Published in final edited form as:

Biomacromolecules. 2008 January ; 9(1): 222–230. doi:10.1021/bm7007982.

In Situ Cross-Linking of Elastin-like Polypeptide Block Copolymers for Tissue Repair

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Abstract

Rapid cross-linking of elastin-like polypeptides (ELPs) with hydroxymethylphosphines (HMPs) in aqueous solution is attractive for minimally invasive in vivo implantation of biomaterials and tissue engineering scaffolds. In order to examine the independent effect of the location and number of reactive sites on the chemical cross-linking kinetics of ELPs and the mechanical properties of the resulting hydrogels, we have designed ELP block copolymers comprised of cross-linkable, hydrophobic ELP blocks with periodic Lys residues (A block) and aliphatic, hydrophilic ELP blocks with no cross-linking sites (B block); three different block architectures, A, ABA, and BABA were synthesized in this study. All ELP block copolymers were rapidly cross-linked with HMPs within several minutes under physiological conditions. The inclusion of the un-cross-linked hydrophilic block, its length relative to the cross-linkable hydrophobic block, and the block copolymer architecture all had a significant effect on swelling ratios of the cross-linked hydrogels, their microstructure, and mechanical properties. Fibroblasts embedded in the ELP hydrogels survived the cross-linking process and remained viable for at least 3 days in vitro when the gels were formed from an equimolar ratio of HMPs and Lys residues of ELPs. DNA quantification of the embedded cells indicated that the cell viability within triblock ELP hydrogels was statistically greater than that in the monoblock gels at day 3. These results suggest that the mechanical properties of ELP hydrogels and the microenvironment that they present to cells can be tuned by the design of the block copolymer architecture.

Introduction

Injectable biomaterials are of significant interest for the rapid, in vivo formation of load-bearing scaffolds and drug delivery reservoirs for tissue repair and regenerative medicine.^{1–5} This approach for regenerative medicine is attractive because cells and bioactive factors can be homogeneously mixed with a liquid and injected into a defect site, followed by in situ formation of a hydrogel scaffold. The rapid formation of an elastomeric network can provide the requisite mechanical properties to the provisional scaffold and can simultaneously entrap cells and bioactive molecules within a three-dimensional microenvironment.^{6–9}

Motivated by the potential of injectable biomaterials for regenerative medicine, elastin-like polypeptides (ELPs)^{10–14} and their fusion proteins^{15–21} have been widely investigated for cartilage and intervertebral disk repair,^{10,12} small-diameter vascular grafts,^{15–18} stem cell

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Supporting Information Available. A figure showing the oscillatory rheological profile for the triblock ELP[KV7F-72]-[VG7A8-64]-[KV7F-72] at 22 °C (below its T_i) after addition of THPP cross-linker to ELP triblock copolymer solution in PBS. This material is available free of charge via the Internet at <http://pubs.acs.org>.

matrices,¹¹ and the generation of stem cell sheets.²² ELPs are artificial repetitive polypeptides that are derived from a repetitive Val-Pro-Gly-Xaa-Gly peptide motif of tropoelastin (where Xaa, is any amino acid other than Pro). ELPs are especially attractive as injectable biomaterials because they undergo an inverse temperature phase transition: ELPs are highly soluble in aqueous solutions, but as their temperature is raised above a critical transition temperature, T_t , they desolvate and become insoluble in aqueous solution in a completely reversible process.^{23–27}

ELPs are useful for tissue engineering for the following reasons: First, ELPs appear to have good biocompatibility as suggested by low cytotoxicity and a lack of antigenicity.^{28,29} Second, ELPs do not appear to interact with encapsulated cells. Thus, the “inertness” of ELPs provides a template into which the desired bioactivity (can be) incorporated into the material.^{25,30–32} Third, because ELPs are genetically encodable, the inclusion of bioactive factors and degradation sites can be programmed in at the sequence level. Fourth, ELPs can be produced at high levels in *E. coli* (200–400 mg/L in shaker flask culture).^{25,30,33,34} Finally, they can be easily purified without the need for chromatography using their soluble–insoluble phase transition behavior.^{25,27,30,33,34} The ease with which gram scale quantities of these polypeptides can be produced and purified in a laboratory setting is an attractive practical feature of these polypeptides for tissue engineering.

In the past few years, different methods to create ELP hydrogels have been investigated; these methods include chemical cross-linking,^{14,15,35–39} enzymatic cross-linking by tissue transglutaminase,¹³ photoinitiated- and γ -irradiation cross-linking,^{40–42} and self-assembly of ELP block copolymers into physically cross-linked networks.^{43–45} The thermally induced coacervation of ELPs is an alternative approach that does not create a hydrogel but provides a viscous mixture that is suitable for the encapsulation of cells.¹² The “coacervate” phase of ELPs has been used to encapsulate chondrocytes¹² and human adipose derived stem cells (hADAS)¹¹ and, in the case of entrapped hADAS cells, was shown to induce chondrogenic differentiation.

