Electrospun formulations of bevacizumab for sustained release in the eye

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Abstract

Medicines based on vascular endothelial growth factor (VEGF) neutralising antibodies such as bevacizumab have revolutionized the treatment of age related macular degeneration (AMD), a common blinding disease, and have great potential in preventing scarring after surgery or accelerating the healing of corneal injuries. However, at present, frequent invasive injections are required to deliver these antibodies. Such administration is uncomfortable for patients and expensive for health service providers. Much effort is thus focused on developing dosage forms that can be administered less frequently. Here we use electrospinning to prepare a solid form of bevacizumab designed for prolonged release while maintaining antibody stability. Electrospun fibers were prepared with bevacizumab encapsulated in the core, surrounded by a poly-ε-caprolactone sheath. The fibers were generated using aqueous bevacizumab solutions buffered at two different pH values: 6.2 (the pH of the commercial product; F_{beva}) and 8.3 (the isoelectric point of bevacizumab; F_{bevaP}). The fibers had smooth and cylindrical morphologies, with diameters of *ca.* 500 nm. Both sets of bevacizumab loaded fibers gave sustained release profiles in an aqueous outflow model of the eye. F_{beva} displayed first order kinetics with $t_{1/2}$ of 11.4 ± 4.4 days, while F_{bevaP} comprises a zero-order reservoir type release system with $t_{1/2}$ of 52.9 ± 14.8 days. Both SDS-PAGE and surface plasmon resonance demonstrate that the bevacizumab in F_{bevaP} did not undergo degradation during fiber fabrication or release. In contrast, the antibody released from F_{beva} had degraded, and failed to bind to VEGF. Our results demonstrate that pH control is crucial to maintain antibody stability during the fabrication of core/shell fibers and ensure release of functional protein.

Keywords: coaxial electrospinning, bevacizumab, controlled release system, core-shell fibers, anti-VEGF, poly-ε-caprolactone

Statement of significance

Bevacizumab is a potent protein drug which is highly effective in the treatment of degenerative conditions in the eye. To be effective, frequent injections into the eye are required, which is deeply unpleasant for patients and expensive for healthcare providers. Alternative methods of administration are thus greatly sought after to produce more effective medicines. In our work, we use the electrospinning technique to prepare fiber-based formulations loaded with bevacizumab. By careful control of the experimental parameters we are able to stabilize the protein during processing and ensure a constant rate of release of the protein over two months. These fibers could thus be used to reduce the frequency of dosing required, reducing cost and improving patient outcomes.

Introduction

Antibody-based medicines targeted to vascular endothelial growth factor (VEGF) are widely used to treat a range of conditions in the eye. These include age related macular degeneration (AMD), the main cause of blindness in the elderly. AMD treatment involves direct injection of the antibody into the vitreous cavity of the eye (intravitreal injection). To be effective, intravitreal (IVT) injections are required every 4-8 weeks, which is considered too frequent as patients require treatment for many years [1], often decades. Further, IVT injections are invasive, carry some risk of retinal detachment and infection, and are also expensive to healthcare providers [2]. Other uses of antibodies in the eye include in aiding healing after injury or surgery. In the case of corneal injury, anti-VEGF antibodies can be applied to inhibit angiogenesis at the point of injury, thus helping to preserve sight. Surgery to make a small channel from the anterior chamber into the subconjunctival space is often required to reduce the intraocular pressure in patients with glaucoma, but frequently scarring occurs in the channel, causing the intraocular pressure to increase and glaucoma to progress. This scarring can be ameliorated through the use of antibodies. Thus, antibodies have enormous potential in treating conditions of the eye, but there remain a number of problems in their delivery: frequent injections to the eye are unpleasant, potentially dangerous, and expensive.

A slowly dissolving solid form of an antibody can potentially be used to slow antibody clearance from the vitreous cavity and subconjunctival space. There are a few clinically approved solid intravitreal ophthalmic drug implant technologies that have been developed for small molecules (*e.g.* steroids) [3], and some steroids are also administered as slow dissolving suspensions [4]. However, proteins are large, flexible molecules that are susceptible to aggregation, resulting in the loss of tertiary structure and function. Developing a solid dosage form designed for the slow dissolution and release of a stable functional antibody poses many challenges.

Bevacizumab is a monoclonal antibody that is widely used unlicensed in the clinic to treat AMD by IVT injection, as well as being potent when applied to the subconjunctival space to mediate healing after glaucoma surgery [5]. It also has a wide range of applications elsewhere in the body, and some sustained release formulations of bevacizumab have been reported (*e.g.* for cartilage tissue engineering [6] or brain tumor therapy [7]). There is a widespread ongoing research effort focused on

developing longer acting dosage forms of bevacizumab for use in the eye, with systems based on for instance hydrogels [8,9] and nanoparticles [10,11] having been reported.

Electrospinning (ES) is a one-step 'top-down' process used to fabricate functional nanomaterials, and which has much promise in the development of new drug delivery systems (DDS) [12]. ES has been extensively applied in various facets of biomedical research such as tissue engineering, wound dressings, imaging and anti-cancer therapeutics [13,14]. In the process, a volatile solvent is first used to dissolve a polymer and a functional component(s). This solution is then loaded into a syringe and ejected through a metal needle (spinneret) towards a metal collector. There is an electrical potential difference applied between the spinneret and collector, typically with a high positive voltage at the spinneret and the collector being grounded. As the ES solution flows through the spinneret, the ejected droplets are stretched as the electrical force overcomes the surface tension of the liquid [15]. This deforms the spherical droplet into a conical shape at the spinneret (the Taylor cone). A polymer jet is ejected from the tip of this cone towards the collector, and the solvent is rapidly evaporated as the jet moves away from the spinneret. This yields solid non-woven fibers on the collector.

