Elastin-mimetic protein polymers capable of physical and chemical crosslinking

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\textbf{Abstract}

We report the synthesis of a new class of recombinant elastin-mimetic triblock copolymer capable of both physical and chemical crosslinking. These investigations were motivated by a desire to capture features unique to both physical and chemical crosslinking schemes so as to exert optimal control over a wide range of potential properties afforded by protein-based multiblock materials. We postulated that by chemically locking a multiblock protein assembly in place, functional responses that are linked to specific domain structures and morphologies may be preserved over a broader range of loading conditions that would otherwise disrupt microphase structure solely stabilized by physical crosslinking. Specifically, elastic modulus was enhanced and creep strain reduced through the addition of chemical crosslinking sites. Additionally, we have demonstrated excellent in vivo biocompatibility of glutaraldehyde treated multiblock systems.

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1. Introduction

Genetic engineering provides a facile route for the design of novel protein polymers composed of repetitive amino acid sequences or peptide blocks whose structural complexity imparts distinct mechanical, chemical or biological properties. To date, the majority of recombinant multiblock protein polymers have been designed with relatively short block sequences that limit structural polymorphism. As a consequence, opportunities to access diverse polymer morphologies are limited and the potential to tune a wide range of functional responses reduced [1,2]. Recently, we have reported a new class of elastin-mimetic multiblock copolymer composed of identical endblocks derived from self-associating, hydrophobic sequences that display plastic-like mechanical responses (Ile-Pro-Ala-Val-Gly), separated by a central block that is both hydrophilic and elastomeric (Val-Pro-Gly-Glu-Gly) [3,4]. Block sizes, typically, exceed 35 kDa, which has allowed us to explore the production of protein-based materials that are structurally polymorphic [3–8].

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damaged at applied stresses lower than those required to disrupt covalent crosslinks.

Native elastin is enzymatically crosslinked upon proper alignment of two pairs of lysine residues between adjacent tropoelastin chains with formation of desmosine or isodesmosine linkages [11,12]. Likewise, most recombinant elastin analogues that have been designed to date have relied on crosslinking through available amino groups, albeit with most reports describing the use of chemical crosslinkers, including isocyanates, NHS-esters, phosphines, aldehydes, or genipin [9,13–23]. In this regard, we have previously reported the design of a synthetic elastin sequence (Val-Pro-Gly-Val-Gly)[4(Val-Pro-Gly-Lys-Gly)], in which lysine residues were chemically crosslinked using bis(sulfo succinimidyl) suberate and disuccinimidyl suberate [20]. Subsequent studies have reported the application of transglutaminase or lysyl oxidase for enzymatic crosslinking [24]. In addition, we have also explored solid-state crosslinking of recombinant elastin-mimetic proteins using both UV and visible light activated photoinitiators [25]. In tropoelastin, lysine residues are often interspersed among alanine repeats (e.g. Ala-Ala-Ala-Lys-Ala-Lys-Ala-Ala), which has suggested that self-association of alanine-rich sequences facilitates crosslinking [26,27]. Several elastin-like proteins have been designed in similar manner [9,10,28].

The capacity of chemical crosslinks to provide an independent mechanism for control of protein mechanical responses and bio-stability is well established. However, in this report we postulated that by chemically locking a multiblock protein assembly in place, functional responses that are linked to specific domain structures and morphologies may be preserved over a broader range of loading conditions that would otherwise disrupt microphase structure solely stabilized by physical crosslinking. We report herein the synthesis of a new class of recombinant elastin-mimetic triblock copolymer capable of both physical and chemical crosslinking. These investigations were motivated by a desire to capture features unique to both physical and chemical crosslinking schemes so as to exert optimal control over a wide range of potential properties afforded by protein-based multiblock materials.

2. Materials and methods

2.1. Synthetic gene construction of elastic- and plastic-like domains

Synthetic methods used to produce the DNA inserts that encode the various elastin-mimetic block copolymers have been described previously [3,5,7,8]. Genes encoding two distinct chemically crosslinkable protein triblock copolymers were synthesized. Briefly, oligonucleotide cassettes encoding elastic- (E) and plastic-like (P) repeat units (Table 1) were independently synthesized and inserted into the BamHI and HindIII sites within the multiple cloning region of pZErO cloning vectors. Specifically, P1 and E1 encode the monomer repeat unit for plastic- and elastic-like domains designed for the triblock protein polymer, referred to as LysB10. A second set of oligonucleotide cassettes, P2 and E2, were designed to encode monomer repeat units for plastic- and elastic-like domains for a second protein triblock copolymer, designated R4. Recombinant clones were isolated after propagation in Escherichia coli strain TOP10F’ E. coli cells. A total of 100 µl of the transformation mixture was spread onto low salt Luria Broth (LSB) agar supplemented with Zeocin (50 µg/ml). The plates were incubated for 12 h at 37°C. Five transformants were selected from each plate to inoculate individual 7 ml cultures of LSB/Zeocin. Cultures were rotated incubated for 12 h at 37°C. Plasmid DNA was recovered through a Qiagen Spin Miniprep protocol (Qiagen, Inc.). DNA was initially screened by BamHI and HindIII double digestion. Positive transformants were verified by agarose gel electrophoresis (4% GTG NuSieve agarose, 1× TBE buffer). Automated DNA sequencing utilizing the M13 forward and reverse primers confirmed correct DNA products. Plasmids containing the correct sequence for the Lysine Insert and Lysine Adaptor are identified as pL and pR, respectively.

