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Molecular Engineering of Silk-Elastinlike Polymers for Matrix-Mediated Gene Delivery: Biosynthesis and Characterization

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Abstract: The unique advantage of genetic engineering techniques for the design and development of polymers for controlled gene delivery lies in exquisite control over polymer structure. In this article we report the biosynthesis and characterization of a series of new silk-elastinlike protein polymers (SELPs), namely, SELP415K, with larger elastin blocks per monomer unit than SELP47K previously studied for matrix-mediated gene delivery. A new cloning strategy was used, where a block of eight elastin units (8E) was integrated into the existing DNA sequence of SELP47K monomer genes using appropriate restriction endonuclease recognition sites. Following random multimerization, multimer gene segments of desired size were selected, expressed, and purified on Ni-agarose columns. The molecular weight and sequence composition of the purified SELPs were determined by MALDI-TOF and amino acid analysis, respectively. The influence of structural changes on the rheological properties of the polymers was investigated. In addition, hydrogel disks were prepared from 47K and 415K-8mer polymer solutions, and the effects of cure time and environmental conditions on the hydrogel equilibrium swelling ratio as a function of polymer composition were studied. DNA sequencing and agarose gel electrophoresis confirmed the successful cloning of the monomer gene segment of SELP415K consisting of 312 bp. Random concatemerization of SELP415K monomer gene segments resulted in a library of SELP415K multimer sequences of 6, 8, and 10 repeats respectively, each yielding a polymer with exact molecular weight and sequence. Rheometric measurements showed that both complex shear modulus (G^*) and gelation point were influenced by polymer composition. Equilibrium swelling studies on hydrogel disks prepared from 47K and 415K-8mer polymer solutions showed that changes in polymer composition resulted in different gelation patterns and increased sensitivity toward changes in temperature and ionic strength but not pH. Together these results demonstrate the potential of recombinant techniques in engineering polymers with defined structures which allows the study of the structural parameters affecting matrix-mediated delivery of genes and bioactive agents.

Keywords: Genetically engineered polymers; silk-elastinlike protein polymers; gene delivery; hydrogels; drug delivery

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Introduction

Controlled release of bioactive agents including nucleic acids from hydrogels is influenced by polymer structure and composition. Thus far, most of the polymers used as controlled release systems have been synthesized by chemical means. These polymers have shown utility in a multitude of drug and gene delivery applications. Chemical polymeriza-

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tion methods however produce random copolymers with unspecified monomer sequences and statistical distribution of molecular weights and monomer compositions. This limits the ability to correlate polymer structure with properties.

Progress in recombinant DNA technology has enabled the genetic engineering of large molecular weight polymers containing repeating blocks of amino acids with defined composition, sequence, and length (reviewed in ref 1). Control over polymeric architecture at the molecular level results in fine control over physicochemical properties important for the controlled delivery of bioactive agents[1−6] and the fabrication of tissue engineering scaffolds.[7,8] These properties include biorecognition,[9] swelling and drug release,[10−14] sensitivity to environmental conditions such as pH, temperature and ionic strength,[15−18] and biodegradation.[19]

One unique class of genetically engineered biomaterials is the family of silk-elastinlike protein polymers (SELPs). Structurally, SELPs consist of tandem repeats of silk-like (GAGAGS) and elastin-like (GVGVP) peptide blocks.[20] By combining the silk-like and elastin-like blocks in various ratios and sequences, it is possible to produce a variety of biomaterials with diverse material properties. SELP copolymers, with the appropriate sequence and composition, undergo an irreversible sol-to-gel transition, which is accelerated at body temperature.[10−13] The formation of hydrogen bonds between the silk-like blocks is thought to be the primary driving force behind gelation of SELPs serving as points of contact (cross-links) between the polymer chains. The periodic inclusion of elastin-like blocks increases the flexibility and aqueous solubility of the polymer. The polymeric solutions are liquid at room temperature and form a firm yet pliable hydrogel in situ minutes after injection.

Recent research in our laboratory has focused on the potential of SELP hydrogels in drug and gene delivery.[11,13,21] SELP47K (structure, Figure 1A) is one member of the SELP family containing four silk units and seven elastin units, in addition to an elastin unit where a valine residue is replaced by lysine per monomer repeat unit. We have shown that viable plasmid DNA and adenoviral particles can be released from these hydrogels in vitro up to a period of 28 days.[21] Localized in vivo gene delivery from these systems resulted in prolonged gene expression in breast cancer tumor xenografts.[21] DNA release from SELP47K hydrogels was found to be influenced by ionic strength, polymer concentration, hydrogel cure time,[11] and DNA size and conformation.[21] These results point to the potential of this class of recom-

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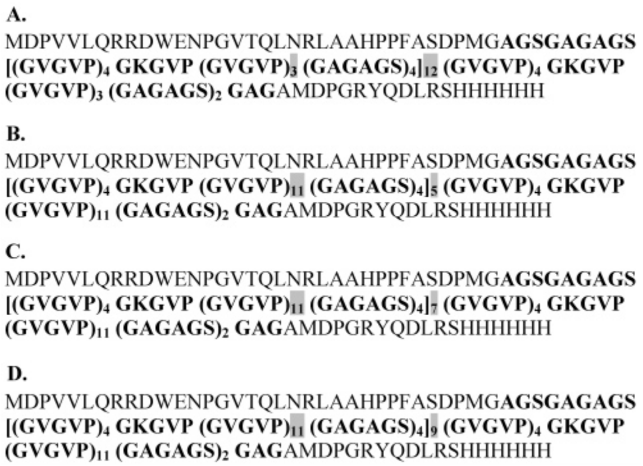


A.

MDPVVLQRRDWENPGVTQLNRLAAHPPFASDPMG**AGSGAGAGS**
[(GVGVP)$_4$ GKGVP (GVGVP)$_3$ (GAGAGS)$_4$]$_{12}$ (GVGVP)$_4$ GKGVP
(GVGVP)$_3$ (GAGAGS)$_2$ GAGAMDPGRYQDLRSHHHHHH

B.

MDPVVLQRRDWENPGVTQLNRLAAHPPFASDPMG**AGSGAGAGS**
[(GVGVP)$_4$ GKGVP (GVGVP)$_{11}$ (GAGAGS)$_4$]$_5$ (GVGVP)$_4$ GKGVP
(GVGVP)$_{11}$ (GAGAGS)$_2$ GAGAMDPGRYQDLRSHHHHHH

C.

MDPVVLQRRDWENPGVTQLNRLAAHPPFASDPMG**AGSGAGAGS**
[(GVGVP)$_4$ GKGVP (GVGVP)$_{11}$ (GAGAGS)$_4$]$_7$ (GVGVP)$_4$ GKGVP
(GVGVP)$_{11}$ (GAGAGS)$_2$ GAGAMDPGRYQDLRSHHHHHH

D.

MDPVVLQRRDWENPGVTQLNRLAAHPPFASDPMG**AGSGAGAGS**
[(GVGVP)$_4$ GKGVP (GVGVP)$_{11}$ (GAGAGS)$_4$]$_9$ (GVGVP)$_4$ GKGVP
(GVGVP)$_{11}$ (GAGAGS)$_2$ GAGAMDPGRYQDLRSHHHHHH

Figure 1. The amino acid sequence of three SELP415K analogues: (A) SELP47K (molecular mass 69,814 Da); (B) SELP415K-6mer (molecular mass 55,130 Da); (C) SELP415K-8mer (molecular mass 71,500 Da); (D) SELP415K-10mer (molecular mass 87,860 Da). All polymers are composed of head and tail portions, and a series of silk-like (GAGAGS) and elastin-like (GVGVP) repeats (primary repetitive sequence in bold, number of repeats highlighted in gray). For amino acids see abbreviations.

binant polymers in localized gene delivery. However, to control release, biodegradation, and ultimately efficacy of gene therapy using these matrices, in addition to the above factors, the influence of polymer structure on these parameters needs to be examined.

