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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; with 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.

Keywords

Protein polymer; crosslinking; mechanical properties; drug release

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

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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 isocyanates, NHS-esters, phosphines, aldehydes, or genipin [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 and genipin 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 mechanical properties, such as strain to failure values that may be as high as 1300% [5]; suitable for matching 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, 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.

MATERIALS and METHODS

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)₂VPGE(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 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 $100 \pm 12 \mu\text{m}$. Films were cut into rectangles measuring $3 \times 19 \text{ mm}$ and weighed. The average film weight was $8.0 \pm 1.7 \text{ mg}$.

Crosslinking

Protein polymer films were crosslinked by glutaraldehyde vapor (GTA_{vap}), glutaraldehyde solution (GTA_{sol}), genipin (GN_{sol}), or disulfide formation (SS_{sol} or SS_{vap}). 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 (GTA_{vol} , GN_{vol} , or SS_{vol}).

For GTA vapor 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. Glutaraldehyde solution crosslinking (GTA_{sol}) was performed by immersing films in glutaraldehyde (0.5%, 25°C, 24 h). Genipin crosslinked (GN_{sol}) protein polymer films were placed in solutions of genipin (6 mg/mL in PBS, 37°C, 24 h). For solution phase disulfide formation (SS_{sol}), LysB10 was chemically modified with cystamine, as previously reported [25]. Cystamine (Sigma Aldrich) was added to the solution at 20-fold molar excess to a cooled solution of LysB10 (10 mg/mL, 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 mM Tris (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 (SS_{vap}). 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 GTA_{vol} , GN_{vol} , or SS_{vol} solutions in a fixed volume, confined state under conditions of mechanical compression (Figure 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.

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 $T = T_{\text{HAC}}/T_{\text{BC}}$, 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 $\text{SR} = W_{\text{H}}/W_{\text{D}}$ and $E = (W_{\text{H}} - W_{\text{D}})/W_{\text{H}}$, where W_{H} and W_{D} correspond to the hydrated and dried weights, respectively.

Extent of crosslinking

Measurements of percent extractable protein were obtained by placing protein films in TFE and shaking for seven days at 37°C. Films were removed, placed in a vacuum chamber for

72 h and weighed. Percent extractable protein was defined as $Ex = (W_D - W_E)/W_E \cdot 100\%$, where W_D and W_E are sample weights before and after solvent extraction.

Degree of crosslink formation was measured by colorimetric assays. Free amino groups were quantified with the ninhydrin assay [26]. 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 with UV–visible spectrophotometer (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 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.

The degree of crosslinking (C) was calculated from the ninhydrin assay for lysine residues and Ellman's reagent for thiol groups, such that $C = (X_U - X_C)/X_U \cdot 100\%$, where X_U and X_C are the number of detected groups in uncrosslinked and crosslinked samples, respectively. The degree of crosslinking was multiplied by the available number of crosslinking sites, which were 8 for amino and 17.9 for thiol-based crosslinking, to estimate the number of crosslinks per macromolecule.

Mechanical analysis of hydrated protein polymer films

Uniaxial stress strain tests were conducted on a dynamic thermal analyzer (DMTA V, Rheometric Scientific, Inc. Newcastle, DE) with a 15-N load cell in the inverted orientation, with specimens immersed in PBS at 37°C. Samples of protein polymer film were cut into a dumbbell shape using a stainless steel die with a gauge length of 13 mm and width of 4.75 mm. Samples were subjected to a preconditioning protocol of 10 cycles to 10% strain with offloading periods of 5 min between each cycle. Following preconditioning, films were stretched to failure at a rate of 5 mm/min. Engineering stress and strain levels were calculated assuming sample thicknesses of 100 µm. Elastic moduli were calculated from two linear regions, 5–15% and 50–175% strain, occurring before and after a yield point consistently noted between 15% and 50% strain. Since the maximum travel distance of the DMTA is 23 mm, more extensible materials were strained to failure on a miniature materials tester (Minimat 2000, Rheometric Scientific) at a rate of 5 mm/min, with testing conducted in air at room temperature. In this system, all samples were fully hydrated in PBS prior to testing and coated with a thin layer of mineral oil to prevent dehydration.