We recently reported the rapid chemical cross-linking of ELPs with an organophosphorous cross-linker, β -[tris(hydroxymethyl)phosphino]-propionic acid (THPP), in aqueous solution by a Mannich-type condensation reaction.⁴⁶ This reaction is of interest for in situ hydrogel formation for tissue engineering applications because: (1) it can be carried out under physiological conditions without an additional reduction procedure;^{46–49} (2) the chemical linkages, aminomethylphosphines, are stable in a physiological milieu and the cross-linking reaction releases water as the sole byproduct;^{47,48,50,51} (3) gelation proceeds rapidly with initial stabilization of the hydrogel in several minutes and continued evolution of the hydrogel properties over an hour;^{46,51} and (4) the cross-linking agent can be mixed with cells without compromising their viability.^{52,53} In addition, the formation of cross-linking sites presents phosphine centers to potentially coordinate transition metals as well as reactive carboxylic acids for the installation of bioactive ligands into the ELP hydrogels.⁵⁴

The ELPs used in our previous study that demonstrated proof-of-concept of this cross-linking method had reactive lysine (Lys) residues that were periodically positioned along a single ELP segment.⁴⁶ As the next step in the development of this cross-linking methodology for the formation of ELP hydrogels, we posed the question of how the placement of the Lys residues within specific blocks of different ELP block copolymer architectures would modulate the mechanical properties and biocompatibility of the cross-linked ELP hydrogels. This question was also motivated by the observation that tropoelastin itself has a blocky structure with a sequence that contains clustered cross-linking sites that are interspersed between hydrophobic blocks.^{55–60}

To answer this question, we synthesized ELP block copolymers that consist of cross-linkable, hydrophobic blocks containing periodic Lys residues that would function as elastic domains (A block) and aliphatic, hydrophilic blocks without cross-linking sites (B block) that would provide viscous domains. Four different block copolymers, A (control), two ABA triblock copolymers with different lengths of the B block, and a BABA tetrablock copolymer, were synthesized in this study. By placing the Lys residues periodically along one or more A segments of the block copolymer versus distributing them along the entire length of the polymer as in our previous study,^{13,14,46} and by varying the ratio of the lengths of the two blocks, we hypothesized that a further degree of control could be gained over the mechanical properties of the hydrogels and potentially their interactions with cells.

We report herein that chemically cross-linking of all ELP block copolymers by THPP proceeds rapidly in aqueous conditions and that the swelling ratios, microstructures, and mechanical properties of THPP cross-linked ELP block copolymer hydrogels can be modulated by incorporation of a hydrophilic block (B block) into a hydrophobic, cross-linkable block (A block) in the form of triblock (ABA) or tetrablock (BABA) copolymers. We also show that cell viability is dependent upon the ELP block copolymer architecture. These results suggest that the mechanical properties of ELP hydrogels and the microenvironment that they present to cells can be tuned by the design of the block copolymer architecture.

Experimental Section

ELP Block Copolymer Notation

All ELP block copolymers are composed of one or more ELP blocks, and each block is named using the following notation: ELP[X_iY_jZ_k-n] where the bracketed capital letters are single letter amino acid codes of a guest residue, an amino acid at the fourth position (X) of the VPGXG pentapeptide, their corresponding subscripts denote the ratios of that guest residue in the monomer, and *n* indicates the number of pentapeptides in the ELP. For example, ELP[KV₇F-72] is an ELP that contains 72 repeats of the pentapeptide unit, in which the ratio of K, V, and F at the fourth, guest residue position (X) is 1:7:1. The ELP block copolymers are named by the composition of each block in square brackets with a hyphen between blocks. Examples of a triblock and an alternating tetrablock copolymer are ELP[KV₇F-72]-[VG₇A₈-32]-[KV₇F-72] and ELP[VG₇A₈-32]-[KV₇F-72]-[VG₇A₈-32]-[KV₇F-72].

ELP Block Copolymer Gene Synthesis

Three pUC19 plasmids, each containing a gene for ELP[KV₇F-72], ELP[VG₇A₈-32], and ELP[VG₇A₈-64], were available from a previous study.^{26,34,46} These genes were then ligated together to create genes that encode block copolymers of different architectures that varied from diblock to tetrablock ELPs.^{12,25,26,30} Scheme 1 shows the steps in the construction of plasmids encoding ELP block copolymers using the available genes of ELP[KV₇F-72] and ELP[VG₇A₈-64]. In order to build the gene of the ELP diblock copolymer, ELP[VG₇A₈-64]-[KV₇F-72], the pUC19 cloning vector harboring ELP[VG₇A₈-64] was doubly digested with Pflm I and Bgl I (New England Biolabs, Beverly, MA), separated from other DNA fragments by gel electrophoresis with low melting agarose, AquaPor LM (National Diagnostics, Atlanta, GA) and was purified using a Qiaquick Gel extraction kit (Qiagen, Inc., Germantown, MD) to isolate a DNA insert. A pUC19 cloning vector harboring ELP[KV₇F-72] was digested with Pflm I, enzymatically dephosphorylated using calf intestinal alkaline phosphatase (CIP) (Gibco BRL-Life Technologies, Grand Island, NY), and separated and purified using the same method as the insert to provide a linearized vector. The linearized ELP[KV₇F-72] vector was ligated with

the ELP[VG₇A₈-64] insert by T4 DNA ligase (New England Biolabs) to create ELP[VG₇A₈-64]-[KV₇F-72]. Next, the plasmid encoding the ELP diblock copolymer, ELP[VG₇A₈-64]-[KV₇F-72], was digested with Pflm I and dephosphorylated to create a linearized vector and the plasmid of ELP[KV₇F-72] was double-digested with Pflm I and Bgl I to isolate an insert. The linearized, ELP[VG₇A₈-64]-[KV₇F-72] vector was ligated with the ELP[KV₇F-72] insert to create a plasmid that encodes for the ELP triblock copolymer, ELP[KV₇F-72]-[VG₇A₈-64]-[KV₇F-72]. Likewise, the plasmid of the ELP tetrablock copolymer, ELP[VG₇A₈-32]-[KV₇F-72]-[VG₇A₈-32]-[KV₇F-72] was constructed using a similar procedure using ELP[VG₇A₈-32]. Chemically competent XL1-Blue *Escherichia coli* (Novagen Inc., Milwaukee, WI) were transformed with each ligation mixture. Transformants with DNA sequences of each ELP block were identified by colony polymerase chain reaction and were verified by fluorescent dye terminator DNA sequencing (ABI 370 DNA sequencer). The size of each ELP block copolymer gene was confirmed by gel electrophoresis after digestion by EcoR I and Hind III, recognition sequences for which are located outside the ELP gene.^{34,61}

