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Long-term biostability of self-assembling protein polymers in the absence of covalent crosslinking

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ABSTRACT

Unless chemically crosslinked, matrix proteins, such as collagen or silk, display a limited lifetime *in vivo* with significant degradation observed over a period of weeks. Likewise, amphiphilic peptides, lipopeptides, or glycolipids that self-assemble through hydrophobic interactions to form thin films, fiber networks, or vesicles do not demonstrate *in vivo* biostability beyond a few days. We report herein that a self-assembling, recombinant elastin-mimetic triblock copolymer elicited minimal inflammatory response and displayed robust *in vivo* stability for periods exceeding 1 year, in the absence of either chemical or ionic crosslinking. Specifically, neither a significant inflammatory response nor calcification was observed upon implantation of test materials into the peritoneal cavity or subcutaneous space of a mouse model. Moreover, serial quantitative magnetic resonance imaging, evaluation of pre- and post-explant ultrastructure by cryo-high resolution scanning electron microscopy, and an examination of implant mechanical responses revealed substantial preservation of form, material architecture, and biomechanical properties, providing convincing evidence of a non-chemically or ionically crosslinked protein polymer system that exhibits long-term stability *in vivo*.

1. Introduction

Elastin-based protein polymers, genetically engineered with or without cell binding motifs, crosslinking domains, or non-natural amino acids, represent a promising new class of biomaterial. Their capacity to be processed as gels [1–3], films [4,5], or nanofibers [6,7] demonstrate the versatility of these recombinant proteins with potential applications in drug delivery [1,8,9], tissue engineering [10,11] or as constituents of implanted medical devices [12,13]. The majority of these proteins have been examined as covalently crosslinked networks [6,14–22].

Recently, we have reported the synthesis of amphiphilic elastin-mimetic protein polymers composed of complex block sequences that self-assemble through the formation of robust physical crosslinks [3,4,7,23]. The biosynthetic scheme for generating self-assembling recombinant proteins has been based upon a convergent strategy for integrating multiple blocks of concatemerized DNA cassettes by sequential ligation [1,7,20]. To date, this strategy has been used to design diblock, triblock, and tetrablock copolymers ranging from 100 to 200 kDa in molecular weight [3–5,7,12,23,24]. The segregation of protein blocks into compositionally, structurally, and spatially distinct domains affords ordered structures on the nanometer to micrometer size range. Significantly, protein polymers that are structurally polymorphic display tunable mechanical, chemical, and biological properties [3,4,7,23].

We have recently synthesized a triblock copolymer, designated **B9**, that contains identical hydrophobic endblocks with $[(IPAVG)_4(VPAVG)]$ repeat sequences, separated by a central hydrophilic block with repeating units of $[(VPGVG)_2(VPGEG)(VPGVG)_2]$ [1]. Phase behavior and mechanical properties of elastin-mimetic polypeptides are critically dependent on the identity of the residues within the pentapeptide repeat unit Val/Ile-Pro-Xaa-Yaa-Gly. Yaa modulates the coacervation or inverse transition temperature (T_t) in

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water in a manner commensurate with the polarity of the amino acid side chain and polymer–solvent interactions. Substitution of Ala for Gly in the third position of the repeat results in a change in mechanical response from elastic to plastic [25]. Thus, the midblock of **B9** displays elastic-like behavior with spectroscopic features consistent with structural conformations of native elastin, including a highly mobile backbone, β-turns, and a loose helical β-spiral. The presence of glutamic acid in the midblock raises the inverse transition temperature, preventing coacervation of the midblock over a temperature range exceeding 75 °C. Nonetheless, these protein polymers reversibly self-assemble from concentrated aqueous solution above the T_t of the hydrophobic endblocks (~ 18 °C) to form a stable, water solvated, interlocking network. Two-dimensional Fourier transform infrared (FTIR) spectroscopy reveals that above the T_t, endblock secondary structure changes from helix to sheet with the assembly of physical crosslinks [24]. Due to the presence of Ala in the third position of the pentapeptide repeat, the hydrophobic endblocks form relatively rigid domains that display plastic-like behavior. Prior investigations from our group have confirmed robust viscoelastic and mechanical responses for this, as well as for other related elastin-mimetic triblock copolymers [3–5,7].