Drug release

As a model system, drug release was evaluated by incorporating rapamycin into the protein solution prior to film casting, at a concentration of 10 µg of drug per milligram of protein polymer. Films were crosslinked, as described above, and subjected to 8 washes in PBS at 37°C. Due to limited solubility of the drug in the aqueous crosslinking and rinsing solutions no drug was lost in the crosslinking process.

To determine drug release, protein polymer films were incubated in 2 mL of release medium (9:1 PBS:ethanol, 37°C) on a shaker, rotating at 500 rpm [27]. Protein polymer film thicknesses and weights were recorded to validate film stability in the release medium. Drug concentration in the release medium was determined by monitoring the absorbance at 278 nm with a UV spectrophotometer with reference to a standard curve. Initial measurements were conducted at 2 h and every 24 h thereafter, until no additional drug release was measured. The drug burst was defined as the amount of drug that had diffused out of the

construct after 2 hours. The drug delivery rate was calculated from the slope of the linear portion of the drug delivery plot, as was the time point at which 50% of the drug was delivered. The final drug delivery time was defined as the time when further elution from the construct could no longer be detected.

Adhesion and proliferation of endothelial cells

Human umbilical vein endothelial cells (HUVECs, Clonetics) were cultured in endothelial growth medium-2 (EGM-2, 2% serum, Clonetics) following standard techniques using cells between passages 4 and 9. Protein polymer surfaces were prepared by adding 75 μ L of 10 wt % protein polymer in TFE into wells of a 96 well plate and evaporating the solvent over 48 h with the lid on, followed by 120 h exposure to vacuum. Films were then subjected to solution phase crosslinking procedures. All samples, including uncrosslinked controls, were then washed 10 times over the course of 48 h (PBS, 25°C). A 50 μ L solution of fibronectin (Fn, 250 μ g/mL, Sigma Aldrich, St. Louis, MO) was adsorbed to the films for 24 h [28]. Samples were washed once with PBS prior to seeding with 100 μ L suspension of HUVECs in EGM-2 at 200,000 cells/mL or 50,000 cells/mL, for adhesion and proliferation assays, respectively.

To determine cell adhesion, plates were incubated for 2 h at 37°C, washed three times with PBS, and frozen at -80°C for 24 h. Prior to proliferation measurement, film surfaces were rinsed 2 h after cell seeding with media three times to remove unbound or loosely adhered cells, followed by an additional 46 h of culture. Quantification of cell adhesion and proliferation was determined with the CyQuant Cell Proliferation Assay Kit (Molecular Probes), following the manufacturer's directions. After thawing frozen cells to enhance lysis, the CyQuant cell lysis buffer and fluorescence reagent were added to each sample. Samples were then measured in a microplate spectrofluorometer and compared to a standard curve created from 100 to 50,000 cells for each set of samples.

Statistical Analysis—Comparisons were made between multiple groups by ANOVA or a paired, two-tailed student's t-test, with $p < 0.05$ considered significant. Results are presented as mean $\pm$ standard deviation. Six or more replicates were processed for all groups.

RESULTS

Extent of crosslinking

The extent of crosslinking was determined by measuring the percent of extractable protein and through colorimetric assays (Table 1). The physical state of the film during solution phase crosslinking, with or without compression influenced the extent of crosslinking. Film confinement during genipin and disulfide crosslinking significantly decreased the degree of crosslinking. In contrast, film confinement had no measurable effect on the degree of crosslinking when crosslinked by glutaraldehyde.

Crosslinking in the vapor phase had varying effects. Glutaraldehyde vapor-phase crosslinking significantly increased the degree of crosslinking compared to solution-phase, but had no significant effect on protein extractables. Vapor phase disulfide crosslinking resulted in less complete crosslinking than when performed in the solution phase, both in degree of crosslinking and extent of extractable protein.

The estimated number of crosslinks per macromolecule reveals that despite a lower extent of crosslinking, disulfide formation afforded the greatest crosslink density suggestive of higher level of intramolecular bond formation. When disulfide crosslinking is compared to

glutaraldehyde and genipin strategies, there are ~60% more crosslinks formed following the vapor phase reaction, and 110% more following solution-based approaches.