Compared to conventional encapsulation routes, ES involves electrical evaporation rather than using elevated temperatures to remove solvent. It can thus avoid any thermally-induced degradation of therapeutic proteins. Aqueous protein solutions need to be used for ES, however, because proteins will unfold or denature when exposed to most organic solvents in all but very dilute concentrations (of both protein and organic solvent) [16]. A modified form of ES called coaxial ES can be used to overcome this problem: this approach uses two concentric needles, one nested inside another, as the spinneret. Coaxial ES can in principle permit the encapsulation of bioactive proteins with minimal contact with any organic solvent. This is achieved by separately feeding an aqueous protein solution through the inner needle and the polymer solution (in a volatile organic solvent) through the outer needle. The coaxial method can also overcome other challenges associated with single-fluid ES, which can include an initial burst of release and the random distribution of encapsulated molecules in the fiber matrices [17,18].

In this work, we have developed solid electrospun forms of bevacizumab which have the potential to be implanted in the eye, either in the vitreous cavity or subconjunctival space. Bevacizumab-loaded fibers were fabricated using coaxial

electrospinning, encapsulating the antibody as the core material inside a poly-ε-caprolactone (PCL) shell. The pH used in the core solution was varied to determine its influence on bevacizumab stability and the release of protein from the fibers. The latter was probed using a flow chamber that mimics aqueous outflow in the subconjunctival space.

Materials and methods

Materials

Poly-ε-caprolactone (PCL; 80 kDa), 2,2,2-trifluoroethanol (TFE), basic fuchsin, Dulbecco's phosphate buffered saline (PBS), fluorescein isothiocyanate isomer I (FITC), sodium bicarbonate, sodium carbonate monohydrate, Trizma® hydrochloride, Trizma® base, InstantBlue and human vascular endothelial growth factor ($VEGF_{165}$) were purchased from Sigma-Aldrich (UK). Bevacizumab (Avastin®, 25 mg/mL, Genentech, US) was obtained from clinical donations that remained after an appropriate dose had been administered to patients. MicroBCA protein assays, Pierce™ Silver Stain kits and PD-10 desalting columns were procured from Thermo Fisher Scientific (UK). Novex bis-tris 4-12% SDS-PAGE gels, NuPAGE MOPS SDS running buffer, Novex Sharp pre-stained protein standard and NuPAGE LDS sample buffer (4X) were obtained from Life Technologies (UK). BIAcore consumables (including an amine coupling kit for ligand immobilisation which contained N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), a regeneration scouting kit, and CM3 sensor chips) were sourced from GE Healthcare (UK). Vivaspin 6® centrifugal concentrators (molecular weight cut-off, MCWO 30 kDa) were purchased from VWR International (UK).

Methods

FITC-bevacizumab conjugation

FITC-conjugated bevacizumab (FITC-beva) was prepared to study its distribution in the fibers. Briefly, reconstituted bevacizumab solution (1.4 mg/mL, 2.5 mL) was prepared in conjugation buffer (bicarbonate buffer, pH 9, 100 mM) using a PD-10 desalting column to obtain the protein in the buffered solution (1.0 mg/mL, 3.5 mL). FITC in conjugation buffer (1 mg/mL, 175 μL) was incubated with the bevacizumab solution for 8 h at room temperature (RT, ~25°C) under gentle stirring and protected from light. The protein labeling ratio was 20:1 (1 mg protein: 0.05 mg

FITC). The reaction mixture was then purified using a PD-10 desalting column to eliminate unreacted FITC. The eluted fraction from the desalting column was subjected to size exclusion chromatography (SEC; Superose 12 10/300 GL column, Amersham Bioscience, US) using a mobile phase of PBS (pH 7.4) at a flow of 1.0 mL/min for 60 mins to purify the FITC-beva (see Supporting Information, **Figure S1**). The chromatographic system was equipped with Jasco (UK) HPLC systems including a pump (PU-980), AS-1555 autosampler and UV detector (UV-1570). Detection was performed at 280 nm (injection volume 300 μL). The HPLC systems was operated with Azur software version 5.0.10.0 (Kromatek, UK). The purified FITC-beva was centrifuged for 3 mins using a Vivaspin 6 centrifugal concentrator at 4000 rpm and stored at 4 °C prior to further use.

SDS-PAGE

The FITC-beva conjugation and purification processes were monitored using SDS-PAGE. In brief, 15 μL of each protein sample was mixed with 5 μL of NuPAGE® LDS sample buffer (4X) and loaded on to Novex bis-tris 4−12% SDS-PAGE gels. The gels were run at 200V for 55 mins and analysed under a UV lamp at 254 / 365 nm (UVLS-28EL series UV Lamp, UVP, UK) to identify the fraction containing fluorescently labeled bevacizumab (see **Figure S2**). Subsequently, the gels were stained with InstantBlue for 35 mins and destained with water for 2 h (**Figure S3**).

