition distance was varied (at 25, 50 and 100 mm) determined as the distance between the tip of the needle to the collection substrate. For the single needle process, NOSC and Ang II solutions (1:1 ratio) were used. For the coaxial ES process the inner needle was supplied with Ang II and NOSC solutions (1:1 ratio) with the tristearin solution perfused through the outer needle. Details of applied voltage, working distance and other parameters are shown in Table 2. The samples for single needle ES were collected in a glass vial (15 ml of heptane) and for the coaxial ES process were collected in water (15 ml). The setup of the ES process utilised in this work is shown in Figure 1a, with a close-up of the processing needle shown in Fig. 1b. Encapsulation material formulations are shown in Fig. 1c. All experiments were carried out at the ambient temperature (23°C). For individual experiments, initially optimised flow rates and applied voltages were determined when stable jetting was achieved (Fig. 1b).

2.5 Particle size, distribution and zeta-potential measurement

Dynamic light scattering (DLS) was used to measure the size distribution of NPs in the collection media (Malvern Zetasizer Nano-ZS, Malvern Instruments Ltd, UK). For imaging purposes, samples were collected on glass slides and dried for 24 h prior to studying by scanning electron microscopy (SEM/JEOL-6060LV, JEOL Ltd, Japan), operated at an accelerating voltage of 5 kV. Following drying, samples were gold coated for 2 min via a rotary-pumped sputter coater (Q150R ES, Quorum Technologies Ltd, East Sussex, UK) for

improving the conductivity of the sample surface. Transmission electron microscopy (TEM) was performed using TEM/JEOL-2000FX (JEOL Ltd, Japan) with an operating voltage of 80 kV. TEM grids (Formvar films–200 Mesh Copper) were coated using the osmium tetroxide and uranyl acetate solution (1 wt.% and 2 wt.% respectively) and air-dried before addition of samples and imaging. Zeta-potential was measured using a Nanobrook Omni analyser (Nanobrook Omni, Brookhaven, US) (ten replicates) and the mean value was obtained. For this NPs were collected and analysed in PBS (pH 7.4).

2.6 Peptide stability through preliminary ELISA and HPLC analysis

Human Ang II was used in all experiments. The stability of Ang II under ES conditions was assessed using High Performance Liquid Chromatography (HPLC/ Agilent Technologies, US) and enzyme-linked immunosorbent assay (ELISA/ Enzo Life Sciences Ltd, UK). Samples were prepared using flow rates of; 5, 10 and 20 $\mu\text{l}/\text{min}$ and each flow rate was subjected to applied voltages of 20 and 30 kV. For HPLC a Phenomenex Aeris™ Peptide column (Phenomenex Ltd, UK) was used (250 $\times$ 4.6 mm, 3.6 $\mu\text{m}/100 \text{ \AA}$, using an acetonitrile/water (50:50 ratio) solvent system with 0.1 v/v% trifluoroacetic acid). A calibration curve was constructed with Ang II concentrations of 50,100,200,400,600 and 800 $\mu\text{g}/\text{ml}$. Peptide analysis was performed at 214 nm. In addition, the stability of Ang II was also studied through direct quantitation by ELISA as per manufacturer's instructions. A standard series of Ang II (3.91-10,000 pg/ml) was prepared. The assay kit employed immobilised anti-Ang II antibodies to capture the samples or standard Ang II, which compete for binding with later added biotinylated Ang II, the presence of which is then detected through amplification by streptavidin conjugated to horseradish peroxidase (HRP), followed by colorimetric measurement of the HRP-catalysed reaction at 450 nm. Thus, in this assay,

the amount of signal detected was inversely proportional to the level of Ang II in the sample. Graph Pad Prism 6.01 software was used for data evaluation.