Gene Expression and Purification of ELP block Copolymers

The pET-25b(+) expression vector (Novagen) was previously modified to introduce a unique Sfi I restriction site for insertion of the ELP block copolymer genes and codons for a C-terminal Trp for spectrophotometric detection of ELPs.^{25,26} The ELP block copolymers were expressed by ligating the ELP genes that were excised from the pUC19 vectors by Pflm I and Bgl I digestion, into the pET-25b(+) expression vectors (Novagen) that had been linearized with Sfi I (New England Biolabs) and dephosphorylated using CIP. *E. coli* strain, BLR (DE3) cells (Novagen) were transformed with the pET-25b(+) vectors containing an ELP insert, and grown for 24 h at 37 °C at 200 rpm as 1 L cultures of CircleGrow media (Qbiogene, Carlsbad, CA) supplemented with 100 µg/mL ampicillin. *E. coli* cells were harvested and lysed, and the ELPs were purified by inverse transition cycling (ITC), as previously described.^{25,26}

Physicochemical Characterization of ELP Block Copolymers

The purity and molecular weight of the ELP block copolymers were characterized by SDS-PAGE (BioRad, Inc., Hercules, CA) with copper staining. Their theoretical molecular weights were calculated from their primary amino acid sequences with a software program, Protean (DNA Star), while their actual molecular weights were determined by matrix-assisted laser desorption/ionization mass spectroscopy (MALDI-MS), which was performed on a PE Biosystems Voyager-DE instrument equipped with a nitrogen laser (337 nm). The ELP samples for MALDI-MS measurements were prepared in an aqueous 50% (v/v) acetonitrile solution containing 0.1% (v/v) trifluoroacetic acid, using a sinapinic acid matrix. The inverse phase transition temperatures (T_i) of the ELP block copolymers were measured at a concentration of 25 µM between 10 and 90 °C at a heating rate of 1 °C/min, and the OD₃₅₀ was measured by a UV-visible spectrophotometer (Cary 300 Varian Instruments). The T_i is defined as the temperature at which the first derivative of the turbidity as a function of temperature ($d(OD)/dT$) was the maximum. The ELP concentration was determined by the ELP molar extinction coefficient at 280 nm (5690 M⁻¹cm⁻¹) calculated from primary amino acid sequences of ELPs with a software program, Protean (DNA Star). The absorbance at 280 nm was measured on a spectrophotometer (Shimadzu Scientific Instruments, UC-1601).

Oscillatory Rheology for Gelation Kinetics

The cross-linking kinetics of the ELP block copolymers with THPP (Pierce, Rockford, IL) were measured by oscillatory rheology as a function of time. The rheological behaviors during cross-linking were characterized in dynamic torsional shear mode using a cone-on-

plate configuration (ARES Rheometer, TA Instruments, cone angle = 0.1 rad, plate diameter = 25 mm) at 37 °C over 1 h (1 Hz frequency and shear strain, γ_0 of 0.01). In order to measure the cross-linking kinetics in a physiologically relevant buffer, 60–80 μ L of 100 mg/mL THPP solution in phosphate buffered saline, PBS (10 mM phosphate buffer, 138 mM NaCl, 2.7 mM KCl, pH 7.4), was homogeneously mixed by vigorous pipetting at 4 °C with 400 μ L of 200 mg/mL ELP block copolymer solution to yield a 5–7-fold molar excess of reactive HMP to primary amine of ELPs. The solution was transferred to the bottom platen of the test apparatus, which was held at a constant temperature of 37 °C. The platen was lowered to completely fill the gap with the polymer-cross-linker solution. The dynamic shear experiment was then performed as described in order to obtain values for the storage (G') and loss (G'') moduli over time for 1 h. Time to the intersection of G' and G'' was taken as the time to gelation (τ).

Mechanical Properties of Cross-Linked ELP Block Copolymer Hydrogels

All ELP block copolymers (200 mg/mL) were cross-linked by the same procedure in PBS, as follows: A THPP solution was added to each ELP block copolymer solution in a customized Teflon mold (8 mm of diameter and 2 mm of height) at 4 °C and homogeneously mixed by vigorous pipetting, and then the mixture was incubated at 37 °C for 1 h. The cross-linked ELP hydrogels were immersed in distilled and deionized water at 4 °C overnight in order to remove residual THPP and stored at 4 °C in their swollen state in PBS. The cross-linked ELP block copolymer hydrogels were tested to determine their equilibrium compressive modulus, E , complex shear modulus, $|G^*|$, equilibrium shear modulus, μ , and loss angle, δ . Parallel plate platens of nonporous stainless steel were used (plate radius = 8 mm) with samples and test platens submerged in PBS in a temperature-controlled bath held at 37 °C. Samples were equilibrated under a tare load of 10–20 g and allowed to relax until equilibrium. The reference thickness was recorded as the distance between platens under the compressive tare load. Samples were then compressed to 5% compressive strain, ϵ , and allowed to relax until equilibrium. This protocol was repeated in 5% increments until 15% compressive strain was achieved. The equilibrium compressive modulus, E , was obtained from linear regression of calculated normal stress, σ , at equilibrium on ϵ . At 15% compression, a dynamic strain sweep test was performed at 10 rad/s of frequency (sinusoidal strain 0.0001–0.10 maximum strain amplitude) to determine the range of strain amplitudes where the gels show linear stress–strain behaviors. A dynamic frequency sweep (0.01–50 rad/s) test was executed in torsional shear at a maximum shear strain, γ_0 of 0.05. Linear viscoelastic theory was used to calculate the magnitude of the complex shear modulus, $|G^*|$, and the loss angle, δ , which are measures of the stiffness and internal energy dissipation of the material under dynamic loading, respectively. Samples were then tested in torsional shear to determine the equilibrium shear modulus, μ . Samples were allowed to relax for 5 min between strain increments. Four replicates of each ELP formulation were measured for all mechanical properties.

