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Hydrazone self-crosslinking of multiphase elastin-like block copolymer networks

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ABSTRACT

Biosynthetic strategies for the production of recombinant elastin-like protein (ELP) triblock copolymers have resulted in elastomeric protein hydrogels, formed through rapid physical crosslinking upon warming of concentrated solutions. However, the strength of physically crosslinked networks can be limited, and options for non-toxic chemical crosslinking of these networks are not optimal. In this report, we modify two recombinant elastin-like proteins with aldehyde and hydrazide functionalities. When combined, these modified recombinant proteins self-crosslink through hydrazone bonding without requiring initiators or producing by-products. Crosslinked materials are evaluated for water content and swelling upon hydration, and subject to tensile and compressive mechanical tests. Hydrazone crosslinking is a viable method for increasing the mechanical strength of elastin-like protein polymers, in a manner that is likely to lend itself to the biocompatible in situ formation of chemically and physically crosslinked ELP hydrogels.

1. Introduction

Recombinant elastin-like protein polymers (ELP) represent a promising new class of biomaterials that may be formulated as gels, films, nanofibers or micelles with potential applications in drug delivery, tissue engineering or as components of implanted medical devices [1–8]. We have recently described ELPs with hydrophilic, elastomeric midblock sequences flanked by self-associating, hydrophobic endblocks in an ABA triblock format [1,9,10]. Designs with individual block sizes in excess of 35 kDa have resulted in protein-based biomaterials demonstrating structural polymorphism, providing the opportunity to broadly tune mechanical responses and drug elution rates [11–15]. Notably, due to the self-association of endblock sequences, triblock ELPs form physical, non-covalent crosslinked gel networks in aqueous, physiologic environments (pH 7.4, 37 °C), as reviewed elsewhere [1]. Physical crosslinking is reversible and, in principle, eliminates the need for inflammatory or cytotoxic compounds associated with chemical crosslinking schemes. ELP properties, including physical crosslinking, depend upon repeat sequences of the pentapeptide [(Val/Ile)–Pro–Xaa–Yaa–Gly]. The polarity of the fourth residue (Yaa) dictates the coacervation or inverse temperature transition (T_t) of the polypeptide in aqueous solution. The identity of the third residue (Xaa) influences block mechanical properties, with the consensus glycine enhancing elasticity and the substitution of alanine contributing to plastic mechanical behavior. Consequently, ELP endblocks with a significant fraction of (VPAVG) sequences tend towards plastic behavior and lower T_t while midblocks containing (VPGEG) repeats contribute to elasticity and elevated T_t. Sequences are designed so that cold, aqueous ELP solutions transition to elastomeric gels through endblock self-assembly as they are warmed to physiologic temperature, with T_t in the range of 10–15 °C.

Despite the advantages of physical crosslinking, self-assembled domains can be disrupted at lower mechanical stresses than covalent crosslinks. The majority of reported methods for covalent crosslinking of ELPs rely upon amino groups, and employ either chemical or enzymatic approaches. Chemical methods including isocyanates, NHS-esters, phosphines, aldehydes or genipin have been reported [16–23], whereas enzymatic approaches have consisted of transglutaminase and lysyl oxidase [24]. In addition, we have investigated solid-state crosslinking of recombinant elastin using both UV and visible light-activated photoinitiators [25].

Strategies for combined chemical and physical crosslinking may result in significant reinforcement of properties arising from self-assembled, multiphase polymer network structure. In an ELP designated LysB10, lysine residues were included at the block interfaces, and crosslinked with glutaraldehyde (GTA) after endblock self-assembly [10]. This modification successfully stabilized

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the physically crosslinked multiblock domain structure with covalent crosslinks, and significantly enhanced mechanical properties. As a result of this combination of physical and chemical crosslinks, LysB10 served as an elastomeric component in protein-based biomaterials that matched the mechanical behavior of native artery, heart valve leaflet and abdominal wall connective tissue [26–28]. However, due to toxicity, use of GTA precludes in situ crosslinking, otherwise readily feasible through the injection of cooled ELP solutions that rapidly gel when warmed. Moreover, although subcutaneous and peritoneal implants of GTA-treated ELPs in mice elicited minimal inflammatory responses [10], in many instances the cytotoxic effects of GTA and by-products from other chemical crosslinkers have been firmly established.

In this report, we describe covalently modified ELPs capable of physical and chemical self-crosslinking. This approach relies upon hydrazone bonding, and required the addition of aldehyde or hydrazide functional groups to glutamic acid residues in the ELP polymer backbone. Hydrazone crosslinking proceeds without initiators or by-products, and circumvents toxicity typical of chemical crosslinking. Moreover, the self-crosslinking character of hydrazone bonding creates the potential for in situ covalent stabilization of triblock domain structure through the coinjection of cooled, modified ELP components. Here, the approach is applied to the ELP LysB10 [10], permitting a comparison between hydrazone and GTA crosslinking. In addition, the hydrazone crosslinking is applied to B9 [13], an ELP that cannot be crosslinked with GTA due to an absence of amine functional groups. In comparison to LysB10, B9 has a significantly lower molecular weight, but presents the potential for almost twice the number of aldehyde and hydrazide modifications for hydrazone crosslinking.

2. Materials and methods

2.1. Materials

All solvents and reagents were purchased from commercial sources and were used as received, unless otherwise noted. The biosynthetic strategy for the expression and purification of the recombinant ELP triblock polymers B9 [13] and LysB10 [10] has been described previously. The ABA triblock amino acid sequence of both ELPs is depicted in Scheme 1.