One factor that influences the release of nucleic acids from hydrogels is the degree of cross-linking. The degree of cross-linking of SELP hydrogels is a function of several parameters including polymer concentration, the length and sequence of silk and elastin units, and the polymer molecular weight. By varying these parameters it is possible to control gelation, release, and biodegradation from SELP matrices. In this article, we report the biosynthesis and characterization of three distinct molecular weights of a new silk-elastinlike analogue, namely, SELP415K (structures shown in Figure 1B−D). The monomers in SELP415K contain eight more elastin units than SELP47K studied previously for gene release.[11,21] The three SELP415K polymers reported here vary in the number of monomers per polymer chain with 6, 8, and 10 repeats, respectively. In addition polymer 47K and polymer 415K-8mer (polymers of similar length but different sequence) were used to study the effect of monomer sequence and composition on polymer rheological properties, gel formation, and swelling behavior of hydrogels in response to changes in gelation time and environmental conditions.

Experimental Section

Materials. The following materials were used for the biosynthesis of SELP415K analogues and characterization of the polymers. Template and complimentary oligonucleotides encoding for eight elastin units (8E) were obtained from IDTdna (Coralville, IA). *Escherichia coli* HB101 competent cells were purchased from Promega (Madison, WI). Restriction endonuclease enzymes *Bam*HI, *Ban*I, *Bsa*HI and *Eco*RV and the DNA modification enzyme T4 DNA ligase were purchased from New England Biolab (Beverly, MA). GeneRuler 1 Kbp DNA ladder and shrimp alkaline phosphatase (SAP) were purchased from Fermentas (Hanover, MD). QIAprep Spin Miniprep kits, QIAGEN Plasmid Maxi kits, and QiaQuick gel extraction kits were obtained from Qiagen (Valencia, CA). A ProBond purification system was obtained from Invitrogen (Carlsbad, CA). Bio-Spin 30 Tris columns, Precast Tris-HCl 4−15% linear gradient gels, Tris-glycine SDS buffer, Precision Plus Protein standards, and Bio-Safe Coomassie stain were obtained from Bio-Rad (Hercules, CA). Micropure-EZ and Amicon Ultra 10000 MWCO centrifugal filter devices were obtained from Millipore (Bedford, MA). Slide-A-Lyzer mini dialysis units 7000 MWCO were purchased from PIERCE (Rockford, IL). ProteoMass peptide and protein MALDI-MS calibration kit were obtained from Sigma-Aldrich (St. Louis, MO). pHydrion buffer capsules were obtained from Micro-Essential Laboratory (Brooklyn, NY).

SELP47K was obtained from Protein Polymer Technologies, Inc. (San Diego, CA), as 12 wt % solutions in 3-mL syringes and stored at −80 °C until use.

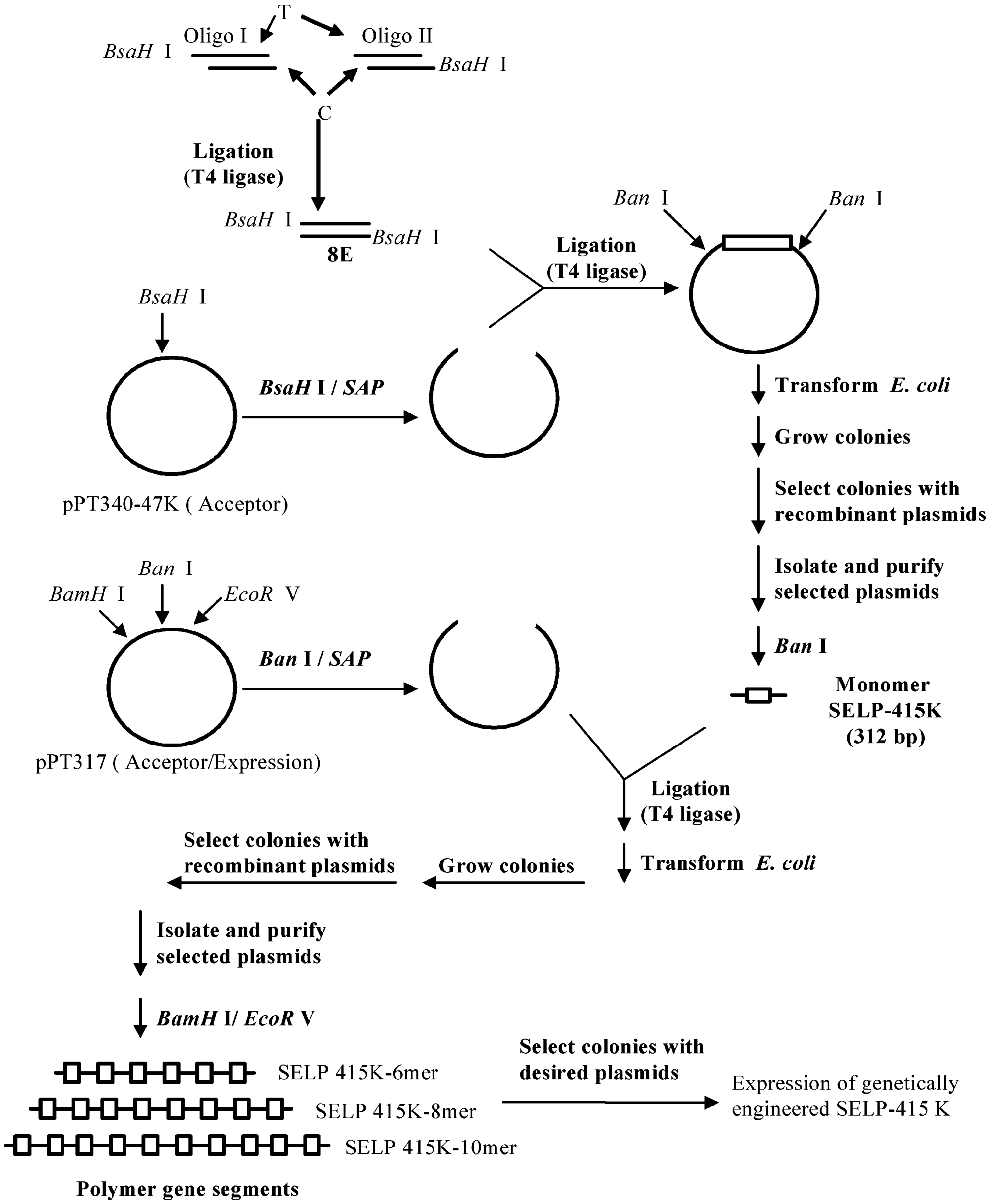
Cloning and Expression of the SELP415K Polymers. The general biosynthetic methodology is outlined in Scheme 1. The details of the methodology are described below.

Digestion and Purification of Acceptor and Expression Vectors. The acceptor and expression vectors, pPT340 and pPT317 (Protein Polymer Technology, Inc., San Diego, CA), were propagated in *E. coli* HB101 and purified using a Qiagen Giga Kit according to manufacturer's instructions. The concentration and purity of the plasmids were verified by UV spectrophotometry (Ultrospec 4000, Amersham Biosciences, Piscataway, NJ) at 260 and 280 nm. The ratio of A_{260}/A_{280} was in the range of 1.8−2.0 for all plasmids. Plasmids were electrophoresed on a 0.9% agarose gel and stained with ethidium bromide to verify the absence of genomic DNA and the integrity of the plasmid.