Table 1

<table>
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<tr>
<th>Coding sequences of oligonucleotide cassettes employed for the construction of crosslinkable protein triblocks, LysB10 (AP1IE1IP1A) and R4 (AP2IE2IP2A).</th>
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2.2. Synthetic gene construction of chemical crosslinking domains

Single stranded oligonucleotides encoding the sense and anti-sense strands of the Lysine Insert (I) and Lysine Adaptor (A) were chemically synthesized (Sigma Genosys, Inc.) (Table 1). The Lysine Insert is a 60-bp DNA cassette encoding the crosslinking sequence, Lys-Ala-Ala-Lys, which was inserted between the plastic- and elastic-like domains. The Lysine Adaptor is a 50-bp DNA cassette designed with restriction enzyme cut sites midway through the cassette to allow for insertion of the assembled triblock gene. The Lysine Adaptor encodes for a single N-terminal lysine residue and two C-terminal lysine residues. Additionally, it allows for facile cloning into the PET24-α expression vector within the multiple cloning region. This ensures correct insertion of the gene in frame with the N-terminal polyhistidine tag. The following procedure detailing the protocol to generate double-stranded DNA was implemented for both the Lysine Insert and Lysine Adaptor. The DNA was suspended in 10 mM Tris buffer (pH 8) to a final concentration of 0.5 µg/µl. A solution of 10 µl of each corresponding oligonucleotide, 4 µl of 5 M NaCl, 4 µl of 1 M MgCl2, and 152 µl of sterile ddH2O was subjected to an annealing procedure initiated at a reaction temperature of 95°C with temperature decrements of 1°C every 5 min to a final reaction temperature of 30°C. The resultant double-stranded DNA cassette was analyzed by agarose gel electrophoresis (4% GTG NuSieve agarose, 1× TBE buffer).

Double-stranded synthetic DNA was phosphorylated through a 2-h incubation with T4 Polynucleotide Kinase (New England Biolabs) in the presence of T4 DNA ligase buffer with 10 mM ATPs (New England Biolabs). The enzymes were removed with phenol/chloroform/isooamy alcohol (25:24:1) and the double-stranded DNA (dsDNA) was recovered through an ethanol precipitation. The pZEO1-α acceptor plasmid (1 µg) was prepared via BamHI and HindIII double digestion, followed by heat inactivation of the enzymes at 65°C and a dilution of the digested plasmid to 10 ng/µl. The Lysine Insert and Lysine Adaptor were designed with BamHI and HindIII overhangs to enable cloning into pZEO1-α at these restriction sites.

The DNA cassette and respective acceptor plasmid were ligated together in the presence of T4 DNA ligase at 16°C for 30 min. A 2-µl aliquot of the ligation reaction mixture was used to transform 40 µl of electrocompetent TOP10F’ E. coli cells. The resultant double-stranded DNA was recovered through a QiaGene Spin Miniprep protocol (Qiagen, Inc.). DNA was initially screened by BamHI and HindIII double digestion. Positive transformants were verified by agarose gel electrophoresis (4% GTG NuSieve agarose, 1× TBE buffer). Automated DNA sequencing utilizing the M13 forward and reverse primers confirmed correct DNA products. Plasmids containing the correct sequence for the Lysine Insert and Lysine Adaptor are identified as pL and pR, respectively.
2.3. Assembly of elastin-mimetic triblock copolymers

The proteins, LysB10 and R4, were designed to contain the Lysine Insert between each plastic-like and elastin-like block, and to be flanked by the Lysine Adaptor (Lysine Adaptor-Plastic-like Domain-Lysine Insert-Elastin-like Domain-Lysine Insert-Plastic-like Domain-Lysine Adaptor). All subcloning steps were performed in the pZEO-1 plasmid using LSLB media under Zeocin antibiotic selection.

Recombinant plasmids encoding the elastin-like (pE) (E1: 2.1 kb, E2: 1.1 kb) and plastic-like (pP) (P2: 2.5 kb, P1: 1.2 kb) domains were constructed, as described above. Each gene was isolated from its respective plasmid with BbsI and BsmBI sequential digestion. The gene fragment was isolated via preparative gel electrophoresis (1% agarose, 0.5x TBE) and purified using Zymoclean Gel Recovery (ZymoResearch, Inc.). Preparative amounts of pl DNA were isolated for two separate reactions. A total of 5 µg of pl was digested with restriction enzyme BbsI and Shrimp Alkaline Phosphatase (SAP) dephosphorylated (1 U SAP per 1 pmol strand ends) to prevent re-ligation. A separate 5 µg of pl was digested with the restriction enzyme BsmBI and SAP dephosphorylated. The linearized plasmids were isolated via preparative gel electrophoresis (1% agarose, 0.5x TBE) and purified using Zymoclean Gel Recovery.

Similar protocols for ligation, transformation, and propagation were followed, as previously described. Two separate ligation reactions were performed between BbsI digested pl and p and BsmBI digested pl and P. Isolated DNA from clones were screened by a BamHI and HindIII double digestion and cleavage fragments analyzed by agarose gel electrophoresis. Correct ligation was confirmed by automated DNA sequence analysis using M13 forward and reverse primers. Plasmids containing the correct sequences are identified as pPIE and pPI.

Analogously, 5 µg of pPI was digested with restriction enzyme BsmBI and Shrimp Alkaline Phosphatase (SAP) dephosphorylated (1 U SAP per 1 pmol strand ends) to prevent re-ligation. The linearized plasmid was isolated via preparative gel electrophoresis (1% agarose, 0.5x TBE) and purified using Zymoclean Gel Recovery. Linearized pPI was ligated with E, followed by transformation and propagation. Isolated DNA from transformants was screened by a BamHI and HindIII double digestion and cleavage fragments analyzed by agarose gel electrophoresis. Correct ligation was confirmed by automated DNA sequence analysis using M13 forward and reverse primers. The plasmid containing the correct sequence was termed pPIE.

Recombinant plasmids pIP and pPIE were digested with BbsI/BamHI and BsmBI/XcmI, respectively. The gene fragment from each of these digestions was isolated via preparative gel electrophoresis (1% agarose, 0.5x TBE) and purified using Zymoclean Gel Recovery. pIP and pPIE fragments were ligated by T4 DNA ligase, transformed into Top10F cells and plated on LSLB/Zeocin plates. As the XcmI site cuts within the Zeocin coding region, only clones containing the correctly assembled triblock (pPIE), and thus, the correctly reassembled antibiotic coding region, were able to propagate. Transformants were confirmed by analysis of BamHI and HindIII restriction digest fragments with agarose gel electrophoresis (1% agarose, 0.5x TBE) and automated DNA sequence analysis using M13 forward and reverse primers. The plasmid containing the correct sequence for the triblock was termed pPIEIP.

pA, containing the Lysine Adaptor, was assembled, as described above. This plasmid was then digested with restriction enzyme BsmBI and SAP dephosphorylated. The triblock, pPIEIP, was excised from the pZEO-1 plasmid via sequential digestion using restriction enzymes BbsI and BsmBI and purified via gel isolation. A ligation reaction was performed to relocate the pPIEIP gene from pPIEIP to pA. The ligation mixture was transformed into competent TOP10F cells and plated on LSLB media under Zeocin antibiotic selection. Isolated DNA from transformants was screened via agarose gel electrophoresis analysis of a BamHI and HindIII double digestion. Automated DNA sequence analysis using M13 forward and reverse primers confirmed correct insertion of the gene pAPIEIPIA and pAPIEIPAP. pAPIEIPIA was identified as the cloning plasmid, plysB10 and pAPIEIPAP as the cloning plasmid, pR4.