2.7 Drug loading and release study

The quantity of drug loaded in NPs was determined using centrifugal filters (Amicon Ultra-4- ultracell membrane 3 kDa MWCO) which were used to separate loaded particles from free Ang II, quantified using HPLC analysis. A known quantity (15 mg) of electrosprayed NPs were added to the centrifugal filters and centrifuged for 30 min at 4000 rpm to separate the NPs from the untrapped peptide in flow-through filtered solutions. Filtered solutions were then analysed by HPLC (using an acetonitrile/water (50:50 ratio) solvent system with 0.1 v/v% trifluoroacetic acid) as described above. Ang II release from electrosprayed NPs was performed using Float-A-Lyzer G2 dialysis filters. The filters were prepared according to the manufacturer's instructions. Electrosprayed NPs were added to the filters and incubated in 15 ml of PBS at 37°C (under agitation). At specified time intervals aliquots of the release medium were taken (1 ml) and an equal volume of fresh PBS was added. Ang II was quantified by HPLC as described above. All studies were performed in triplicate and mean values are reported. A control experiment to determine the release behaviour of the free Ang II was also performed. The release study was carried out over a 48 h period. Encapsulation efficiency was confirmed using equation (2):

$$\text{Encapsulation efficiency (EE)} = \frac{\text{Actual amount of drug loaded in NPs}}{\text{Theoretical amount of drug loaded in NPs}} \times 100\%$$

2.8 *In vitro* cell culture

To evaluate the potential biocompatibility of the electrosprayed NPs, the mouse brain b-End 3 endothelial cell line was used [34]. b-End 3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% foetal calf serum (FCS), 1% non-essential amino acids, L-ascorbic acid (0.150 g/l), 2 mM L-glutamine, 0.02 M HEPES, penicillin (100 U/ml) and streptomycin (0.1 mg/ml) (all purchased from Sigma, Dorset, UK). B-End 3 cells were maintained at 37°C in a humidified incubator with 5% CO₂.

2.9 MTT toxicity assay

Cells were seeded at 1×10^5 cells per well (using a 96 well plate, Nunc, Fisher Scientific, Loughborough, UK) and were incubated for 24 h in culture media. 100 µL of the electrosprayed NPs, unloaded and loaded with Ang II, were added to selected wells. The cells were further incubated for 24 h. 50 µL of MTT reagent (prepared in a physiological solution) was then added to cells and incubated for 2 h as per manufacturer's instructions. The negative control contained media only. After 2 h of MTT incubation, the culture media was removed and cells were washed with 100 µL of Dulbecco's phosphate buffered saline (PBS) prior to the addition of 100 µL of dimethylsulfoxide (DMSO) to each well to dissolve formazan precipitates. The quantity of formazan, which is proportional to the number of viable cells was measured via change in absorbance at 570 nm using a spectrophotometer (POLARstar OPTIMA, BMG Labtech, Bucks, UK). Also, phase-contrast microscopy images of cells were captured using a Leica inverted DMIRB microscope (attached to a Leica DMC 2900 camera, Milton Keynes, UK).

2.10 *In vivo* study using trout model

In vivo experiments were performed on unanaesthetised adult rainbow trout (*Oncorhynchus mykiss*) weighing approximately 250 g (Fig. 1d). The technique used for placement of the electrocardiographic electrodes, recording of the ventilation, cannulation of the dorsal aorta, intra-arterial (IA) injection of the peptide, and data acquisition and analysis of the cardiovascular variables have been described previously in detail [35,36]. The ventilation signal was only used here to monitor the recovery of normal baseline ventilation after surgery and no quantification of the ventilatory variables was performed. The dorsal aortic blood pressure (P_{DA}) and the heart rate (HR) were monitored and quantified continuously. Briefly, after two post-operative days, recording sessions of 30 min were started. 5 min after the beginning of the recordings, the trout firstly received an IA injection of vehicle (Ringer's trout solution, n=13) followed 30 min later with an IA injection of Ang II (50 pmol/trout; ~ 200 pmol/kg, n=8), or tristearin NPs loaded with Ang II (equivalent to 50 pmol/trout, n=6). The dorsal aortic blood pressure (P_{DA}) and the heart rate were monitored continuously (n=8-13).