2.2. Nuclear magnetic resonance (NMR)

^{1}H NMR spectra was used to confirm the chemical modification of ELPs. Typically the modified samples were dissolved in D_2O (8 mg ml^{-1}) and the spectrum was recorded at 4 °C using a Varian INOVA 600 spectrometer with magnetic field strengths of 600 MHz.

2.3. Synthesis of hydrazide-modified ELPs

To solutions of B9 (600 mg, 34.8×10^{-4} mmol) in 20 mM HEPES buffer (pH 6.0) was added 10 equivalents of amino-dPEG$_4$-*t*-boc-hydrazide (Quanta Biodesigns, Powell, OH; 632.95 mg, 1.71 mmol), hydroxysulfosuccinimide (Sulfo-NHS) (36 mg, 0.17 mmol) and 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC) (132 mg, 0.69 mmol) at 4 °C. The reaction mixture was stirred for 72 h at 4 °C and then purified by dialysis (MWCO 10000) against ddH_2O for 3 days (two water changes a day) at 4 °C to afford B9-tBoc-hydrazide (570 mg, 50%) after lyophilization.

To solutions of LysB10 (600 mg, 28.8×10^{-4} mmol) in 20 mM HEPES buffer (pH 6.0) was added 10 equivalents of amino-dPEG$_4$-*t*-boc-hydrazide (300 mg, 0.81 mmol), Sulfo-NHS (21.54 mg, 0.102 mmol) and EDC (79.2 mg, 0.414 mmol) at 4 °C. The reaction mixture was stirred for 72 h at 4 °C and then purified by dialysis (MWCO 10000) against ddH_2O for 3 days (two water changes a day) at 4 °C to afford LysB10-tBoc-hydrazide (576 mg, 54%) after lyophilization.

For both modified LysB10 and B9, cleavage of the acid-labile Boc protection group was carried out in a mixture of trifluoroacetic acid (TFA), water and triisopropylsilane (TIS) (TFA:H_2O:TIS, 95:2.5:2.5). Excess TFA was removed under high vacuum and the crude mixture was diluted with water and exhaustively dialyzed (MWCO 10000) against ddH_2O for 3 days. The solutions were finally lyophilized to yield either B9-hydrazide or LysB10-hydrazide as a white product in 85–90% yield.

The conjugation efficiency was determined by ^{1}H NMR spectroscopy based on comparison of the integration of methyl protons of the *t*-Boc group with the β-methyl protons of alanine in Boc protected ELP-hydrazides. B9 and LysB10 have 164 and 465 alanine groups and 48 and 28 glutamic acid groups per macromolecule, respectively. Using the intensity of the alanine peak as a reference, the number of methyl protons of *t*-Boc group per macromolecule of ELP was determined from the ^{1}H NMR spectrum. The percent of modified glutamic acid residues was then calculated as $a = (b/(9 \cdot c)) \cdot 100\%$, where a is the percent modification, b is the number of methyl protons of *t*-Boc, 9 is the number of methyl protons per *t*-Boc group and c is the number of glutamic acid residues per macromolecule.

2.4. Synthesis of aldehyde-modified ELPs

B9 (630 mg, 36.54×10^{-4} mmol) was dissolved in distilled, deionized water and aminoacetaldehyde dimethylacetal (40 equivalents, 565 μl) was added. The pH of the reaction mixture was adjusted to pH 7.5 with 0.1 M NaOH/0.1 M HCl, and Sulfo-NHS (37.8 mg, 0.179 mmol) and EDC (138.6 mg, 0.725 mmol) were added. The reaction mixture was stirred for 72 h at 4 °C and then purified by dialysis (MWCO 10000) against ddH_2O for 3 days (two water changes a day) at 4 °C to afford B9-dimethylacetal (605 mg, 58%) after lyophilization.

For aldehyde modification of LysB10, the ELP (600 mg, 28.8×10^{-4} mmol) in 20 mM HEPES buffer (pH 6.0) was added aminoacetaldehyde dimethylacetal (240 μl, 40 equiv.), Sulfo-NHS (21.54 mg, 0.102 mmol) and EDC (79.2 mg, 0.414 mmol). The reaction mixture was stirred for 48 h at room temperature and then purified by dialysis (MWCO 10000) against ddH2O for 3 days (two water changes a day) at 4 °C to afford LyB10-dimethylacetal (576 mg, 52%) after lyophilization.

For both ELPs, cleavage of the acid-labile acetal protection group was carried out in a mixture of TFA-H_2O (95:5). Excess of TFA was removed under high vacuum and the crude mixture was

B9	A-B-A	midblock (B)	VPGVG[(VPGVG)$_2$VPGEG(VPGVG)$_2$]$_{48}$VPGVG
		endblock (A)	VPAVG[(IPAVG)$_4$(VPAVG)]$_{16}$IPAVG
LysB10	A_N-B-A_C	midblock (B)	(IPAVG)KAAK(VPGAG)[(VPGAG)$_2$VPGEG(VPGAG)$_2$]$_{28}$(VPAVG)KAAK(VPGAG)
		n-terminal endblock (A_N)	VPAVGK[(VPAVG)(IPAVG)$_4$][(IPAVG)$_5$]$_{33}$
		c-terminal endblock (A_C)	[(VPAVG)(IPAVG)$_4$][(IPAVG)$_5$]$_{33}$IPAVGKAAKA

Scheme 1. Amino acid sequence of protein-based block copolymers. Glutamic acid (E) residues employed for chemical modification are located in the ELP midblocks.