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Rapid Crosslinking of Elastin-like Polypeptides with Hydroxymethylphosphines in Aqueous Solution

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Abstract

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 crosslinked elastin-like polypeptide (ELP) hydrogels can be rapidly formed in aqueous solution by reacting lysine containing ELPs with an organophosphorous crosslinker, β -[tris(hydroxymethyl) phosphino]-propionic acid (THPP) under physiological conditions. The mechanical properties of the crosslinked ELP hydrogels were largely modulated by the molar concentration of lysine residues in the ELP and the pH at which the crosslinking reaction was carried out. Fibroblasts embedded in ELP hydrogels survived the crosslinking 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 crosslinking may be a biocompatible strategy for the *in situ* formation of crosslinked hydrogels.

Introduction

Biologically inspired peptide-based materials^{1,2} are of increasing interest for application as biomaterials³⁻⁸ 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 cytotoxicity¹¹; 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

We¹⁴⁻¹⁸ 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 culture²⁴⁻²⁶ 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

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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 provides the rationale for the development of crosslinking strategies.

In the past few years, different crosslinking methods of ELPs^{15,30,31} have been investigated; chemical methods include crosslinking by radiation³²⁻³⁴, photoinitiation³⁵, chemical crosslinking^{15,30,31,36-38} and enzymatic crosslinking by tissue transglutaminase¹⁶, while the formation of physically crosslinked 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 crosslinked state, which provides a secondary variable to tune their mechanical behaviors by modulating the degree of solvation of the crosslinked ELP hydrogel.¹⁵

Despite the variety of crosslinking 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 by-products, the need for organic solvents or the sub-optimal kinetics of the crosslinking reactions¹⁶. Motivated by these considerations, the objective of this study was to develop a crosslinking strategy that permits a mixture of soluble ELP and cells to be injected and crosslinked *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 objective of this study was to evaluate the potential to vary the physical properties of the crosslinked gels by the density of crosslinkable, functional groups in the ELPs as well as the pH of the crosslinking reaction.

We introduce in this paper the Mannich-type condensation of hydroxymethylphosphines (HMPs) with primary- and secondary- amines of amino acids as a new crosslinking 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 metals⁴⁵⁻⁴⁹, but has not been used to crosslink polypeptides, to the best of our knowledge. In this study, we demonstrate that chemically crosslinked 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-crosslinked ELP hydrogels can be modulated by the concentration of Lys residues in the ELP and by the pH of the crosslinking reaction. Finally, we show that murine fibroblasts can be encapsulated in the ELP hydrogels in a biocompatible process and that they survive the crosslinking procedure and *in vitro* culture for 3 days.

Experimental

ELP Notation

All ELPs are 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, 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-9] is an ELP that contains 9 repeats of the VPGXG pentapeptide, in which one pentapeptide contains K at the 4th, guest residue position (X), another pentapeptide has F at the 4th position, and 7 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[KV₆-7] 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[KV₇F-9] was constructed by ligation of the plasmid-borne gene for ELP[KV₆-7] with a plasmid-borne gene that encoded ELP[VF-2] while the gene for ELP[KV₂F-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[KV₇F-18, 36, 72, 144] and ELP[KV₂F-16, 32, 64, 128], which encode for ELPs with molecular weight (MW) ranging from 7.7 kDa to 61.1 kDa.

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 Pflm I and Bgl I into the modified pET-25b(+) expression vector (Novagen Inc., Milwaukee, WI) digested with Sfi I (New England Biolabs, Beverly, MA) that had been dephosphorylated using CIP. The *E. Coli* strain, BLR (DE3) cells (Novagen Inc., Milwaukee, WI) transformed with the modified pET-25b(+) vector containing an ELP insert, were grown in 1 L cultures of CircleGrow™ media supplemented with 100 g/ml of ampicillin for 24 h at 37 °C at 200 rpm (Qbiogene, Carlsbad, CA). The expressed ELPs were purified by inverse transition cycling (ITC), as previously described.²⁴⁻²⁹

Physicochemical characterization of ELPs

The purity 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_i*) of ELPs was measured by heating a 25 μM solution of the ELP between 10 °C 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* 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. The *T_i* is defined as the temperature at which the change in optical density with respect to temperature (*dOD/dT*) reaches its maximum. The ELP concentration was determined by the ELP molar extinction coefficient at 280 nm (5690 M⁻¹cm⁻¹) calculated from the primary amino acid sequence of the ELPs using the software program, Protean (DNA Star).