2.11 Statistical analysis

The data from MTT assay was analysed statistically using one-way ANOVA with Bonferroni *post hoc* test; $P < 0.05$ was considered as statistically significant. The *in vivo* data were expressed as means $\pm$ S.E.M for each 5 min period, for which two-way ANOVA for repeated measures followed by the Bonferroni test for comparisons between groups at selected time-points. The criterion for statistical difference between groups was $P < 0.05$. Two-way ANOVA was also employed for ELISA and HPLC analysis. The statistical tests were performed using Prism 6.01 (GraphPad, San Diego, CA).

3 Results and Discussion

The ES process (Fig. 1) was successful in generating spherical core-shelled NPs with a narrow size distribution. At specific ES parameters, through electrified capillary needles (single and coaxial), the liquids adopted a conical shape (Fig. 1b). The ES process is highly dependent on solution (process material) properties in obtaining a stable cone-jet for controlled generation. This process requires the existence of an induced electric field at the tip of the needle plus a minimum electrical conductivity of the solution to be electrosprayed. However, the surface tension is a competing force and it must be overcome by the solution's electrical conductivity, thus permitting the atomisation process. Furthermore, the viscosity of the solution is known to influence the morphology and size of the resulting structures; for concentric double-layered particles, the viscosity of the outer-needle liquid should be optimised in order to accommodate cone formation and break-up (e.g. Reynold number) efficiently through electrical stress flow homogeneity throughout the solution and to the inner phase [37,38]. All solutions employed in the present study were characterised (Table 1). All values for surface tension were below that of water, making them suitable for ES [39]. However, in coaxial mode conventional single needle limits are overcome. For example Loscertales et al. (2002) sprayed oil (not viable for ES alone) with a conducting liquid [40]. All solutions displayed low viscosity, which alludes to the potential of particle generation. Finally all materials demonstrated electrical conductivity and those exhibiting the lowest values (e.g. DCM, $\sim 1.0 \times 10^{-5} \text{ Sm}^{-1}$) have been utilised in previous studies [41]. This also suggests current formulations prepared from tristearin and Ang II are able to provide the electrical stress required for jet formation.

The encapsulation of proteins (e.g. insulin, BSA and lysozyme) and peptides in polymeric microparticles and NPs has been studied previously. However, there are limited studies

focusing on the effect of the applied electrical field on the stability of the peptide under investigation [42,43,41]. In addition, reported electrical field values for peptide/protein encapsulation have generally been below 20 kV [41,44]. In this study, the stability of Ang II was investigated using conventional HPLC (Fig. 2a) and ELISA (Fig. 2b). The stability of Ang II under ES conditions with different controlled parameters (flow rates at 5, 10 and 20 $\mu\text{l}/\text{min}$; applied voltage at 20 and 30 kV) were studied. For higher flow rates it was found that there was no observable change to the peptide even at high voltages (Fig. 2) by means of detection using HPLC. However, with ELISA (Ang II amount recognised by antibody) there is a difference between samples collected at different applied electric fields ($P < 0.0001$). In this instance the flow rate had no significant impact. Based on these findings, and by providing a stable cone-jet for the collection of NPs, an applied electric field below 20 kV was selected. As the ES process is largely driven by flow rate and applied electric field, exploratory conditions were used to optimise the process.