Characterization of Swelling Property and Microstructural Morphology

The swelling ratio by weight, Q_w , defined as the ratio of swollen gel weight, W_s , to freeze-dried gel weight, W_d ($Q_w = W_s/W_d$) was measured in PBS as a function of temperature (4, 23, 30, 37, and 50 °C) to determine the volume change associated with the phase transition of the cross-linked ELP block copolymers between their swollen and collapsed states ($n = 4$). The cross-linked ELP hydrogels were freeze-dried for 3 days and immersed in PBS at 4 °C overnight to attain their swollen state. The cross-linked hydrogels were equilibrated at each temperature for 1 h and weighed. Equilibrium was defined as the steady state at which there was no change in volume of the ELP hydrogels. Volume transition at each temperature occurred within 5 min.

In order to investigate the microstructure of the cross-linked ELP block copolymers, ELP hydrogels were immersed in PBS at 37 °C for a day then instantaneously plunged into liquid nitrogen, and freeze-dried. The lyophilized ELP hydrogels were physically fractured by tweezers, and their internal microstructures were imaged by scanning electron microscopy (SEM) (Philips FEI XL30) at an accelerating voltage of 1.0 kV without any coating procedures. Each chamber in SEM images of the freeze-fractured ELP hydrogels was randomly selected, and its diameter was determined by using a scale bar in each SEM image ($n = 10$).

Fluorescent Cell Viability/Cytotoxicity Assay and DNA Quantification

A suspension of mouse NIH-3T3 fibroblasts in HEPES-buffered saline were mixed with ELP[KV₇F-144] or ELP[KV₇F-72]-[VG₇A₈-64]-[KV₇F-72] and THPP at room temperature at a 1:1 molar ratio of HMP and Lys residues at a final concentration of 200 mg/mL of the ELP, and 10×10^6 cells/mL for fluorescent cell viability and DNA quantification. The mixture was injected into a custom mold via a syringe at room temperature and then incubated at 37 °C for 1 h in a 5% CO₂ incubator. The fibroblast embedded, cross-linked ELP gel constructs were excised by a biopsy punch (Miltex, York, PA) with a 5 mm diameter to produce multiple cellular ELP gels having the same dimension. Each gel was cultured at 37 °C in a 5% CO₂ incubator for 1 h or 3 days. Fluorescent cell viability/cytotoxicity assay was performed by using the Live/Dead cell assay kit (Molecular Probes, Eugene, OR). Cellular ELP hydrogels were removed at each time point ($n = 4$ or 5 per time point) from the culture medium and placed in 48-well plates. The hydrogels were washed three times in PBS, followed by addition of 500 μ L of the staining solution (2 μ M calcein and 2 μ M ethidium homodimer-1 (EthD-1)) and incubated for 30 min. The calcein/EthD-1 solution was discarded, and the hydrogels were washed three times with PBS. Cell survival within the gels was analyzed by laser scanning confocal fluorescence microscopy (Zeiss LSM 510, Carl Zeiss, Inc., Thornwood, NY).

DNA content of the encapsulated fibroblasts in each construct was determined by the Quant-iT Picogreen dsDNA assay (Molecular Probes). Four or five cellular ELP hydrogels were removed at each time point from the culture medium and digested in PBS containing 125 μ g/mL papain (Sigma), 0.05% trypsin (Invitrogen, Carlsbad, CA), 2 mM EDTA, and 2 mM L-cysteine at 37 °C for 1 day and at 65 °C for another day, and then centrifuged at 13000g for 10 min. Picogreen reagent was added to each digest solution and DNA controls in a 96-well plate and then incubated for 5 min. Total DNA content in each digest solution was analyzed by a fluorometric assay on a plate reader (Tecan GENios, Phenix Research Products, Candler, NC) using 520–530 nm/480–485 nm emission/excitation.

Statistical Analysis

A one-factor analysis of variance (ANOVA) and Fisher's post hoc tests (Statview, SAS Institute, Cary, NC) were used to test for the effect of ELP architecture on the swelling ratio, Q_w as well as various mechanical properties (E , $|G^*|$, and μ) and to evaluate differences among ELP block copolymers. Two-factor ANOVA and Fisher's post hoc tests were used to test for the effect of ELP architecture and the culture time on the DNA content. Statistical significance was determined at a value of $p < 0.05$.

Results and Discussion

Design and Characterization of ELP Block Copolymers

A series of ELP block copolymers were synthesized from plasmid-borne genes in *E. coli* as follows: (1) a monoblock, ELP[KV₇F-144]; (2) an ABA triblock, ELP[KV₇F-72]-[VG₇A₈-32]-[KV₇F-72]; (3) another ABA triblock, ELP[KV₇F-72]-[VG₇A₈-64]-

[KV₇F-72]; and (4) an alternating tetrablock, ELP-[VG₇A₈-32]-[KV₇F-72]-[VG₇A₈-32]-[KV₇F-72]. The ELP-[KV₇F-72] block was chosen as the hydrophobic and cross-linkable domain (A block) to provide periodically spaced Lys guest residues (one Lys every nine pentapeptide repeats) for chemical cross-linking as well as hydrophobic Phe guest residues at the same ratio as Lys residues in the ELP repeat (one Phe every nine pentapeptide repeats) to counterbalance the hydrophilicity imparted to this block by the Lys residues. The ELP[VG₇A₈] repeat was selected as the hydrophilic block (B block) because the T_i values of the ELP[VG₇A₈] series are greater than 90 °C, due to the large fraction of glycine and alanine guest residues.²⁶ Two different block lengths, ELP-[VG₇A₈-32] and ELP[VG₇A₈-64] were chosen to examine the ratio of the hydrophilic/hydrophobic block length on the mechanical properties of the cross-linked hydrogels. The block architecture and lengths chosen provide a set of orthogonal design variables to investigate the mechanical properties of these cross-linked hydrogels.