Hydration of crosslinked protein polymers

Fixed volume crosslinking had a significant effect on the water uptake in all cases, with significant reductions in the thickness ratio, equilibrium water content, and swelling ratio (Figure 2). Crosslinking in the vapor phase reduced water content further, with a significant difference in thickness ratio for both GTA and SS schemes. However, water content and swelling ratio were not significantly different between these groups.

Mechanical Analysis

The tensile stress-strain behavior for all samples consisted of an initial region of high-modulus deformation followed by yielding and a second, lower modulus deformation region (Figure 3). In all cases, crosslinking significantly increased Young's modulus and UTS, and reduced strain at failure (Table 2). High-strain modulus also tended to increase significantly after genipin and disulfide crosslinking when performed under fixed volume conditions. Vapor-phase methods also led to significantly higher high-strain modulus as compared to solution-phase crosslinking. In the case of GTA_{vap} , UTS was also significantly increased.

Comparing across the chemical crosslinkers, GTA and SS schemes had similar mechanical effects on films for a given reaction type (solution, fixed volume, or vapor). However, in both confined and unconfined solution-phase reactions, genipin-crosslinked films tended to have lower stiffness than the other two schemes.

Drug Elution

All crosslinking strategies, except GN_{sol} , significantly reduced the drug burst level, slowed release rates, and increased the time to 50% of drug release and total drug release, when compared to uncrosslinked controls (Table 3, Figure 4). In both the GN and SS crosslinking strategies, fixed volume, confinement during crosslinking significantly reduced amount of drug released in the initial burst. The burst effect was significantly higher for uncrosslinked polymers than for all other groups, and almost 10 times higher than observed for GTA_{vap} crosslinking. The GTA_{sol} samples displayed a similar burst effect as observed for samples crosslinked using either vapor or by a confined volume approach.

The rate of drug delivery was significantly greater for uncrosslinked and solution phase, genipin crosslinked samples as compared to all others. Within the GTA samples, release rates were significantly different, with solution phase crosslinked samples having the fastest release rate and vapor phase crosslinked samples having the slowest release rate. The same trend was observed for SS crosslinked samples, as well. Compared to GTA, all SS crosslinked samples had significantly slower release rates.

Time to 50% drug delivery occurred over a period of less than 24 hours without crosslinking, which was a significantly shorter interval than for crosslinked samples. GTA samples released 50% of the drug over a period exceeding three days. Within each crosslinking scheme, reaction set-up had a significant effect on time to 50% drug release. In all three cases, solution phase crosslinked samples was associated with the shortest period to 50% drug delivery, followed by fixed volume crosslinked samples, and vapor-phase samples. Time to total drug release followed a similar trend. Notably, SS crosslinking extended the time to total drug delivery by 50% and 100% for solution phase and vapor phase crosslinked samples, as compared to controls. In general, vapor crosslinking tended to slower lease more than solution phase techniques.

Endothelialization

The effect of crosslinker on the adhesion and proliferation of endothelial cells was assessed qualitatively with optical microscopy. Without adsorbed fibronectin, protein polymer films displayed little cell attachment, as compared to fibronectin treated films (Figure 5a–f). Fibronectin treated, genipin crosslinked films afforded high cell densities with well-spread morphology and proliferation after 48 h (Figure 5h,k). Fibronectin treated, GTA films displayed similar initial morphology and cell density, but with somewhat limited proliferation (Figure 5g,j). Disulfide crosslinked samples displayed robust adhesion and proliferation (Figure 5i,l). These observations were supported quantitatively by the CyQuant assay (Figure 6). Specifically, cell adhesion was normalized to the fraction of adherent cells on non-tissue culture treated polystyrene with adsorbed Fn. Adsorbing Fn on the protein polymer, doubled the number of adherent cells, but crosslinked samples had significantly higher adhesion, with the genipin samples displaying the highest level of cell adhesion, followed by SS and GTA crosslinked films. To explore cell proliferation, the number of adherent cells was quantified at 2 and 48 hours. Similar to adhesion studies, Fn adsorbed crosslinked films exhibited the highest levels of proliferation with somewhat lower levels on GTA crosslinked films, perhaps due to intrinsic low level toxicity associated with glutaraldehyde.