An ES single needle process was employed (flow rate of 3 $\mu\text{l}/\text{min}$ and applied voltage of 15-19 kV) to generate NPs containing Ang II embedded in NOSC matrix. Ang II loaded NPs were collected in heptane (working distance 100 mm). Following analysis (using DLS) they were found to have an average size of $105.7 \text{ nm} \pm 4 \text{ nm}$ (Fig. 3a). However, when the same initial matrix material NOSC with Ang II was used (inner needle) during the coaxial ES process (tristearin in the outer layer), two peaks resulted, giving rise to a bimodal distribution. The first peak demonstrated a mean particle diameter of $\sim 250 \text{ nm}$. The second peak occurred on the micron scale ($\sim 5 \text{ }\mu\text{m}$) which was a clearly different to the former (Fig. 3b). This correlated with the particle generating ES process, which exhibited intermittent jetting instabilities during the preparation of NOSC-Ang II and tristearin particles (coaxial ES). The polydispersity of the particles also increased appreciably ($\sim 73\%$). The encapsulation (coaxial

ES) of Ang II (in water) alone into a tristearin core-shell resulted in a more uniform particle size and distribution (178.28 nm). By decreasing the working distance from 50mm to 25mm resulted in an increase in the average particle size from 178.28 nm (± 20 nm) to 267.48 nm (± 30 nm) (Fig. 3c and Fig. 3d). The parameters and outcomes have been summarised in Table 2.

To assess the morphology of NPs, SEM and TEM were used. Micrographs (Fig. 4a) show spherical particles for electrosprayed samples (Table 2) and the size of particles correlated with values obtained using DLS. Fig. 4b shows particles generated using the coaxial ES process (tristearin in outer layer, NOSC and Ang II in inner layer) where a broad size of particles can be observed. However, coarse 5 μ m particles detected on DLS are not evident, but in this instance smaller (1 μ m-500 nm) particles can be observed. This may also suggest some degree of agglomeration during DLS analysis. However, even within these samples there exists a coarser distribution which can also be attributed to the unstable jetting observed using NOSC in the coaxial ES process. Removal of NOSC from the formulation (using tristearin in the outer layer and Ang II in inner layer) gave rise to a more uniform particle size distribution, which also exhibited spherical morphologies (Fig. 4c). TEM was used to assess the degree of core-shell encapsulation. Both samples c and d (Table 2/ Fig. 4d and 4e) demonstrated well defined core-shelled structures with hollow centres. The layering of tristearin and encapsulated Ang II was visible. The effect of deposition distance, was confirmed using particle size analysis and a clear difference in size distribution and uniformity was observed (Fig. 4f). In some artefacts during the consolidation process (droplet to particle formation), the inner Ang II formed bridges (Fig. 4g), which remained intact once solidified. This again confirmed the hollow nature of the particles. The well defined representative structure of NPs is shown in Fig. 4h. HPLC analysis of the peptide content in NPs samples were calculated to be $\sim 92\% \pm 1.8$. These values, however, showed a disparity

between those obtained when using ELISA (Fig. 2), where a significant reduction in Ang II stability/retention was detected at high voltage ($P < 0.0001$) indicating chemical change or alteration of the peptide during the ES process at high electric fields (20 kV or greater). The zeta potential of NPs (in PBS, pH 7.4) was -4.58 ± 2.52 mV (range between -7.06 to -2.03 mV), which suggests these structures will have a tendency to agglomerate. The parametric optimisation through mode-map selection (preliminary optimisation) was below 20 kV, and in all instances HPLC was used further to assess release of encapsulated Ang II from core-shelled tristearin NPs.

In vitro validation using free Ang II (as a control) demonstrated a release of at least 90% of peptide across dialysis membranes under 4 h and total release at 6 h (99% of the control concentration). In contrast, the nano-encapsulation of Ang II within tristearin core-shells from nanocapsules demonstrated a triphasic release profile, which has been observed in other encapsulation methods [45]. Upto 5 h the cumulative release quantity of Ang II was ~5%, and during this time the release was sustained, this being the first phase. From 5 h onwards, the second phase demonstrated a rapid release of an additional ~15% of Ang II, which could be due to free Ang II (non-embedded or adsorbed onto the inner core-shell tristearin matrix) diffusing out of nanocapsules due to fractures or pores arising from the hydrolysis of ester groups within its structure [46,47]. The final phase demonstrated a typical matrix-based release profile where adsorbed Ang II in the inner core was released and then subsequent embedded Ang II (into the matrix) was also released. This process however was gradual, with 50% Ang II release at 15 h and 75% release at 24 h. A further ~15% Ang II was released by 48 h (Fig. 5). Based on *in vitro* release findings, the encapsulation of Ang II using coaxial ES is a highly successful method to control release, or moreover, a method to encapsulate the drug and permit release once localised in the desired tissue. This is a common problem with