The ELP block copolymers were expressed in *E. coli* and purified by ITC, as previously described for other ELPs and their fusions.^{25,30,31,34} A copper-stained SDS-PAGE gel in Figure 1A shows that the ELP block copolymers could be purified to at least 95% homogeneity by three to four rounds of ITC. All ELP block copolymers migrated approximately 20% larger than their calculated molecular weights based on the migration pattern of protein standards, as reported previously.^{14,26,34} MALDI-MS confirmed that the sizes of the ELP block copolymers were close to that predicted by their primary amino acid sequence.

Figure 1B shows the thermal behavior of the ELP block copolymers. Introduction of the hydrophilic middle block, ELP[VG₇A₈-32] ($T_i > 90$ °C) in the center of the hydrophobic block, ELP[KV₇F-144] (T_i : 26.1 °C) increased the T_i of the triblock, ELP[KV₇F-72]-[VG₇A₈-32]-[KV₇F-72] by approximately 2 °C compared to the parent monoblock polypeptide, ELP[KV₇F-144]. Likewise, the T_i of the triblock, ELP[KV₇F-72]-[VG₇A₈-64]-[KV₇F-72] was ~4 °C higher than that of ELP[KV₇F-144] by introduction of the 2× longer middle block, ELP[VG₇A₈-64] ($T_i > 90$ °C). Interestingly, this triblock copolypeptide ELP[KV₇F-72]-[VG₇A₈-64]-[KV₇F-72] and the BABA tetrablock ELP[VG₇A₈-32]-[KV₇F-72]-[VG₇A₈-32]-[KV₇F-72] show very different thermal behaviors that are related to their different architectures, despite having identical amino acid composition. In contrast to the tetrablock, which simply displays a single transition from soluble monomers to bulk aggregate, as is typically seen for monoblock ELPs,^{14,26} the triblock ELP has two inflection points in its thermal turbidity profile at approximately 26 and 30 °C. These inflection points are typically indicative of temperature-dependent mesoscale self-assembly, based on similar behavior observed for ELP diblock copolymers.²⁶ These results highlight that in addition to the primary variables of composition and molecular weight,²⁷ the thermal behavior of ELPs can be further tuned by the guest residue sequence and block architecture of the ELP.

Chemical Cross-Linking of ELP Block Copolymers

Figure 2A shows schematics of the architecture of the monoblock ELP[KV₇F-144] and the triblock ELP[KV₇F-72]-[VG₇A₈-64]-[KV₇F-72] and photographic images of hydrated, cross-linked hydrogels synthesized from these ELPs at room temperature. Visually, the triblock hydrogel appeared more swollen than the monoblock gel due to the inclusion of a hydrated, ELP[VG₇A₈-64] middle block that more effectively traps water in the hydrogel. Figure 2B shows the swelling ratio (Q_w), defined as the ratio of swollen gel weight (W_s) to dried gel weight (W_d) ($Q_w = W_s/W_d$) of the cross-linked ELP block copolymers as a function of temperature. In general, as the ELP block copolymer hydrogels were heated from 4 to 50 °C, a continuous 4.2–6-fold decrease of the Q_w was observed, similar to other chemically cross-linked ELP gels,^{14,37,38,42} presumably because of dehydration of the ELP. This picture is consistent with MD simulations of the conformational collapse and desolvation of

ELP, which show that a single solvated ELP gradually contracts and sheds water molecules with an increase in solution temperature over the entire temperature range of the simulation. 62–64

Figure 2B also shows that at all temperatures the swelling ratio appears to be related to two structural parameters: First, the swelling ratio appears to directly scale with the ratio of the hydrophilic/hydrophobic block in the copolymers, so that the ABA triblock with the longer ELP[VG₇A₈-64] middle block (labeled triblock (64-mers) in Figure 2B) has the largest swelling ratio at all temperatures, while the monoblock ELP had the smallest swelling ratio. Second, this effect is modulated by the block copolymer architecture, in that the swelling ratio is also controlled by the length of hydrophilic block. This effect is apparent by comparing the Q_w of the BABA tetrablock copolymer, ELP[VG₇A₈-32]-[KV₇F-72]-[VG₇A₈-32]-[KV₇F-72] with that of the ABA triblock, ELP[KV₇F-72]-[VG₇A₈-64]-[KV₇F-72] that has a ELP[VG₇A₈-64] hydrophilic middle block. Both copolymers have an identical hydrophobic/hydrophilic chain length ratio, but the hydrophilic B block is broken into two shorter 32-pentamer segments in the BABA tetrablock copolymer, in contrast to the ABA triblock, where it is present as an uninterrupted 64-pentamer segment embedded within adjoining hydrophobic A blocks. The Q_w for the BABA tetrablock copolymer is significantly lower than the ABA triblock (with a 64-pentamer B block), despite an identical overall hydrophilic/hydrophobic chain length ratio, which clearly demonstrates that the architecture plays a critical role in controlling the swelling of these block copolymers.