Preparation of bevacizumab loaded core shell fibers

Bevacizumab-loaded core-shell fibers were formulated using different pH conditions for the protein core solution, to investigate the impact of this on the fibers' properties. Reconstituted Avastin® solutions (pH 6.2, 12.5 mg/mL, 1.0 mL) and bevacizumab in Trizma® buffer (pH 8.3, 12.5 mg/mL, 1.0 mL, 50 mM) were prepared prior to ES. The PCL shell (see **Table 1**) and bevacizumab core solutions were then separately loaded into 5.0 and 1.0 mL plastic syringes respectively. The syringes were mounted to feed a stainless steel coaxial spinneret (inner / outer needle internal diameters: 0.5 / 1.0 mm). The spinneret was connected to a high voltage DC power supply (HCP 35-35000, FuG Elektronik, Germany). The solutions were ejected through the spinneret using two separate syringe pumps (78-9100C, Cole Parmer, UK) at constant flow rates. The fibers were collected on a grounded plate collector covered with aluminum foil.

Coaxial ES was performed using parameters as given in **Table 1**. After the ES process, each electrospun fiber mat (ca. 500 mg) was stored at 2-8 ^{o}C before further characterization.

Table 1

Scanning electron microscopy (SEM)

An approximately 0.5 × 0.5 cm^2 section of each fiber sample was sputter coated with a 20 nm gold layer (Q150T coater, Quorum, UK) and analysed with a field emission scanning electron microscope (Quanta 200 instrument, FEI, USA) connected to a secondary Everheart-Thomley electron detector. The average fiber diameter was measured by using the ImageJ software version 1.49 (National Institutes of Health, USA) to determine the fiber dimensions at ca. 150 points of measurement in three SEM images.

Transmission electron microscopy (TEM)

A small amount of fibers was collected on a TEM grid by spinning directly onto the grid. The samples were then analysed using a field emission transmission electron microscope (Philips/FEI CM 120 Bio-Twin, FEI, USA).

Digital microscopy

Fibers were collected onto a glass slide and analysed using an inverted digital microscope (EVOS XL Cell Imaging System, Thermo Fisher Scientific, UK).

Differential scanning calorimetry (DSC)

DSC analysis was conducted using a Q2000 instrument (TA Instruments, USA). Samples (approximately 1 mg) were prepared in Tzero aluminum pans (T130425, TA instruments, USA) and sealed with pin-holed aluminum lids. The samples were heated from -70 to 150 ^{o}C at a rate of 10 ^{o}C/min. The DSC instrument was purged with nitrogen gas at a flow rate of 50 mL/min throughout the measurements. The resultant data were analysed using the TA Universal Analysis software version 4.5A (TA Instruments, USA).

Thermogravimetric analysis (TGA)

TGA analysis was performed using a Discovery TGA instrument (TA Instruments, USA). Samples (approximately 1 mg) were heated at 30 ^{o}C for 2 h in open aluminum pans. The instrument was purged with nitrogen gas at a flow of 25.0 mL/min throughout. Data were analysed using the Trios software version 3.3.0.4055 (TA Instruments, USA).

Fourier-transform infrared spectroscopy (FTIR)

FTIR spectra of fiber samples (approximately 0.2 × 0.2 cm^2) were obtained using a Spectrum 100 spectrometer (Perkin Elmer, USA). The spectral data were analysed with the Essential FTIR v3.10.016 software (Operant LLC, USA). Data were collected over the wavenumber range from 650-4000 cm^{-1}, with resolution 1 cm^{-1} and 4 scans obtained.

Determination of protein distribution in core-shell fibers

The purified FITC-beva solutions (see Supporting Information, **Figure S2**) were prepared in phosphate buffer (pH 6.2) and Trizma® buffer (pH 8.3) using a PD-10 desalting column. Both solutions were then electrospun using the same method as described above. The resulting FITC-beva fibers were analysed using an inverted digital microscope, employing a GFP filtered-fluorescence mode in bright field, and TEM.

Encapsulation efficiency

Bevacizumab encapsulation efficiency was quantified using a modified method from the literature [19]. Approximately 20 mg of protein-loaded fibers was extracted with 0.5% w/v sodium dodecyl sulfate (SDS) in 10/90 v/v dimethyl sulfoxide (DMSO): water with shaking for 2 h at RT. The supernatant was then collected and analysed using the microBCA assay. Encapsulation efficiency is presented as a percentage the theoretical protein loading.

In vitro release study

Release studies on the F_{beva} and F_{bevaP} fibers were performed in an in-house flow rig model (see **Figure 1**). A cylindrical sample chamber with a diameter of 8.8 mm, thickness of 3.27 mm, and capacity of 200 μL was employed for these studies. The rigs were rinsed, cleaned and dried prior to each experiment. The model was disassembled by removing the screws. Approximately 52.0 mg (5.2 × 7.2 × 1.8 mm^3 of each sample

was placed in each of the rigs, which were then reassembled. All rigs used in the experiment were placed in a pre-heated oil bath at 37°C. An inlet portin the rigs allows a liquid flow similar to that in the front of the eye (2.0 μL/min) to be maintained [20,21], and a constant flow of PBS (pH 7.4, at 37°C) supplemented with sodium azide (0.05%) was provided using a 16-channel Ismatec peristaltic pump (Michael Smith Engineers Ltd, UK). An outlet port is present to allow easy sample collection at predetermined time points, and experiments were performed over 3 weeks and 2 months for F_{beva} and F_{bevaP}, respectively. The bevacizumab aliquots from each experiment were filtered with 0.22 μm Millex-GP syringe filter units (Fisher Scientific, UK) prior to quantification using the MicroBCA assay. The results are presented as percent cumulative release:

$$\% \text{ Cumulative protein release } = \frac{M_t}{M_¥} \times 100 \qquad \textbf{Equation 1}$$

where M_t is the cumulative amount of bevacizumab released at time t and $M_¥$ is the actual bevacizumab loading of each fiber. Further, the release profiles of the bevacizumab fibers were mathematically modeled using equations including the zero-order, first-order, and Korsmeyer-Peppas equations [22]

