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Effects of crosslinking on the mechanical properties, drug release and cytocompatibility of protein polymers

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ABSTRACT

Recombinant elastin-like protein polymers are increasingly being investigated as component materials of a variety of implantable medical devices. This is chiefly a result of their favorable biological properties and the ability to tailor their physical and mechanical properties. In this report, we explore the potential of modulating the water content, mechanical properties, and drug release profiles of protein films through the selection of different crosslinking schemes and processing strategies. We find that the selection of crosslinking scheme and processing strategy has a significant influence on all aspects of protein polymer films. Significantly, utilization of a confined, fixed volume, as well as vapor-phase crosslinking strategies, decreased protein polymer equilibrium water content. Specifically, as compared to uncrosslinked protein gels, water content was reduced for genipin (15.5%), glutaraldehyde (GTA, 24.5%), GTA vapor crosslinking (31.6%), disulfide (SS, 18.2%) and SS vapor crosslinking (25.5%) ($P < 0.05$). Distinct crosslinking strategies modulated protein polymer stiffness, strain at failure and ultimate tensile strength (UTS). In all cases, vapor-phase crosslinking produced the stiffest films with the highest UTS. Moreover, both confined, fixed volume and vapor-phase approaches influenced drug delivery rates, resulting in decreased initial drug burst and release rates as compared to solution phase crosslinking. Tailored crosslinking strategies provide an important option for modulating the physical, mechanical and drug delivery properties of protein polymers.

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1. Introduction

Recombinant elastin-like protein polymers (ELPs) represent a promising class of biomaterials that can be tailored to meet the specific needs of diverse applications ranging from drug delivery devices [1,2] to medical device coatings [3,4]. Altering the processing conditions of these materials allows researchers to fabricate these biopolymers into gels [5–7] and films [8,9], thereby increasing the potential utility of ELPs as scaffolds with applications in tissue engineering. We have reported the design and development of ELPs with a hydrophilic, elastomeric midblock sequence flanked by hydrophobic endblocks in an ABA triblock format [6,10,11]. As a result of the self-association of endblock sequences, triblock ELPs form physical, non-covalent crosslinked gel networks in physiological environments (pH 7.4, 37 °C), detailed elsewhere [6]. Fabrication strategies that employ this physical crosslinking possess several advantages, such as the lack of exogenous crosslinking components and the reversibility of the process. However, physical crosslinking resulting from self-assembled domains can be disrupted at sufficiently high mechanical stresses [8].

Native elastin is enzymatically crosslinked via the formation of desmosine or isodesmosine linkages upon proper alignment of two pairs of lysine residues between adjacent tropoelastin chains [12,13]. Similarly, the majority of ELPs that have been designed to date rely on crosslinking through available amino groups, and employ either isocynates, NHS-esters, phosphines, aldehydes or genipin (GN) [14–21]. The utilization of different crosslinkers has enabled the tailoring of mechanical strength, drug elution, cell compatibility and biocompatibility of recombinant materials. To this end, the current study has investigated the mechanical, physical and biological properties of a recombinant protein crosslinked with different crosslinking agents at different crosslinking sites. Glutaraldehyde (GTA) and GN utilize free amines found on the lysine residues located at block interfaces and endpoints within the protein backbone. A third crosslinking strategy explored the addition of cystamine residues to the carboxyl groups of the glutamic acid residues within the elastomeric midblock to allow for an additional set of disulfide-bond-forming crosslinking sites [22].

Elastin-like protein polymers have been chiefly processed, via their inverse transition temperature, into hydrogels with elastic

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mechanical properties—such as strain-to-failure values as high as 1300% [5]—that match the mechanical properties of soft tissues [23,24]. In this study, we focused on variants that display less extensibility but higher degrees of ultimate tensile strength (UTS), which can be achieved when these ELPs are processed with solvents that lead to substantial microphase mixing of the hydrophobic, plastic blocks with hydrophilic, elastic blocks [9]. This was accomplished by utilizing trifluoroethanol as the solvent from which these films were cast. In addition to this solvent, a selection of crosslinking methods and processing conditions were investigated to further enhance the stiffness and strength of the protein polymers yielding mechanically robust films. In addition to different crosslinking modalities, we also explored the potential of fixed-volume, "confined" crosslinking, in which polymer swelling during the solution-phase crosslinking process was restrained, to yield stiffer films with decreased water content. The ability to modulate the physical, mechanical and biological properties of protein polymers, such as swelling ratio, strain to failure and drug delivery rates will assist in the future development of these materials as coatings or as stand-alone devices.

2. Materials and methods

2.1. Protein polymer films

The recombinant, elastin-mimetic protein polymer LysB10 has been described elsewhere [11]. Briefly, LysB10 consists of a 58 kDa hydrophilic central midblock composed of 28 repeats of the elastic sequence [$(VPGAG)_2$VPGEG$(VPGAG)_2$] flanked by 75 kDa hydrophobic endblocks composed of 33 repeats of the pentapeptide sequence $[IPAVG]_5$. To allow for enhanced crosslinking, residues [KAAK] were located at the C terminus and at both the midblock–endblock interfaces. These sequences, in combination with the N-terminal amines, provided a total of eight amine groups per macromolecule.

