Rapid Cross-Linking of Elastin-like Polypeptides with (Hydroxymethyl)phosphines in Aqueous Solution

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In situ gelation of injectable polypeptide-based materials is attractive for minimally invasive in vivo implantation of biomaterials and tissue engineering scaffolds. We demonstrate that chemically cross-linked elastin-like polypeptide (ELP) hydrogels can be rapidly formed in aqueous solution by reacting lysine-containing ELPs with an organophosphorous cross-linker, β-[tris(hydroxymethyl)phosphino]propionic acid (THPP) under physiological conditions. The mechanical properties of the cross-linked ELP hydrogels were largely modulated by the molar concentration of lysine residues in the ELP and the pH at which the cross-linking reaction was carried out. Fibroblasts embedded in ELP hydrogels survived the cross-linking process and were viable after in vitro culture for 3 days. DNA quantification of ELP hydrogels with encapsulated fibroblasts indicated that there was no significant difference in DNA content between day 0 and day 3 when ELP hydrogels were formed with an equimolar ratio of THPP and lysine residues of the ELPs. These results suggest that THPP cross-linking may be a biocompatible strategy for the in situ formation of cross-linked hydrogels.

Introduction

Biologically inspired peptide-based materials[1,2] are of increasing interest for application as biomaterials[3−8] because (1) their sequence can be programmed at the gene level, (2) they can be readily synthesized by recombinant DNA techniques in bacterial expression systems, (3) they yield monodisperse polymers with precisely defined molecular properties, (4) they can be processed to display a range of useful physical and mechanical behaviors,[9,10] (5) they display the potential for good biocompatibility and low cytotoxicity,[11] and (6) they can be designed to degrade at controlled rates in vivo through a wide variety of proteolytic mechanisms to yield amino acid degradation products that can be readily excreted or resorbed.[11−13]

We[14−18] and others[11,19−21] are especially interested in a specific class of repetitive polypeptides termed elastin-like polypeptides (ELPs). ELPs are artificial polypeptides that are derived from a repetitive Val-Pro-Gly-Xaa-Gly peptide motif in tropoelastin (where Xaa is any amino acid other than Pro). For applications that require large quantities (∼grams in a laboratory setting) such as biomaterials and tissue engineering scaffolds, ELPs have two advantages compared to many other repetitive polypeptides.[22,23] First, ELPs can be routinely expressed at >200 mg/L in shaker flask culture[24−26] and when optimized to levels as high as 1.6 g/L.[27] Second, ELPs are stimulus-responsive polypeptides, as 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 reversible process. This phase transition behavior of ELPs is useful because it enables the rapid purification of gram quantities of ELPs by a simple, nonchromatographic batch purification process, inverse transition cycling (ITC).[24−29]

ELPs are also promising scaffold materials for musculoskeletal and cardiovascular tissue engineering, as their peptide sequences are native to smooth and skeletal muscle, ligaments, and other musculoskeletal tissues, and they show low cytotoxicity and no antigenic response in vivo.[11,19] In addition, ELPs may confer some benefits for cartilage tissue engineering application, as we have shown that the thermally induced aggregated, "coacervate" phase of ELPs allows encapsulation of chondrocytes and human adipose derived stem cells while promoting a chondrogenic phenotype and cartilage matrix synthesis.[14,17] Although these studies have indicated the promise of ELPs for cartilage tissue engineering, these materials exhibited a narrow range of mechanical properties, which may limit their utility as scaffolds for cell-assisted regeneration and provide the rationale for the development of cross-linking strategies.

In the past few years, different cross-linking methods of ELPs[15,30,31] have been investigated; chemical methods include cross-linking by radiation,[32−34] photoinitiation,[35] chemical cross-linking[15,30,31,36−38] and enzymatic cross-linking by tissue transglutaminase,[16] while the formation of physically cross-linked networks has also been demonstrated for ELP block copolymers.[5,9,10,39] Furthermore, the phase transition behavior of ELPs is also maintained in their cross-linked state, which provides a secondary variable to tune their mechanical behaviors by modulating the degree of solvation of the cross-linked ELP hydrogel.[15]

Despite the variety of cross-linking approaches that have been proposed in the literature, many of these methods cannot be used to create injectable ELP scaffolds in which the liquid precursors can be readily injected into a defect site, followed by in situ formation of a conformal hydrogel, because of the cytotoxicity of the reactants or byproducts, the need for organic solvents, or the suboptimal kinetics of the cross-linking reactions.[16] Motivated by these considerations, the objective of this study was to develop a cross-linking strategy that permits a mixture of soluble ELP and cells to be injected and cross-linked in vivo with optimal kinetics (<5 min gelation time), with minimal cytotoxicity to provide a hydrogel whose mechanical properties match those of cartilaginous tissues. A secondary

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objective of this study was to evaluate the potential to vary the physical properties of the cross-linked gels by the density of cross-linkable, functional groups in the ELPs as well as the pH of the cross-linking reaction.

We introduce in this paper the Mannich-type condensation of (hydroxymethyl)phosphines (HMPs) with primary and secondary amines of amino acids as a new cross-linking method for polypeptide-based biomaterials that satisfies these requirements.[40−44] This reaction has been previously used to incorporate phosphines into peptides to coordinate with transition metals,[45−49] but has not been used to cross-link polypeptides, to the best of our knowledge. In this study, we demonstrate that chemically cross-linked ELP hydrogels can be formed rapidly in an aqueous solution by reaction of ELPs containing periodic lysine residues (Lys or K, single-letter amino acid code) with β-[tris(hydroxymethyl)phosphino]propionic acid (THPP). We present data showing that the mechanical properties of THPP-cross-linked ELP hydrogels can be modulated by the concentration of Lys residues in the ELP and by the pH of the cross-linking reaction. Finally, we show that murine fibroblasts can be encapsulated in the ELP hydrogels in a biocompatible process and that they survive the cross-linking procedure and in vitro culture for 3 days.