Figure 1

Silver staining SDS-PAGE

Selected aliquots from the release experiments were assessed by SDS page to determine the protein integrity at each timepoint. The protocol from the Pierce™ Silver Stain Kit was followed when gels were analysed by silver staining. The solutions required were (i) 30% ethanol : 10% acetic acid in distilled water, (ii) 10% ethanol, (iii) sensitiser working solution (50 μL sensitiser with 25.0 mL water), (iv) working stain solution (0.5 mL enhancer with 25.0 mL stain), (v) working developer solution (0.5 mL enhancer with 25.0 mL developer) and (vi) stop solution (5% acetic acid in distilled water). After Coomasie blue staining and destaining, the gel was thoroughly washed twice with ultrapure water for 5 mins and then the gel was fixed with 30% ethanol: 10% acetic acid solution for 15 mins. The gel was then washed twice with 10% ethanol and twice with water (5 mins per wash). After washing, the gel was incubated with the sensitiser working solution for 1 min and washed twice with ultrapure water (1 min each). The silver stain working solution was added to the gel, which was then incubated

for 30 mins. The gel was washed twice with ultrapure water (1 min each) before the developer working solution was added and the gel incubated until protein bands appeared (usually within 2-15 mins). When the required band intensity was achieved, the stop solution (5 % acetic acid) was added for 10 mins and the final gel was washed with water.

SPR Binding assay using Biacore

Human $VEGF_{165}$ (38 kDa) was immobilized on a CM3 chip to provide an immobilisation level of 50.4 RU. Briefly, the CM3 chip was first washed for 60 s with aqueous sodium hydroxide (50.0 mM). The surface of the chip was then activated for 200 s with NHS (300 μL) / EDC (300 μL), followed by immersion in a solution of VEGF (0.1 μg/mL) in acetate buffer (pH 5.5) for 150 s. The active groups on the surface of the CM3 chip were subsequently deactivated with ethanolamine-HCl for 180 s.

Samples were first quantified by microBCA to determine the protein concentrations, and the final concentrations to be tested were prepared in HBS-EPS buffer (containing 10.0 mM HEPES at pH 7.4, 150 mM NaCl, 3.0 mM EDTA, and 0.005% P20) for BIAcore. Binding assays were performed at 25 °C at a flow rate of 30.0 μL/min, with association and disassociation times of 180 and 1200 s respectively and regeneration with glycine-HCl (pH 2.0) for 30 s. All data generated by BIAcore were evaluated with the BIAevaluation software version 2.1 (GE Healthcare, Sweden). The binding affinity was assessed by examining the relative response value (RU) obtained from the software.

Statistical analysis

Quantitative data are presented as mean ± standard deviation, and have been statistically analysed using the Prism software version 6.0 (GraphPad, USA). Unpaired two-way T-tests were performed to calculate p values for comparisons between two groups. A significant difference is defined when $p < 0.05$. Half-lives ($t_{1/2}$) were calculated according to the best fitting model in Prism. First-order kinetic rate constants (k) were derived from the monoexponential curve and $t_{1/2}$s calculated as $0.693/k$. The rate constants (k) of zero-order release profiles were calculated as concentration–time and $t_{1/2}$s were obtained from the initial concentration [A] as $[A]/2k$.

Results

Preparation and characterisation of bevacizumab-loaded core-shell fibers

Fiber morphology

Bevacizumab (beva)-loaded PCL core-shell fibers were fabricated using the optimized parameters shown in **Table 1.** A 90% v/v TFE: deionized water solution was found to be the optimum shell solvent to obtain reproducible core-shell fibers. The water co-solvent was used as this can lower the interfacial tension between the PCL shell and bevacizumab core fluids, facilitating the fabrication of core-shell fibers [23].

We also varied the pH of the protein core solution, as this has been reported to have an effect on the distribution of the encapsulated molecules in the fibers [24] and can influence the release mechanism and the stability of the protein guest. Fibers were first prepared in buffer at pH 6.2 (F_{beva}), which is the pH of the reconstituted Avastin® solution [25]. A second set of fibers was also fabricated at pH 8.3 (F_{bevaP}), the isoelectric point of bevacizumab [26,27]. SEM images of the fibers are shown in **Figure 2a and b.** Both sets of fibers show relatively smooth uniform structures, although there are some surface wrinkles visible in F_{bevaP}. The diameter of F_{beva} was significantly larger than that of F_{bevaP} (520 ± 120 nm vs 469 ± 83 nm, $p < 0.05$;see **Figure 2c and d)**.