The pET24-a plasmid (1 µg, Invitrogen) was prepared via BamHI and HindIII double digestion, followed by gel isolation and purification. The APEIEIPA gene was released from the pZEO-1 vector at analogous sites. Adaptor and plasmid were ligated together in the presence of T4 DNA ligase at 16 °C for 30 min. A 2-µl aliquot of the ligation reaction mixture was used to transform 40 µL of electrocompetent TOP10 E. coli cells. A 100-µl aliquot of the transformation mixture was spread onto LB agar supplemented with kanamycin (50 µg/mL). The plates were incubated for 12 h at 37 °C. Five transformants were selected from each plate to inoculate individual 7 mL cultures of LB/kanamycin media. Cultures were rotary incubated for 12 h at 37 °C. Plasmid DNA was isolated according to a Quagen Spin Miniprep protocol (Quagen, Inc.). DNA was screened by a BamHI and HindIII double digestion. Positive transformants were verified by agarose gel electrophoresis (4% TAE NuSieve agarose, 1x TBE buffer). Automated DNA sequencing utilizing the T7 promoter and T7 terminator primers confirmed the correct DNA product. The resultant plasmid was identified as expression plasmids, plysB10 (APIEIPAP) and pR4 (APIEIPAP). DNA agarose gels in Figs. 1 and 2 depict gene products at each subcloning step in the assembly of plysB10 and pR4, respectively.

2.4. Isolation and purification of protein triblock copolymers

The plasmid, plysB10, encoding the protein LysB10 as a single contiguous reading frame within plasmid pET24-a was used to transform the E. coli expression strain BL21(DE3). This afforded a protein triblock of sequence (Table 2):
Fig. 2. Analytical restriction digests, 13% TAE (Tris–acetate–EDTA) agarose gel, depicting gene and vector sizes at each stage of the R4 assembly process with corresponding digestion schemes. DNA standard used was a 1-kb DNA ladder (NEB). A. Lane 1: Nol digest pE2 (1.1 kb), pZerO-1 (2.8 kb). Lane 2: Nol digest pP2 (1.2 kb), pZerO-1 (2.8 kb). Lane 3: Nol digest pP4 (1.36 kb), pZerO-1 (2.8 kb). Lane 4: Nol digest pP4 (1.26 kb), pZerO-1 (2.8 kb). Lane 5: Nol digest pPIL (2.42 kb), pZerO-1 (2.8 kb). B. Lane 1: Nol digest pP4 (3.7 kb), pZerO-1 (2.8 kb). Lane 2: Nol digest pP4 (3.7 kb), pZerO-1 (2.8 kb). Lane 3: BamHI/HindIII digest pRO (3.7 kb), PET24-a (5.3 kb).

The plasmid, pR4, encoding the protein R4 as a single contiguous reading frame within plasmid PET24-a was used to transform the E. coli expression strain BL21 (DE3). This afforded a protein triblock of sequence (Table 3):

**Table 3**

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2.5. Identification of elastin-mimetic proteins

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed a single protein band at 250 kDa and 100 kDa corresponding to LysB10 (Fig. 1A) and R4 (Fig. 1B). A total of 10 μg of the elastin-mimetic polypeptide along with molecular weight markers (Precision Plus Protein Kaleidoscope, Bio-Rad) were run on a 7.5% gel and negatively stained with a Copper stain.
Amino acid sequence of **R4** and related nucleic acid coding sequence [VPAVGKPVAVG](IPAVG)_{16} [IPAVGIPAVG]KAA(VPGAGVPG) [(VPGIG)_{5}] [VPAGVPAG]KAAK(VPGAGVPG) [(IPAVG)_{16} IPAVGIPAVG]KAAK.

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Table 3

Amino acid sequence of **R4** and related nucleic acid coding sequence [VPAVGKPVAVG](IPAVG)_{16} [IPAVGIPAVG]KAA(VPGAGVPG) [(VPGIG)_{5}] [VPAGVPAG]KAAK(VPGAGVPG) [(IPAVG)_{16} IPAVGIPAVG]KAAK.

2.6. Rheological analysis of concentrated protein polymer solutions

Rheological data were acquired on an Advanced Rheological Expansion System III rheometer (ARES III, TA instrument, NJ) in parallel plate geometry with a plate diameter of 25 mm. The testing protocol for rheological analysis has been detailed elsewhere [4]. In brief, 100 mg/mL protein solutions were prepared by adding distilled, deionized water to lyophilized protein at 4°C, shaking the solution for 48 h, and then allowing the solution to equilibrate for 72 h. The gap between parallel plates was adjusted between 0.2 and 0.35 mm and dynamic mechanical experiments were performed in shear deformation mode. An initial strain amplitude sweep was performed at 4°C and 37°C at a frequency of 1 Hz to confirm the linear viscoelastic range for the protein polymer.

The gelation temperature was determined by heating samples from 4°C to 37°C at a rate of 1°C per minute. Following temperature equilibration at 37°C, viscoelastic properties were examined by a strain sweep at a fixed frequency of 1 Hz and a frequency sweep at fixed strain amplitude of 2%. Experiments were repeated on six samples and representative data presented.