Experimental Section

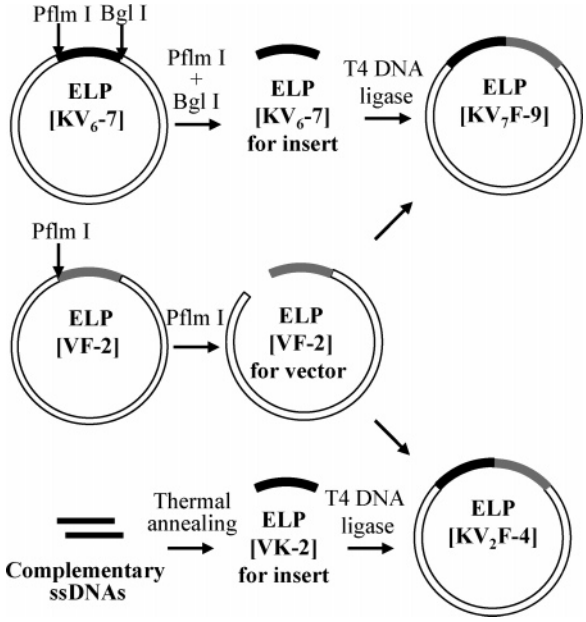
ELP Notation. All ELPs are named using the following notation: ELP[$X_iY_jZ_k$-*n*], where the capital letters in brackets are single-letter amino acid codes of a guest residue, their corresponding subscripts denote the ratios of that guest residue in the monomer, and *n* indicates the number of pentapeptides in the ELP. For example, ELP[KV_7F-9] is an ELP that contains nine repeats of the VPGXG pentapeptide, in which one pentapeptide contains K at the fourth guest residue position (X), another pentapeptide has F at the fourth position, and seven pentapeptides have V at the same position.

Monomer ELP Gene Synthesis and Its Oligomerization. Standard molecular biology protocols were used for gene synthesis of the monomer ELP gene and its oligomerization by recursive directional ligation (RDL) to synthesize genes that encoded for longer ELPs.[25] Two pUC19 plasmids separately containing ELP[KV_6-7] and ELP[VF-2] were previously cloned and used for this study.[15] Scheme 1 shows the method by which monomers for the two ELPs were assembled: the gene for ELP[KV_7F-9] was constructed by ligation of the plasmid-borne gene for ELP[KV_6-7] with a plasmid-borne gene that encoded ELP[VF-2], while the gene for ELP[KV_2F-4] was constructed by ligation of a plasmid-borne gene of ELP[VF-2] with a synthetic oligonucleotide insert that encoded ELP[VK-2]. Multiple rounds of RDL were performed with these monomer genes to create genes for ELP[KV_7F-18,36,72,144] and ELP[KV_2F-16,32,64,128], which encode for ELPs with molecular weight (MW) ranging from 7700 to 61100.

Gene Expression and Purification of ELPs. The pET-25b(+) expression vector (Novagen Inc., Milwaukee, WI) was previously modified to introduce a unique *Sfi*I restriction site for insertion of an ELP gene and codons that encode for a C-terminal Trp for spectrophotometric detection of ELPs.[15,25] ELPs were expressed by inserting ELP genes restricted with *Pfl*mI and *Bgl*I into the modified pET-25b-(+) expression vector (Novagen Inc.) digested with *Sfi*I (New England Biolabs, Beverly, MA) that had been dephosphorylated using CIP. The *E. coli* strain, BLR (DE3) cells (Novagen Inc.) transformed with the modified pET-25b(+) vector containing an ELP insert, were grown in 1 L cultures of CircleGrow medium supplemented with 100 μg/mL ampicillin for 24 h at 37 °C at 200 rpm (Qbiogene, Carlsbad, CA). The expressed ELPs were purified by ITC as previously described.[24−29]

Physicochemical Characterization of ELPs. The purities of ELPs were characterized by SDS−PAGE (BioRad, Inc., Hercules, CA) and visualized by a copper stain. Their MWs were determined by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), PE Biosystems Voyager-DE instrument equipped with a nitrogen laser, 337 nm. ELP samples were dissolved in an aqueous 50% acetonitrile solution containing 0.1% trifluoroacetic acid, and a sinapinic acid matrix was used for MALDI-MS. The inverse phase transition temperature (T_t) of ELPs was measured by heating a 25 μM solution of the ELP between 10 and 90 °C at a heating rate of 1 °C/min, and the OD_{350} was measured by a UV−vis spectrophotometer (Cary 300 Varian Instruments). The T_t of ELPs (0.625 mg/mL) as a function of pH was measured by dissolving freeze-dried ELPs into different 20 mM phosphate buffers at pH 7.5, 10.0, and 12.5. T_t is defined as the temperature at which the change in optical density with respect to temperature (d(OD)/d*T*) reaches its maximum. The ELP concentration was determined by the ELP molar extinction coefficient at 280 nm (5690 M^{-1} cm^{-1}) calculated from the primary amino acid sequence of the ELPs using the software program Protean (DNA Star).

Scheme 1. Schematic of the Construction of Plasmids Encoding ELP[KV_7F-9] and ELP[KV_2F-4] Monomer Genes[a]



[a] The plasmid containing ELP[KV_6-7] is doubly digested with *Pfl*mI and *Bgl*I, and the complementary single-stranded oligonucleotides are thermally annealed to form a double-stranded DNA oligonucleotide cassette encoding ELP[VK-2] with *Pfl*mI compatible overhangs. The plasmid of ELP[VF-2] is digested with *Pfl*mI and then enzymatically dephosphorylated. The linearized, dephosphorylated ELP[VF-2] vectors are separately ligated with the ELP[KV_6-7] insert and the oligonucleotide cassette of ELP[VK-2].

Gelation Kinetics by Oscillatory Rheology. The cross-linking kinetics of ELPs with THPP (Pierce, Rockford, IL) was measured by oscillatory rheology as a function of time. Their 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 35 °C over 1 h (1 Hz frequency and 0.01 shear strain). To ensure homogeneous mixing of the reactants, 75 μL of 200 mg/mL THPP in 20 mM phosphate with 700 mM NaCl at pH 7.5 was mixed by vigorous pipetting with 450 μL of 200 mg/mL ELP[KV_7F-144] in 20 mM phosphate at pH 7.5 to yield an 8−9-fold molar excess of reactive HMP of THPP to primary amine of ELPs as well as a final NaCl concentration of 100 mM. The bottom platen of the test apparatus was held at a constant temperature of 35 °C. Values for the storage (G') and loss (G'') moduli were obtained from the torque data.