In vivo studies have demonstrated excellent blood contacting properties in a primate arteriovenous shunt model when the triblock copolymer, **B9**, is coated as a thin film on the lumen of a small diameter vascular graft [12]. However, long-term biocompatibility and biostability for any of the members of this new class of physically crosslinked protein-based material has yet to be fully defined. Indeed, it is possible that amphiphilic protein hydrogels would display significant *in vivo* instability, given the existence of naturally occurring amphiphiles in biological fluids, such as phospholipids, glycolipids, and lipoproteins. Such amphiphiles could act as surfactants destabilizing the virtual crosslinks of protein-based material whose structural integrity is based on the association of hydrophobic domains. While structural instability may be acceptable for biodegradable systems, it has the potential to severely limit the longevity of biomaterials whose integrity is related to hydrophobic interactions. Many *non-covalently* crosslinked self-assembled systems, such as amphiphilic peptides, lipopeptides, or glycolipids that form thin films or fiber networks are useful for drug delivery where the half-life of the system is on the order of hours. In general, none of these approaches demonstrate stability beyond a few days [26–28].

We report herein that a virtually crosslinked elastin-mimetic triblock copolymer exhibits long-term biostability and exceptional biocompatibility over a period exceeding 1 year. In conducting these studies, we have employed serial quantitative magnetic resonance imaging, evaluation of pre- and post-explant ultrastructure by cryo-high resolution scanning electron microscopy, and an examination of implant mechanical responses to characterize changes in form, material architecture, and biomechanical properties. All told, these studies reveal a non-chemically or ionically crosslinked protein polymer system that exhibits long-term stability *in vivo*.

2. Materials and methods

2.1. Synthesis and purification of the elastin-mimetic triblock copolymer *B9*

The recombinant protein polymer **B9** was derived from concatemerization of elastin-mimetic peptide sequences, as previously described [1]. The structure consists of an ABA triblock where:

A block: VPAVG[(IPAVG)$_4$(VPAVG)]$_{16}$IPAVG
B block: VPGVG[(VPGVG)$_2$VPGEG(VPGVG)$_2$]$_{48}$VPGVG.

Individual colonies of **B9** in pET24-a in *E. coli* strain BL21 (DE3) were used to inoculate 30 mL of Circle Grow liquid media (Q-BIOgene) supplemented with the antibiotic kanamycin (50 μg/mL) and grown overnight at 37 °C with shaking. A total of 5% vol/vol of the overnight culture was used to inoculate large expression flasks containing 500 mL of Circle Grow media and antibiotic, followed by a 24 h expression at 37 °C with shaking.

Cells were harvested through centrifugation in sterile tubes at 1660 RCF for 20 min at 4 °C. The supernatant was carefully decanted, cell pellets were resuspended in cold, sterile phosphate buffered saline (PBS; 20 mL per large culture flask pellet) and frozen at −80 °C. Three freeze (−80 °C) / thaw (25 °C) cycles were employed for the initial cell fracture with equilibration back to cold temperatures following the cycles. Once cells were completely resuspended, six cycles of sonication, consisting of 20 s pulses with 20 s between each pulse in an ice bath, was employed to thoroughly break the cells. To recover any unbroken cells, a preparative centrifugation step was used at 1660 RCF for 10 min at 4 °C. Unbroken cells, which pelleted out during the spin, were resuspended in cold, sterile PBS and re-sonicated, as described above.