The swelling ratio of the block copolymers at the physiologically relevant temperature of 37 °C quantitatively follows these trends as shown in Figure 2C. The ABA triblock copolymer with 64 pentamers of the B block, has a Q_w that is significantly greater than that of the other polymers (one asterisk indicates $p < 0.001$ and two asterisks indicate $p < 0.01$, Fisher's). Notably, its Q_w is almost 1.5-fold greater than the BABA tetrablock copolymer that has the same overall composition, which highlights the critical role of block architecture in controlling the hydration of these copolymers. The tetrablock copolymer was observed to have a Q_w that is significantly greater than that of the monoblock (three asterisks indicate $p < 0.05$, Fisher's) but was not significantly different from the ABA triblock copolymer with the shorter 32-pentamer block ($p > 0.05$, Fisher's).

In an effort to further examine the microstructure of the cross-linked ELP block copolymers, the ELP hydrogels were incubated in PBS at 37 °C and then instantaneously plunged into liquid nitrogen, freeze-fractured, and examined by SEM. Figure 3 shows the microstructures of the freeze-fractured surfaces of the ELP block copolymer hydrogels. In general, each cross-linked ELP hydrogel exhibited porous microstructures with different chamber diameters and distributions. The monoblock ELP in panels A and B of Figure 3 had the smallest chamber diameters, ranging from ~5 to 20 μm ($10.4 \pm 4.9 \mu\text{m}$) and the triblock with the shorter middle block, ELP[VG₇A₈-32] in panels C and D of Figure 3 had larger chamber diameters than that of the monoblock and a higher distribution of chambers with a diameter of ~20–30 μm ($23.2 \pm 5.7 \mu\text{m}$). The ABA triblock with the longer ELP[VG₇A₈-64] middle block had the largest ~100–200 μm ($133.3 \pm 30.5 \mu\text{m}$) chamber diameters as shown in panels E and F of Figure 3. Interestingly, this triblock hydrogel also had evenly distributed, unique beadlike micro-structures ~1.0–1.6 μm ($1.3 \pm 0.2 \mu\text{m}$) in diameter. The tetrablock copolymer, which is the closest architecture to the cross-linked form of tropoelastin,^{55,65} exhibited intermediate chamber diameters varying from ~35 to 90 μm ($61.7 \pm 20 \mu\text{m}$) in panels G and H of Figure 3. The larger chamber diameters qualitatively correlate with the degree of swelling of the hydrogels, indicating that the capacity of the materials to imbibe and retain water is related to their microstructure.

The evolution of viscoelastic properties during isothermal cross-linking of the ELP block copolymers with THPP in PBS at 37 °C was measured by oscillatory rheology as a function of time (Figure 4). The gelation point (τ), defined by the crossover point of the dynamic storage (G') and loss (G'') modulus is an operational measure of the evolution of elastic properties from a viscous material and is often used as a metric of cross-linking kinetics.^{66,67} As shown in Figure 4A, the gelation time was <1 min for all ELP block copolymers and multiple measurements provided similar values, indicating that the initial formation of a loosely cross-linked elastic network occurred rapidly. The data in Figure 4B clearly show, however, that cross-linking continued for a significantly longer period of time, as the G' continued to increase for all block copolymer architectures, up to the end of the rheological measurements. Although the G'' values remained constant at approximately 100 Pa, the G' values increased from 4000 to 6000 Pa within 30 min depending on different domain interactions between the cross-linked, aggregated A block, ELP[KV₇F-72] and the hydrated, hydrophilic B middle block, [VG₇A₈-32, 64] of the different ELP block architectures. Generally, the gelation kinetics of ELP block copolymers with THPP in aqueous solution largely depends on temperature, protein concentration, ionic strength, and pH (unpublished data). Temperature was found to significantly affect the gelation kinetics of ELPs with THPP as well as hydrogel evolution (Supporting Information) because of its effect on coacervation (formation of the desolvated, aggregated phase). The effect of coacervation on cross-linking kinetics was significant, as gelation of the triblock, ELP[KV₇F-72]-[VG₇A₈-64]-[KV₇F-72] at room temperature (22 °C < T_l) occurred approximately ~23 min and the properties of the gel very slowly evolved with low values of G' (~430 Pa after 1 h reaction). In contrast, when the cross-linking of the same ELP was carried out at 37 °C, conditions under which the cross-linking domain undergoes its phase transition, gelation occurred within 1 min.

These results indicate that initial formation of a loosely cross-linked network of the cross-linkable, hydrophobic A block with measurable elastic properties proceeds rapidly but that ELPs continue to cross-link over a longer period of time. Furthermore, the evolution of the mechanical properties is not simply driven by cross-linking but is also driven by the spontaneous induction of the volume phase transition of the ELP, as cross-linkable Lys residues are consumed in the reaction, which lowers the T_l of the ELPs below physiological temperature and stiffens the hydrogels because of expulsion of bound water from the network. As shown previously, the change in T_l of the monoblock, ELP[KV₇F-144] when the pH is raised from pH 7.5 to 12.5 is 23 °C due to deprotonation of Lys residues, which supports the notion that evolution of the mechanical properties is accelerated by the lowered T_l of this ELP block as charged Lys groups are consumed in the cross-linking reaction.⁴⁶

Mechanical Properties of ELP Block Copolymer Hydrogels

The complex shear modulus, $|G^*|$, equilibrium shear modulus (μ) and equilibrium compressive modulus (E) of all three ELP block copolymers and the monoblock control were measured in PBS at 37 °C. In general, all ELP hydrogels are largely elastic materials, as the loss angles (δ) indicating the dissipation inherent in the materials ($\delta = 0^\circ$ for an elastic solid; $\delta = 90^\circ$ for Newtonian viscous fluid) ranged from $2.7 \pm 0.5^\circ$ for the monoblock to $9.5 \pm 2.5^\circ$ for the triblock with the longer ELP[VG₇A₈-64] middle block. As expected, the ELP monoblock without an un-cross-linked viscous segment was the most elastic, while the ELP triblock with the longest uninterrupted viscous segment was the least elastic of the four hydrogels. There was evidence of differences in the magnitude of δ among all evaluated ELP hydrogels ($p < 0.01$, ANOVA). Specifically, the values of δ of the triblock with ELP[VG₇A₈-64] middle block were significantly different from those of the monoblock and the triblock with the shorter ELP[VG₇A₈-32] middle block ($p < 0.01$, Fisher's).