Mechanical Properties of Cross-Linked ELP Hydrogels. ELP-[KV_7F-72] and ELP[KV_2F-64] were cross-linked in 20 mM phosphate, 100 mM NaCl at different pH values (7.5 and 12.0) as follows. A THPP solution was mixed by vigorous pipetting of each ELP solution in a customized Teflon mold (8 mm diameter and 2 mm height) at 4 °C,

and the mixture was then incubated at 37 °C for 1 h. The fully cross-linked ELP hydrogels were immersed in distilled, deionized water at 4 °C overnight to remove salts, free phosphates, and unreacted THPP and stored at 4 °C in their swollen state in 20 mM phosphate at pH 7.5. The cross-linked hydrogels of ELP[KV_7F-72] and ELP[KV_2F-64] were tested in 20 mM phosphate at pH 7.5 to determine the equilibrium compressive modulus (E), complex shear modulus ($|G^*|$), equilibrium shear modulus (μ), and loss angle (δ). Parallel plate platens of porous stainless steel were used (plate radius 10 mm, 50% porosity, 40−60 μm pore size) with samples and test platens submerged in 20 mM phosphate at pH 7.5 held at room temperature. Samples were equilibrated under a tare load of 5−10 g, and the reference thickness was recorded as the distance between platens at equilibrium. Samples were then compressed to 5% compressive strain, ϵ, and allowed to relax until equilibrium. This protocol was repeated in 5% increments until 15% strain was achieved. Linear regression of calculated normal stress, σ, at equilibrium on ϵ was performed to calculate the equilibrium compressive modulus, E. At 15% compression, a dynamic frequency sweep test was performed in torsional shear at a maximum shear strain of 0.01 (0.1−50 rad/s). Linear viscoelastic theory was used to calculate the magnitude of the complex shear modulus, $|G^*|$, and the loss angle representing stiffness and internal energy dissipation of the material under dynamic loading. The equilibrium shear modulus (μ) was also determined from the relationship between torsional shear strain measurements of 0.05 and the resulting shear stress. Four ELP hydrogels per formulation were measured for all mechanical properties in 20 mM phosphate at pH 7.5.

Characterization of Swelling Properties. The swelling ratio by weight, Q_w, defined as the ratio of the swollen gel weight, W_s, to the freeze-dried gel weight, W_d ($Q_w = W_s/W_d$), was measured in 20 mM phosphate at pH 7.5 as a function of temperature to measure the dimensional changes between the swollen and collapsed states of the thermoresponsive ELP hydrogels. The swelling ratios of ELP[KV_7F-72] and ELP[KV_2F-64] hydrogels cross-linked at pH 7.5 and 12.0 were measured using four ELP hydrogel constructs per formulation.

Microstructural Morphology. Freeze-dried ELP hydrogels were physically fractured by tweezers, and their microstructures were imaged by a Philips FEI XL30 field emission scanning electron microscope at an acceleration voltage of 30 kV. Four ELP hydrogels of each formulation were analyzed to investigate the morphology as a function of cross-linkable Lys density of the ELPs.

Cell Viability and DNA Quantification. A suspension of mouse NIH-3T3 fibroblasts was mixed with ELP[KV_7F-144] and THPP at room temperature in a 1:1 HMP:Lys molar ratio to achieve a final concentration of 200 mg/mL ELP and 10×10^6 cells/mL in HEPES-buffered saline (25 mM HEPES, 150 mM NaCl). The solution was injected into a custom mold by a syringe at room temperature and then incubated for 1 h at 37 °C in a 5% CO_2 incubator. The fibroblast-embedded, cross-linked ELP slabs of 2 mm thickness were cut out by a 5 mm diameter biopsy punch (Miltex, York, PA) to produce multiple cellular ELP hydrogels having identical dimensions. Each cellular ELP hydrogel was immersed in growth medium in individual wells of 24-well plates and then cultured at 37 °C in a 5% CO_2 incubator for 3 days. A cell viability assay was performed using the live/dead cell viability/cytotoxicity assay kit (Molecular Probes, Eugene, OR). Four cellular ELP hydrogels were removed at each time point from the culture medium, placed in 48-well plates, and washed three times in PBS to remove serum esterase activity. Each construct was incubated in a staining solution, which contained 2 μM calcein and 2 μM ethidium homodimer-1 (EthD-1), for 30 min. The calcein/EthD-1 solution was discarded, and each hydrogel was washed three times with PBS. Cell survival within the ELP hydrogels was visualized via fluorescence laser scanning confocal microscopy (Zeiss LSM 510, Carl Zeiss, Inc., Thornwood, NY).

The DNA content of encapsulated fibroblasts in each ELP hydrogel construct was determined with four or five different constructs at each time point by using the Quant-iT Picogreen dsDNA assay (Molecular Probes). ELP hydrogels with encapsulated fibroblasts were digested in PBS containing 125 μg/mL papain (Sigma, St. Louis, MO) and 0.05% trypsin (Invitrogen, Carlsbad, CA) at 37 °C for 1 day, followed by consecutive incubation at 65 °C for 1 day, and then centrifuged at 13000g for 10 min. Picogreen reagent was added to aliquots of each digest solution and DNA controls in a 96-well plate, followed by incubation for 5 min. The total DNA content per digest solution was measured using a plate reader (Tecan GENios, Phenix Research Products, Candler, NC) using 480−485 nm/520−530 nm excitation/emission.

Statistical Analysis. Two-factor analysis of variance (ANOVA) and Fisher's post hoc tests in Statview software (SAS Institute, Cary, NC) were used to test for the effect of ELP type and cross-linking pH on various mechanical properties (E, $|G^*|$, and μ). A one-factor ANOVA was used to test for the effect of culture time on the DNA content of the cells. Statistical significance was determined at a value of $p < 0.05$.