Gelation kinetics by oscillatory rheology

The crosslinking kinetics of ELPs with THPP (Pierce, Rockford, IL) was measured by oscillatory rheology as a function of time. Their rheological behaviors during crosslinking 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 μ l of 200 mg/ml THPP in 20 mM phosphate with 700 mM of NaCl at pH 7.5, was mixed by vigorous pipetting with 450 μ l of 200 mg/ml of ELP [KV₇F-144] in 20 mM phosphate at pH 7.5, to yield a 8-9 fold molar excess of reactive hydroxymethylphosphine (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'') modulus were obtained from the torque data.

Mechanical properties of crosslinked ELP hydrogels

ELP[KV₇F-72] and ELP[KV₂F-64] were crosslinked in 20 mM phosphate, 100 mM NaCl at different pHs (pH 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 of diameter and 2 mm of height) at 4 °C, and the mixture was then incubated at 37 °C for 1 hr. The fully crosslinked 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 crosslinked hydrogels of ELP [KV₇F-72] and ELP[KV₂F-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; stainless steel porous platens; 50 % porosity; 40-60 μ m 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 grams 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/sec). 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, respectively. The equilibrium shear modulus (μ) was also determined from relationship between torsional shear strain measurements of 0.05 and resulting shear stress. Four ELP hydrogels per each 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 swollen gel weight, W_s , to 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 in order to measure the dimensional changes between the swollen and collapsed states of the thermo-responsive ELP hydrogels. The swelling ratio of ELP[KV₇F-72] and ELP [KV₂F-64] hydrogels crosslinked at pH 7.5 and 12.0 were measured using four ELP hydrogel constructs per each 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 microscopy (SEM) at an

acceleration voltage of 30 kV. Four ELP hydrogels of each formulation were analyzed in order to investigate the morphology as a function of crosslinkable Lys density of the ELPs.

Cell viability and DNA quantification

A suspension of mouse NIH-3T3 fibroblasts was mixed with ELP[KV₇F-144] and THPP at room temperature at a 1:1 HMP:Lys molar ratio of to achieve a final concentration of 200 mg/ml of ELP and 10×10^6 cells/ml in HEPES buffered saline (25mM 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% CO₂ incubator. The fibroblast embedded, crosslinked 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% CO₂ 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 3 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 3 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, Eugene, OR). ELP hydrogels with encapsulated fibroblasts were digested in PBS containing 125 µg/ml papain (Sigma, St. Louis, Mo), 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 13,000 g for 10 mins. Picogreen reagent was added to aliquots of each digest solution and DNA controls in a 96 well plate and then incubated for 5 min. 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 crosslinking pH on various mechanical properties (E , $|G^*|$, and μ). A 1-factor ANOVA was used to test for the effect of culture time on DNA content of cells. Statistical significance was determined at a value of $p < 0.05$.

Results and Discussion

Design and Characterization of ELPs

Two ELP libraries, ELP[KV₇F-9, 18, 36, 72, 144] and ELP[KV₂F-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 crosslinking. The different concentrations of Lys were chosen to examine the effect of crosslinking density on the mechanical properties of the crosslinked 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 crosslinking reaction does not proceed to completion, the fraction of uncrosslinked Lys residues in the ELP hydrogels increases the temperature range over which the volume phase

transition occurs. Phe, a hydrophobic guest residue, was incorporated in 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[KV₇F] and ELP[KV₂F] 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 based on 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 in molecular weight between their calculated and the experimentally measured MW ranged between 0.01 % and 0.5 %.

