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Research Paper

Protein-loaded emulsion electrospun fibers optimized for bioactivity retention and pH-controlled release for peroral delivery of biologic therapeutics

Hannah Frizzell, Tiffany J. Ohlsen, Kim A. Woodrow*

Department of Bioengineering, University of Washington, 3720 15th Ave NE, Seattle, WA 98195, USA

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ABSTRACT

Biologics are the most rapidly growing class of therapeutics, but commonly suffer from low stability. Peroral administration of these therapeutics is an attractive delivery route; however, this route introduces unique physiological challenges that increase the susceptibility of proteins to lose function. Formulation of proteins into biomaterials, such as electrospun fibers, is one strategy to overcome these barriers, but such platforms need to be optimized to ensure protein stability and maintenance of bioactivity during the formulation process. This work develops an emulsion electrospinning method to load proteins into Eudragit® L100 fibers for peroral delivery. Horseradish peroxidase and alkaline phosphatase are encapsulated with high efficiency into fibers and released with pH-specificity. Recovery of protein bioactivity is enhanced through reduction of the emulsion aqueous phase and the inclusion of a hydrophilic polymer excipient. Finally, we show that formulation of proteins in lyophilized electrospun fibers extends the therapeutic shelf life compared to aqueous storage. Thus, this platform shows promise as a novel dosage form for the peroral delivery of biotherapeutics.

1. Introduction

While the development of biological products has increased dramatically in the last three decades, the delivery of protein therapeutics remains particularly challenging (Mitragotri et al., 2014). Proteins are large macromolecules that require a precise 3D structure to carry out their function. Due to this structural complexity, loss of protein activity can occur in response to a variety of factors that cause protein stress and changes in conformation such as temperature, pH, ultraviolet light, and interaction with organic solvents. In the clinical application of such biopharmaceutics, the route of administration introduces a variety of these unique challenges. Peroral administration, through swallowing of a solution or pill, is one of the most attractive routes for therapeutic delivery, as patient comfort is increased, the use of needles is eliminated, and local delivery to the gastrointestinal tract can be achieved, reducing systemic side effects (Levine and Dougan, 1998). However, peroral delivery of proteins is difficult due to the inherent barrier of the acidic environment of the stomach, which can result in protein degradation, leading to low bioavailability and the need for multiple doses (Goldberg and Gomez-Orellana, 2003; Pawar et al., 2014). Recently, a variety of nanotechnology platforms utilizing biomaterials have been developed to overcome these challenges in biologic formulation and peroral delivery to the gut (Gupta et al., 2013; Yu et al., 2016).

Electrospinning is a technique that has been explored in the pharmaceutical nanotechnology field to develop materials as scaffolds for tissue engineering and for the delivery of small molecule therapeutics (Sill and von Recum, 2008). The electrospinning platform has many advantages compared to other formulations such as particulates, films, and tablets, including the versatility of polymers that can be employed to tailor release kinetics and therapeutic targeting for specific applications, high encapsulation efficiency, which is critical when loading high-cost therapeutics, high surface-area-to-volume ratio for enhanced interaction with the tissue of interest, and ease of production (Agarwal et al., 2008; Hu et al., 2014; Sill and von Recum, 2008). In the last 10 years, electrospun nanofibers have been investigated for the delivery of larger, less stable therapeutics such as protein biologics and have proven to be advantageous in terms of controlled delivery and enhancing protein stability (Ji et al., 2011). While protein encapsulation into nanofibers has been primarily developed for the production of bioactive scaffolds, we expect this platform holds promise for the targeted delivery of proteins to the gut with the proper design and optimization.

Proteins can be loaded into electrospun nanofibers by a method called emulsion electrospinning, which forms core-shell nanofibers

* Corresponding author.
E-mail address: woodrow@uw.edu (K.A. Woodrow).

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from an emulsion comprised of an organic phase (containing polymer), surfactant, and aqueous phase (containing protein) (Yarin, 2011). In the emulsion electrospinning process, a high voltage is applied to the solution as it is pumped out of a needle and syringe until electrostatic forces overcome droplet surface tension. This results in the formation of a Taylor cone and the ejection of a polymer jet that is electrically charged (Reneker and Chun, 1996; Yarin et al., 2001). As this charged jet moves through an electric field to be deposited onto a grounded collector as elongated fibers, the organic solvent evaporates more quickly than water and the emulsion droplets move inward and are stretched to form fibers with a continuous core surrounded by a viscous polymer shell (Sanders et al., 2003; Shastri et al., 2009). This architecture is advantageous for enhancing protein stability and controlled protein release compared to blend electrospinning, which directly exposes proteins to organic solvents and exhibits burst release kinetics (Ji et al., 2011). However, since in emulsion electrospinning proteins in the aqueous phase may still interact with organic solvents throughout the process, maintenance of protein bioactivity and stability must be optimized to prevent loss of protein structure, and thus function. In many studies that load non-enzymatic proteins into electrospun fibers, function is demonstrated but protein activity is not able to be quantified (Briggs et al., 2015; Choi et al., 2015; Hu et al., 2016; Li et al., 2010; Wang et al., 2015), and only a few studies have investigated the effects of emulsion electrospinning solution and processing parameters on maintaining protein bioactivity (Briggs and Arinzeh, 2014; Pinto et al., 2015; Place et al., 2016). Learning from these studies, it is clear that many parameters can significantly affect protein loading and activity and that the main challenges of high protein loading efficiency and bioactivity preservation still remain. In particular, previous studies have elucidated that each delivery system must be optimized based on electrospinning method, processing parameters, and the polymers and proteins used to ensure appropriate formulation of active, stable proteins in nanofibers. Additionally, the stability of therapeutic proteins loaded in electrospun fibers over time has not been widely studied. This shelf life is critical to evaluate, understand, and improve for the translation of such protein delivery platforms to clinical use.