Results and Discussion

Design and Characterization of ELPs. Two ELP libraries, ELP[KV_7F-9,18,36,72,144] and ELP[KV_2F-8,16,32,64,128], were synthesized from plasmid-borne genes in *E. coli*. Periodic Lys (K) residues were incorporated in their primary amino acid sequence to provide sites for chemical cross-linking. The different concentrations of Lys were chosen to examine the effect of cross-linking density on the mechanical properties of the cross-linked hydrogels. The incorporation of Lys residues raises the T_t at physiological pH, which has two undesirable consequences: first, it makes purification of ELPs by ITC difficult within a reasonable range of temperatures, necessitating use of high concentrations of NaCl to trigger the phase transition of the ELPs during ITC. Second, because the cross-linking reaction does not proceed to completion, the fraction of un-cross-linked Lys residues in the ELP hydrogels increases the temperature range over which the volume phase transition occurs. Phe, a hydrophobic guest residue, was incorporated into the ELPs at the same ratio as Lys residues in the ELP repeat to counterbalance the effect of the Lys residues, as it lowers the T_t of the ELPs.

Members of the ELP[KV_7F] and ELP[KV_2F] libraries were expressed in *E. coli* and purified by ITC.[25] Copper-stained SDS−PAGE gels in Figure 1 show that the ELPs could be purified to at least 95% homogeneity by multiple rounds of ITC.[15,25] All ELPs migrated approximately 20% larger than their calculated molecular weights on the basis of the migration pattern of protein standards, as previously reported for other ELPs.[15,25] MALDI-MS, however, confirmed that the size of the ELPs was close to that predicted by their amino acid sequence, as the difference between their calculated and experimentally measured MWs ranged between 0.01% and 0.5%.

Figure 2A shows that the T_t values of the ELP[KV_2F] series are 16−42 °C higher than those of ELP[KV_7F] of similar molecular weight because of the greater fraction of Lys residues in the ELP[KV_2F] family. Similarly, the T_t values of polypeptides of the ELP[KV_7F] series are more than 20 °C lower than the those of ELP[KV_6] polypeptides of similar molecular weight due to the introduction of hydrophobic Phe residues.[50,51] Within each series, T_t is also inversely proportional to the MW of the ELP, as previously seen for other ELPs.[52] These results highlight the degree of control that can be exercised over the T_t of ELPs simply by control of the amino acid sequence and MW of the polypeptides.

The results in Figure 2B,C show the effect of ionizable groups on the T_t of ELPs. Figure 2B shows that the salt sensitivity, defined by the parameter $\Delta T_t/M$(NaCl) (defined as the decrease

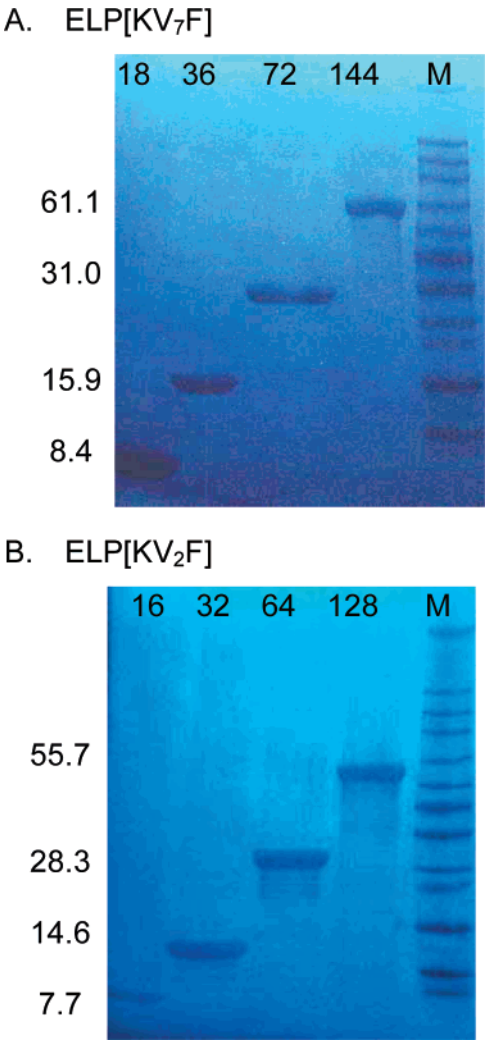


Figure 1. Copper-stained SDS−PAGE gels of (A) ELP[KV_7F] and (B) ELP[KV_2F]. The lane marked "M" contains molecular size markers (205, 116, 97, 84, 66, 55, 45, 36, 29, 24, 20, 14, and 7 kDa from top to bottom). The numbers of pentapeptide repeats of ELPs are labeled above each lane, and the expected molecular weights of the ELPs are shown on the left.