non-core shelled (non-layered matrix) technologies providing inadequate layered encapsulation. While tristearin is prone to degradation factors (e.g. water and light when using PBS in an open system), an *in vivo* model would also introduce further host environmental factors such enzymatic, chemical and mechanical actions [48,49]. An *in vivo* trout model was used (Fig. 1d) to assess the activity of the NPs. Results demonstrated that IA injection of vehicle (trout's ringer solution) induced no change in the trout's cardiovascular variables, as expected (Fig. 6 a and b). In contrast, the IA injection of free Ang II provoked an increase in the blood pressure (Fig. 6a), indicating successful delivery of nano-encapsulated Ang II. A two-fold increase in baseline value peaked 3 min after the injection. This increase in blood pressure was transient since baseline P_{DA} was reached by the end of the recording. IA injection of tristearin-encapsulated Ang II was as potent as free Ang II for inducing a sharp increase in P_{DA} . Interestingly, no delayed response could be observed in the effect of tristearin-encapsulated Ang II in this study, as the speed of response of encapsulated Ang II appeared to mirror that of the free peptide. However, tristearin-encapsulated Ang II had a more sustained physiological effect since its hypertensive effect persisted until the end of the recording ($P<0.05$). After IA injection of vehicle, free Ang II and tristearin-encapsulated Ang II, the change in HR was not statistically significant between groups (Fig. 6b). This could be explained due to two reasons. The first relates to the administration method of the NP formulation. The shearing effect encountered during infusion may have lead to fracture of the NP core-shell; which presents an opportunity for immediate release of Ang II. Secondly, potential enzymatic, physiological and anatomical features in the trout model which could not be accounted for in the PBS drug-release study may have led to damaged NP core-shell systems (either mechanical or chemical). This again would have led to rapid release. Only changes in blood pressure (P_{DA}) were significant (i.e. based on differences in P_{DA} after administration of free Ang II, vehicle and tristearin-Ang II NPs) at time-points 10,

15, 20 and 25 minutes (* $P < 0.05$). In addition, at the 30 minute time-point, P_{DA} was significantly elevated for the tristearin-Ang II NPs when compared to the vehicle and free Ang II (+ $P < 0.05$). There is no change in HR at any time.

For the rapid and accurate determination of cell viability, a method that was originally developed as an assay for survival of mammalian lymphoma cells is based on the transformation and colorimetric quantification of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] [50]. Mitochondria reduce tetrazolium salts and thereby form non-water-soluble violet formazan crystals within the cell. The amount of these crystals can be determined spectrophotometrically and serves as an estimate for the number of mitochondria and hence the number of living cells in the sample [51]. These features are taken advantage of in cytotoxicity or cell proliferation assays and are widely to study cell viability in toxicology and cell biology. The results of the MTT assay for loaded and unloaded NPs demonstrated significant cellular toxicity only at the highest concentration of electrosprayed NPs (1 mg/ml) compared to the control (cells and media). No significant differences in toxicity were observed between loaded vs. unloaded NPs. This effect could relate to a real physiological effect through some unknown NP interaction mechanism, or may relate to residual levels of DCM in NP base solvents which could accumulate at higher concentrations of NPs. Phase contrast imaging of cell culture tests with NPs after 24 h incubation are shown in Fig. 7a-c, respectively. In some areas, localised agglomerations of NPs could be observed (Fig. 7c), but in general NPs either loaded or unloaded were well tolerated causing no obvious morphological changes in cells. NP concentrations lower than < 0.5 mg/ml only resulted in very limited levels of toxicity as measured by MTT assay (Fig. 7d). However, no significant differences in toxicity were observed between loaded and

unloaded NPs, and this confirms cellular tolerance to NPs loaded with Ang II (Bonferroni* = $P < 0.05$, (Fig. 7d)).