2.7. Fabrication of water cast protein films

For mechanical property analysis, films were cast from protein solutions in water at room temperature. In brief, lyophilized proteins were dissolved at a concentration of 100 mg/mL in water at 4°C. The protein solution was then poured into Teflon casting molds and regulated solvent evaporation performed at 23°C. Subsequently, the films were rinsed in PBS for 48 h with a change of PBS at 24 h. Test samples were referred to as crosslinked or non-crosslinked indicating whether GTA vapor phase crosslinking. Specifically, films were enclosed in a 2-L chamber containing a 10 mL of 25% glutaraldehyde (GTA) solution. Films were placed on a platform 4 cm above the GTA solution and exposed to GTA vapor for 24 h. Subsequently, the films were rinsed in PBS for 48 h with a change of PBS at 24 h. Test samples were referred to as crosslinked or non-crosslinked indicating whether GTA treatment was used. Prior to testing, films were hydrated in PBS at 37°C, which contained NaN₃ at 0.2 mg/mL to prevent biological contamination. Samples were cut into a dumbbell shape using a stainless steel die with gauge dimensions of 13 mm x 4.75 mm. Hydrated film thickness, as measured by optical microscopy, was typically 0.07 mm for non-crosslinked and crosslinked **LysB10** films and 0.1 mm for **R4** films.

2.8. Evaluation of water content in protein films

For evaluation of water content, 200 μL of a 10-wt% protein solution was cast as a disk measuring 1 cm in diameter. Dried films were vapor phase crosslinked with a 25% GTA solution for 24 h, fully dehydrated under vacuum, and the dehydrated weight obtained using a Mettler balance. Films were subsequently incubated in PBS at 37°C for 24 h and fully hydrated weights were obtained. A total of six films were evaluated for each protein. The equilibrium water content and equilibrium swelling ratio were determined according to Eqs. (1) and (2), respectively, and expressed as mean ± standard deviation [30].

\[
\text{Equilibrium water content} = \frac{\text{hydrated weight} - \text{dehydrated weight}}{\text{hydrated weight}} \tag{1}
\]

\[
\text{Equilibrium swelling ratio} = \frac{\text{hydrated weight}}{\text{dehydrated weight}} \tag{2}
\]
2.9. Characterization of non-crosslinked extractables

To determine the percent of potentially extractable protein polymer, 200 μL of a 10 wt% protein solution was cast as a disk measuring 1 cm in diameter. Dried films were vapor phase crosslinked with a 25% GTA solution for 24 h, rinsed in PBS and incubated at 4°C, below the inverse transition temperature of the protein, for a period of 7 days. Every 48 h films were dehydrated and dry weight was monitored for material losses. Six films were investigated for each protein. The percent extractable was determined by Eq. (3) and expressed as a mean ± standard deviation.

\[
\% \text{ Extractables} = 1 - \frac{\text{final dehydrated weight}}{\text{initial dehydrated weight}}
\]

\[
\% \text{ Resilience} = \frac{\text{area under loading curve} - \text{area under unloading curve}}{\text{area under loading curve}} \times 100
\]

Crep analysis was performed on 6–12 specimens for each film type subjected to varying levels of constant engineering stress for periods of up to 11 h.

2.10. Mechanical analysis of hydrated protein films

A preconditioning protocol was employed for LysB10 samples that consisted of a single cyclic stretch to 50% strain for one cycle followed by 20 cycles of 30% strain with off-loading periods of 5 min between each cycle. Due to the plasticity of R4 protein films, preconditioning was not conducted.

Uniaxial stress–strain properties of protein films were determined on at least 3–6 individual specimens using a dynamic mechanical thermal analyzer (DMTA V, Rheometric Scientific, Inc., New Castle, DE) with a 15-N load cell in the inverted orientation, so that samples could be immersed in a jacketed beaker filled with PBS at 37°C. The maximum travel distance of the drive shaft was 23 mm, which limited maximum strain to 70% of engineering strain. Given the extensibility of these materials, uniaxial stress–strain responses were also characterized using a miniature materials tester (Minimat 2000, Rheometric Scientific) in tensile deformation mode at a rate of 5 mm/min conducted in air at room temperature. All samples were coated with a thin layer of mineral oil to prevent dehydration. For both DMTA and Minimat testing, samples were cut into a dumbbell shape using a stainless steel die with gauge dimensions of 13 mm × 4.75 mm. In addition, to calculating Young's modulus (E), ultimate tensile stress (UTS), and strain at failure (ε), resilience was determined from Eq. (4).

\[
\% \text{ Extractables} = \frac{\text{initial dehydrated weight - final dehydrated weight}}{\text{initial dehydrated weight}} \times 100
\]

\[
\% \text{ Extractables} = \frac{m_m}{m_m + m_C14 + m_C12 + m_C6 + m_C0 + m_LysB10 + m_{R.E.}}
\]

\[
\% \text{ Extractables} = \frac{m_Lys}{m_Lys + m_{R.E.} + m_{H2O}}
\]

2.11. In vivo evaluation of crosslinked protein gels

2.11.1. Syringe casting method for creating cylindrical implants

In order to minimize sample manipulation and the risk of cross-contamination, the following protocol was used for preparation of samples for in vivo implant studies. A 10 wt% cold protein solution was drawn into a chilled sterile 1 mL syringe (Becton Dickinson) and subsequently gelled at 37°C. The tip of the syringe was diluted to 1 μL/50 μL/100 μL [4]. The implant was then immediately immersed in 10 mL of cold Hank’s Balanced Salt Solution containing 10 U/mL heparin and 1% BSA (Mediatech, Inc.). Typically, 6–7 mL of lavage solution was retrieved and cells immunostained for flow cytometry with PE-conjugated rat monoclonal anti-mouse CD11b for macrophages, FITC-conjugated hamster anti-mouse CD3 for total T cells, FITC-conjugated rat monoclonal anti-mouse CD4 for helper T cells, FITC-conjugated rat monoclonal anti-mouse CD8 for cytotoxic T cells, FITC-conjugated rat monoclonal anti-mouse CD19 for B cells, and FITC-conjugated rat monoclonal anti-mouse Gr-1 for neutrophils (BD Bioscience Pharmingen). Typically, antibodies were diluted to 1 μg/50 μL/100 μL in PBS containing 1% BSA and 0.1% sodium azide. Cells were incubated in the dark for 30 min on ice, then washed three times in staining buffer, and fixed in 1% paraformaldehyde. Analysis was performed on an FACSscan using CellQuest (Becton Dickinson) and FlowJo software (Tree Star) [31]. Comparison between groups was analyzed via a Student’s t-test and p < 0.05 was considered to be significant. Results are presented as mean ± SEM. Two control groups were employed; one that did not undergo surgery and another in which surgery was performed without sample implantation. At least five mice were enrolled in each experimental and control group.