Figure 2A shows that the T_t of the ELP[KV₂F] series are 16 to 42 °C higher than those of ELP[KV₇F] of similar molecular weight because of the greater fraction of Lys residues in the ELP[KV₂F] family. Similarly, the T_t of polypeptides of the ELP[KV₇F] series are more than 20 °C lower than the those of ELP[KV₆] polypeptides of similar molecular weight due to the introduction of hydrophobic Phe residues.^{50,51} Within each series, the 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 and Figure 2C 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 in T_t caused by the addition of 1 M NaCl to a 25 μ M ELP solution in PBS) ranges between 30-50 °C and 15-25 °C, for ELP[KV₂F] and ELP[KV₇F], respectively. The increased salt sensitivity of the ELP[KV₂F] series as compared to the ELP[KV₇F] series is consistent with the greater density of Lys residues in ELP[KV₂F]. Furthermore, Figure 2C shows that the change in T_t of ELP[KV₂F-128] and ELP[KV₇F-144] when the pH is raised from 7.5 to 12.5 are 64 °C 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 crosslinking reaction of ELP[KV₇F-72] and ELP[KV₂F-64] with THPP was carried out in 20 mM phosphate, 100 mM NaCl at pH's that ranged from 2-13 (Figure 3A). Figure 3B shows that ELP[KV₇F-72] crosslinked 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 crosslinked ELP chains. In contrast, ELP[KV₂F-64], crosslinked under the same conditions, exhibited less collapse than ELP[KV₇F-72] hydrogels at 37 °C. To examine the differences in their microstructure, freeze-dried and fractured ELP hydrogels was imaged by scanning electron microscopy (SEM). The ELP[KV₂F-64] hydrogel exhibits a denser microstructure than the ELP[KV₇F-72] hydrogel that was crosslinked under identical conditions. 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 ELP[KV₇F-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[KV₂F-64] hydrogels crosslinked 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[KV₂F-64] at 4 °C compared to ELP[KV₇F-72] as well as its denser microstructure is presumably due to its higher crosslinking density than ELP[KV₇F-72] hydrogels because of the greater concentration of Lys (K) residues in ELP[KV₂F-64]. The higher densities of chemically conjugated amines, aminomethyl-phosphines as well as the higher ratio of hydrophobic Phe guest residue of ELP[KV₂F-64] hydrogels dramatically decrease the LCST of crosslinked ELP hydrogels, making the gels appear opaque at 4 °C.

Related studies of the stability of ELP hydrogels show that the wet weight of THPP-crosslinked His₆-tagged ELP hydrogels in PBS at 37 °C did not change significantly within 60 days, suggesting THPP crosslinked gels were stable for at least two months under physiological conditions.¹⁸

The gelation kinetics of THPP-crosslinked ELP[KV₇F-144], measured by oscillatory rheology as a function of time, showed that ELP hydrogels formed in less than one minute under physiological conditions, with hydrogel formation defined by the crossover point of the dynamic storage (G') and loss (G'') modulus (Fig. 4A).⁵⁵ After this cross-over 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 an hour while G'' remained constant, at approximately 0.1 kPa. These results suggest that Lys residues within the ELP hydrogels continued to be crosslinked until G' reached its maximum value (Fig. 4B).¹⁵ The fully crosslinked ELP hydrogels of ELP[KV₇F-72] and ELP[KV₂F-64] were highly elastic as demonstrated in dynamic torsional test by the values of G' exceeding G'' over several decades of frequency (0.01 to 50 rad/sec). The crosslinking kinetics of ELP[KV₇F-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-crosslinked 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 one minute solid after 5 minutes. Thus, all surgical sites were closed at 5 minutes after injection into the cartilage defect site. The results of that study led us to conclude that gelation occurs rapidly enough, so that diffusion of unreacted THPP within < 5 minutes from the defect site is not a concern about 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 to a successful procedure for filling a cartilage defect.

Mechanical Properties of ELP Hydrogels

The final mechanical properties of the fully crosslinked 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 in order to indirectly estimate the degree of effective crosslinking. The mechanical properties of ELP[KV₇F-72] and ELP[KV₂F-64] with different Lys densities were significantly different under identical crosslinking conditions, and were also strongly influenced by the crosslinking pH (7.5 and 12.0). Average values for $|G^*|$, the dynamic modulus of ELP[KV₂F-64] hydrogels varied from 25.8 to 45.8 kPa, which were 3.6 - 4.8 times greater than those of comparable ELP[KV₇F-72] hydrogels at the two different pHs, pH 7.5 (* p <0.001) and pH 12.0 (** p <0.01) used to carry out the crosslinking reaction. The values for $|G^*|$ of ELP[KV₇F-72] and ELP[KV₂F-64] hydrogels crosslinked at pH 12.0 are about 1.7 - 2.2 times greater than those crosslinked at pH 7.5 and this effect was statistically significant for both the ELP[KV₇F-72] hydrogels (** p <0.05) and the ELP[KV₂F-64] hydrogels (** p <0.01) (Figure 5A). This observation indicates that degree of crosslinking of ELPs by THPP is pH dependent, possibly due to the high ratio of $[\text{NH}_2]/[\text{NH}_3^+]$ 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 and 5C). The results suggest that a wide range of the mechanical properties of the crosslinked ELP hydrogels can be achieved by crosslinking ELPs with different Lys densities and by carrying out the crosslinking reaction at different pHs. Furthermore, values of the loss angle (δ) which is