Figure 5 shows that the mechanical properties of the ELP block copolymer hydrogels were largely controlled by the different lengths of the hydrophilic ELP[VG₇A₈] middle block at identical ELP concentration (200 mg/mL). A constant ELP concentration was used to measure mechanical properties because protein concentration significantly affects the mechanical properties of cross-linked hydrogels.¹⁴ In general, the average value of $|G^*|$, μ , and E in Figure 5 followed the trend: monoblock > ABA triblock with ELP[VG₇A₈-32] middle block > triblock with ELP[VG₇A₈-64] = tetrablock BABA copolymer. The $|G^*|$ for the triblock with ELP[VG₇A₈-32] was significantly different from that of the monoblock (one asterisk indicates $p < 0.001$, Fisher's) (Figure 5A). Furthermore, the $|G^*|$ for the triblock with ELP[VG₇A₈-64] (two asterisks indicate $p < 0.01$, Fisher's) and the tetrablock (one asterisk indicates $p < 0.001$, Fisher's) were statistically lower than that of the triblock with a ELP[VG₇A₈-32] midblock. However, the $|G^*|$ values of the tetrablock were not significantly different from those of the triblock with ELP[VG₇A₈-64] hydrophilic block ($p > 0.05$, Fisher's), suggesting that different block copolymer architectures with identical amino acid composition have similar dynamic stiffness after cross-linking, possibly due to a large effect of the viscous domain of the hydrated, hydrophilic middle blocks positioned between the hydrophobic, cross-linked blocks, ELP[KV₇F-72] on the $|G^*|$. The only exception to this general trend for $|G^*|$ and μ shown in parts A and B of Figure 5 was for the compressive modulus in Figure 5C, where similar values of E between the monoblock and the triblock with ELP[VG₇A₈-32] were measured, indicating that the short middle block has little effect on the equilibrium compressive properties of the hydrogels.

In general, these trends are the opposite of those observed for the swelling ratio and highlight that the mechanical properties are a function of solvation of the gels, which are themselves related to the differences in their microstructure. Unlike the solvation behavior of the block copolymers, however, these properties appear to be solely controlled by the ratio of the chain length of the hydrophobic, cross-linked domains to the hydrophilic, and viscous domains of the block copolymers and not by the block architecture as there was no difference between the ABA triblock with the 64-pentamer B block or the BABA tetrablock, which has the overall same length of the hydrophilic block broken down into two separate 32-pentamer segments.

In order to rule out that these differences in mechanical properties were not solely due to the differences in the concentration of cross-linking sites between the different ELPs, we compared the mechanical properties of cross-linked hydrogels of the ABA triblock with the ELP[VG₇A₈-64] middle block with that of the monoblock ELP at the same concentration of Lys residues. In order to do so, the triblock ELP was cross-linked at 1.4× higher concentration (278 mg/mL) than the monoblock ELP (200 mg/mL solution) to compare the mechanical properties at the same Lys concentration. The values of the mechanical properties ($|G^*|$, μ , and E) of the cross-linked, triblock ELP at the higher concentration were not significantly different than those of the same polymer cross-linked from solutions at 200 mg/mL ($p > 0.05$, Fisher's). However, these values of mechanical properties on $|G^*|$, μ , and E were significantly different from the values of the monoblock ELP (one asterisk indicates $p < 0.001$, Fisher's) as also observed for the swelling ratio's at 37 °C ($p < 0.01$, Fisher's). These results strongly suggest that the mechanical properties of the block copolymers are not simply a function of the density of cross-linkable Lys residues but are modulated by the length of the hydrophilic block within the ELP block copolymer.

Cell Viability within Cross-Linked ELP Hydrogels

Mouse fibroblasts were mixed at room temperature with the ELP block copolymers (the monoblock, ELP[KV₇F-144] and the triblock, ELP[KV₇F-72]-[VG₇A₈-64]-[KV₇F-72]) and THPP at a 1:1 molar ratio of HMP to Lys residues of the ELPs to investigate different cell viability based on the hydrogel networks of the cross-linked ELP block copolymers. The

hydrogels formed rapidly within several minutes after incubation at 37 °C as observed in the oscillatory rheology tests. The cells embedded in the ELP hydrogels were cultured at 37 °C for 1 h or 3 days. The live and dead cells in the hydrogels were stained by a cell viability/cytotoxicity assay, and their fluorescence images in Figure 6 ((A, B) the monoblock and (C, D) the triblock with 64 mers) were visualized in situ at room temperature by confocal fluorescence microscopy at day 0 (A, C) and day 3 (B, D). Figure 6 shows that most cells at day 0 and day 3 fluoresced green, indicating that they were alive, while a small number of cells in the monoblock hydrogels at day 3 (B) fluoresced red, which is an indication of cell death due to infiltration of EtHD-1 through compromised cell membranes and its intercalation into the DNA in the nucleus.