2.11.2. Subcutaneous and peritoneal implant models

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Emory University. Eight-week-old inbred male C57BL/6 mice weighing 25–30 g were obtained from the Jackson Laboratory (Bar Harbor, ME). Under ketamine (95 mg/kg, IM) and xylazine (5 mg/kg, IM) anesthesia, a 1-cm dorsal midline incision was performed and a single test sample implanted in the subcutaneous space, parallel to the longitudinal body axis of each mouse. Each implant type was implanted in at least three separate mice. Three weeks after implantation, animals were sacrificed and samples explanted with overlying skin. For peritoneal implants, a 1-cm long midline incision was made along the linea alba of the abdominal wall and a single implant placed into the peritoneal cavity. After closing the abdominal muscle with 4-0 absorbable surgical suture (Vicryl, Ethicon, Inc., NJ), the skin incision was closed with wound clips. Each recombinant protein was implanted in at least five mice. Mice were sacrificed one week later and, prior to sample removal, a peritoneal saline lavage was performed to harvest cells for FACS analysis.

2.11.3. Histological examination

Retrieved samples were processed for histological and immunohistochemical evaluation to characterize the local cellular response and to determine the extent and thickness of fibrous capsule formation. All samples were fixed in 10% neutral buffered formalin overnight and processed for paraffin embedding. Sections were prepared at a thickness of 5 μm and stained with hematoxylin and eosin (H&E) or rat anti-mouse monoclonal F4/80 (CI:A3-1, Abcam) to identify infiltrating macrophages. In all cases, multiple sections were examined in three to five separate samples for each protein polymer type.

2.11.4. Fluorescent-activated cell sorting (FACS) of peritoneal lavage

Prior to harvesting implants, each peritoneal cavity was initially lavaged with 10 mL of cold Hank’s Balanced Salt Solution containing 10 U/mL heparin and 1% BSA (Mediatech, Inc.). Typically, 6–7 mL of lavage solution was retrieved and cells immunostained for flow cytometry with PE-conjugated rat monoclonal anti-mouse CD11b for macrophages, FITC-conjugated hamster anti-mouse CD3 for total T cells, FITC-conjugated rat monoclonal anti-mouse CD4 for helper T cells, FITC-conjugated rat monoclonal anti-mouse CD8 for cytotoxic T cells, FITC-conjugated rat monoclonal anti-mouse CD19 for B cells, and FITC-conjugated rat monoclonal anti-mouse Gr-1 for neutrophils (BD Bioscience Pharmingen). Typically, antibodies were diluted to 1 μg/50 μL/100 μL in PBS containing 1% BSA and 0.1% sodium azide. Cells were incubated in the dark for 30 min on ice, then washed three times in staining buffer, and fixed in 1% paraformaldehyde. Analysis was performed on an FACSscan using CellQuest (Becton Dickinson) and FlowJo software (Tree Star) [31]. Comparison between groups was analyzed via a Student’s t-test and p < 0.05 was considered to be significant. Results are presented as mean ± SEM. Two control groups were employed; one that did not undergo surgery and another in which surgery was performed without sample implantation. At least five mice were enrolled in each experimental and control group.

3. Results and discussion

3.1. Synthesis of triblock protein copolymers capable of both chemical and physical crosslinking

We have recently reported the design of a new class of recombinant elastin-mimetic triblock copolymer that has the capacity to form physical or virtual crosslinks, which stabilize protein network structure [3–5]. Moreover, through selective engineering of block structure, including the design of block size or sequence, and choice of film casting conditions, microphase structure can be manipulated and, as a consequence, material properties, such as drug elution characteristics and mechanical behavior tailored over a wide range of responses [4,7,8]. However, physical crosslinks formed as a result of hydrophobic aggregation may be deformed or disrupted under external stresses lower than that required to disrupt covalent crosslinks. This feature may limit the capacity of physically crosslinked protein-based materials to retain material integrity under loading conditions operative for a number of potential applications in tissue engineering or regenerative medicine. Given these considerations, we have postulated that chemically locking a multiblock protein assembly in place may provide a strategy to preserve functional responses that are linked to specific domain structures and morphologies over a broader range of externally applied loads. Further, it would also provide an additional approach for altering material strength and compliance, as well as stress induced creep behavior.

In this report, elastin-mimetic triblock copolymers were designed with endblock sequences, encoded by a hydrophilic acid sequence, and a central elastomeric block of varying amino acid sequence. The endblock sequence was designed with functional responses that are linked to specific domain structures and morphologies over a broader range of externally applied loads. Further, it would also provide an additional approach for altering material strength and compliance, as well as stress induced creep behavior.
endblocks, each with a mass of approximately 37 kDa that contained 16 repeats of [IPAVG]5, separated by a 35-kD mid-block comprised of 15 repeats of [VPGIG]5 (Table 3). Likewise, a total of eight potential crosslinking sites were engineered into the protein sequence; positioned predominantly as lysine pairs (KAAK) that flank each block. The substitution of isoleucine for glutamic acid in the midblock yielded a protein that was largely hydrophobic with little difference in block polarity. While VPGEG and VPGIG are both reported to form β-spiral structures that display elastic responses when crosslinked as gels, polypeptides from each sequence differ significantly in their inverse transition temperature. Specifically, by incorporating the hydrophobic residue, isoleucine, into the fourth position of the repeat sequence, the inverse transition temperature of VPGIG is much lower than that associated with VPGEG [32–36].