The methacrylic acid and methyl methacrylate co-polymer Eudragit® L100 has been used in clinical settings as a capsule for the peroral delivery of therapeutics as it demonstrates pH-dependent solubility in neutral and basic conditions. This polymer has been applied to electrospinning and Eudragit®-based nanofibers have been used for small molecule drug delivery, stent coatings, antibacterial mats, and contrast agents for imaging (Aguilar et al., 2015; Bruni et al., 2015; Hamori et al., 2014; Jin et al., 2016; Shen et al., 2011). In the space of protein therapeutics, one study loaded human growth hormone into Eudragit® fibers using blend electrospinning for buccal drug delivery and demonstrated successful fabrication and wound healing function, but did not investigate how protein function was affected by the process (Choi et al., 2015).

The encapsulation of more diverse proteins, such as larger protein antigens or complex therapeutic antibodies, into Eudragit® fibers has not been investigated nor has the emulsion electrospinning of protein-loaded Eudragit® fibers, which can address problems in loss of protein function during formulation. The development of such a system would be advantageous for the delivery of proteins to targets in the intestinal tract, either to improve patient comfort in therapeutic administration, to access the mucosal immune system, or to address intestine-specific diseases (Date et al., 2016; Davitt and Lavelle, 2015; Ilan, 2016). Thus, in this study we aimed to optimize the electrospinning of two diverse proteins into Eudragit® L100 nanofibers. Specifically, we investigated the solution and processing parameters of emulsion electrospinning to achieve high protein loading into nanofibers, retention of protein bioactivity, extended formulation stability, and pH-controlled protein release for future applications in peroral therapeutic delivery to the intestinal tract.

2. Materials and methods

2.1. Materials

Eudragit® L100 was a gift from Evonik Industries (Essen, Germany). *N,N*-Dimethylformamide (DMF) (anhydrous, 99.8%), poly(vinyl alcohol) (PVA) (30,000–70,000 g mol^{-1}, 87–95% hydrolyzed), Tween® 20 (polysorbate 20), albumin–fluorescein isothiocyanate conjugate (FITC-BSA), alkaline phosphatase (AP) from bovine intestinal mucosa (≥10 DEA units mg^{-1} solid), sodium hydroxide (NaOH) (50% in H_2O), alkaline phosphatase yellow (pNPP) liquid substrate system, and magnesium chloride ($MgCl_2$) (anhydrous, ≥98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Horseradish peroxidase (HRP), Micro BCA™ Protein Assay Kit, and Karl Fischer titration reagents AQUASTAR™ CombiMethanol and Combititrant 5 were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Hydrochloric acid (HCl) (36.5%-38.0%) was purchased from Avantor Performance Materials (Center Valley, PA, USA). Dulbecco's Phosphate-Buffered Saline (PBS) was purchased from Corning Inc. (Corning, NY, USA). TMB substrate set was purchased from BioLegend (San Diego, CA, USA). Tris-HCl (Tris-hydrochloride, molecular biology grade) was purchased from Promega (Madison, WI, USA). Sodium chloride (NaCl) was purchased from EMD Millipore (Gibbstown, NJ, USA). Ethanol (EtOH) (200 proof) was purchased from the University of Washington's chemistry supply store.

2.2. Emulsion electrospinning

Eudragit® L100 was dissolved at 15% (wt/vol) in a solution of 4:1 (vol:vol) EtOH:DMF. Non-ionic surfactant Tween® 20 was added to the stirring Eudragit® solution at 0.4% (vol/vol) in 20 mL glass scintillation vials. Aqueous protein solutions were dropped into the stirring Eudragit® and surfactant solution at either 5% or 20% (vol/vol). Protein loading was kept constant at 1 wt%. The solution was emulsified for 10 min by constant stirring at high speed then loaded into a 1 mL glass syringe with a 21G stainless steel blunt needle for electrospinning on a needle rig set-up utilizing a syringe pump and voltage source. We chose high speed stirring over sonication for emulsification, as sonication has been shown to have an effect on secondary protein structure (Yang et al., 2008a). The solution was electrospun onto a wax paper substrate on a grounded metal collector at a distance of 10–12 cm. A constant voltage of 12.5 kV was applied and the flow rate was varied between formulations to determine its effect on protein bioactivity. Low and high flow rate formulations were electrospun at 25 and 40 μL/min, respectively. Electrospinning was performed at room temperature in a fume hood. Protein-loaded fibers were stored in sealed bags at room temperature unless stated otherwise.

2.3. Characterization of protein-loaded electrospun fibers

2.3.1. Microscopy of protein-loaded electrospun fibers

Electrospun fibers were characterized using scanning electron microscopy (SEM) using a Sirion XL30 SEM and transmission electron microscopy (TEM) using a Tecani G2 F20 Supertwin operating at 200 kV (Molecular Analysis Facility, University of Washington). For SEM imaging, fiber samples were mounted on carbon tape and sputter coated with gold and palladium before imaging. Image settings of 5 kV, spot size of 2, and a working distance of 5 mm were used. Fiber diameters were measured using ImageJ software (NIH) by bisectioning each image diagonally with a line and manually measuring diameters for fibers that intersected the line. At least 30 fibers were measured per fiber formulation sample. Samples for TEM imaging were prepared by electrospinning fibers onto copper-coated TEM grids.

The location of protein within the fibers was determined by electrospinning FITC-BSA into Eudragit® L100 as described above. Briefly, FITC-BSA was dissolved in PBS at a concentration of 1 mg mL^{-1} and dropped into a spinning solution of 15 wt% Eudragit® L100 in 4:1

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