The spatial location of bevacizumab in the fibers was probed by conjugating FITC to bevacizumab (**Figure 2e and f**). The F_{beva} fibers display greater fluorescence intensity than the F_{bevaP} materials, suggesting there is a greater tendency for the protein to migrate to the fiber shell when the core solution has a pH of 6.2. The TEM results (**Figure 2g and h**) indicate the F_{bevaP} system has a clearly defined core/shell structure, while this is irregular and discontinuous with F_{beva}. The bevacizumab in F_{beva} (pH 6.2) has a net positive charge, but in F_{bevaP} (pH 8.3) it will be neutral. Clearly, the charge of the protein during ES has a profound effect on the protein distribution in the resultant fibers.

Figure 2

Physical characterisation

TGA analysis was used to analyze both sets of fibers after heating at 30 ºC in isothermal mode. This temperature is lower than the melting point of PCL [28] and the

degradation temperature of bevacizumab [29]. Therefore, degradation of the two materials is thus unlikely to occur during the TGA experiment. The thermograms (see **Figure S4**) show the mass loss was around 0.01% in both fiber sets, ten times lower than the sensitivity of the instrument (± 0.1% mass change; Discovery TGA User Manual [30]) throughout the heating process. This means that the mass remained unchanged during heating, suggesting that both TFE and water from the shell and core compartments were completely evaporated during the ES process. Further physical characterisation of the bevacizumab fibers by DSC and FTIR (**Figure S5 and S6**) showed only the characteristic peaks of the PCL material, and the antibody signal was obscured. This is attributed to the low loading of the antibody in both fiber formulations, which results in the protein signals being overwhelmed by those from the polymer.

Encapsulation efficiency and in vitro release study

Prior to investigating the release kinetics, the encapsulation efficiencies of F_{beva} and F_{bevaP} were determined. The theoretical loading was approximately 24 μg bevacizumab in 1 mg of the fabricated fibers. The encapsulation efficiency of F_{beva} was 72.64 ± 1.05 % (approx. 908 μg bevacizumab), significantly higher than that observed for F_{bevaP} (63.15 ± 0.30%; approx. 789 μg bevacizumab; $p < 0.05$).

Release studies were conducted in an in house *in vitro* flow rig model [31]. A constant flow rate of 2.0 μL/min was used to mimic the rate of aqueous turnover at the front of the eye [32,33]. The release media was supplemented with 0.05% sodium azide to prevent microbial growth. This apparatus was selected over the USP I or IV instruments because it more closely mimics the relevant parts of the eye. The microBCA assay was used to quantify the amount of bevacizumab in each aliquot collected and to calculate the cumulative release (see **Figure 3**). The dimensions of the fiber samples was equivalent to a 68 μL volume, which is approximately three times smaller than the sample chamber.

Figure 3

Sustained release profiles were observed with both formulations throughout the study. F_{beva} exhibited a $t_{1/2}$ of 11.4 ± 4.4 days with cumulative release of 60.6 ± 7.3% (*ca.* 662 μg) of the actual loading over 19 days (**Figure 3a**). The F_{bevaP} formulation

displayed a much more prolonged release profile, with $t_{1/2}$ of 52.9 ± 14.8 days (p = 0.0096). The amount of bevacizumab released from F_{bevaP} was 55.6 ± 16.8% (*ca.* 439 μg) after 60 days (**Figure 3b**). Conversely, the Avastin® solution was tested in the *in vitro* flow rig using the same dose (1.25 mg in 200 μl phosphate buffer) and displayed a much shorter $t_{1/2}$ of ~ 4 h (see **Figure S7)**

Considering the profiles in more detail, it is clear that the concentration profile of F_{beva} is a biphasic release profile beginning with a burst phase over the first day (see **Figure 3c**). After 24 h, we observed a bevacizumab concentration of 60.04 ± 45.48 μg/mL (248 μg, accounting for 24.6 ± 19.3% of the total release). This is followed by a close-to-constant rate of release with the concentration ranging from 5.29-17.00 μg/mL after 1 week of release and also over the remainder of the study period. Conversely, F_{bevaP} displayed a continuous, monophasic, release profile over two months, with the concentration of the antibody ranging from 0.78 ± 0.34 to 7.38 ± 6.67 μg/mL (**Figure 3d**).

The $t_{1/2}$ of bevacizumab clearance in a similar flow model has been found to be 1.2 ± 0.1 days in PBS, or 10.7 ± 0.7 days in simulated vitreal fluid [34]. Its clearance from the vitreous cavity in humans has been reported to have a $t_{1/2}$ of 4.9-10 days [32–36]. Both the formulations prepared in this work are able to extend the residence time of the antibody considerably. As convective flow is responsible for drug elimination from the aqueous humor [37], the longer $t_{1/2}$ observed with the fibers over a bevacizumab solution suggests that the fibers can overcome rapid clearance by the aqueous flow in the eye. The bevacizumab-loaded fibers therefore have the potential to reduce very significantly the frequency of treatment required.

To elucidate the drug release mechanism, the release profiles were fitted with mathematical models. We hypothesized that drug diffusion would be a key mechanism contributing to the release of bevacizumab from the fibers. Given the slow hydrolytic degradation of PCL [28], it is unlikely that this polymer would undergo complete degradation in the conditions used to study release. Correlation coefficients (R^2), slopes after linear regression, release rate constants (k) and other kinetic parameters obtained are given in **Table 2** (kinetic fitting plots are provided in the Supporting Information, **Figures S8-S13**).