The cold cell lysate was centrifuged at 20,000*g* for 40 min at 4 °C. The supernatant was transferred to a cold, sterile tube and poly(ethyleneimine) (PEI) was added to a final concentration of 0.5%. This solution was centrifuged again at 20,000*g* for 40 min at 4 °C to remove all nucleic acids and contaminating cellular material precipitated by the PEI. The supernatant was transferred to 50 mL Falcon tubes and NaCl was added to a final concentration of 2 M. The elastin-mimetic protein was salted out of solution at 25 °C for 30–45 min. This solution was centrifuged at 9500*g* for 15 minutes at 25 °C to recover the protein product ('hot-spin'). The supernatant was discarded and the protein pellet was resuspended in cold, sterile PBS on ice for up to 10–20 min to avoid solubilizing unwanted contaminates. The resuspended solution was then subjected to a 'cold spin' at 20,000*g* for 40 min at 4 °C. The supernatant was transferred to sterile 50 mL tubes and salting precipitation repeated. The hot (25 °C)/cold (4 °C) spin cycles were repeated until a contaminating pellet was no longer observed after the cold spin. Typically, 6–10 cycles were required followed by a hot spin.

For *in vivo* studies, **B9** underwent a secondary treatment with sodium hydroxide. The protein pellet was resuspended in cold, sterile PBS at approximately 50 mg per 20 mL. Sterile sodium hydroxide was added to a final concentration of 0.4 N and mixed gently by hand. The mixture was incubated on ice for fifteen minutes, after which 5 M sodium chloride was added to a final concentration of 2 M. The protein was precipitated from solution at 25 °C, centrifuged at 8500*g* for 20 min at 25 °C, and resuspended in cold PBS. This treatment was repeated three times. Following the third treatment, the protein solution was adjusted to pH 6–8. A cold spin was performed at 20,000 rpm for 40 min at 4 °C and the supernatant was desalted using a PD-10 desalting column (GE Healthcare Lifesciences) with molecular grade water (Cellgro). The end product was passed through 0.2 μm filter, eluted into autoclaved Lyoguard freeze drying trays (Gore), frozen at −80 °C, and lyophilized. This procedure afforded **B9** as a white fibrous protein product with isolated yields of 50 mg/L of expression culture.

Lyophilized **B9** was resuspended in sterile molecular grade water at 1 mg/mL and endotoxin levels were assessed using the Limulus Amoebocyte Lysate (LAL) assay (Cambrex). Levels of endotoxin were typically 0.1 EU/mg of protein polymer (1 EU = 100 pg of endotoxin).

2.2. Structural characterization of the elastin-mimetic triblock copolymer *B9*

2.2.1. Gel electrophoresis

Protein size and purity were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A total of 10 μg of the elastin-mimetic polypeptide was run on a 7.5% gel along with Precision Plus Protein Kaleidoscope (Bio-Rad) molecular weight markers and negatively stained with Copper (Bio-Rad). MALDI-TOF mass spectrometry identified a 165 kDa protein and the sequence was confirmed by amino acid compositional analysis [3].

2.2.2. Evaluation of water content in protein gels

For evaluation of water content, 200 μL of a 10 wt% protein solution was cast as a disk measuring 1 cm in diameter. Samples were first dried at room temperature under controlled water evaporation conditions and then fully dehydrated under vacuum. Specimens were subsequently incubated in PBS at 37 °C for 24 h and fully hydrated weights were obtained. The equilibrium water content and equilibrium swelling ratio were determined according to Eqs. (1) and (2), respectively, and expressed as mean ± standard deviation.

$$\text{Equilibrium water content} = [(\text{hydrated weight} - \text{dehydrate weight})] / (\text{hydrated weight}) \quad (1)$$

$$\text{Equilibrium swelling ratio} = (\text{hydrated weight})/(\text{dehydrated weight}) \quad (2)$$

2.2.3. Rheological analysis of concentrated *B9* 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 [7]. In brief, protein solutions were prepared at 100 mg/mL by adding distilled, deionized water to lyophilized protein at 4 °C, shaking the solution for