Lysine crosslinking domains were engineered with an appreciation of the structure of similar crosslinking sites in native elastin and a consideration of the ‘N-end rule’, such that the identity of the N-terminal residue of a recombinant protein may influence degradation in bacterial expression systems. In native elastin, crosslinking domains contain paired lysines within polyalanine repeats (e.g. Ala-Ala-Ala-Lys-Ala-Ala-Lys-Ala-Ala) [26,27], which promotes formation of an alpha-helix that has been reported to facilitate intermolecular crosslinking [37–40]. Thus, for both protein triblock copolymers, a lysine-containing insert was designed encoding two lysine residues separated by two alanine residues (Lys-Ala-Ala-Lys) that were inserted between component blocks. Additionally, lysine-containing adaptor sequences were designed to encode for two C-terminal, as well as a single lysine residue near the N-terminus. Lysine was not incorporated as an N-terminal residue, as previous efforts to encode lysine in this position have lead to a 10-fold decrease in protein yield [1,10,41,42]. This design afforded eight free amines for crosslinking, seven from lysine and one from the N-terminal amine (Fig. 3C).

3.2. Rheological analysis confirms formation of viscoelastic protein gels

The gelation point of protein solutions can be determined by measurement of $G'$ and $G''$ as a function of temperature at a fixed frequency. Above 13 °C, the shear storage ($G'$) and loss ($G''$) modulus of concentrated aqueous solutions of LysB10 increased by a factor of approximately 10³ and 10 (Pa), respectively, while tan δ ($G'/G''$) decreased, consistent with the formation of a viscoelastic gel (Fig. 4A). Above 15 °C, R4 solutions displayed an increase in shear storage ($G'$) and loss ($G''$) modulus to 10⁴ and 10³ (Pa), respectively, with only a modest reduction in tan δ (Fig. 4C). For both LysB10 and R4 protein solutions at 37 °C, $G'$ and $G''$ were independent of frequency between 1 to 10 rad/s at a fixed strain amplitude of 2% (Fig. 4B,D). In addition, the complex viscosity ($η^*$) was a linear function of the logarithm of frequency with a slope of 1.0, however, as evident by a significantly higher tan δ and complex viscosity, aqueous gels of R4 were more viscous than those of LysB10. This difference highlights the significance of the midblock structure in triblock design. Despite similar endblock structure and the presence of an elastomeric midblock sequence in both R4 and LysB10 triblocks, the R4 midblock is considerably more hydrophobic and coacervates along with the endblock at 37 °C. Indeed, when expressed as single blocks the inverse transition temperatures of the R4 endblock and midblock proteins

---

Fig. 4. Rheological behavior of triblock protein polymers in water. (A) LysB10 dynamic shear storage ($G'$) and loss modulus ($G''$) are plotted as a function of temperature ($γ$ 2%, ω 1 Hz). (B) LysB10 dynamic shear storage ($G'$), loss modulus ($G''$), and complex viscosity ($η^*$) are plotted as a function of frequency ($γ$ 2%, 37 °C). (C) R4 dynamic shear storage ($G'$) and loss modulus ($G''$) are plotted as a function of temperature ($γ$ 2%, ω 1 Hz). (D) R4 dynamic shear storage ($G'$), loss modulus ($G''$), and complex viscosity ($η^*$) are plotted as a function of frequency ($γ$ 2%, 37 °C).
were 26 °C and 16 °C, respectively, while the comparable transition temperatures for LysB10 blocks were 21 °C and >80 °C (data not shown). Thus, significant mixing of the elastomeric and plastic-like blocks occurs only in the case of gels produced from the R4 protein polymer, which limits its elastomeric response. We have previously demonstrated that selected changes in midblock size and amino acid sequence result in significant changes in viscoelastic mechanical properties. Indeed, a more viscous response, similar to that observed for R4, was displayed by a protein composed entirely of hydrophobic plastic-like endblock sequences [4].

3.3. Glutaraldehyde crosslinking of elastin-mimetic triblock copolymer films

Investigations by our group and others have demonstrated that covalent crosslinks can enhance the mechanical stability of a variety of elastin analogues (Table 4). Aldehyde crosslinking agents, such as glutaraldehyde, have been commonly used to process implanted tissues and proteins because of their capacity for covalent crosslinks can enhance the mechanical stability of elastomeric; displaying an elastic modulus of 0.49 ± 0.38 MPa, an ultimate tensile strength of 2.8 ± 0.91 MPa, a strain at failure of 430 ± 34%, and a resilience of 53 ± 2%. In contrast, preconditioned crosslinked samples exhibited a two- to three-fold increase in Young’s modulus and a 50% decrease in strain at failure with a modest increase in ultimate tensile strength, as compared to their non-crosslinked counterparts (Table 5, Figs. 5 and 6). We speculate that these differences are largely related to the stabilization of semi-rigid endblocks by chemical crosslinking. While crosslinking enhanced strength and modulus, a small reduction in resilience was noted. Since crosslinking was performed prior to preconditioning, the capacity of chain entanglements between midblock and endblock sequences to structurally rearrange in response to the conditioning protocol may have been restricted. In other words, in addition to restricting the mobility of the elastomeric

The equilibrium water content observed for R4 and LysB10 films was 32.0 ± 3.7% and 54.3 ± 3.8%, respectively; values that are similar to the 32% water content reported for hydrated elastin at 36 °C[45]. Likewise, water swelling ratios were 1.5 ± 0.1 for R4 and 2.4 ± 0.2 for LysB10, consistent with the higher proportion of hydrophobic amino acids in R4.

3.4. Mechanical responses of LysB10 copolymer films

Most biomolecular constructs represent dynamic systems of hydrated biopolymer chains whose entanglements and structural interrelationships may be altered in response to an external load. As a consequence, initial mechanical properties may change in response to repetitive loading forces until stable behavior is observed. Thus, mechanical preconditioning is presumed to induce changes in microstructure that lead to fixed structural rearrangements of the constituent polymer chains and, as a consequence, stable material properties under a given loading environment[46–49]. Preconditioned, non-crosslinked LysB10 films were robust and elastomeric; displaying an elastic modulus of 0.49 ± 0.03 MPa, an ultimate tensile strength of 2.88 ± 0.91 MPa, a strain at failure of 430 ± 34%, and a resilience of 53 ± 2%. In contrast, preconditioned crosslinked samples exhibited a two- to three-fold increase in Young’s modulus and a 50% decrease in strain at failure with a modest increase in ultimate tensile strength, as compared to their non-crosslinked counterparts (Table 5, Figs. 5 and 6). We speculate that these differences are largely related to the stabilization of semi-rigid endblocks by chemical crosslinking. While crosslinking enhanced strength and modulus, a small reduction in resilience was noted. Since crosslinking was performed prior to preconditioning, the capacity of chain entanglements between midblock and endblock sequences to structurally rearrange in response to the conditioning protocol may have been restricted. In other words, in addition to restricting the mobility of the elastomeric
phase, crosslinked-fixed mixing between rigid and elastomeric domains may have contributed to this effect.