DISCUSSION

The ability to modulate the properties of ELPs with chemical crosslinkers is well documented. In this study, we examined three chemical crosslinking schemes, as well as different crosslinking processes. Glutaraldehyde is a well known crosslinker that has been utilized extensively, although its use has been linked to calcification [29] and cytotoxicity [30, 31]. Genipin is less cytotoxic but yields constructs with lower strength [32]. Disulfide-mediated crosslinking affords post process modification and is amenable to a range of crosslinking processes [33]. Here, we evaluated protein polymer films subjected to crosslinking strategies designed to enhance mechanical strength, reduce water content, and slow drug release, without compromising cell adhesion and proliferation. Although both solution phase and vapor phase crosslinking resulted in protein polymers with a high degree of crosslinking, their physical and mechanical properties were significantly different. A novel fixed volume, confined solution phase crosslinking method was explored to examine the potential of obtaining constructs with enhanced mechanical strength, as observed with the vapor phase technique, by functionally increasing polymer chain density.

Degree of Crosslinking

Almost all techniques resulted in degrees of crosslinking in the range of 90%, with the exception of SS_{vap}. The efficiency of glutaraldehyde and genipin crosslinking of protein-based materials has been reported [26, 34, 35]. The disulfide reaction may have been less efficient in the vapor phase due to limited chain mobility in the dehydrated protein network. In contrast to the disulfide vapor reaction, exposure to glutaraldehyde vapor resulted in a high degree of crosslinking, probably due to the potential of glutaraldehyde to form polymeric crosslinks [36].

Disulfide crosslinking techniques led to the greatest number of crosslinks per molecule as compared to techniques based on amine crosslinking because more thiol groups were available for reaction. However, the higher number of crosslinks per molecule was not associated with a reduction in the percentage of extractables, which is likely related to intramolecular crosslink formation. For the SS and GN schemes, extractable protein tended to be greater and degree of crosslinking lower, when crosslinking was performed in a

confined fixed volume, which may be attributable to the limited mobility of the reactive groups.

Hydration Level of Protein Polymers

Crosslinking in a confined state influenced protein polymer swelling ratio and water content yielding denser protein networks with lower water content and greater strength. The properties obtained through GN and GTA solution phase crosslinking were consistent with other reports [37, 38]. Protein based materials that display less swelling upon hydration can be machined or shaped in the dry state with less geometrical distortion upon hydration.

Mechanical Analysis

Samples were initially preconditioned to afford reproducible material properties [39, 40]. In uniaxial tension, all samples displayed deformation behavior typical of triblock protein polymers cast from solvents that promote interpenetration of hydrophobic and hydrophilic domains [9]. An initial high modulus deformation response was followed by yielding at 2 to 6 MPa and then an extended low modulus deformation regime until failure at 200 to 400% strain. The interpenetration of hydrophobic and hydrophilic domains may be partly reversed when the protein polymer swells upon hydration. Therefore, limiting swelling through confinement or avoiding hydration entirely by vapor-phase crosslinking likely promotes a greater degree of interpenetration between domains, which could account for the higher modulus and yield stress observed when crosslinks were generated in the vapor or confined state.

Glutaraldehyde yields crosslink through multiple mechanisms and often has the greatest effect on mechanical properties as compared to other crosslinkers [36, 41]. However, under controlled reaction conditions, SS based crosslinking strategies can have an equivalent effect [33]. Furthermore, an increase in stiffness at high strains when crosslinking was performed under confined conditions was observed for both SS and GN groups, suggesting that the effect may be attainable for a variety of solution-phase crosslinkers. As opposed to GTA, crosslinking sites for SS and GN must be in close proximity to ensure intermolecular crosslinks. Although the degree of crosslinks is lower in these systems, the short crosslinks may limit water content and lead to stiffer films

Drug Release

The type and mode of crosslinking had a significant impact on the elution of rapamycin, as a model hydrophobic drug. Rapamycin was selected because of its hydrophobicity and widespread application in treatment of both cancer and cardiovascular disease. Typically, the drug diffusion coefficient of a hydrogel decreases as the crosslinking density increases due to increased microstructural tortuosity and decreased space between macromolecular chains [42, 43]. Decreasing porosity and water content is also associated with slowed release [44]. Drug release is characteristically inversely related to polymer swelling ratio and water content and directly related to the degree of crosslinking or physical chain entanglements. Indeed, lower water content and swelling ratio, along with slower drug release kinetics were observed when crosslinking was performed under confined conditions as compared to polymers subjected to solution state crosslinking. Small differences in chemical crosslink density may have been offset by a greater level of chain entanglement in the confined state.