The purified plasmids pPT340 and pPT317 were digested with *Bsa*HI and *Ban*I restriction endonucleases, respectively, followed by treatment with SAP to dephosphorylate 5′ terminals and prevent recircularization. The digestion mixtures were deproteinized using Micropure-EZ columns and desalted by Bio-Spin 30 Tris columns according to manufacturer's specifications. Complete digestion of the plasmid was confirmed by electrophoresis on 1% agarose gels with 0.5 μg/mL ethidium bromide followed by visualization of the bands corresponding to the linearized vectors under UV light at 302 nm.

Synthesis of SELP415K Monomer Gene Segments. A gene segment encoding for eight elastin units (8E) with the structure 5′-CGTACCAGGAGTAGGCGTACCGGGAG TAGGAGTGCCGGGTGTAGGAGTTCCTGGAGTTGGC GTACCAGGAGTAGGCGTACCGGGAGTAGGAGTGC CGGGTGTAGGAGTTCCTGGAGTTGG-3′, was synthe-

Scheme 1. Biosynthetic Strategy for SELP415K Analogues[a]



[a] Oligo: oligonucleotide. T: template strand. C: complementary strand. 8E: gene segment encoding eight elastin units. *Bsa*HI, *Ban*I, *Bam*HI, and *Eco*RV: restriction endonuclease enzymes. SAP: shrimp alkaline phosphatase.

sized as two complementary segments (Oligo1 and Oligo2) using an automated oligonucleotide synthesizer. The complementary oligonucleotides were designed with a 15 bp overhang that only allows unidirectional assembly to form the entire 8E sequence. In addition, the 5′ terminal of the 8E gene segment was designed with *Bsa*HI digest recognition sites (underlined) to allow further ligation to acceptor vector pPT340 and construction of monomer gene segment 415K. The 8E gene segment was constructed after each template was annealed to its corresponding complementary strands followed by overnight ligation of the two oligonucleotides for 16 h at 16 °C using T4 DNA ligase.

The acceptor plasmid pPT340 with a built-in SELP47K construct was digested with *Bsa*HI with recognition site located inside the 47K construct and purified as described above. Linearized acceptor vector and purified monomer gene segment were allowed to react in a 1:1 molar ratio overnight for 16 h at 16 °C in the presence of T4 DNA ligase. The ligation mixture was then transformed into *E. coli* HB101 and propagated on agar plates treated with chloramphenicol. Plasmids were extracted from positive colonies using the QIAGEN Plasmid Maxi kit, and the monomer gene segment 415K was isolated by preparative agarose gel electrophoresis after digestion with *Ban*I, using the QiaQuick gel extraction kit. The structure of the 415K monomer gene segment was confirmed by fluorescence-based automated DNA sequencing using appropriate sequencing primers.

Construction of the Polymer Genes. The ability of the monomer gene segments to self-assemble was tested by incubating monomer inserts (120 ng and 50 ng) at room temperature in the presence of T4 DNA ligase for 1 h. The mixture was separated by agarose gel electrophoresis.

Expression vector, pPT317 containing λ_{PL} promoter, was restriction digested with *Ban*I, dephosphorylated with SAP, and purified as described above. The purified monomer gene segments and the linearized expression vector were mixed at a high monomer-to-vector molar ratio in the presence of T4 DNA ligase, to allow multimerization and ligation of the resulting multimer gene segments to the expression vector to occur in a single step. The ligation mixture was then transformed into *E. coli* HB101 and plated on Kanamycin containing agar plates. The plates were incubated overnight at 30 °C, and the DNA was extracted from the colonies using QIAprep Spin Miniprep kits. Agarose gel electrophoresis subsequent to *Bam*HI and *Eco*RV double digestion was used to screen the colonies for the presence and size of the polymer gene.

Optimization and Analysis of Small-Scale Polymer Expression. Bacteria from one of the colonies containing the polymer gene were inoculated into 5 mL of LB broth with kanamycin and incubated overnight at 30 °C while being shaken at 280 rpm. A 50-mL flask containing 10 mL of LB broth with kanamycin was inoculated with 100 μL of the polymer gene-containing culture and incubated as before. Once the optical density of the culture at 600 nm (OD_{600}) reached 0.6, the culture was transferred to an incubator maintained at 42 °C and shaken at 200 rpm for different time intervals (0.5, 1, 2, and 3 h) in order to optimize induction of λ_{PL} promoter for protein expression. An aliquot of the culture corresponding to 2 OD_{600} units was removed for polyacrylamide gel electrophoresis on Precast Tris-HCl 4−15% linear gradient gels.

Expression and Purification of SELP415K Analogues. The polymers were expressed in 250-mL LB broth cultures. Bacterial cells were harvested cells from the inducted cultures by centrifugation at 6000*g* for 15 min and then resuspended in native binding buffer from ProBond purification kit. The cells were lysed by incubation in lysozyme (5 mg/mL) on ice for 30 min followed by sonication for 6 × 10 s bursts on ice. Cell debris was removed by centrifugation, and the supernatant was purified using ProBond purification system according to manufacturer's instructions. The purified polymers were eluted, concentrated by centrifugation using Amicon Ultra (10000 MWCO) centrifugal filter devices, and dialyzed against distilled water using Slide-A-Lyzer mini dialysis units (7000 MWCO). Yields of 25 mg/L were obtained. Polymers 47K and 415K-8mer were produced in *E. coli* by fermentation and purified to 99% purity determined by amino acid analysis.

Molecular and Structural Characterization of SELP415K Polymers. The polymers were analyzed by gel electrophoresis, mass spectroscopy, and amino acid analysis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was carried out using Tris-HCl 4−15% linear gradient gels run in tris-glycine SDS buffer and stained with Bio-Safe Coomassie stain. Matrix-assisted laser desorption ionization mass spectrometry (MALDI-TOF-MS) was carried out using OmniFLEX (Bruker Daltonics Inc., Billerica, MA) in the positive ion mode in a sinapinic acid matrix. Amino acid content analysis of the polymers was carried out by Commonwealth Biotechnologies, Inc. (Richmond, VA), by chromatographic measurement of derivatized peaks after hydrolysis of the sample in 6 N HCl at 110 °C for 20 h.

Determination of the Rheological Properties of Polymer Solutions. Polymers 47K and 415K-8mer were produced in 1X phosphate buffered saline solution with pH and osmolality of 7.4 and 300 mosm/kg, respectively. The rheological properties of the polymer solutions were determined using a rheometer with Peltier plate assembly, model no. AR2000 (TA Instruments−Waters LLC, New Castle, DE).

The Peltier plate was set at 35 °C, and 0.63 mL of the protein solution was placed directly onto the Peltier plate. A 40-mm stainless steel plate was lowered until the gap reached 500 μm and the polymer solution completely filled the space between the plates. The exposed perimeter of the solution was covered with light mineral oil to prevent evaporation. A time sweep was run at a frequency of 1 Hz and a constant strain of 0.2%.

Preparation of Hydrogels. Frozen syringes containing 12 wt % SELP47K or 12 wt % 415K-8mer solutions were thawed in a beaker containing 500 mL of water for 5 min at room temperature. Polymer solutions having 6 wt % and 9 wt % polymers were prepared by adding appropriate amounts of Dulbecco's PBS. Two hundred microliters of each polymer solution was then transferred to a separate disposable syringe (1 mL), covered with Para film, and incubated at 37 °C for hydrogel formation. At specific time points (4, 24, and 48 h) the hydrogels were extruded from the syringes and cut into 40-mm^3 cylindrical disks using a razor blade.