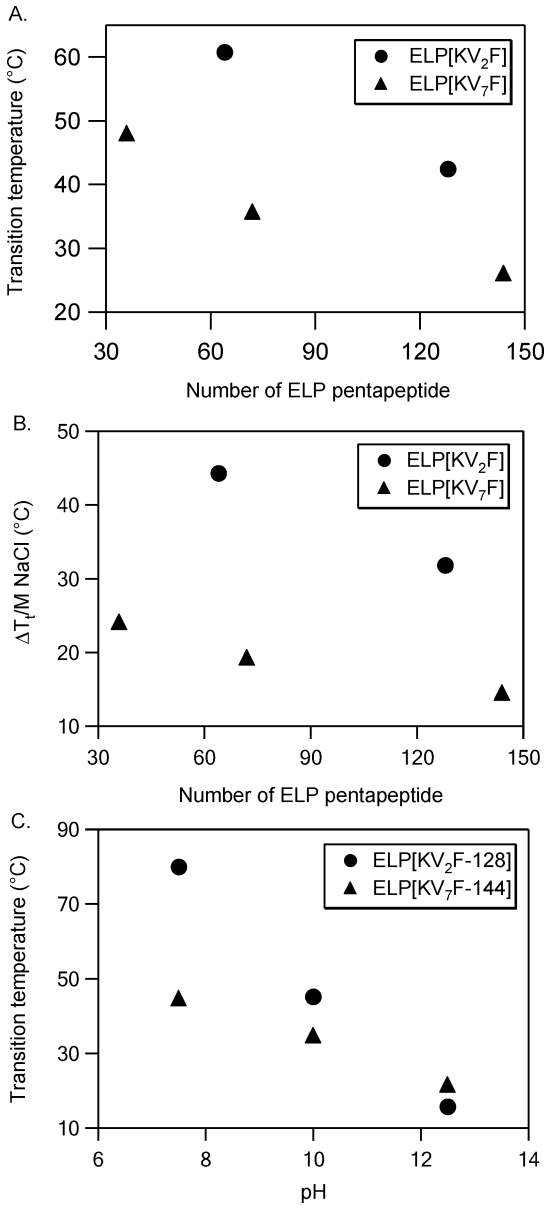


Figure 2. (A) Transition temperature, T_t, and (B) $\Delta T_t/M$(NaCl) of ELPs as a function of the number of ELP pentapeptides for 25 μM ELP solutions in phosphate-buffered saline. (C) T_t of ELP[KV_7F-144] and ELP[KV_2F-128] as a function of pH for 0.625 mg/mL (10.2−11.2 μM) ELP solutions in 20 mM phosphate buffer.

in T_t caused by the addition of 1 M NaCl to a 25 μM ELP solution in PBS) ranges between 30 and 50 °C and between 15 and 25 °C for ELP[KV_2F] and ELP[KV_7F], respectively. The increased salt sensitivity of the ELP[KV_2F] series as compared to the ELP[KV_7F] series is consistent with the greater density of Lys residues in ELP[KV_2F]. Furthermore, Figure 2C shows that the changes in the T_t of ELP[KV_2F-128] and ELP[KV_7F-144] when the pH is raised from 7.5 to 12.5 are 64 and 23 °C, respectively. This observation is consistent with the biophysical properties of other charged ELPs[50,53,54] and can be attributed to the marked difference in the ionization of the ELPs as the pH is raised above the pK_a of their Lys residues.

Chemical Cross-Linking of ELPs. The cross-linking reaction of ELP[KV_7F-72] and ELP[KV_2F-64] with THPP was carried out in 20 mM phosphate, 100 mM NaCl at pH from 2 to 13 (Figure 3A). Figure 3B shows that ELP[KV_7F-72] cross-linked in phosphate buffer at pH 7.5 was swollen at 4 °C, but was in a collapsed state at 37 °C due to the thermally triggered volume phase transition behavior of the cross-linked ELP chains. In contrast, ELP[KV_2F-64], cross-linked under the same conditions, exhibited less collapse than ELP[KV_7F-72] hydrogels at 37 °C. To examine the differences in their microstructure, freeze-dried and fractured ELP hydrogels were imaged by scanning electron microscopy (SEM). The ELP[KV_2F-64] hydrogel exhibits a denser microstructure than the ELP[KV_7F-72] hydrogel that was cross-linked under identical conditions. The swelling ratio (Q_w), defined as the ratio of the swollen gel weight (W_s) to the dried gel weight (W_d), $Q_w = W_s/W_d$, of ELP[KV_7F-72] hydrogels as a function of temperature ranged from 12.3 ± 0.5 at 4 °C to 3.7 ± 0.2 at 37 °C. In contrast, ELP[KV_2F-64] hydrogels cross-linked at pH 7.5 exhibited a Q_w that ranged from 8.2 ± 0.5 at 4 °C to 4.2 ± 0.7 at 37 °C. The lower swelling ratio of ELP[KV_2F-64] at 4 °C compared to ELP[KV_7F-72] as well as its denser microstructure is presumably due to its higher cross-linking density than that of ELP[KV_7F-72] hydrogels because of the greater concentration of Lys (K) residues in ELP[KV_2F-64]. The higher densities of chemically conjugated amines, (aminomethyl)phosphines, as well as the higher ratio of the hydrophobic Phe guest residue of ELP[KV_2F-64] hydrogels dramatically decrease the LCST of cross-linked ELP hydrogels, making the gels appear opaque at 4 °C. Related