It is evident that the release profiles of both the F_{beva} and F_{bevaP} fibers are governed by diffusion controlled mechanisms, as indicated by R^2 being close to 1 for diffusion-release models. The kinetic profile of F_{beva} was best fitted with a first-order

equation (R^2 = 0.99). This is consistent with the results reported for most drug-loaded PCL fiber formulations, such as those containing tetracycline hydrochloride [38], bovine serum albumin [39], or alkaline phosphatase [19]. In contrast, the release profile from F_{bevaP} displayed zero-order kinetics, which is highly desirable in the design of controlled release systems. A zero-order release profile is very difficult to achieve with electrospun systems, because there is usually a significant burst release of drug in the initial stages of the process [40].

Table 2

To gain further understanding of the release behavior, we employed the Korsmeyer-Peppas (or Ritger-Peppas) equation [41]. The n exponent in this model gives information on the release phenomena from different geometrical dosage forms. It can be assumed that the release mechanism of polymeric systems follow Fick's law, in which drug diffusion is concentration-gradient dependent [42]. In the Korsmeyer-Peppas model, ideal Fickian diffusion is observed when n = 0.5 for a thin film, 0.45 for a cylindrical geometry, and 0.43 for a sphere; polymeric swelling is predominant when n is equal to 1.0, 0.89 and 0.85, respectively.

The n exponents from F_{beva} and F_{bevaP} were 0.60 and 0.82, respectively. These values are between 0.45 and 0.89, which is indicative of non-Fickian diffusion (given that the fibers are cylindrical), suggesting a more complex drug release mechanism is operational here. However, care should be taken with interpretation because the Korsmeyer-Peppas model is applicable only for systems with homogeneous drug distribution, which it is not the case in F_{bevaP} (as is clear from the TEM images). Srikar *et al.* [43] proposed that the desorption of solute from nanopores generated during the ES process is a key release mechanism for PCL nanofibers. This is consistent with the result from Gandhi's study [44] which demonstrated a similar mechanism for the release of protein from PCL fibers. We believe that this is also the case for both bevacizumab fiber formulations explored in this study, since our findings are wholly consistent with such a model. However, further investigation is required to unravel the details of the bevacizumab release mechanism from the PCL core-shell fibers.

In vitro stability of encapsulated bevacizumab

The bevacizumab released from the *in vitro* flow rigs was evaluated by SDS-PAGE with silver stain detection. Aliquots from days 1, 5, 10, 15 and 19 of the F_{beva} release study, and those from days 1, 5, 10, 15, 20, 30, 40, and 60 from the F_{bevaP} release study were evaluated (**Figure 4**)**.** Bevacizumab showed a band at approximately 150 kDa (see control lane, **Figure 4**). Reconstituted Avastin® solution displayed aggregates (band > 160 kDa), intact bevacizumab (150 kDa) and fragmented bevacizumab (< 160 kDa; lane 1). A trace amount of antibody fragment was also present at 50 kDa, along with other unidentified fragments. This indicates that the bevacizumab molecules underwent chemical degradation during incubation [45]. Similar degradation profiles were observed from all fractions released from F_{beva} (see lane 2-6, **Figure 4**). The F_{beva} fibers hence do not appear to stabilize bevacizumab. However, all the release fractions from the F_{bevaP} formulation displayed only the band for the intact antibody at 150 kDa (see lanes 7-14, **Figure 4**). There did not appear to be any protein fragmentation or aggregation here, and F_{bevaP} thus successfully maintains the stability of the antibody.

Figure 4

Preliminary binding screening using surface plasmon resonance (SPR) was used to determine if the released bevacizumab would bind to immobilized VEGF (50.4 relative response (RU)). The response value was calculated from the sensorgram; a positive RU value means that bevacizumab underwent binding to VEGF, whereas a zero or negative value indicates no binding. Non-specific binding from the buffer (HPS-EPS; negative control) should also give a RU value of zero.

The binding results are given in **Figure 5**. For F_{beva}, positive RU values were observed in the samples collected up to day 10, but RU values close to zero or negative were obtained at day 15 and 19 (see **Figure 5a**). Thus, only the samples collected in the first 10 days of release retained their ability to bind VEGF. In contrast, all aliquots from the F_{bevaP} study showed positive RUs (**Figure 5b**). Indeed, a quantitative binding analysis showed that the average RU remains approximately constant from the samples collected after 30, 40 and 60 days of F_{bevaP} release (see **Figure 5c**). The F_{bevaP} formulation hence retains its anti-VEGF activity throughout the release period. The SPR results are in good agreement with the SDS-PAGE analysis in the case of F_{bevaP},

confirming that the bevacizumab molecules remain intact over two months of release. However, the SPR and SDS-PAGE results for F_{beva} are more complicated as there is a combination of intact, aggregated and fragmented antibody in the collected aliquots (**Figure 4**), and the fragments could contribute to antibody binding in the SPR study if the binding site was not cleaved [45]. Even though some fractions from F_{beva} show affinity, the inconsistent aggregation and fragmentation of bevacizumab visible in SDS-PAGE raises concerns regarding this formulation.