The approximate hoop stress of a blood vessel with an inner diameter of 4.5 mm and wall thickness of 0.8 mm is 45 kPa, when subjected to an intraluminal pressure of 16 kPa (120 mmHg). Crosslinked LysB10 films demonstrated limited creep (<10%) over

11 h at stress levels at or below 45 kPa. Increasing the applied stress by a factor of ten increased creep to ~30% (Fig. 7A). In contrast, non-crosslinked films showed a four-fold greater level of creep strain in response to 45 kPa loading stress (Fig. 7B).

3.5. Mechanical responses of R4 copolymer films

Hydrated R4 films revealed plastic-like deformation behavior. Specifically, stress increased linearly with increasing strain until a yield point was observed at 1.72 MPa and 4.49 MPa in non-crosslinked and crosslinked films, respectively (Fig. 8A). Corresponding values of Young’s modulus were 48.6 ± 0.9 MPa and 67 ± 5.14 MPa for non-crosslinked and crosslinked samples, respectively. These values are four- to ten-fold greater than those noted for LysB10 (Table 5). As compared to LysB10, the more hydrophobic character of R4 is associated with reduced water uptake, which contributes to an increase in material rigidity and tensile strength. Moreover, given the similarity of endblock and midblock polarity, size, and transition temperature, we speculate that a greater degree of block mixing occurs in films composed of R4 with rigid, plastic-like domains sustaining a higher level of the external load. Crosslinking appears to have a relatively greater effect on the yield point (2.6-fold increase) than Young’s modulus (1.4-fold increase). Likely, the effect of block mixing has a more profound effect on the mobility of the elastomeric phase than crosslinking, which predominantly stabilizes the semi-rigid endblocks. Consistent with these observed uniaxial stress–strain properties, crosslinked R4 films demonstrated limited creep strain (<10%) over an 11-h period, despite applied stresses as high as

![Table 5](image)

Summary of mechanical parameters in crosslinked and non-crosslinked films

<table>
<thead>
<tr>
<th>Protein Treatment</th>
<th>Resilience at 30% strain (%)</th>
<th>Young’s modulus DMTA (MPa)</th>
<th>Young’s modulus Minimat (MPa)</th>
<th>UTS (MPa)</th>
<th>Strain at failure (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LysB10 GTA</td>
<td>39 ± 1</td>
<td>1.10 ± 0.45*</td>
<td>1.60 ± 0.48*</td>
<td>3.62 ± 0.98</td>
<td>223 ± 30*</td>
</tr>
<tr>
<td>Xlinked</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LysB10 Non-</td>
<td>52 ± 2*</td>
<td>0.49 ± 0.03*</td>
<td>0.53 ± 0.02*</td>
<td>2.88 ± 0.91</td>
<td>430 ± 34*</td>
</tr>
<tr>
<td>Xlinked</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R4 GTA</td>
<td>–</td>
<td>67.4 ± 5.14*</td>
<td>–</td>
<td>4.49 ± 0.27</td>
<td>8 ± 2*</td>
</tr>
<tr>
<td>Xlinked</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R4 Non-Xlinked</td>
<td>–</td>
<td>48.6 ± 0.90*</td>
<td>–</td>
<td>1.72 ± 0.30</td>
<td>4.9 ± 1*</td>
</tr>
<tr>
<td>B10 Water</td>
<td>67 ± 1</td>
<td>0.71 ± 0.12</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Average values obtained from 3 to 10 replicates. Resilience and Young’s Modulus determined from DMTA testing. Young’s modulus, UTS, and % strain determined from Minimat testing.

*p < 0.05 between crosslinked and non-crosslinked samples.

* B10 Data obtained from Wu et al. [72].
400 kPa. Substantial deformation, however, was noted at stresses of nearly 1 MPa (Fig. 8B).

3.6. In vivo responses to crosslinked elastin-mimetic protein hydrogels

Using a murine model, crosslinked LysB10 and R4 hydrogels were implanted into either the subcutaneous space or the peritoneal cavity. FACS analysis demonstrated no difference in either the cell number or cell type identified within peritoneal lavage fluid, harvested one week after either sham surgery or protein polymer implantation (Fig. 9). Samples implanted within the peritoneal cavity and subcutaneous tissue were explanted one and three weeks after implantation, respectively, without evidence of protein gel dissolution (Figs. 10 and 11). Macrophages were identified along the periphery of the fibrous capsule and in the surrounding tissue without infiltration into the implant. The fibrous capsule thickness of LysB10 samples within the subcutaneous space was 25.3 ± 16.1 μm, while the capsule measured 14.5 ± 5.3 μm for those placed in the peritoneal cavity. R4 implants displayed a similar response, with a fibrous capsule thickness of 24.2 ± 6.0 μm and 8.4 ± 1.2 μm for subcutaneous and peritoneal cavity implants, respectively.