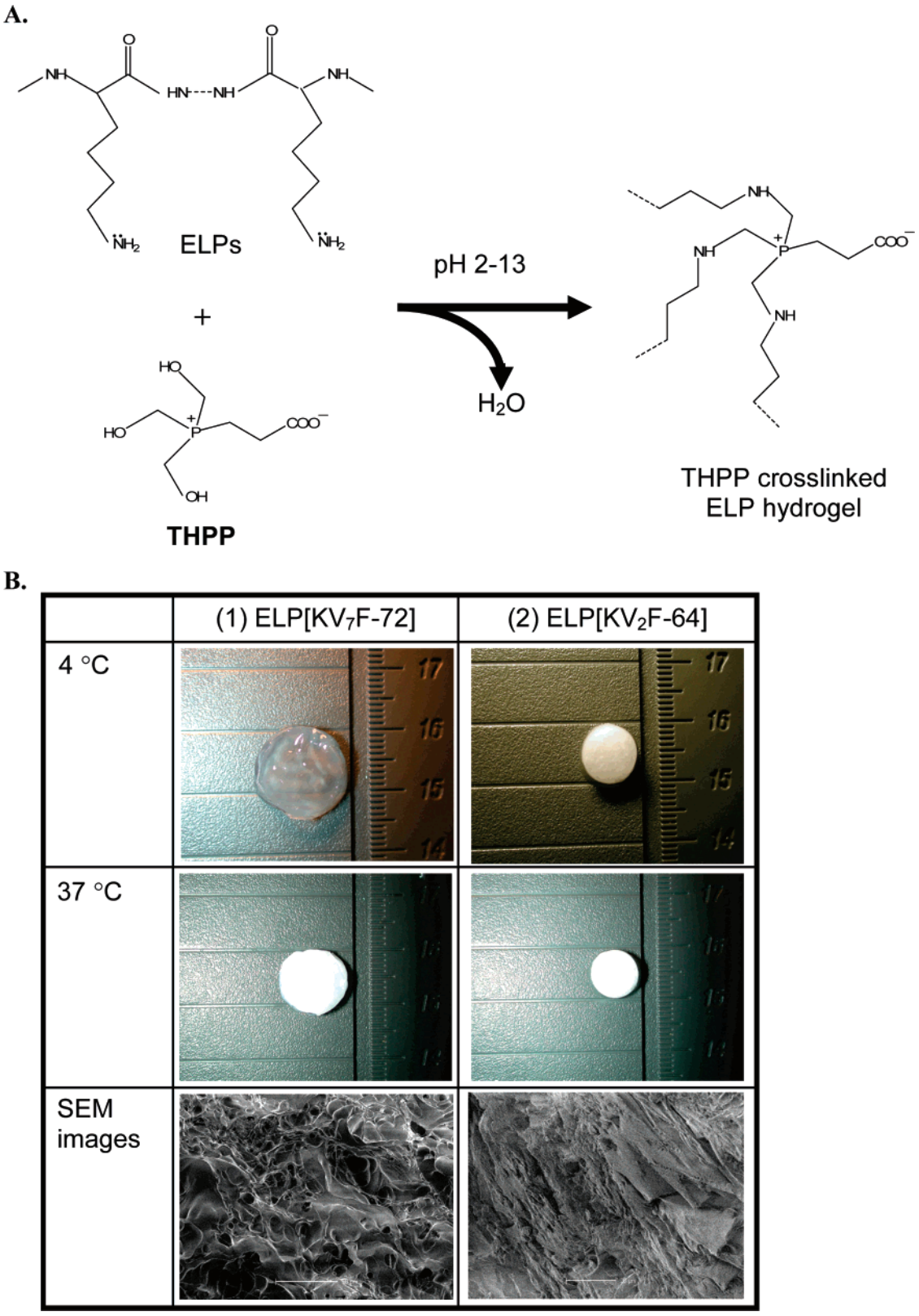


Figure 3. (A) Schematic of the inter- or intramolecular cross-linking mechanism between Lys residues of ELPs and THPP. (B) Photographs of thermoresponsive swollen ELP[KV_7F-72] and ELP[KV_2F-64] hydrogels cross-linked by THPP in 20 mM phosphate buffer at pH 7.5 at 4 and 37 °C and their cross-sectional SEM images. Scale bars in the SEM images represent 50 μm (1) and 20 μm (2).

studies of the stability of ELP hydrogels show that the wet weight of THPP-cross-linked His_6-tagged ELP hydrogels in PBS at 37 °C did not change significantly within 60 days, suggesting THPP cross-linked gels were stable for at least 2 months under physiological conditions.[18]

The gelation kinetics of THPP-cross-linked ELP[KV_7F-144], measured by oscillatory rheology as a function of time, showed that ELP hydrogels formed in less than 1 min under physiological conditions, with hydrogel formation defined by the crossover point of the dynamic storage (G') and loss (G'') moduli

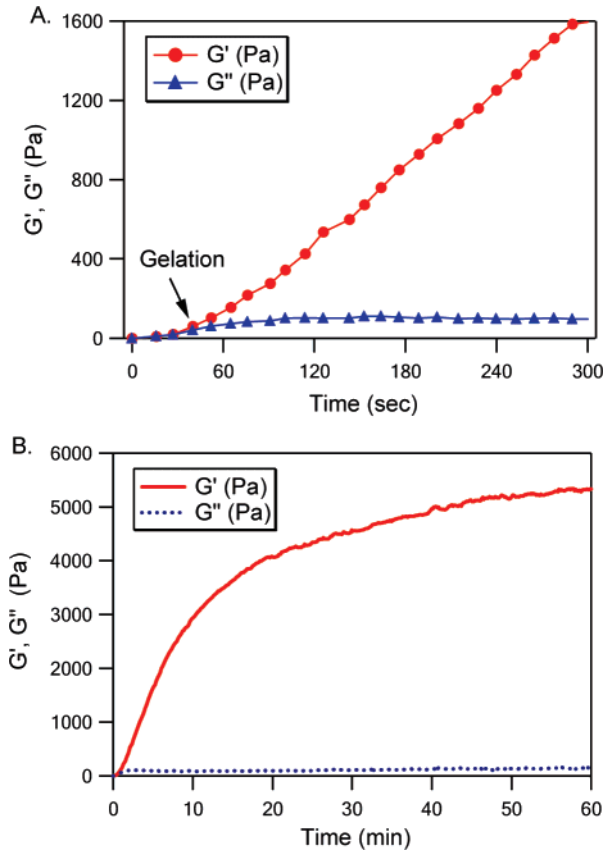


Figure 4. Oscillatory rheological profiles for ELP[KV_7F-144] and THPP mixtures. Aliquots of THPP (200 mg/mL, 20 mM phosphate, 700 mM NaCl, pH 7.5) were added to ELP[KV_7F-144] (200 mg/mL ELP, 20 mM phosphate, pH 7.5) placed in the stage of a cone-on-plate rheometer equilibrated at 35 °C. The elastic component of the dynamic modulus, G', and viscous component, G'', as a function of time are shown in (A) and (B). Dynamic shearing was performed at 1 rad/s and $\gamma_0 = 0.01$.

