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Bioactive electrospun fish sarcoplasmic proteins as a drug delivery system

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ABSTRACT

Nano-microfibers were made from cod (*Gadus morhua*) sarcoplasmic proteins (FSP) (M_w < 200 kDa) using the electrospinning technique. The FSP fibers were studied by scanning electron microscopy, and the fiber morphology was found to be strongly dependent on FSP concentration. Interestingly, the FSP fibers were insoluble in water. However, when exposed to proteolytic enzymes, the fibers were degraded. The degradation products of the FSP fibers proved to be inhibitors of the diabetes-related enzyme DPP-IV. The FSP fibers may have biomedical applications, among others as a delivery system. To demonstrate this, a dipeptide (Ala-Trp) was encapsulated into the FSP fibers, and the release properties were investigated in gastric buffer and in intestinal buffer. The release profile showed an initial burst release, where 30% of the compound was released within the first minute, after which an additional 40% was released (still exponential) within the next 30 min (gastric buffer) or 15 min (intestinal buffer). The remaining 30% was not released in the timespan of the experiment.

Introduction

Electrospinning processing is a straightforward technique suitable for the production of continuous and functional nano-microfibers from a wide range of (bio)polymers [1–3]. This electrostatic technique involves the use of a high-voltage electrostatic field to charge the surface of a polymer solution droplet, thereby inducing ejection of a liquid jet through a spinneret. On the way to the collector, the jet will be subjected to forces that allow it to stretch immensely. Simultaneously, the jet will solidify through solvent evaporation, and electrically charged nano-microfibers will remain, directed by electrical forces toward the collector.

Several proteins have been electrospun [4]. To name a few, the structural proteins collagen and gelatin were electrospun, among others, in an attempt to mimic the extra cellular matrix [5–7]. Dror et al. presented a study in which the globular protein bovine serum albumin was electrospun into nano-microfibers [8], and Barnes et al. have electrospun two other globular proteins, hemoglobin and myoglobin, aiming at the development of a biologic construct with the potential of being used as an oxygen delivery system [9]. In a study by Neal et al., laminin proteins were electrospun in order to mimic the basement membranes [10], and Woerderman et al. demonstrated that it was possible to electrospin a heterogeneous protein mixture, such as the commercial wheat gluten [11]. Moreover, electrospun silk proteins have been studied, especially for biomedical applications [12–15]. In many cases, however, proteins need a carrier system in order to be electrospun, and crosslinking to achieve, for instance, the desired mechanical properties, or to become insoluble. For example, crosslinking of hemoglobin and myoglobin fibers as well as collagen and gelatin have been studied [9,16,17]. Poly vinyl alcohol (PVA) has been used as a carrier system to achieve egg albumin spinnability [18], and poly(ethylene oxide) (PEO) facilitate electrospinning of whey proteins [19]. In a study by Jiang et al., poly (ε-caprolactone) (PCL) was co-axially electrospun together with zein proteins (PCL as the core material and zein as the shell), to increase the mechanical strength of the zein fibers [20].

Fish sarcoplasmic proteins account for 25–30% of the fish muscle proteins. They are easily accessible, and comprise peptides and proteins of up to ~200 kDa in molecular weight. Fish protein hydrolysates have been shown to comprise bioactive properties [21–23]. One example is inhibitory effects against dipeptidyl peptidase-4 (DPP-IV) [24], an enzyme with an essential role in glucose metabolism and linked to type 2 diabetes, and DPP-IV inhibitors may be used as a potential new treatment for type 2 diabetes [25,26]. Additionally, dietary cod proteins have been shown to improve insulin sensitivity in insulin-resistant subjects [27,28].

The usage of electrospun nano-microfibers for biomedical application has gained widespread interest [29–31], and more specific in the drug delivery fields of bio-functional scaffolds for

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regenerative medicine [32,33], wound dressing [34,35], as well as sublingual drug delivery systems [36]. Electrospun nano-microfibers can be used to enhance the solubility and release of poorly soluble drugs, due to the large aspect ratio of fibers. For instance the poorly water soluble drug nabumetone has been encapsulated into polyethylene oxide nano-microfibers [37], while itraconazole has been encapsulated into hydroxypropyl methyl cellulose [38]. Likewise, lipophilic drugs such as rifampicin and paclitaxel have also been encapsulated into polymeric nano-microfibers [39].

Few studies have been published on the application of drug-loaded nano-microfibers as oral drug delivery systems, which is the desired route of administration from a patient convenience point of view. These systems are mainly polymer-based matrices, such as the system described by Shen et al., where Eudragit® L 100-55 nano-microfibers were loaded with diclofenac sodium for colon-targeted delivery [40], or the loading of caffeine and riboflavin into polyvinyl-alcohol fibers, as described by Li et al. [41].

In this study, FSP were electrospun into nano-microfibers and characterized with respect to fiber morphology, protein composition, fiber solubility, fiber degradability and bioactivity. To illustrate how the FSP fibers may be used as a delivery system, a dipeptide (Ala-Trp) was encapsulated into the fibers and the release properties were investigated. Rhodamine B (RhdB) was also encapsulated to study the distribution of an encapsulated compound in the FSP fibers.