To remove soluble polymer fractions remaining in the hydrogels postgelation, disks were extensively washed for 1 week in 1X PBS (10 mM PBS, pH 7.4, $\mu = 0.16$) with 0.01 w/v% sodium azide under mild agitation (speed = 120 rpm) in a temperature-controlled convection incubator (VWR, model 1575, Bridgeport, NJ) set at 37 °C. Gel washing was performed at 37 °C to simulate physiological conditions and to ensure that the gelation process was complete prior to storage and use. Fresh buffer was replaced daily throughout the washing period. Prior to use, washed hydrogel disks were stored at 2−8 °C in 1X PBS with 0.01 w/v% sodium azide.

Determination of Equilibrium Swelling Ratio. The influence of environmental conditions on weight equilibrium swelling ratio (q) was experimentally determined using eq 1, where W_s is the weight of swollen hydrogels following a specific environmental treatment and W_d is the dry hydrogel weight.

$$q = \frac{W_s}{W_d} \tag{1}$$

At study completion, the hydrogels were removed from solutions, extensively washed with deionized water, and gently blotted on lint-free paper for the removal of excess solvent on hydrogel surface. Dry hydrogels were obtained after incubation of swollen hydrogels in a desiccator containing Drierite (W. A. Hammond DRIERITE Co. LTD, Xenia, OH) for 5 days. The weights of the hydrogels were determined with a precision microbalance, and the average values of three measurements were taken for each sample for calculation of q.

The effect of cure time on q was determined by placing hydrogel disks cured for various time periods (4, 24, and 48 h) in closed vials containing 1X PBS with 0.01% sodium azide. The vials were placed in a temperature-controlled water bath and equilibrated for 24 h at 37 °C prior to weight determination.

The influence of temperature on q was determined by placing hydrogel disks that were cured at 37 °C for 24 h in closed vials containing 1X PBS with 0.01% sodium azide. The vials were then equilibrated for 24 h at different temperatures over the range 4−47 °C, prior to weight determination.

The effect of ionic strength on q was determined by placing swollen hydrogels cured at 37 °C for 24 h in closed vials containing 10 mM PBS solution (pH 7.4) with 0.01% sodium azide. The ionic strength of each solution was adjusted with NaCl, and q was determined over the range 0.016−1.6 M.

The influence of pH on q was determined by placing swollen hydrogel disks cured at 37 °C for 24 h into closed vials containing buffer solution (pH 2.4−12). The buffer solutions were prepared from pHydrion capsules, and the ionic strength of each solution was adjusted to 0.16 M with NaCl. Hydrogels were equilibrated for 24h at 37 °C in each buffer solution prior to weight determination.

Statistical Analysis. Statistical analysis of data was performed by one-way analysis of variance (ANOVA) and the Tukey HSD procedure for post hoc comparison using SPSS 5.0 for Windows. $p < 0.05$ was considered statistically significant. All studies were performed in triplicate.

Results

Isolation and Characterization of Monomer Gene Segment. DNA sequencing of acceptor vector pPT340 was used to verify the structure of the gene construct after insertion of the 8E gene segment (Figure 2A). The sequencing data showed the presence of a 318 bp DNA construct that encodes for the required SELP415K monomer unit and has the 8E segment inserted at the center of the sequence and a *Ban*I restriction recognition site at each end of the monomer construct.

Agarose gel electrophoresis of *Ban*I digestion of the expression vector pPT317 containing the monomer gene segment 415K (lane: 2, Figure 2B) showed the presence of a band at 312 bp. This band corresponds to the required 415K gene monomer segment flanked by a *Ban*I restriction endonuclease enzyme recognition site.

Isolation and Characterization of SELP415K Polymer Gene Segments. The self-ligation of 415K monomer gene segments is shown in Figure 2B (lanes: 3−5). The monomer gene segments were self-ligated as evidenced by the appearance of high molecular weight products larger than 312 bp following the double digestion of the expression vector pPT317 containing multimer gene segments with *Bam*HI and *Eco*RV restriction endonuclease enzymes. Lane 3 shows the formation of a polymer gene segment containing 6 repeats of the monomer gene (415K-6mer) and resulting in a segment of 1990 bp ([312 × 6] + 118 bp representing the flanking sequence). Lanes 4 and 5 show the formation of multimer gene segments of 8 and 10 monomer units (415K-8mer and 415K-10mer) with bands at 2614 bp and 3230 bp, respectively.

A.

5’GGTGCCGGTTCTGGAGCTGGCGCGGGCTCTGGTGTTGGAGTG
CCAGGTGTCGGTGTTCCGGGTGTAGGCGTTCCGGGAGTTGGT
GTACCTGGAAAAGGTGTTCCGGGGGTAGGTGTGCCGGGCGTT
GGAGTACCAGGTGTAGG**CGTACCAGGAGTAGGCGTACCGG**
GAGTAGGAGTGCCGGGTGTAGGAGTTCCTGGAGTTGGCG
TACCAGGAGTAGGCGTACCGGGAGTAGGAGTGCCGGGTG
TAGGAGTTCCTGGAGTTGGCGTCCCGGGAGCGGGTGCTGGT
AGCGGCGCAGGCGCGGGCTCTGGAGCGGGTGCC 3’

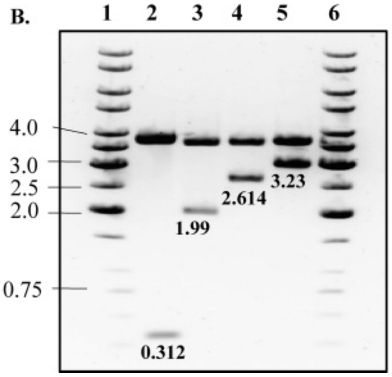


Figure 2. Analysis of monomer gene segments. (A) DNA sequencing data for SELP415K monomer gene segment constructed from insertion of eight elastin units 8E (underlined) inside SELP47K sequence resulting in a 318 bp segment flanked by *Ban*I restriction endonuclease recognition sites (highlighted in gray). (B) Analytical gel electrophoresis of digests of plasmid pPT317 containing polymer gene. Lanes 1 and 6: GeneRuler 1 Kbp DNA ladder (0.25−10 Kbp). Lane 2: monomer gene segment resulting from plasmid-*Ban*I digest. Lanes 3−5: plasmid-*Bam*HI/*Eco*RV digest (lane 3, 6-mer; lane 4, 8-mer; lane 5, 10-mer).

DNA sequencing was carried out on the multimer gene segment containing plasmid, and the identity of the terminal fragments of the inserted sequence was confirmed. These results indicate the successful synthesis and cloning of the SELP415K multimer gene segments of exact molecular weight and sequence.

Structural Characterization of SELP415K Analogues. SDS−PAGE data (Figure 3) indicate that the expressed polymers 415K-6mer, 415K-8mer, and 415K-10mer have apparent molecular masses of 55.5, 71.5, and 87.5 kDa, respectively. MALDI-TOF data show the presence of peaks at 55,369, 71,726, and 87,927 for polymers 415K-6mer, 415K-8mer, and 415K-10mer, respectively (Figure 4). In addition, the MALDI-TOF spectrum for polymer 415K-6mer

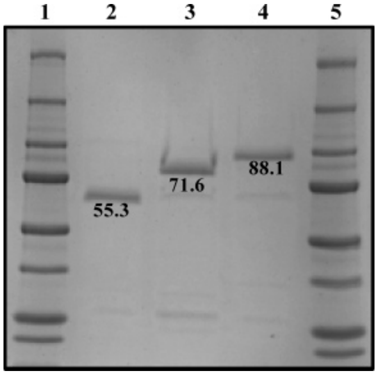


Figure 3. SDS−PAGE analysis of expressed SELP415K polymers of different molecular weights (values shown as molecular weight × 10^{-3}). Lanes 1 and 5: molecular mass markers (20−250 kDa). Lane 2: SELP415K-6mer. Lane 3: SELP415K-8mer. Lane 4: SELP415K-10mer.

showed a peak at 22752, which can be explained as the doubly charged molecular ion at half the *m*/*z* value of the parent 55369. Similarly, MALDI-TOF data for polymer 415K-8mer showed the presence of a peak at 35999 and that of polymer 415K-10mer showed a peak at 43331 resulting from doubly charged molecular ions at approximately half the *m*/*z* values. The theoretical amino acid sequences of polymers 415K-6mer, 415K-8mer, and 415K-10mer (Figure 1) predict the molecular masses to be 55,130 Da, 71,500 Da, and 87,860 Da, respectively.