Endothelial Cell Compatibility

Prior studies have demonstrated that cell adhesion and proliferation on protein polymer surfaces is enhanced by surface adsorbed fibronectin [45]. We observed that higher levels of cell adhesion and proliferation are observed on crosslinked than non-crosslinked samples

after absorption of fibronectin. Ongoing studies are evaluating whether this is related to higher levels of ligand density, conformational changes in protein structure, or alterations in material stiffness [46]. GN and SS crosslinking were associated with higher levels of cell adherence and proliferation than GTA-crosslinked structures. Differences in the cytocompatibility of genipin and GTA have been previously noted [47, 48].

CONCLUSIONS

The ability to tailor the physical and mechanical responses of protein polymers, as well as drug delivery rates and the capacity for endothelialization was achieved through modifying crosslinking schemes involving GTA, GN, or SS based reaction strategies. Solution-phase crosslinking was compared to crosslinking under a confined state. In the case of GTA and SS, crosslinking of dehydrated protein polymer films was also applied in the vapor phase. Fixed volume and vapor phase reactions decreased water uptake, slowed drug release, and increased mechanical stiffness, demonstrating that crosslinking under confined conditions offers an approach to enhance and tune a wide range of properties when vapor phase crosslinking reactions are not feasible. Crosslinking increased adhesion and proliferation of endothelial cells as compared to uncrosslinked films with GN films displaying the highest levels, closely followed by SS and GTA films.

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Figure 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.

Figure 2.

Thickness ratio (A), water content (B), and swelling ratio (C) of crosslinked films. Unconfined samples are shown in black, confined illustrated in light gray, and vapor presented in dark gray. Glutaraldehyde (GTA), genipin (GN), or disulfide formation (SS).

Figure 3. Stress strain plots for (A) uncrosslinked, (B) glutaraldehyde crosslinked, (C) genipin crosslinked, and (D) thiol crosslinked films.

Figure 4. Drug delivery profiles for (A) controls, (B) glutaraldehyde crosslinked, (C) genipin crosslinked, and (D) sulfide crosslinked films.

Figure 5. Photomicrographs of endothelial cells 2h and 48 h after cell seeding. Non-crosslinked protein polymer films with (a, d) and without (b, e) surface adsorbed fibronectin. Non-tissue culture treated polystyrene surfaces (f, c). All crosslinked surfaces, including GTA (g, j), GN (h, k), and SS (i, l) were exposed to fibronectin prior to cell seeding (pp = protein polymer LysB10).

Figure 6.

(A) Endothelial cell adhesion on crosslinked samples treated with fibronectin. Cell adhesion was normalized to adhesion on fibronectin (Fn) coated polystyrene. (B) Cell adhesion and proliferation on crosslinked surfaces (* $p > 0.05$, pp = protein polymer LysB10, ps = polystyrene).

Table 1

Percent protein extractable, degree of crosslinking, and estimated crosslink density

Crosslinking Scheme	Extractable Protein (%)	Degree of crosslinking (%)	Estimated crosslinks per macromolecule
Uncrosslinked	100 ^{†*}	3.27 ± 2.74 ^{†*}	0.26 ± 0.19
Uncrosslinked, fixed volume	100 ^{†*}	2.56 ± 1.54 ^{†*}	0.20 ± 0.11
GTA solution	7.55 ± 2.19	90.64 ± 2.01	7.24 ± 0.15
GTA fixed volume	8.98 ± 1.96	91.93 ± 1.58	7.37 ± 0.11
GTA vapor	9.38 ± 1.66	95.68 ± 0.26 [*]	7.66 ± 0.02
GN solution	6.93 ± 1.51 [*]	92.61 ± 0.50 [*]	7.41 ± 0.04
GN fixed volume	11.74 ± 2.59 [*]	88.53 ± 1.65 [*]	7.08 ± 0.12
SS solution	6.81 ± 1.28	94.89 ± 0.46 [*]	16.99 ± 0.08
SS fixed volume	8.90 ± 2.18	87.91 ± 1.23 [*]	15.73 ± 0.21
SS vapor	16.62 ± 1.96 ^{†*}	66.29 ± 2.59 ^{†*}	11.89 ± 0.45