Figure 5

Discussion

The results presented above clearly highlight that the pH of the protein solution used for ES has profound effects on the resultant fiber properties (F_{beva} *cf* F_{bevaP}). pH changes cause the net charge of bevacizumab to change, which affects the migration of the protein from the core to the shell fluid during ES. As the isoelectric point of bevacizumab is 8.3 [26,27], the protein in F_{beva} was positively charged at pH 6.2. There will be no net charge on the antibody when ES was conducted with Trizma buffer at pH 8.3. Tang *et al*. reported that the migration of macromolecules is driven by dielectrophoretic movement in the non-uniform electrical field generated during the ES process [24]. This means that any polarisable species may migrate toward the area where the strongest field is present, regardless of its electrostatic charge.

Coaxial ES is more complicated than single-fluid ES because a compound Taylor cone is developed. Luo and Edirisinghe [46] pointed out that charges can localize at the external interface between the shell polymer and air, creating an electrical field. This means that the outer cone will possess higher electric field intensity than the inner cone. Thus, it is expected that more charged macromolecules would accumulate in the shell than the core. In contrast, neutral moieties would be localized in the core. It can hence be hypothesized that bevacizumab will migrate to the shell in the spinning of F_{beva} fibers, whereas in F_{bevaP} the protein would be completely encapsulated in the core.

The charged protein at pH 6.2 can therefore migrate to the PCL fluid during Taylor cone development, and become immobilized there upon fiber solidification (see **Figure 6a**). This results in an uncontrolled distribution of bevacizumab throughout the

F_{beva} fibers, which may contribute to the release behavior of the fibers being similar to a monolithic system. In contrast, neutral bevacizumab at the pI was localised in the core of the F_{bevaP} fibers (**Figure 6b**), resulting in zero-order release kinetics consistent with reservoir systems reported in the literature [47]. The results from this study thus show that zero-order release devices can be fabricated using coaxial ES by controlling the charge on the active ingredient being explored. This approach could be applied widely to other therapeutic payloads, where zero-order systems are highly desirable [40].

Figure 6

The protein distribution proposed for the F_{beva} and F_{bevaP} fibers also explains the observed stabilities. Some positively charged bevacizumab in F_{beva} may be exposed to TFE in the shell fluid upon migration during Taylor cone formation. This could in turn lead to the protein becoming unfolded. Despite the possible unfolding mediated by TFE [48], however, the loss in structural integrity observed in this study is mostly ascribed to heavy-light chain fragmentation, as evidenced by the SDS-PAGE analysis. We assume that some bevacizumab molecules exposed to TFE were unfolded and immobilized in the polymer shell after ES. After elution and diffusion out of the fibers, the unfolded state of these proteins facilitated degradation during the release study. In contrast, the neutral bevacizumab remained in the core solution during F_{bevaP} fabrication, thus precluding exposure to TFE (see **Figure 6b**). Therefore, the antibody remained intact after fabrication, and during the release study no fragmentation was detected in SDS-PAGE. Electrospinning at the pI of the antibody thus clearly improves the in-process stability of therapeutic proteins.

It should be noted that the encapsulation efficacy is another attribute influenced by the charge of the encapsulated bevacizumab. As previously described, charge generation on the polymer surface arising during ES contributes to the transportation of charged and uncharged species in the spinning fluid. Positively charged species are repelled from the inner surface of the needle and migrate toward the grounded electrode whereas neutral species remain in the bulk [15]. In our study, the positively charged protein in F_{beva} results in more bevacizumab being embedded in the polymer shell while the neutral species present during production of F_{bevaP} does not appear to be subject to such forces. This leads to higher encapsulation efficacies with F_{beva} than F_{bevaP}. However, further investigation is required to elucidate in more detail the effects of

charge transport on the encapsulation efficacy, as this is a complex process and there is an interplay of several different factors which should be taken into account.

Our intent for this study was to prepare formulations for use intraocularly. The importance of the subconjunctiva part of the eye in this context relates to the clinical observation that the action of bevacizumab mediates healing after glaucoma surgery [5]. To reduce the intraocular pressure in patients with glaucoma, surgery is conducted to make a small channel from the anterior chamber (front of the eye) into the subconjunctival space, to allow the aqueous outflow to drain into the conjunctiva and the circulation. Often scarring occurs in this channel, blocking the aqueous outflow and causing the intraocular pressure to increase and glaucoma to progress. In previous work, we developed an implantable tablet form of bevacizumab that has been evaluated in the flow rig used in this study [49]. We found that the *in vitro* release results correlated with local pharmacokinetics in experimental glaucoma surgery, and the tablets were able to effectively mediate healing. The fibers prepared in this work have the potential to prolong bevacizumab release for a longer period of time than our existing formulation, and thus should result in further improvements to patient outcomes. Additionally, we have found that bevacizumab mediates healing after injury to the cornea, and placement of fibers near the point of injury in the cornea would help to maintain local bevacizumab concentrations, inhibiting angiogenesis within the cornea and preserving a patient's sight.

Our fibers thus have a wide range of potential applications as ocular implants. Since the fibers have very high surface areas, their degradation will be much more rapid that solid PCL implants (which last for *ca.* 3 years in vivo). PCL is FDA-approved, and its degradation products are safe in humans. Thus, in the case of one-shot administration of the formulations (*e.g.* to prevent scarring after corneal injury) the fibres could safely be left in place to degrade after their therapeutic effects had been exhausted. In the case of multiple administrations, for instance for AMD treatment, then the surgeon could remove the currently placed implant and replace it with a fresh one. The *in vivo* performance of our materials will be explored in future work.