indicative of the dissipation inherent in the materials ($\delta = 0^\circ$ for an elastic solid; $\delta = 90^\circ$ for Newtonian viscous fluid) are between 1.0° and 3.0° irrespective of 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 crosslinked ELP[KV₂F-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[KV₇F-144] and THPP at a 1:1 molar ratio of HMP to Lys residues of the ELP, and the resulting gel 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 and 6B show that the cells survived the THPP-crosslinking 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 crosslinked 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 7C). Related studies of encapsulated human chondrocytes in THPP-crosslinked ELP hydrogels show that cells survived for up to 12 weeks, even for aged human cells of degenerated tissues⁵⁷, 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 crosslinked 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 crosslinked 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 crosslinking of environmentally responsive ELPs with a biologically benign crosslinker, THPP in aqueous solution. Crosslinking 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 crosslinking reaction releases water as the sole by-product; (3) the crosslinking agent can be mixed with cells without significantly compromising their viability; (4) gelation proceeds rapidly, with initial stabilization of the gel achieved within 1 minute; (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 crosslinking sites presents reactive carboxylic acids of THPP for additional introduction of bioactive moieties into the hydrogels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Scheme 1.

Schematic of construction of plasmids encoding ELP[KV₇F-9] and ELP[KV₂F-4] monomer genes. The plasmid containing ELP[KV₆-7] is doubly-digested with Pflm I 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 Pflm I compatible overhangs. The plasmid of ELP[VF-2] is digested with Pflm I and then enzymatically dephosphorylated. The linearized, dephosphorylated ELP[VF-2] vectors are separately ligated with the ELP[KV₆-7] insert and the oligonucleotide cassette of ELP[VK-2].

A. ELP[KV₇F] B. ELP[KV₂F] **Figure 1.**

Copper stained SDS-PAGE gels of (A) ELP[KV₇F] and (B) ELP[KV₂F]. The lane marked (M) lane contains molecular size markers (205, 116, 97, 84, 66, 55, 45, 36, 29, 24, 20, 14, 7 kDa from top to bottom). The numbers of pentapeptide repeats of ELPs are labeled above each lane and the expected molecular weight of the ELPs are shown on the left.

Figure 2.

(A) Transition temperature, T_t and (B) $\Delta T_t/M$ of NaCl of ELPs as a function of number of ELP pentapeptide for 25 μ M ELP solutions in phosphate buffered saline. (C) T_t of [KV₇F-144] and ELP [KV₂F-128] as a function of pH for 0.625 mg/ml (10.2 -11.2 μ M) ELP solutions in 20 mM phosphate buffer.

Figure 3.

(A) Schematic of inter-, or intra- molecular crosslinking mechanism between Lys residues of ELPs and THPP (β -[tris(hydroxymethyl)phosphino]-propionic acid). (B) photographs of thermo-responsive swollen ELP[KV₇F-72] and ELP[KV₂F-64] hydrogels crosslinked by THPP in 20 mM of phosphate buffer at pH 7.5 at 4 °C and 37 °C, respectively and their cross-sectional SEM (Scanning electron microscopy) images. Scale bars in the SEM images are shown in 50 μm (1) and 20 μm (2).

Figure 4.

Oscillatory rheological profiles for ELP [KV₇F-144] and THPP mixtures. Aliquots of THPP (200 mg/ml, 20 mM phosphate, 700 mM NaCl, pH 7.5) were added to ELP [KV₇F-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. Elastic component of 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 5.

Mechanical properties of ELP[KV₇F-72] and ELP[KV₂F-64] hydrogels crosslinked 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 were reported as mean $\pm$ SD ($n=4$). *, $p < 0.001$; **, $p < 0.01$; ***, $p < 0.05$, significantly different between ELP types and crosslinking pHs.

Figure 6. Fluorescence microscopy of fibroblasts encapsulated in rapidly crosslinked ELP[KV₇F-144] hydrogels and their DNA content per each 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 were reported as mean ± SD (n=5). $p > 0.05$, no difference was detected between day 0 and day 3 in DNA content of the constructs.

Table 1

Complex Shear Moduli of Polymer Network

Polymer Networks	MW (kDa)	Lys periodicity	Concentration (mg/ml)	G* (kPa) ^a	Reference ^b
ELP coacervate, 37 °C	36.0	0	324	0.08	14
tTG-crosslinked ELP	47.1	7	100	0.26	16
TSAT-crosslinked ELP	42.7, 47.1	17, 7	180	8 - 10	15
THPP-crosslinked ELP	31.0, 28.3	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

Abbreviations: ELP, elastin-like polypeptide; tTG, tissue transglutaminase; TSAT, tris-succinimidyl aminotriacetate; THPP, β-[tris(hydroxymethyl)phosphino]-propionic acid, NA, not available.

^a Angular frequency of 10 rad/s.

^b Some data estimated from graphical plots.