On the basis of peak width at half-height calculations, the error of the TOF instrument was calculated to be approximately 692, 454, and 587 and the actual difference in molecular weights observed was 239, 226, and 67 for polymers 415K-6mer, 415K-8mer, and 415K-10mer, respectively. Hence, the molecular weights predicted by MALDI-TOF analysis agree with the theoretical molecular weights.

The results of amino acid content analysis are listed in Table 1. The molar ratios of amino acids constituting the backbone of the polymer are listed in Table 2. In general, the observed amino acid compositions for polymers 415K-6mer, 415K-8mer, and 415K-10mer agree with the expected amino acid compositions based on the expected amino acid sequences.

Table 1. Amino Acid Composition Analysis of the Polymers

	415K-6mer		415K-8mer		415K-10mer	
amino acid	R/M Th[a]	R/M Ob[a,b]	R/M Th[a]	R/M Ob[a,b]	R/M Th[a]	R/M Ob[a,b]
G	267	246.8	355	335.1	443	416.6
V	189	172.6	251	233.0	313	282.1
P	102	106.2	134	116.0	166	143.3
A	52	52.3	68	68.9	84	86.0
S	26	24.3	34	31.8	42	41.0
K	6	8.6	8	13.7	10	13.4
H	7	7.9	7	10.8	7	9.6
T	1	2.9	1	3.6	1	5.6
R	5	7.1	5	8.0	5	9.8
Y	1	1.8	1	2.5	1	3.7
C	0	0	0	0	0	0
M	3	3.7	3	3.6	3	5.0
W	1	*c*	1	*c*	1	*c*
F	1	2.3	1	3.2	1	3.9
I	0	1.3	0	2.3	0	3.8
L	4	5.9	4	8.4	4	10.36

[a] R/M Th, theoretical number of residues per mole; R/M Ob, observed number of residues per mole. [b] Based on observed molecular weight. [c] Not determined.

Effect of Polymer Structure on Complex Shear Modulus. The complex shear modulus (*G**) for solutions of polymers 47K (with shorter elastin units) and 415K-8mer (12 wt %, pH 7.4 and 300 mosm/kg) (similar molecular weight but longer elastin units in the monomer) was measured as a funtion of time at 37 °C at a frequency of 1 Hz and a constant strain of 0.2%. The results are shown in Figure 5. The complex shear modulus for both polymers showed a rapid initial increase as a result of gel formation. Both polymer solutions achieved a *G** of 260−285 Pa, a nonflowable form at 10 and 120 min, respectively. The complex shear modulus, *G**, for polymer 47K and polymer

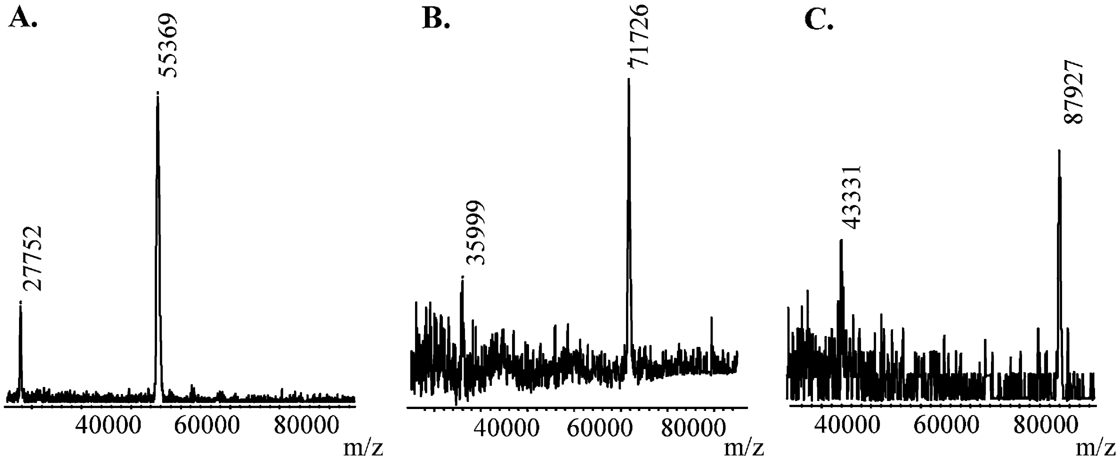


Figure 4. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) spectra: (A) SELP415K-6mer; (B)SELP415K-8mer; (C) SELP415K-10mer.

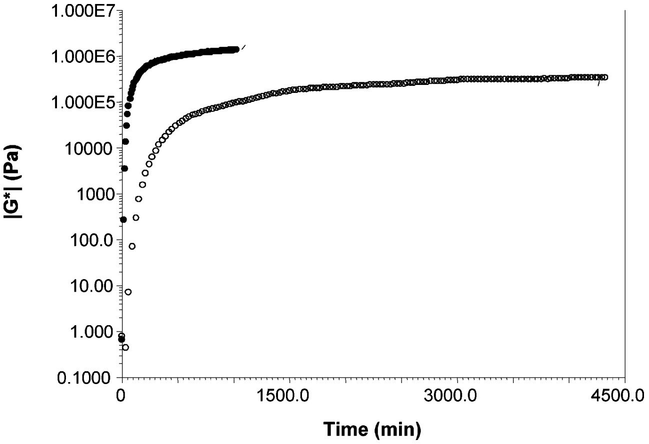


Figure 5. Determination of complex shear modulus versus time at 35 °C: (○) polymer 415K-8mer; (●) polymer 47K.

Table 2. Molar Ratios (with Respect to Valine) for the Amino Acids Constituting the Backbone of the Polymers

molar ratio	415K-6mer		415K-8mer		415K-10mer	
	Ex[a]	Ob[a]	Ex[a]	Ob[a]	Ex[a]	Ob[a]
G/V	1.41	1.43	1.41	1.44	1.42	1.48
P/V	0.54	0.62	0.53	0.50	0.53	0.51
A/V	0.28	0.30	0.27	0.30	0.27	0.30
S/V	0.14	0.14	0.14	0.14	0.13	0.15

[a] Ex: expected molar ratios. Ob: observed mole ratios.

Table 3. Effect of Polymer Composition on Formation of 47K and 415K-8mer Hydrogels[a]

cure time (h)	415K-8mer			47K		
	6 wt %	9 wt %	12 wt %	6 wt %	9 wt %	12 wt %
4	−	−	+	+	+	+
24	−	−	+	+	+	+
48	−	+	+	+	+	+

[a] (+) formation of physically robust hydrogels. (−) absence of physically robust hydrogels.

415K-8mer at gelation point was 6.08×10^5 and 9.39×10^4 Pa, respectively. The maximum G^* values at the ends of the analyses were 1.33×10^6 and 3.37×10^5 Pa for polymers 47K and 415K-8mer, respectively. Since both polymers have comparable molecular weights, the reduced shear modulus and greater gelation time of polymer 415K-8mer is likely due to the difference in the sequence of the two polymers. The decreased number of silk-like block domains of polymer 415K-8mer relative to polymer 47K reduces the number of potential physical cross-links between polymer chains.