The DNA content of cells within identical gel constructs (2 mm thickness and 5 mm diameter) was identical at day 0 for the two ELPs ($p > 0.05$, Fisher's), indicating that the level of cell encapsulation was initially similar. At day 3, in contrast, the DNA content in the triblock gels was significantly greater than that of the monoblock ($p < 0.05$, Fisher's) in Figure 6E. These results clearly show that: (1) the cells survived the THPP cross-linking system with the ELP block copolymers at day 0; (2) the cells lived for at least 3 days with a fairly uniform distribution of live cells; and (3) notably, that the triblock copolymer gels provided an environment that appears to support greater cell proliferation compared to the monoblock ELP. Although our results do not enable us to conclude reasons for the enhanced cell numbers in the triblock ELP copolymer at day 3 compared to the monoblock ELP, they suggest that it might be possibly related to the difference in microstructure and hydration provided by the un-cross-linked, hydrophilic middle block in the triblock copolymer architecture as compared to the monoblock control.

Conclusions

This study demonstrates that the various ELP block copolymer architectures with THPP rapidly form hydrogel scaffolds under physiological conditions. The major findings of this study are that swelling ratio and mechanical properties of the hydrogels are controlled by the various block architectures comprising a hydrophilic middle block as the viscous domain and a hydrophobic, cross-linked block as the elastic domain as well as the ratio of their block length. Furthermore, the triblock copolymers with a hydrophilic middle block enhance cell viability as compared to hydrogels synthesized from a single block suggesting that segregation of the cross-linking regions within an ELP has beneficial effects on gel hydration and cellular interaction. In conclusion, the results reported in this study identify a new variable—block copolymer architecture—to control the mechanical properties and potentially functional outcomes in tissue engineering via design of block copolymer architecture and underscores the utility of recombinant DNA approaches for polymer design for functional tissue engineering and regenerative medicine, given their exquisite ability to provide absolute control over these variables.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded by NIH EB002263. We would like to thank Jared Gardner, a Pratt fellow at Duke University for ELP purification and helpful discussions.

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Figure 1.

(A) Copper-stained SDS-PAGE gel and (B) thermal profiles of ELP block copolymers. Lane (M) contains molecular size markers (205, 116, 97, 84, 66, 55, 45, 36, 29, 24, 20, 14, and 7 kDa from top to bottom). The numbers of ELP block copolymers are labeled above each lane and their molecular weights are shown on the right: 1, ELP monoblock, ELP[KV₇F-144]; 2, ELP triblock with a short middle block (32 pentamers), ELP[KV₇F-72]-[VG₇A₈-32]-[KV₇F-72]; 3, ELP triblock with a longer middle block (64 pentamers), ELP[KV₇F-72]-[VG₇A₈-64]-[KV₇F-72]; and 4, ELP tetrablock, ELP[VG₇A₈-32]-[KV₇F-72]-[VG₇A₈-32]-[KV₇F-72].

Figure 2.

(A) Schematics of architecture of ELP block copolymers and photographic images of the cross-linked ELP gels in a swollen state at room temperature. (B) Swelling ratio (Q_w) of the hydrogels in PBS as a function of temperature. (C) Q_w at 37 °C of the different ELP hydrogels. Data were reported as mean $\pm$ standard deviation ($n = 4$). Key: *, $p < 0.001$; **, $p < 0.01$; ***, $p < 0.05$, by Fisher's post hoc test.

Figure 3. Cross-sectional SEM images of freeze-fractured ELP hydrogels: (A, B) ELP[KV₇F-144]; (C, D) ELP[KV₇F-72]-[VG₇A₈-32]-[KV₇F-72]; (E, F) ELP[KV₇F-72]-[VG₇A₈-64]-[KV₇F-72]; (G, H) ELP[VG₇A₈-32]-[KV₇F-72]-[VG₇A₈-32]-[KV₇F-72]. Scale bars are 50 μm in (A) and (C), 200 μm in (E) and (G), and 10 μm in (B), (D), (F), and (H).

Figure 4.

Oscillatory rheological profiles for ELP block copolymers for (A) 90 s and (B) 300 s, after addition of THPP cross-linker to solutions of ELP block copolymers in PBS. Elastic modulus (G') and viscous modulus (G'') are shown as a function of time.

Figure 5. Mechanical properties of ELP block copolymer hydrogels cross-linked by THPP in PBS at pH 7.5. (A) Complex shear modulus, $|G^*|$, (B) Equilibrium shear modulus, μ , and (C) Equilibrium compressive modulus, E . Data were reported as mean $\pm$ standard deviation ($n = 4$). Key: *, $p < 0.001$; **, $p < 0.01$; ***, $p < 0.05$, by Fisher's post hoc test.

Figure 6.

(A–D) Fluorescent cell images showing cell survival of fibroblasts encapsulated in cross-linked monoblock, ELP[KV₇F-144] and triblock, ELP[KV₇F-72]-[VG₇A₈-64]-[KV₇F-72] hydrogels and (E) DNA content in each hydrogel at day 0 and day 3. Cell survival of encapsulated fibroblasts was determined by a fluorescent cell viability/cytotoxicity assay; (A, B) the monoblock hydrogels at day 0 (A) and day 3 (B); and (C, D) the triblock hydrogels at day 0 (C) and day 3 (D). Data were reported as mean $\pm$ standard deviation ($n = 5$). Key: ***, $p < 0.05$, by Fisher's post hoc test in (E).

Scheme 1.

Schematic of Recursive Directional Ligation To Construct Plasmids Encoding ELP Diblock and Triblock Copolymers Using Synthetic Genes that Encode ELP[KV₇F-72] and ELP[VG₇A₈-64]