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Effect of shear on physicochemical properties of matrix metalloproteinase responsive silk-elastinlike hydrogels

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ABSTRACT

Silk-elastinlike protein polymers (SELPs) have been fabricated as matrices for controlled delivery of bioactive agents. In this application the need for an environmentally responsive, degradable polymer has risen to improve treatment outcomes. To satisfy this need, we have designed, synthesized, and expressed SELPs with matrix metalloproteinase (MMP) degradable sequences inserted in distinct regions of the polymer backbone. Upon characterization of the physicochemical properties of newly synthesized analogs, it was determined that conditioning of the polymers was necessary for normalization of batch properties, and to generate a more robust polymer network suitable for delivery. In this report we have examined the use of shear stress to condition synthesized material prior to application as a controlled release matrix. The application of high shear to SELPs results in significant changes in physiochemical properties as assayed by swelling ratio, soluble fraction release, rate of gel formation, stiffness of hydrogels, and nanoparticle release from matrices. These observed changes in material characteristics may be caused by the removal of semi-stable secondary and tertiary structures from single polymer strands leading to a more robust hydrogel with greater intermolecular interaction.

1. Introduction

Silk-elastinlike protein polymers (SELPs) are block copolymers consisting of repeating amino acid sequences modeled from silkworm silk fibroin (GAGAGS) and mammalian elastin (GVGVP) synthesized using recombinant DNA technology [1]. SELPs possess the unique property of transitioning from an aqueous solution into a physical network triggered by increase in temperature and dictated by the specific sequence and ratio of silk to elastin blocks. SELPs are liquid at room temperature to facilitate mixing with bioactive agents. When raised to body temperature they form insoluble hydrogels suitable for controlled release. The use of recombinant DNA technology in synthesis allows for a high degree of control over the sequence and length of the polymers formed, as well as the amino acid sequence-level precision at which modifications can be made to existing polymers [2–7]. SELPs have undergone extensive characterization of physicochemical and release properties [8–14]. In the context of intratumoral gene delivery, it has been demonstrated that one particular analog of SELPs, namely SELP815K (Fig. 1), holds promise for localized release of adenoviruses in the treatment of head and neck solid tumors [15,16].

While SELPs including SELP815K have shown potential for use as controlled release matrices, their observed *in vivo* durability as biomaterials in murine models leads to progressive encapsulation over 12 weeks [17]. In order to promote more rapid degradation, a SELP analog with sequences degradable by matrix metalloproteinases (MMPs) was synthesized [18]. It was demonstrated that these hydrogels degrade in the presence of MMPs, a family of naturally occurring proteases that function to breakdown extracellular matrix proteins that are over expressed in a variety of solid tumors [19]. The influence that the location of MMP responsive sequences in the SELP backbone may have on physicochemical properties of the resulting hydrogels and release characteristics from these matrices is however unknown. In this work several analogs of SELPS were biosynthesized by inserting the MMP responsive sequences into two structurally distinct regions of each monomer repeat, namely the silk block and the elastin block (Fig. 1). Following synthesis and initial experiments on the physicochemical properties of new analogs, it was determined that mechanical conditioning of the synthesized material could be used for normalization of properties and to obtain a more robust polymer network.

Shear stress has long been postulated to contribute to protein denaturation and has been shown to be utilized in living systems to effect conformational change in circulating proteins [20,21]. Experimentally, conformational change can be induced mechanically via high fluid flow, particularly in molecules such as DNA whose conformation depends on weaker non-covalent bonds [22]. Applying mechanical shear

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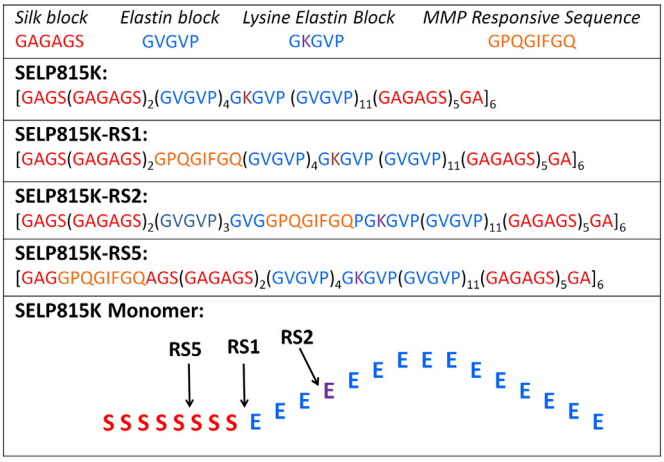

Fig. 1. Single letter amino acid structures of SELP815K, SELP815K-RS1, SELP815K-RS2, and SELP815K-RS5 with graphical representation of matrix metalloproteinase responsive sequence insertion sites into the SELP815K monomer.

stress to stable globular proteins, however, has shown to be less effective than expected in completely breaking secondary and tertiary conformational bonds due to the combination of weak interactions together with covalent bonds [23]. Proteins with weaker, less defined single strand tertiary structure such as SELPs may be more susceptible to the denaturing effects of shear stress. This will render linearized polymer strands that can form a higher density of intermolecular interactions. Here we report the biosynthesis and characterization of new MMP responsive SELPs and the influence of high shear rate on the physicochemical properties of these polymers in the context of controlled release.