Tissue–material interactions, including biopolymer stability, are integral to assessing the suitability of elastin-like protein polymers for implant related applications. To date, reports documenting in vivo responses to elastin-mimetic protein implants have been limited; largely confined to studies performed 15–20 years ago on proteins synthesized chemically and subject to radiation crosslinking [36,50]. In these investigations, homopolymers or copolymers composed of VPGVG, VPGKG, VPGEG, IPAVG, and VPAVG reportedly did not induce significant inflammatory or allergic reactions [50–53]. The most thoroughly characterized elastin variant, chemically synthesized poly(GVGVP), was subjected to in vitro toxicity and mutagenicity assays and was administered via intravenous, intraocular, intramuscular, intraperitoneal, and subcutaneous routes without toxic effect [36,50]. A fibrous capsule was noted three weeks after intramuscular implantation of a radiation crosslinked sample [36]. In a more recent report, elastin microparticles prepared from chemically synthesized poly(VPAVG) were evaluated following subcutaneous and intravitreal injection. No inflammatory response was observed after 28 days. However, tractional retinal detachment was noted [51]. The failure to detect an immune mediated reaction to these polymers is consistent with other studies that have sought to identify potentially immunogenic epitopes on native elastin. While polyclonal and monoclonal antibodies can be raised against peptides derived from the hydrolysis of native elastin, neither VPGVG nor VPAVG peptides have been among the recognized sequences [53]. Moreover, VPGVG peptides were unable to competitively inhibit the binding of any of the antibodies raised against native elastin, which further supports the notion that this pentapeptide is not present among antigenic elastin epitopes [53].
Recently, genetically engineered elastin-mimetic protein polymers have been investigated in vivo as non-thrombogenic coatings [54,55], targeted drug delivery vehicles [56–58], and as an implantable material [59]. In the latter instance, after a 13-week implant period in the subcutaneous space, recombinant human tropoelastin ‘sponges’, chemically crosslinked with bis(sulfosuccinimidyl) suberate, were surrounded by a fibrous capsule with a minimal to moderate inflammatory response [59]. Non-chemically crosslinked recombinant elastin-like proteins have also been administered within the intra-articular space as a 650-µM protein solution [59]. Although the biological response was not evaluated, this study revealed a 3-h half-life for non-aggregating VPGVG proteins and a 3 day half-life for aggregating VPCXG proteins, where X = V:G:A at a ratio of 1:7:8. As a related material, a 47-kDa recombinant silk-elastin-like protein (SELP), comprised of GAGAS silk-like [S] and GVGVP elastin-like [E] amino acid sequences ([S]₄[E]₄[EK][E]₃) has been studied after injection into the subcutaneous space. Histological analysis revealed minimal fibrous encapsulation after four weeks with a mild degree of inflammation that included the presence of macrophages in the surrounding tissue [60]. SELPs have been also used for adenoviral gene delivery and demonstrate prolonged and localized expression of adenoviruses for up to 15 days [61,62].

The evaluation of in vivo biocompatibility is largely based on characterizing local tissue responses to subcutaneously implanted materials where the intensity and duration of inflammation and wound healing, including capsule formation, is evaluated histologically [63,64]. Although histological studies of biopolymers containing elastin-mimetic sequences have previously noted the presence of ‘mild inflammation’ [50,51] and ‘a reaction that was limited to a typical cell mediated response to the presence of a foreign body’, the extent of fibrous capsule formation has not been reported [59]. Fibrous capsule thickness has been investigated for a variety of polymeric and ceramic implants designed for tissue

Fig. 9. FACS analysis of peritoneal cells one week after implantation of LysB10 and R4 cylindrical hydrogels (n = 5 for each group). Experimental groups displayed an identical cell profile to non-operated and sham surgery groups.

Fig. 10. (A) Hematoxylin and eosin staining of subcutaneous LysB10 implants demonstrates the presence of a mild foreign body reaction along the periphery. (B) F4/80 staining of subcutaneous LysB10 implants demonstrate the presence of macrophages along the periphery of the fibrous capsule. (C) H&E staining of peritoneal LysB10 implants demonstrates the presence of a mild foreign body reaction along the periphery. (D) F4/80 staining of peritoneal LysB10 implants demonstrates the presence of macrophages along the periphery. Images are oriented so that the LysB10 gel is located in the bottom right corner, 20x magnification.
repair, cell encapsulation, or as drug delivery systems \[65–68\]. Capsule thicknesses are dependent on implant site and material type and typically vary between 2 and 150 \(\mu m\) over implantation periods of one to three months. For example, greater capsule thickness has been observed for materials within intraperitoneal sites compared to those in subcutaneous sites over identical implant durations \[66\]. As an illustration of the effects of surface chemistry, implants comprised of poly(alkyl methacrylate) (PAMA) with short alkyl side chains exhibited a thicker fibrous capsule than those with long side chains (140 \(\mu m\) vs 120 \(\mu m\)) \[65\]. Additionally, self-assembled monolayers (SAMs) of alkanethiols on gold with different terminal functional groups displayed surface dependent inflammatory responses after one week with extremely hydrophobic methyl terminated surfaces inducing thick fibrous capsules (130 \(\mu m\)) and higher recruitment of inflammatory cells compared to hydrophilic COOH- and OH- terminated surfaces (80 \(\mu m\) and 70 \(\mu m\), respectively) \[69\]. Likewise, functionalized polypropylene implants revealed similar foreign body reactions to surface modifications, with –OH surfaces triggering a stronger response (\(~100\ \mu m\)) compared to –COOH rich surfaces (37 \(\mu m\)) \[70\]. In contrast, surface topography does not appear to have a significant effect on capsule thickness, although it may influence local inflammatory responses \[71\]. In this report, there were no observable differences in biological responses for either chemically crosslinked triblock elastin-mimetic protein polymer. Both R4 and LysB10 implants initiated limited local inflammatory activity and displayed relatively thin fibrous capsules.

4. Conclusions

A new class of recombinant elastin-mimetic protein polymer has been designed that is capable of both physical and chemical crosslinking. We have demonstrated that chemical crosslinking provides an independent mechanism for control of protein mechanical responses. Specifically, elastic modulus can be enhanced and creep strain reduced through the addition of chemical crosslinking sites. Additionally, we have demonstrated exceptional biocompatibility of glutaraldehyde treated multiblock systems. By chemically locking a multiblock protein assembly in place, unique structures and morphologies are preserved and stabilized, which provides the capacity to modulate a wide range of functional responses, such as mechanical behaviors, permeability, and drug elution characteristics. We anticipate that these materials will find utility in a number of vascular and non-vascular biomedical applications.

Acknowledgements

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Conflict of interest

Drs. Chaikof and Conticello are entitled to a portion of milestone fees and royalty derived from BioSequent’s sale of products related to the research described in this report. The terms of this arrangement have been reviewed and approved by Emory University in accordance with its conflict of interest policies.

Appendix

Figures with essential colour discrimination. Certain figures in this article, in particular Figs. 9 and 10, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2008.09.040.

References