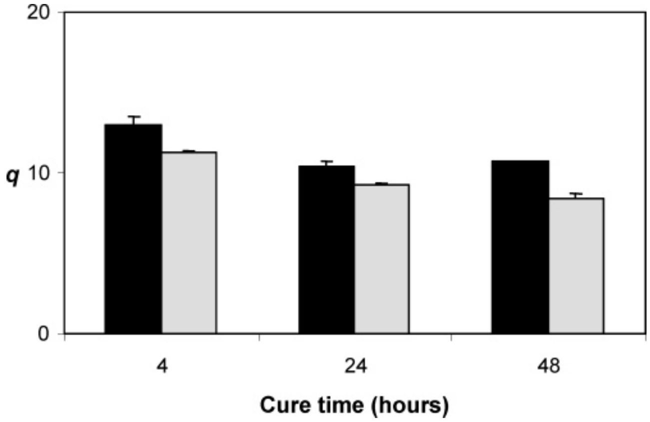


Figure 6. Effect of gelation (cure) time on the weight equilibrium swelling ratio (*q*) as a function of polymer structure: (black bars) 415K-8mer; (gray bars) 47K. Bars represent mean value ± 1 standard deviation (*n* = 3).

Effect of Polymer Composition on Formation of 47K and 415K-8mer Hydrogels. The ability of 47K and 415K-8mer to form physically robust hydrogels using different initial polymer concentrations and cure times is shown in Table 3. Polymer 47K formed a hydrogel after 4 h of cure time at 37 °C at 6, 9, and 12 wt % initial polymer concentrations. In contrast, firm hydrogel disks were obtained only from 12 wt % 415K-8mer polymer solutions after incubation for 4 h at 37 °C, while those prepared from 6 and 9 wt % 415K-8mer polymer solution failed to achieve an intact three-dimensional structure even after cure periods were extended up to 24 h and 48 h, respectively. The inability of 415K-8mer polymer chains to assemble into a defined three-dimensional structure at concentrations less than 12 wt % probably indicates an insufficient number of silk-like blocks for polymer cross-linking and formation of hydrogels at lower polymer concentrations.

Effect of Polymer Composition on Swelling Behavior of Hydrogels Prepared at Different Cure Times. The influence of polymer composition on the weight equilibrium swelling ratio for 12 wt % 47K and 415K-8mer hydrogels disks cured for 4, 24, and 48 h is presented in Figure 6. The equilibrium swelling ratios of polymer 415K-8mer hydrogels were significantly greater than those of 47K hydrogel disks at each cure time. At 24 and 48 h, sensitivity to cure time was observed for 47K hydrogel disks but not for those prepared from polymer 415K-8mer. Hydrogels prepared from polymer 47K displayed a decreasing trend in their equilibrium swelling ratio with increase in cure time while 415K-8mer showed no significant decrease in *q* at cure times greater than 24 h. The greater degree of swelling of 415K-8mer hydrogels compared to 47K hydrogels at all cure times corresponds with the greater cross-linking density of 47K probably attributable to the greater number of silk-like block domains in the polymer backbone. The lack of change in the swelling of 415K-8mer at cure times greater than 24 h (compared to the continued decrease in swelling of 47K as cure time increased to 48 h) is an indication that at 24 h of cure time cross-linking has reached a maximum.

Effect of Temperature, Ionic Strength, and pH on Hydrogel Swelling Behavior as a Function of Polymer Composition. The effect of temperature on the weight equilibrium swelling ratio of 12 wt % 47K and 415K-8mer hydrogel disks cured for 24 h is shown in Figure 7A. While the equilibrium swelling ratio for 47K hydrogels is not significantly different over the temperature range investigated, 415K-8mer hydrogels showed a significant change

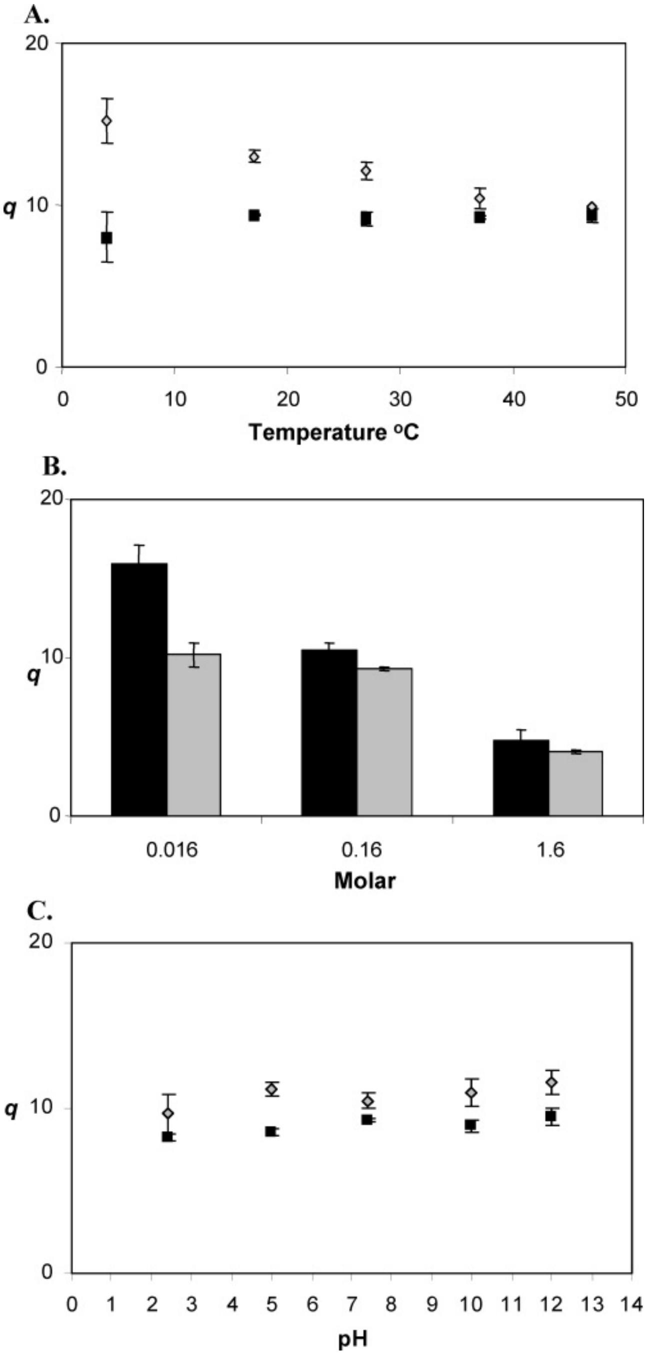


Figure 7. Influence of environmental conditions on the weight equilibrium swelling ratio, *q*, of 12 wt % polymer hydrogels cured for 24 h at 37 °C: (A) effect of temperature in 1X PBS (pH 7.4, $\mu = 0.16$); (B) effect of ionic strength at 37 °C in phosphate buffer solution (pH 7.4); (C) effect of pH at 37 °C ($\mu = 0.16$); (black symbols and bars) 415K-8mer; (gray symbols and bars) 47K. Symbols and bars represent mean value ± 1 standard deviation ($n = 3$).

in their equilibrium swelling ratios in response to temperature. Raising the temperature from 4 °C to 27, 37, and 47 °C resulted in 20%, 31%, and 34% decreases in equilibrium swelling ratios, respectively.