4 Conclusions

In this work, successful encapsulation of Ang II into tristearin core-shell nanoparticles (average size of 267.48 nm and 178.28 nm) *via* a coaxial ES technique is demonstrated. The single needle process proved to be viable in the preparation of matrix (non-core shelled) particles (~105 nm) using advanced polymeric materials e.g. NOSC. However, when used in a coaxial ES system a bimodal particle distribution resulted with a large size difference. The stability of Ang II, when subjected to high-voltage electric fields, was evaluated using ELISA and HPLC, indicated a potential use of ELISA as a preliminary analytical tool for peptide stability during ES encapsulation. These results indicated that the structure of Ang II was not affected below 20 kV and low flow rates (5 μ L) was applied ($P < 0.0001$). The *in vitro* release of Ang II from tristearin NPs appeared to show a triphasic release pattern which was sustained. This was in sharp contrast to NP behaviour using *in vivo* models. Upon delivery of free Ang II and loaded NPs, very similar physiological activity was observed, although NPs demonstrated prolonged activity towards the end of the study. This also suggests some anatomical or environmental (chemical) features of the trout model that need to be taken into account. MTT toxicity assay effectively determined an acceptable loading concentration for loaded or unloaded encapsulated NPs, generating no acute toxicity or morphological effects *in vitro*. Furthermore, *in vivo* experiments (using trout model) indicated a sustained hypertensive action when Ang II was administered in tristearin-encapsulated form and consequently confirmed the bioavailability of the peptide. The outcomes of both *in vitro* and *in vivo* release studies indicate the substantial potential of single/coaxial ES techniques for

the fabrication of NPs or multi-layered nanocapsules that can be used in the development of a variety of applications for the delivery of water-soluble peptides.

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Figure Captions

Figure 1 Experimental setup showing (a) the electrospraying device and the peripheral components (b) close-up of the processing needle (stable cone-jet mode) (c) with encapsulated material formulations (d) setup for *in vivo* trout model.

Figure 2 Stability studies of Ang II subject to high-voltage electric field at different flow rates: results obtained by (a) HPLC and (b) ELISA.

Figure 3 Size distribution report (intensity) of encapsulated NPs generated by DLS; (a) sample a (single needle) containing the model peptide Ang II embedded in NOSC matrix with average size of $105.7 \text{ nm} \pm 4 \text{ nm}$ (b) sample b (coaxial ES) containing NOSC and Ang II (inner layer) and tristearin (outer layer) with coarse particles (micron scale $\sim 5 \text{ }\mu\text{m}$) (c) sample c (coaxial ES) containing Ang II (inner layer) and tristearin (outer layer) with average size of 178.28 nm and working distance of 50 mm (d) sample d with the same formulation of sample c and working distance of 25 mm , average size of 267.48 nm .

Figure 4 Scanning and Transmission electron microscopy (SEM, TEM) images showing Ang II loaded NOSC and tristearin NPs; (a) electron micrograph of NOSC and Ang II using single needle at operating voltage of $15\text{-}19 \text{ kV}$ and flow rate of $3 \text{ }\mu\text{l/min}$ (b) electron micrograph of NOSC and Ang II (inner layer) and tristearin (outer layer) using coaxial ES needles at operating voltage of $15\text{-}17.9 \text{ kV}$ and flow rate of 5 and $11 \text{ }\mu\text{l/min}$ for inner and outer layer respectively (c) electron micrograph of Ang II (inner layer) and tristearin (outer layer) using coaxial ES needles at operating voltage of $15\text{-}17.9 \text{ kV}$ and flow rate of 5 and $11 \text{ }\mu\text{l/min}$ for inner and outer layer respectively and (d-f) TEM images showing the generated encapsulated

NPs with core-shell structures and hollow centres (g) encapsulated Ang II bridging across the NP core (h) illustrated structure of encapsulated NPs.