[†] $p < 0.05$ compared with other chemical strategies in the same phase (solution, fixed volume, vapor)

^{*} $p < 0.05$ compared with other methods using the same crosslinker

Table 2

Mechanical parameters

Crosslinking Scheme	Modulus (MPa) (5–15% Strain)	Modulus (MPa) (50–175% Strain)	Strain at Failure (%)	Ultimate Tensile Strength (MPa)
Uncrosslinked	19.93 ± 1.19	0.37 ± 0.04	389.24 ± 43.00	4.05 ± 0.33
Uncrosslinked, fixed volume	19.67 ± 2.51	0.39 ± 0.05	375.81 ± 31.00	3.89 ± 0.38
GTA solution	61.67 ± 2.16	1.02 ± 0.19	302.28 ± 31.00	6.77 ± 0.63
GTA fixed volume	72.66 ± 6.95	1.22 ± 0.15	254.81 ± 22.00	7.51 ± 0.48
GTA vapor	68.67 ± 7.96	1.72 ± 0.21 *	231.41 ± 25.00	8.39 ± 0.53
GN solution	32.67 ± 7.23	0.53 ± 0.13 *	319.92 ± 31.00	5.78 ± 0.45
GN fixed volume	36.70 ± 5.54	0.82 ± 0.10 *	273.72 ± 26.00	6.25 ± 0.46
SS solution	43.51 ± 5.58 *	0.95 ± 0.12 *	281.72 ± 28.00	6.61 ± 0.61
SS fixed volume	61.58 ± 7.20	1.23 ± 0.11 *	238.39 ± 21.00	7.43 ± 0.57
SS vapor	63.55 ± 6.66	1.82 ± 0.22 *	216.19 ± 19.00	7.60 ± 0.55

* $p < 0.05$ compared with other methods with the similar crosslinker.

Table 3

Drug release parameters

Crosslinking Scheme	Burst Amount (μg)	Rate (% days ⁻¹)	50% Drug Release (days)	Total Drug Release (days)
Uncrosslinked	$13.98 \pm 1.26^{\dagger}$	26.07 ± 1.15	$0.49 \pm 0.08^{\dagger*}$	6.80 ± 0.84
Uncrosslinked, fixed volume	$14.01 \pm 1.10^{\dagger}$	25.97 ± 0.97	$0.50 \pm 0.07^{\dagger*}$	7.00 ± 0.70
GTA solution	1.77 ± 0.09	$16.00 \pm 0.96^{\dagger*}$	$2.69 \pm 0.10^*$	$11.80 \pm 0.84^*$
GTA fixed volume	1.58 ± 0.05	$13.20 \pm 0.79^{\dagger*}$	$3.22 \pm 0.29^*$	13.40 ± 0.55
GTA vapor	1.44 ± 0.07	$11.83 \pm 0.21^{\dagger*}$	$3.75 \pm 0.09^*$	13.80 ± 0.84
GN solution	$4.55 \pm 0.69^{\dagger*}$	$25.30 \pm 0.35^*$	$1.26 \pm 0.09^{\dagger*}$	$8.20 \pm 0.84^{\dagger*}$
GN fixed volume	$2.90 \pm 0.54^*$	$19.93 \pm 0.58^{\dagger*}$	$1.80 \pm 0.08^{\dagger*}$	$9.80 \pm 0.55^*$
SS solution	$9.43 \pm 0.93^{\dagger*}$	$10.27 \pm 0.12^{\dagger}$	$2.15 \pm 0.21^{\dagger*}$	$10.56 \pm 0.55^*$
SS fixed volume	$6.83 \pm 0.63^{\dagger*}$	$8.97 \pm 0.21^{\dagger}$	$2.95 \pm 0.17^{\dagger}$	11.98 ± 0.84
SS vapor	$2.08 \pm 0.78^*$	$8.60 \pm 0.78^{\dagger}$	$3.75 \pm 0.14^*$	12.65 ± 0.55

[†] $p < 0.05$ compared with other chemical strategies in the same phase (solution, compressed, vapor)

^{*} $p < 0.05$ compared with other methods using the same crosslinker.