To investigate the effect of ionic strength on equilibrium hydrogel swelling ratio, the ionic strength of a phosphate buffer solution (pH 7.4) was adjusted from 0.016 to 1.6 M with NaCl. Figure 7B shows that the equilibrium swelling ratio for both polymer hydrogels at 12 wt % decreased significantly with increase in ionic strength. However this decrease was more pronounced for 415K-8mer hydrogels than 47K hydrogels. The hydrogel swelling ratios decreased by 34% and 70% upon increasing the ionic strength from 0.016 M to 0.16 and 1.6 M, respectively. For 47K, there was no significant decrease in *q* when the ionic strength of the media increased from 0.016 to 0.16 M. However, when the ionic strength was increased to 1.6 M, the equilibrium swelling ratio significantly decreased by 62%. The reasons for this observation need to be further investigated. One possible explanation is the increase in the outflow of water molecules from the hydrogel under the influence of a large osmotic pressure gradient.

Figure 7C presents the effect of pH on the equilibrium swelling ratio of 12 wt % hydrogels cured for 24 h. The results demonstrate that, although *q* of 415K-8mer hydrogels was greater than that of 47K hydrogels in the full range of pH, neither polymer displayed pH sensitivity.

Discussion

The release of bioactive agents from hydrogels depends in part on their degree of swelling. The degree of swelling of these three-dimensional polymeric networks in turn depends on the interactions between and within the polymer chains, polymer−solute interactions, and polymer−solvent interactions.[22] A decrease in cross-linking density can result in an increase in the degree of swelling. An increase in molecular weight of the polymer chains can result in an increase in inter- and intrapolymer interactions and entanglements, leading to increased cross-linking density and decreased degree of swelling and drug/gene release. These important phenomena for controlled delivery from hydrogels have been reviewed extensively and are well established for synthetic hydrogels.[23] The determining factor for the physicochemical properties of the hydrogels is the structure of monomers and the number of repeating units of the polymer chains that constitute the three-dimensional matrix.

Matrix-mediated gene delivery from biocompatible genetically engineered SELPs provides several distinct advantages. Depending on the length and sequence of the silk and elastin units, the gelation properties and biodegradation of these matrices can be controlled.[1,5] These protein-based polymers can be mixed with plasmid DNA and adenoviral particles in aqueous media in the absence of toxic solvents and monomer residues, injected into the site where gene transfer is required, and allow release and transfection over a

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prolonged period of time. In addition, precise control over monomer sequence and polymer length provided by advances in recombinant DNA technology for protein-based polymer synthesis allows a high degree of control for tailor-making SELP hydrogels for matrix-mediated delivery.

To date, one SELP composition, SELP47K (structure shown in Figure 1A), has been evaluated for matrix-mediated gene delivery.[11,21] Previously it was shown that, by varying the concentration and cure time of SELP47K hydrogels, their degree of swelling[12] and plasmid DNA, adenoviral release, and in vivo transfection efficiency[11,21] can be influenced. Variations in these parameters as well as the size and conformation of plasmid DNA, ionic strength of the media, and hydrogel geometry were shown to provide a range of conditions by which gene delivery from SELP matrices can be modulated. However, the influences of polymeric structural parameters such as the length of silk and elastin units within the monomer repeats and the number of monomer repeats on the physicochemical properties of the resulting SELP hydrogels and subsequent gene release, transfection, biodegradation, and elimination are poorly understood and merit investigation.

As a first step toward this goal, the design, synthesis, and characterization of a series of novel structurally related SELPs are reported in this article. The monomer gene segment was designed to encode for 4 silk units and 16 elastin units with one elastin unit containing a lysine residue yielding the monomer repeat of SELP415K. This polymer has longer elastin-like repeating units per monomer than the previously studied SELP47K, which has eight elastin units with a lysine residue in the monomer backbone. By varying the length of the elastin block, the distance between silk-like blocks necessary for formation of hydrogen bonds among the polymer chains will also vary. An increase in the length of elastin repeating units in the polymer backbone, while maintaining the length of silk repeating units constant, can result in an increased degree of swelling and potentially increase the cumulative amount and rate of release from SELP hydrogels. The monomer units were assembled to form polymer gene segments of varying, but precise, lengths, namely, 6, 8, and 10 repeating units. These multimer genes were then expressed to form three 415K analogues of exact molecular weights and monomer sequence.

A novel strategy was used to clone and express the new polymers. Instead of designing and constructing a full-length 318 bp monomer unit, we started with a cloning vector pPT340 containing a gene segment encoding for SELP47K monomer flanked by *Ban*I recognition sites and made use of a *Bsa*HI recognition site located near the 5′ end of that segment exactly at the end of the sequence encoding for the lysine-containing elastin unit as a means to extend the length of the elastin-like blocks. Then oligonucleotides encoding for eight elastin units (8E) having *Bsa*HI recognition sites at the 5′ termini of the oligonucleotide were designed and constructed. The incorporation of 8E into cloning vector pPT340 treated with *Bsa*HI resulted in a new gene construct for SELP415K with the desired sequence and length of the monomer gene segment as confirmed by DNA sequencing and agarose gel electrophoresis (Figure 2). Construction of SELP415K polymer gene segments was accomplished by random multimerization techniques after treatment of pPT340 plasmid containing the new construct encoding for SELP415K monomer with *Ban*I restriction enzyme. Random multimerization techniques have successfully produced, in a single step, a library of clones each containing plasmids with genes encoding structurally similar polymers of SELP415K of different specific molecular weights.

Three different clones containing polymer gene segments encoding for 415K-6mer, 415K-8mer, and 415K-10mer were selected for further expression. Polymer 415K-8mer was expected to have a molecular mass of 71.5 kDa, which makes it a candidate of choice for comparison with SELP47K (molecular mass = 69.8 kDa) to study the effect of polymer sequence on the physicochemical properties of the hydrogels. The expected molecular masses for polymers 415K-6mer and 415K-10mer were 55.1 and 87.9 kDa, respectively. The molecular weights of the expressed protein-based polymers were confirmed by SDS−PAGE (Figure 3) and MALDI-TOF measurement (Figure 4).

The composition of the 415K polymers was confirmed by amino acid composition analysis. As the purification methods are expected to yield no greater than 85−95% purity, the additional protein impurities were derived from the host, *E. coli*, as evidenced by the detected isoleucine (I) which is not contained in the polymer composition. The molar ratio data of the amino acid residues forming the backbone of the polymer compared to valine (Table 2) confirms the identity of the polymer composition with regard to G, A, S, V, and P, the amino acids constituting 91% of the theoretical polymer composition. These results are consistent with structurally similar SELPs.[16,18]

The physicochemical properties of hydrogels such as shear modulus, gel formation, degree of swelling, and sensitivity to environmental stimuli such as pH, temperature, and ionic strength depend on the structure, molecular weight, and composition of the polymers in the network. We chose SELP47K (69.8 kDa) evaluated for gene delivery previously[11,21] and SELP415K-8mer (71.5 kDa) polymers synthesized here for further investigation of these properties since they have similar molecular weights but vary in the length and ratio of elastin units in the monomers. Investigation of the dynamic rheological properties of polymer 47K and polymer 415K-8mer solutions showed the dependence of both the rate and extent of gelation on the polymer composition (Figure 5). The extent of formation of hydrogen bonds between the silk-like segments on adjacent polymer chains likely affects the viscosity and elasticity of the polymer hydrogels as shown by the reduced shear modulus and the delayed formation of nonflowable gel state observed for polymer 415K-8mer containing a reduced number of silk units per polymer chain. The ability to control the rheological properties through the accurate design and biosynthesis of polymers using genetic engineering techniques can be used in applications that involve in situ hydrogel formation such as delivery of macromolecules and tissue engineering.