2. Materials and methods

2.1. Materials

SELP815K [9] and SELP815K-RS2 with MMP responsive sequence in the middle of elastin blocks [18] were synthesized and characterized as previously described (Fig. 1). SELP815K-RS1 with MMP responsive site located in the junction between silk and elastin blocks, and SELP815K-RS5 (RS5) with MMP responsive sequence in the middle of silk blocks (Fig. 1) were synthesized and characterized using the methods previously described for SELP815K-RS2. All restriction enzymes, T4 ligase, and phosphatases were purchased from New England Biolabs (Ipswich, MA) or Fermentas (Vilnius, LT). C5 Emulsilex was purchased from Avestin (Ottawa, ON) and modified using high pressure valves and fittings from Autoclave Engineers (Erie, PA). Fluorescently labeled polystyrene beads were acquired from Invitrogen (Carlsbad, CA). Materials for Lowry assay were purchased from Thermo Fisher Scientific (Waltham, MA).

2.2. Methods

2.2.1. SELP815K-RS5 synthesis

SELP815K-RS1 and SELP815K-RS5 were synthesized and characterized using methods previously described for SELP815K-RS2 [18]. Briefly DNA oligomers encoding the MMP responsive amino acid sequence GPQGIFGQ were inserted into the SELP815K monomer in a construction plasmid using restriction endonucleases to linearize plasmid, and T4 DNA ligase to recircularize with responsive sequence inserted. MMP responsive monomer population was then expanded and digested from the construction plasmid using BanI. The population of MMP-responsive monomers was multimerized by random concatemerization into a high efficiency expression plasmid using T4 DNA ligase and screened by size using XcmI, NcoI double digest run on a DNA agarose gel. Expression was confirmed using shake flask culture and SDS-PAGE protein gel. Protein was then expressed at 10 L fermentation scale and purified from 1 kg wet cell pellet using several precipitation, ion exchange chromatography, and ultra filtration steps. The product was characterized via SDS-PAGE protein gel, matrix-assisted laser desorption/ionization time of flight (MALDI-ToF, University of California at Los Angeles proteomics core, Los Angeles, CA), and vapor phase hydrolysis amino acid analysis (the University of Nebraska Medical Center protein structure core facility, Omaha, NE).

2.2.2. Shearing

Protein polymers were lyophilized and stored at −80 °C following synthesis and purification. Lyophilized stocks were reconstituted completely in cold 0.7× PBS and kept on ice. Avestin C5 Emulsiflex homogenizer was washed thoroughly using depyrogenated deionized water and PBS. Homogenizer was then submerged in an ice bath to maintain low temperature during shear processing. Reconstituted protein polymer solution was pipetted aseptically into sample vessel and pressurized to 80 psi using nitrogen. The homogenizing valve was opened to allow a flow rate of approximately 5 mL/min and sample fluid was pumped through a needle valve at 17,000 psi. Material was then collected using a sterile luer connection. Sample fluid was aliquoted and snap frozen in liquid nitrogen. Frozen sheared polymers were stored at −80 °C.

2.2.3. Minimum gel forming concentration

Minimum gel forming concentration was determined by qualitative observation of mechanical stability following extrusion from the barrel of a 1 mL tuberculin syringe with the tip removed. Frozen polymer samples were thawed in a room temperature water bath and diluted in PBS to concentrations with a 1% step from 0% wt/wt polymer to 15% wt/wt polymer. Resulting solutions were drawn into 1 mL slip tip syringes and incubated overnight at 37 °C. Syringe tips were then removed with an autoclaved razor blade and contents were extruded and sectioned into 50 μL gel disks. Minimum gel forming concentration was defined as the concentration at which no lower concentration would hold a stable disk shape following sectioning.

2.2.4. Swelling ratio

Swelling ratio was assayed on gel disks formed by extrusion from 1 mL syringes in the same method as minimum gel forming concentration. Gel cylinders were extruded from syringe barrels and sectioned into 50 μL disks using an autoclaved razor blade. Subsequently, disks were incubated in 500 μL release medium composed of 1× PBS + 0.2 mM sodium azide for 7 days to remove soluble fraction. Following incubation, gels were removed from release medium, blotted dry with a lint free wipe, and weighed. Gels were then frozen and lyophilized for 3 days to remove water content and weighed again. Swelling ratio was calculated by dividing wet weight by dry weight.

2.2.5. Soluble fraction

Soluble fraction was assayed using chromogenic modified Lowry assay and UV–vis spectrophotometer with standard curve consisting of serial dilution of SELP and MMP-responsive SELP protein to determine amount of protein in release medium collected in swelling ratio experiment. Soluble fraction was calculated by dividing soluble protein by soluble protein plus dry weight of respective hydrogel.

2.2.6. Nanoparticle release

Model nanoparticle release was assayed using 50 μL gel disks loaded with 110 nm fluorescently labeled polystyrene spheres prepared in the same method as detailed above. Gel disks were incubated for 28 days in 500 μL release medium composed of 1× PBS + 0.2 mM azide. Release medium was collected, gels were washed, and media replaced on days 1, 3, 5, 9, 15, 21, 28 to simulated infinite sink conditions. Bead release was assayed by fluorescence quantification of FITC label after excitation

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