(Figure 4A).[55] After this crossover point, the elastic response of ELP hydrogels started to dominate the viscous response and the structure of the hydrogels continued to evolve, as G' increased to greater than 5.3 kPa within 1 h while G'' remained constant at approximately 0.1 kPa. These results suggest that Lys residues within the ELP hydrogels continued to be cross-linked until G' reached its maximum value (Figure 4B).[15] The fully cross-linked ELP hydrogels of ELP[KV_7F-72] and ELP[KV_2F-64] were highly elastic as demonstrated in a dynamic torsional test by the values of G' exceeding G'' over several decades of frequency (0.01−50 rad/s). The cross-linking kinetics of ELP[KV_7F-144] and the other ELPs in a physiologically relevant buffer with isotonic salt concentration (PBS) were similar and are hence not shown. In addition, the integrity and survival of THPP-cross-linked ELPs following injection into a tissue site in a goat model were evaluated as injectable hydrogels for articular cartilage repair of an osteochondral defect.[56] We observed that the solution mixture of THPP and ELP became turbid within 1 min and solid after 5 min. Thus, all surgical sites were closed at 5 min after injection into the cartilage defect site. The results of that study led us to conclude that gelation occurs rapidly enough that diffusion of unreacted THPP within <5 min from the defect site is not a concern with regard to gelation in vivo. Sufficient THPP remained in the defect over time to allow for gel formation and retention at 7 days and even at 3 months.[56] Preliminary studies also show that the reacting THPP with other amines in the injection site would facilitate integration of the ELP gel with the existing tissue structure, which is presumably necessary for a successful procedure for filling a cartilage defect.

Mechanical Properties of ELP Hydrogels. The final mechanical properties of the fully cross-linked ELP hydrogels were characterized at room temperature by their (1) complex shear modulus, $|G^*|$, (2) equilibrium compressive modulus, E, and (3) equilibrium shear modulus, μ, to indirectly estimate the degree of effective cross-linking. The mechanical properties of ELP[KV_7F-72] and ELP[KV_2F-64] with different Lys densities were significantly different under identical cross-linking conditions and were also strongly influenced by the cross-linking pH (7.5 and 12.0). Average values for $|G^*|$, the dynamic modulus of ELP[KV_2F-64] hydrogels, varied from 25.8 to 45.8 kPa, which were 3.6−4.8 times greater than those of comparable ELP[KV_7F-72] hydrogels at the two different pH values, 7.5 (one asterisk indicates $p < 0.001$) and 12.0 (two asterisks indicate $p < 0.01$), used to carry out the cross-linking reaction. The values for $|G^*|$ of ELP[KV_7F-72] and ELP[KV_2F-64] hydrogels cross-linked at pH 12.0 are about 1.7−2.2 times greater than those cross-linked at pH 7.5, and this effect was statistically significant for both the ELP[KV_7F-72] hydrogels (three asterisks indicate $p < 0.05$) and the ELP[KV_2F-64] hydrogels (two asterisks indicate $p < 0.01$) (Figure 5A). This observation indicates that the degree of cross-linking of ELPs by THPP is pH dependent, possibly due to the high ratio of $[NH_2]$ to $[NH_3^+]$ in the ELPs at pH 12.0.

This general pattern is also observed for E and μ, which provide measures of the material moduli of the gel in compression and shear, respectively (Figure 5B,C). The results suggest that a wide range of mechanical properties of the cross-linked ELP hydrogels can be achieved by cross-linking ELPs with different Lys densities and by carrying out the cross-linking reaction at different pH values. Furthermore, values of the loss angle (δ), which is indicative of the dissipation inherent in the materials ($\delta = 0°$ for an elastic solid; $\delta = 90°$ for a Newtonian viscous fluid), are between 1.0° and 3.0°, irrespective of the ELP formulations, suggesting that all ELP hydrogels are highly elastic, energy-storing solids, as previously reported.[15,16] It is noteworthy that the shear modulus of the cross-linked ELP-

Table 1. Complex Shear Moduli of the Polymer Network[a]

Polymer network	MW	Lys periodicity	concn (mg/mL)	$\|G^*\|$[b] (kPa)	ref[c]
ELP coarcervate, 37 °C	36000	0	324	0.08	14
tTG-cross-linked ELP	47100	7	100	0.26	16
TSAT-cross-linked ELP	42700, 47100	17, 7	180	8−10	15
THPP-cross-linked ELP	31000, 28300	9, 4	200	5.8−45.8	present study
nucleus pulposus	NA	NA	NA	11.0	58
articular cartilage	NA	NA	NA	440.0	59

[a] Abbreviations: ELP, elastin-like polypeptide; tTG, tissue transglutaminase; TSAT, trissuccinimidyl aminotriacetate; THPP, β-[tris(hydroxymethyl)phosphino]propionic acid; NA, not available. [b] Angular frequency of 10 rad/s. [c] Some data estimated from graphical plots.

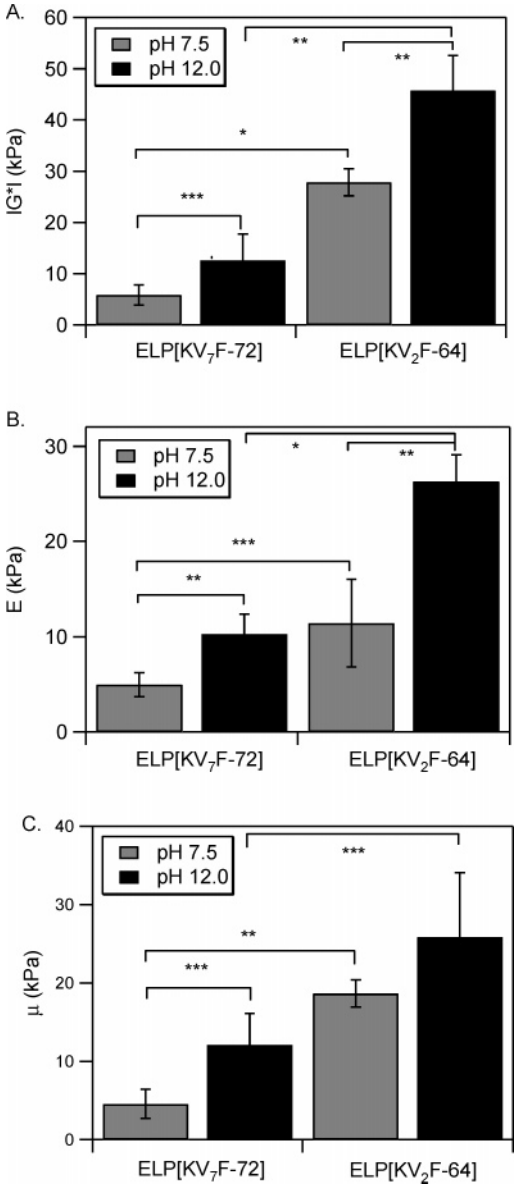


Figure 5. Mechanical properties of ELP[KV_7F-72] and ELP[KV_2F-64] hydrogels cross-linked by THPP in 20 mM phosphate at pH 7.5 and 12.0: (A) complex shear modulus, $|G^*|$, (B) equilibrium compressive modulus, E, and (C) equilibrium shear modulus, μ. Data are reported as the mean ± SD ($n = 4$). Key: *, $p < 0.001$; **, $p < 0.01$; ***, $p < 0.05$, significantly different between ELP types and cross-linking pH values.