Conclusions

In this study sustained release systems for bevacizumab were fabricated using coaxial electrospinning. Core/shell systems were generated with an aqueous protein solution forming the core and PCL as the shell polymer. The pH of the core solution

(6.2 or 8.3 (the pI of bevacizumab)) used for fabrication was varied and found to affect profoundly the release mechanism and stability of the protein in the fibers. While both sets of fibers were cylindrical in shape, those prepared at pH 6.2 did not have a clear core/shell structure, and it is believed that some of the protein migrated to the shell during electrospinning. In contrast, the materials generated at pH 8.3 had very distinct core and shell compartments. Sustained release profiles were seen from both sets of fibers. However, the release behavior of the fibers formed at the pI follows zero order kinetics, while those prepared at the lower pH show an initial burst release and first order kinetics. Moreover, electrospinning at the protein pI enhances its stability during release. Therefore, coaxial electrospinning shows great promise for the design of novel prolonged protein release devices. In the example system explored in this work, developing sustained release anti-VEGF formulations could profoundly improve patient health and wellbeing by reducing the requirement for frequent invasive dosing.

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Tables

Table 1 Coaxial ES parameters for the preparation of bevacizumab loaded core-shell fibers.

Parameters	F_{beva}	F_{bevaP}
Core solution	Reconstituted Avastin® solution in phosphate buffer (pH 6.2, 12.5 mg/mL)	Bevacizumab in Trizma ® buffer (pH 8.3, 12.5 mg/mL)
Shell solution	10% PCL in 90% v/v TFE: deionized water	
Sheath/core flow rates	1.5 : 0.3 mL/h	
Spinneret to collector distance	16 cm	
Voltage	15 kV	

Table 2 Kinetic parameters for bevacizumab release from the fibers.

Sample	Release paramaters			Korsmeyer-Peppas		
	k	$t_{1/2}$ **(days)**	R^2	**n**	k_{kp}	R^2
F_{beva}	0.13[a]	5.9[a]	0.99[a]	0.60	13.30	0.99
F_{bevaP}	0.84[b]	62.5 [b]	0.99[b]	0.82	1.73	0.99

Figure captions

Figure 1 A schematic diagram illustrating the home-made dynamic flow cell apparatus used for dissolution studies. A dispensing pump continuously supplies a sample chamber with PBS buffer (pH 7.4, supplemented with 0.05% sodium azide) at flow rate of 2.0 μL/min. The chamber is placed into a pre-heated oil bath to maintain the temperature at 37 °C. Aliquots were removed from the outlet tube at predetermined time points for further analysis.

Figure 2 SEM images of **a)** bevacizumab-PCL core/shell fibers spun with sodium phosphate buffer, pH 6.2 (F_{beva}) and **b)** bevacizumab-PCL core/shell fibers spun with Trizma buffer at pH 8.3 (F_{bevaP}), together with the size distributions of **c)** F_{beva} and **d)** F_{bevaP} (mean ± SD), and fluorescent microscopy images of **e)** F_{beva} and **f)** F_{bevaP} prepared with FITC-beva. TEM images illustrating the internal structures are also given for **g)** F_{beva} and **h)** F_{bevaP} fibers. Arrows indicate the encapsulated bevacizumab in the fibers.

Figure 3 Cumulative release profiles of **a)** F_{beva} (n=3) and **b)** F_{bevaP} (n=3) in a rig model mimicking the aqueous turnover of the anterior segment of the human eye. The inset in b) shows the release profile in the first 5 days of the release experiment of F_{bevaP}. Also depicted are concentration vs time profiles of **c)** F_{beva} and **d)** F_{bevaP}. Data are shown as mean ± SD.

Figure 4 Silver staining of a Novex Bis-Tris 4-12% gel loaded with bevacizumab collected from release studies (lane 1-14). *Lane M*: molecular weight standard; *Control*: freshly-prepared Avastin® solution; *lane 1*: Avastin® solution after incubation in the flow rig; *lanes 2-6*: F_{beva} at day 1, 5, 10, 15 and 19 respectively; and *lanes 7-14*: F_{bevaP} at day 1, 5, 10, 15, 20, 30, 40 and 60 respectively.

Figure 5 SPR results showing VEGF binding by bevacizumab, compared with HPS-EPS buffer (negative control). Preliminary data are shown for bevacizumab released from **a)** F_{beva} (n = 1) and **b)** F_{bevaP} (n = 1), together with **c)** a detailed SPR binding analysis of aliquots collected from F_{bevaP} at day 40, 50 and 60, with the concentration of bevacizumab quantified by the MicroBCA assay (n=3; data presented as mean ± SD).

Figure 6 A schematic diagram illustrating the effect of bevacizumab migration during electrospinning on the fiber properties. **a)** During the fabrication of F_{beva}, the protein carries a net positive charge at pH 6.2; this causes it to migrate uncontrollably from the aqueous core to the polymer shell and become unfolded upon exposure to TFE. The resultant distribution of both intact bevacizumab and unfolded bevacizumab within the F_{beva} core and shell contributes to the first-order release behavior observed. **b)** In contrast, the uncharged protein remains in the aqueous core for F_{bevaP} (prepared at the pI, pH 8.3), which leads to zero-order release and intact bevacizumab.