Protein polymer films were solvent cast from 100 mg ml^{-1} of lyophilized protein dissolved in 1 ml 2,2,2-trifluoroethanol (TFE). Solutions were cast in Teflon molds and the solvent removed by evaporation, yielding films with a thickness of 100 ± 12 μm. Films were cut into rectangles measuring 3 mm × 19 mm and weighed. The average film weight was 8.0 ± 1.7 mg.

2.2. Crosslinking

Protein polymer films were crosslinked by glutaraldehyde vapor (GTAvap), glutaraldehyde solution (GTAsol), genipin (GNsol) or disulfide formation (SSsol or SSvap). In addition to the solution and vapor-phase experimental groups, some films were subject to solution-phase crosslinking in a fixed-volume, confined state, as detailed below (GTAvol, GNvol or SSvol).

For GTAvap crosslinking, protein polymer films were suspended above a reservoir of 25% (w/v) GTA (Sigma Aldrich, St Louis, MO) in water in a closed chamber for 48 h. GTAsol crosslinking was performed by immersing films in GTA (0.5%, 25 °C, 24 h). GNsol crosslinked protein polymer films were placed in solutions of GN (6 mg ml^{-1} in phosphate-buffered saline (PBS), 37 °C, 24 h). For solution-phase disulfide formation (SSsol), LysB10 was chemically modified with cystamine, as previously reported [22]. Cystamine (Sigma Aldrich) was added to the solution at 20-fold molar excess to a cooled solution of LysB10 (10 mg ml^{-1}, 4 °C, PBS), followed by *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide (EDC) at 5-fold molar excess relative to cystamine. After stirring (72 h, 4 °C) cystamine-modified LysB10 polymer was purified by dialysis and lyophilization (81% yield). Cystamine-LysB10 was processed into films, as described above, and thiol groups were reduced by submerging the films in 26 mMTris (2-carboxyethyl)phosphine (TCEP) for 6 h. Films were then placed in a 0.1% H_2O_2 solution made from 30% sodium hydroxide (Sigma Aldrich) and PBS (pH 7.3). In addition to solution-phase disulfide crosslinking, air oxidation was utilized to form disulfide bonds in dehydrated films (SSvap). In brief, cystamine-LysB10 films were reduced, as previously described, dried and exposed to air in a ventilated dish for 5 days.

Specimens were also crosslinked in GTAvol, GNvol or SSvol solutions in a fixed-volume, confined state under conditions of mechanical compression (Fig. 1). In brief, protein polymer films were placed between two pieces of filter paper (Millipore, Inc.) and two glass slides. U-shaped plastic connectors were placed over the slides and the assembly was submerged in a crosslinking solution. All films were rinsed in PBS (5 bath changes, 25 °C, 48 h) to remove unbound crosslinker.

2.3. Water content of protein polymer films

Surface area, thickness and film weight were measured in the dried and hydrated state before and after crosslinking using optical microscopy and a precision mechanical balance (Mettler-Toledo, Columbus, OH). The thickness ratio was defined as $T = T_{HAC}/T_{BC}$, where T_{HAC} is the hydrated thickness after crosslinking and T_{BC} is the dried thickness before crosslinking. The swelling ratio, *SR*, and equilibrium water content, *E*, were defined as $SR = W_H/W_D$ and $E = (W_H - W_D)/W_H$, where W_H and W_D correspond to the hydrated and dried weights, respectively.

2.4. Extent of crosslinking

Measurements of per cent extractable protein were obtained by placing protein films in TFE and shaking for 7 days at 37 °C. Films were removed, placed in a vacuum chamber for 72 h and weighed. Per cent extractable protein was defined as $Ex = [(W_D - W_E)/W_E] \times 100\%$, where W_D and W_E are the sample weights before and after solvent extraction.

The degree of crosslink formation was measured by colorimetric assays. Free amino groups were quantified with the ninhydrin assay [25]. Protein polymer films were weighed, heated with a ninhydrin solution (Sigma Aldrich, St Louis, MO) for 20 min at 85 °C, cooled to room temperature and diluted in 95% ethanol. The optical absorbance of the solution was quantified by UV–visible spectrophotometry (Cary 50, Varian Inc., Palo Alto, CA) at 570 nm using a standard curve derived from glycine solutions.

The degree of thiol modification was determined by incubation in Ellman's reagent in phosphate buffer (4 mg ml^{-1} Ellman's reagent, 0.1 M sodium phosphate, 1 mM EDTA, pH 8.0) for 15 min at room temperature. Absorbance at 412 nm was measured and concentration values were obtained from comparison of measurements to a standard curve generated from cysteine dilutions in phosphate buffer.

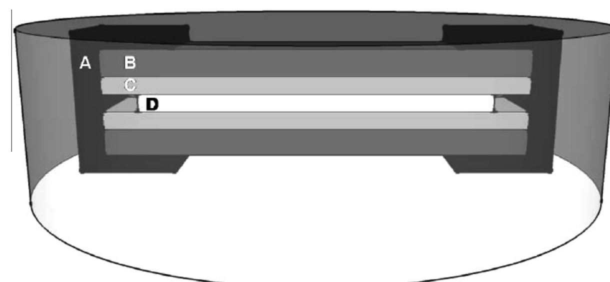

Fig. 1. Illustration of confined crosslinking system consisting of (A) compression clips, (B) glass microscope slides, (C) filter paper and (D) protein polymer submerged in a crosslinking solution.

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