Figure 5 *In vitro* release profile of tristearin NPs loaded Ang II (vs. free peptide used as control).

Figure 6 Time-course curves for (a) the *in vivo* dorsal aortic blood pressure (P_{DA} ; in kilopascal/kPa) and (b) heart rate (HR, beats per minute/bpm) effects of vehicle (Ringer solution, in black, n=13), free Ang II (50 pmol/ per trout; ~200 pmol/kg, in red, n=8), and tristearin-encapsulated Ang II (equivalent to 50 pmol/ per trout, in blue, n=6) in the unanaesthetised trout. The arrow indicates the time of intra-arterial injection. The curves represent mean ($\pm$ S.E.M at selected times). * $P < 0.05$ free Ang II and tristearin-encapsulated Ang II vs vehicle at corresponding time points. + $P < 0.05$ tristearin-encapsulated Ang II vs vehicle and free Ang II. No statistically significant change occurred for HR between groups.

Figure 7 MTT toxicity assay of unloaded and Ang II loaded NPs and phase contrast microscopy images of the cells after 24 h incubation (a) phase contrast micrograph of media only (control) (b) phase contrast micrograph of mouse brain endothelial cells after 24 h incubation without NPs and (c) with added NPs (d) MTT toxicity assay data using media only, unloaded and loaded NPs with Ang II after 24 h incubation the cells (data were analysed using one way ANOVA with Bonferroni test * = $P < 0.05$ relative to control).

[illegible]

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

[Ang II = Angiotensin II, P_{DA} = dorsal aortic blood pressure, kilopascal = kPa, Heart Rate = HR, bpm = beats per minute]

Figure 6

[NPs = Nanoparticles, Ang II = Angiotensin II]

Figure 7

Tables

Table 1 Physical properties of the solutions and solvents used in this study

Solvent or Solution	Surface Tension (mN m ⁻¹)	Viscosity (mPa s)	Electrical Conductivity 10 ⁻² (Sm ⁻¹)
NOSC (0.1w/v %) in DMSO	42.1 ±2.2	2.65 ±0.07	1.050 ±0.0200
Angiotensin II (0.1w/v %) in water (HPLC grade)	56.0 ±1.8	1.39 ±0.05	1.160 ±0.0100
Tristearin (1w/v %) in DCM	25.8 ±1.2	2.51 ±0.04	0.002 ±0.0001
Water (HPLC grade)	71.8 ±0.5	1.40 ±0.02	0.070 ±0.0020
DCM	28.4 ±2.1	1.84 ±0.03	0.001 ±0.0003
DMSO	44.0 ±1.5	2.31 ±0.04	0.012 ±0.0004

[DCM = dichloromethane, DMSO = Dimethylsulfoxide, NOSC= *N*-octyl-*O*-sulphate chitosan]

Table 2 Size distribution data for single and layered (NOSC, Ang II and tristearin) structures

Samples	Applied Voltage (kV)	Inner flow rate (µl/min)	Outer flow rate (µl/min)	Working distance (mm)	Average Size (nm)	SD (±nm)	Polydispersity (%)
a (Single ES)	15-19	3	×	100	105.7	3.64	18
b (Coaxial ES)	15-17.9	5	11	50	255.56 & 5490	48.09 & 155.4	73
c (Coaxial ES)	15-17.9	5	11	50	178.28	11.9	24
d (Coaxial ES)	15-17.9	5	11	25	267.48	31.68	29

[Ang II = Angiotensin II, NOSC= *N*-octyl-*O*-sulphate chitosan, ES = Electrospray]