Gelation occurs when the polymer concentration exceeds the critical gel concentration (CGC) above which the physical junctions in the system are sufficiently strong to yield a nonflowing gel phase. The CGC is generally inversely related to the molecular weight of the polymer used.[24] In our study, direct determination of CGC for polymer 47K and 415K was not performed; however Table 3 shows the effect of polymer composition on hydrogel formation from different polymer concentrations. SELP47K hydrogels were obtained from solutions containing as low as 6 wt % polymer after 4 h of cure time while hydrogel formation was only observed from 12 wt % 415K-8mer solution after similar cure time. The increase in length of the elastin block resulted in a decrease in the cross-linking density, and therefore more polymers are required in a unit volume to achieve the sol−gel transition. When cure time was increased to 48 h, 415K-8mer formed hydrogels at 9 wt %, indicating that over time as a result of the mobility of some polymer chains more silk units participate in cross-link formation.

The sensitivity of cross-linked polymer networks to duration of cure time or environmental conditions may cause changes in the equilibrium swelling ratio that can have an effect on their ability to be used as controlled delivery systems. Our results indicate that the sensitivity of physically cross-linked networks of SELPs to length of cure time or environmental conditions after they form gels is composition-dependent. Figure 6 indicates that, after 24 h, cure time had no significant effect on the weight equilibrium swelling ratio of 415K-8mer hydrogels, while 47K hydrogels showed a decrease in swelling with increase in cure time. The presence of more silk-like blocks per polymer chain in polymer 47K apparently increases the formation of physical cross-links between polymer chains, therefore imparting more rigidity to the hydrogel cured for longer durations.

Water-soluble SELPs containing elastin repeat units exhibit an inverse temperature solubility transition.[16,18] In addition, previous research showed that the equilibrium weight swelling ratio of covalently cross-linked elastin-mimetic hydrogels is temperature dependent.[25−27] However, our studies with 47K demonstrated that after gel formation these hydrogels are insensitive to temperature even at low polymer concentration, which may be due to the formation of the rigid structure resulting from the presence of an increased number of silk units.[12] Results of this study show that 415K-8mer hydrogels exhibited a significant change in their equilibrium swelling ratios in response to temperature (Figure 7A), where q decreased by 34% over a range of 4−47 °C. The temperature sensitivity effect exhibited by 415K-8mer hydrogels, compared to 47K hydrogels, is possibly due to the formation of less rigid, physical cross-links as caused by a decrease in the ability of the silk-like blocks to impart physical robustness to the hydrogels by increasing in the length of the elastin block and in the repeat structure. This is significant in that new stimuli-sensitive SELP hydrogels are synthesized that can be used for drug and gene loading and release in response to changes in environmental conditions such as temperature.

For protein-based polymers, hydrophobic self-assembly is due to the competition between apolar (hydrophobic) and polar residues that are restrained by sequence in the polymer backbone such that their proximity allows them to compete for the same water molecules for hydration.[15] Therefore, at a constant pH, an increase in ionic strength leads to counterion shielding of polar residues and results in a decrease in the water uptake and the hydrogel equilibrium swelling ratio. Hydrogels prepared from polymer 47K and polymer 415K-8mer showed significant sensitivity to ionic strength over the range of 0.016−1.6 M at pH 7.4 (Figure 7B). The extent of sensitivity to change in ionic strength was more prominent for 415K hydrogels especially when the ionic strength was increased from 0.016 to 0.16 M, where a 34% decrease in q was observed compared to an insignificant change in swelling for 47K hydrogels. This highlights the influence of increased number of elastin units between silk-like domains in stimulus sensitivity of SELPs where longer distances between the cross-links allow for higher flexibility of the hydrogel network.

Although the hydrogel swelling ratio was different between the polymers, neither polymer 47K nor 415K-8mer hydrogels displayed pH sensitivity over a pH range of 2.4−12 despite the net positive charge of both polymers at pH 7.4 due to the presence of lysines in the repeat units of the polymer backbone. These results are consistent with previous characterization of polymer 47K hydrogels.[12] Hydrophobicity-induced pK_a shifts in elastin protein-based polymers have been reported[15] suggesting that the actual pK_a of lysine in the 47K and 415K-8mer polymer backbones may be reduced relative to typical protein lysines. The insensitivity to changes in pH may be due to the contributions of amino acid residues adjacent to lysine in the monomeric units to the net pK_a of the lysine residues, contributions of the polymer head and tail amino acid sequences, and/or the nature and the extent of physical cross-linking between the silk units.

In addition to practical applications such as controlled gene delivery, we believe that the design and synthesis of polymeric constructs with exact molecular weights and sequences can provide the basis for improving the theoretical prediction of the influence of polymeric architecture on the physicochemical properties of hydrogels such as pore size, pore volume, cross-linking density, degree of ionization, and polymer−solvent interaction. To date, prediction of param-

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eters such as equilibrium degree of swelling,[28,29] phase transition in swollen polymer networks,[30] and solute diffusion in hydrogels[31] has utilized structural models that are based on statistically defined polymer lengths and sequences. Advances in genetic engineering of polymers with exact molecular weights and sequences such as those reported in this article provide the basis for new models that can predict more precisely hydrogel properties and solute release.

In summary, a cloning strategy was designed for the biosynthesis of new SELP analogues, namely, SELP415K. Multimer gene segments encoding 6, 8, and 10 repeats of SELP415K were isolated. The multimers were expressed and purified yielding three SELP415K polymers with an incremental increase in molecular weight. The physicochemical properties of hydrogels made from polymer 415K-8mer were compared with those of a previously studied SELP hydrogel for gene delivery, namely, SELP47K. Compared to polymer 47K, hydrogels prepared from 415K-8mer showed a less rigid structure, different gelation patterns, and increased sensitivity toward temperature and ionic strength. The next steps are to examine the influence of polymer molecular weight on network properties and evaluate gene release and the corresponding transfection efficiencies using the hydrogels described here. Results of this study and others[10,11,13,21] show the potential of recombinant techniques where well-defined polymers can be engineered at the molecular level for controlled drug and gene delivery.

Abbreviations Used

8E, gene segment encoding eight elastin units; A, adenosine (deoxyribonucleic acid base); A (Ala), alanine (amino acid); ANOVA, analysis of variance; bp, base pairs; C, cytosine (deoxyribonucleic acid base); C (Cys), cysteine (amino acid); CGC, critical gel concentration; D (Asp), aspartic acid; Da, Daltons; DNA, deoxyribonucleic acid; E (Glu), glutamic acid; *E. coli* HB 101, *Escherichia coli* strain HB 101; Ex, expected values; F (Phe), phenylalanine; G, guanosine (deoxyribonucleic acid base); G (Gly), glycine (amino acid); G^*, complex shear modulus; GAGAGS, silk-like repeat; GVGVP, elastin-like repeat; H (His), histidine; I (Ile), isoleucine; K (Lys), lysine; L/Leu, leucine; LB, *Luria-Bertani* broth; λ_{PL}, bacteriophage lambda promoter; M (Met), methionine; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MW, molecular weight; MWCO, molecular weight cutoff; N (Asn), asparagine; Ob, observed values; P (Pro), proline; Q (Gln), glutamine; Q, weight equilibrium swelling ratios of hydrogels (q); R (Arg), arginine; R/M, residue per mole; S (Ser), serine; SAP, shrimp alkaline phosphatase; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SELP, silk-elastinlike polymers; T, thymine (deoxyribonucleic acid base); T (Thr), threonine (amino acid); Th, theoretical values; V (Val), valine; W (Trp), tryptophan; W_d, dry hydrogel weight; W_s, weight of swollen hydrogels; Y (Tyr), tyrosine.

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