Experimental

Materials

Cod (*Gadus morhua*) from the North Sea was obtained from Hanstholm Fisk, Denmark. 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP), pepsin (activity of 3.260 units/mg protein), pancreatin (activity of 8× U.S.P. specifications), DPP-IV, diprotin, Gly-Pro-Nitroanilid and Ala-Trp were obtained from Sigma-Aldrich Company, St. Louis, USA. The remaining reagents were obtained from Sigma-Aldrich Company, St. Louis, USA, and used without further purification.

Methods

Preparation of FSP

FSP extract: fresh cod was filleted and frozen at −30 °C. The frozen fillet was defrosted, chopped, placed into centrifuge tubes and centrifuged for 15 min, 18,000 × *g*, 5 °C (4K15, Sigma Laboratory centrifuges, Germany).

FSP: FSP extract was transferred to a petri dish, frozen, freeze-dried (FD) and stored at −60 °C.

Electrospinning

FSP was dissolved in HFIP, added to a syringe and placed in a syringe pump (New Era Pump Systems, Inc., USA). A 22 G needle (Proto Advantage, Canada) was used. The syringe pump delivered the FSP solution with a flow rate of 0.02 ml/min. Using a high voltage power supply (Gamma High Voltage Research, USA), an electric field of 20 kV was applied between the spinneret of the syringe and a 5 × 5 cm collector plate made of stainless steel with alumina foil wrapped around it. The distance between the syringe tip and the collector plate was 15 cm. The electrospinning was conducted at room temperature, and samples were stored in an exicator until further analysis.

Protein composition of FSP

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [42] using pre-cast 4–20% acrylamide Tris–glycine Novex gels (Life Technologies, USA). FSP extract: 0.5 ml FSP extract was added to a 1.5 ml Eppendorf tube, centrifuged, and the supernatant was saved. FD FSP in water: 27.5 mg FD FSP was added to an Eppendorf tube together with 0.5 ml demineralized water, dissolved and centrifuged. This amount was chosen to obtain a FSP concentration of 55 mg/ml, which is the approximate protein concentration in the FSP extract. The supernatant was saved. FD FSP in HFIP: 27.5 mg FD FSP was added to an Eppendorf tube together with 0.5 ml HFIP, dissolved and centrifuged. The supernatant was saved.

10 μl of each sample was added to Eppendorf tubes together with 60 μl of SDS sample buffer (4.8% SDS, 1 mM EDTA, 0.1 M DTT, 20% (v/v) Glycerol, 125 mM Tris–HCl pH 6.8, 0.05% (w/v) Brom Phenol Blue) and heated for 2 min at 95 °C. 10 μl was loaded on the gel. In addition to the samples, 10 μl Mark12™ (Life Technologies, USA) was loaded. The gel was run at 125 V, 200 mA and 150 W.

Microscopy analysis

Fiber morphology was analyzed with scanning electron microscopy (SEM) (FEI Inspect, USA). A small area of the samples was cut out, placed on carbon tape and sputter coated with gold, 10 s, 40 mA. The diameters of 60 FSP fibers were analyzed with ImageJ [43]. For clarity reasons only images containing few fibers are reported. The polymer jet was stable and continuous, and the fibers shown here represent the collection of fibers obtained after further electrospinning.

The RhdB distribution in FSP fibers was analyzed with confocal microscopy (Zeiss LSM 780, Germany). FSP fibers with 0.05 wt% RhdB were prepared by mixing RhdB into the protein solution (125 mg/ml FSP) prior to electrospinning. The electrospinning was carried out as previously described. Rhodamine fluorescence was detected at 626 nm after excitation with 543 nm, and FSP fiber auto fluorescence was detected at 481 nm after excitation with 405 nm. The images were processed with the ZEN microscopy software (Zeiss, Germany).

Solubility and degradation of FSP fibers

1 ± 0.2 mg fiber was added to 4 × 7 Eppendorf tubes. 250 μl solvent (gastric buffer (2 mg/ml NaCl, 84 mM HCl), simulated gastric fluid (gastric buffer with 0.32% (w/v) pepsin), intestinal buffer (6.8 mg/ml KH_2PO_4, 15.4 mM NaOH, pH 6.8), or simulated intestinal fluid (intestinal buffer with 1% (w/v) pancreatin) was added to Eppendorf tubes and placed on an orbital shaker (90 RPM). At the times 1 min, 30 min, 1 h and 4 h after the addition of solvent, the tubes were centrifuged and supernatants were transferred to another Eppendorf tube containing SDS sample buffer. The samples were treated and SDS-PAGE was performed as described above.

The degradation of FSP fibers by enzymes was studied further in a similar set-up; with low activity simulated fluids (low activity simulated gastric fluid (gastric buffer with 0.0032% (w/v) pepsin) and low activity simulated intestinal fluid (intestinal buffer with 0.01% (w/v) pancreatin).

The degradation pattern of FD FSP was studied as in the case of the FSP fibers, except that the time point at 10 h was omitted and a control consisting of solvent without enzyme was added.

Bioactivity of the FSP fibers

Inhibitor dilution series: 60 mg of FSP fibers was added to an Eppendorf tube together with 1 ml simulated gastric fluid or simulated intestinal fluid. The Eppendorf tubes were placed on an orbital shaker (90 RPM). After 3 h, the Eppendorf tubes were centrifuged and the supernatants were transferred to another 1.5 ml tube and heated at 95 °C for 4 min. The Eppendorf tubes were subsequently centrifuged and the supernatants were saved. In parallel, samples containing only simulated fluids were prepared for control. From the samples, 3-fold dilution series were made using water. The