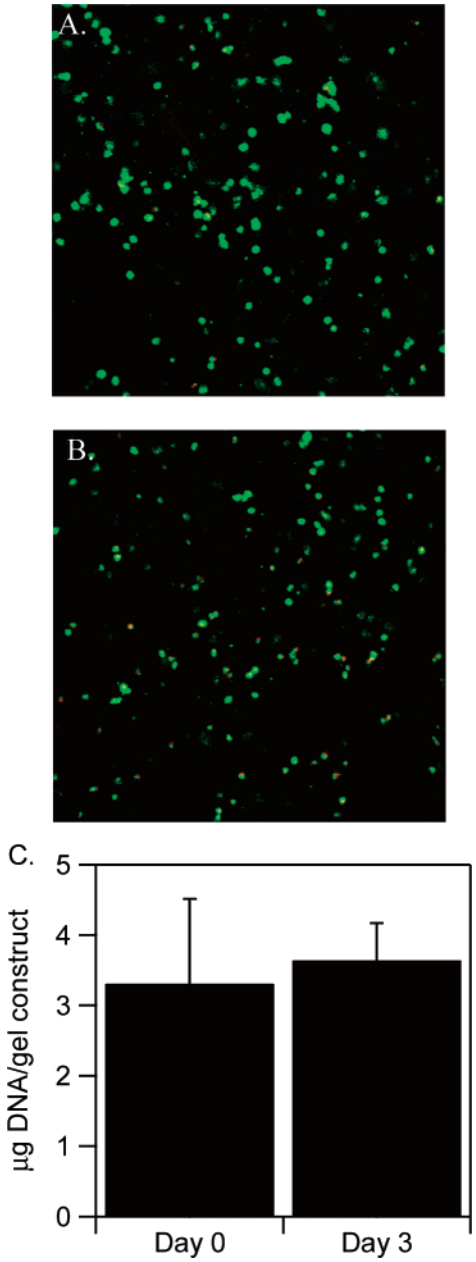


Figure 6. Fluorescence microscopy of fibroblasts encapsulated in rapidly cross-linked ELP[KV_7F-144] hydrogels and their DNA content per hydrogel. Cell survival of encapsulated fibroblasts was evaluated at (A) day 0 and (B) day 3 via a fluorescent cell viability/cytotoxicity assay. The DNA content of fibroblasts within each hydrogel was quantitatively measured by Picogreen DNA assay. Data are reported as the mean ± SD ($n = 5$). $p > 0.05$; no difference was detected between day 0 and day 3 in the DNA content of the constructs.

[KV_2F-64] hydrogels is comparable to or higher than that of some connective tissues, such as nucleus pulposus or meniscus (see Table 1). This suggests their potential utility as a functional scaffold that may assist in cartilage tissue repair.[14,16]

Biocompatibility of Cross-Linked ELP Hydrogels. Mouse fibroblasts were mixed at room temperature with ELP[KV_7F-144] and THPP at a 1:1 molar ratio of HMP to Lys residues of the ELP, and the resulting gel was cultured at 37 °C. Cell viability was examined from the time of encapsulation up to 3 days in culture. Fluorescent cell images obtained via live/dead staining in Figure 6A,B show that the cells survived the THPP-cross-linking of ELPs with a uniform cell distribution and remained viable at day 0 and day 3. A small number of cells demonstrated red fluorescence at staining at 3 days, however, indicating infiltration of ethidium homodimer-1 through compromised cell membranes and subsequent binding to nucleic acids. This finding shows that not all encapsulated cells survived in the THPP-cross-linked gels. The DNA content of encapsulated fibroblasts in each hydrogel was not significantly different between day 0 and day 3 ($p > 0.05$), however, indicating that the cell death was not a significant limiting factor (Figure 6C). Related studies of encapsulated human chondrocytes in THPP-cross-linked ELP hydrogels show that cells survived for up to 12 weeks, even for aged human cells of degenerated tissues,[57] a clinically relevant finding, given that endogenous cells would likely contribute to populating these gels in vivo over this time scale. Together, these results show that THPP-cross-linked ELP hydrogels at a 1:1 molar ratio of HMP and Lys residue are not cytotoxic and these hydrated ELP hydrogels can maintain cell survival in vitro for 3 days and likely much longer periods of

time. Together with the results of mechanical testing, these observations suggest that ELPs cross-linked in this manner provide a biocompatible and injectable system with the potential to support tissue regeneration in a load-bearing environment.

Conclusions

This study demonstrates the rapid chemical cross-linking of environmentally responsive ELPs with a biologically benign cross-linker, THPP in aqueous solution. Cross-linking of ELPs with THPP is of potential interest for ELP hydrogel formation in situ for biomaterials and tissue engineering applications because (1) it can be carried out in aqueous solution, (2) the cross-linking reaction releases water as the sole byproduct, (3) the cross-linking agent can be mixed with cells without significantly compromising their viability, (4) gelation proceeds rapidly, with initial stabilization of the gel achieved within 1 min, (5) continued evolution of the hydrogel over a few hours results in hydrogels with mechanical properties that approach those of cartilaginous tissues, and (6) the formation of cross-linking sites presents reactive carboxylic acids of THPP for additional introduction of bioactive moieties into the hydrogels.

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Supporting Information Available. Text describing the monomer ELP gene synthesis and its oligomerization. This material is available free of charge via the Internet at http://pubs.acs.org.

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