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The overall purpose of the project is to use silkelastin-like polymers (SELPs) for the development of controlled gene delivery systems for localized breast cancer gene therapy. The rationale is that by controlling the structure of the polymer, it is possible to control DNA release, duration of transgene expression and the corresponding reduction in tumor size. In year 2 progress was made in the following areas: i) Finished the biosynthesis of three SELP 415K analogs with incremental increase in molecular weight and started on the biosynthesis of SELP 815K, ii) Compared the physicochemical characteristics of hydrogels made from SELP 415K and SELP 47K, iii) Compared the DNA release characteristics of hydrogels made from SELP 415K and SELP 47K and evaluated the interaction of DNA with these polymers. The next logical steps are to finish the biosynthesis of the third analog (815K), conduct characterization and release studies using hydrogels made from this analog and evaluate the delivery systems in murine models of breast cancer as proposed in the application.

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Polymers, drug delivery, breast cancer, gene delivery, implants

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INTRODUCTION:

The **overall purpose** of the project is to use silk elastin-like polymers (SELPs) for the development of controlled gene delivery systems for localized breast cancer gene therapy. The **rationale** is that by controlling the structure of the polymer, it is possible to control DNA release, duration of transgene expression and the corresponding reduction in tumor size. Three Specific Aims were proposed:

- 1) To synthesize and characterize a series of SELP hydrogels.
- 2) To examine the influence of polymer structure on DNA release *in vitro*.
- 3) To evaluate the influence of polymer structure on transfection efficiency and therapeutic efficacy *in vivo*.

In year 2 of the project progress was made to partially accomplish Aims 1 and 2. In addition related areas were explored as outlined in the body of this report.

BODY:

A) Biosynthesis of the proposed polymers:

For this portion of the project the following task and subtasks were proposed:

Task 1. Synthesis of linear polymers

- a. Design and synthesis of oligonucleotides encoding polymers
- b. Synthesis of monomer gene segments
- c. Synthesis of multimer gene segments
- d. Small scale expression and analysis of polymers
- e. Large scale fermentation of polymers
- f. Dissemination of data in a conference

We made progress in items (d)-(f) for the synthesis of the polymeric analog SELP 415K and disseminated results in a publication and several conferences (last year we had made progress for items (a)-(c) for this polymeric analog). This progress is summarized in Appendix 1 (See Scheme I, Tables 1-2 and Figures 1-4 of this Appendix and related methods, results and discussions). In addition to what was proposed, namely the biosynthesis of polymer C in Fig 1-Appendix 1, we made two other molecular weight analogs of the same polymer (Polymers B and D, Fig. 1, Appendix 1). Factors that influence hydrogel properties are not only polymeric structure (as proposed in the grant application) but also polymeric molecular weight. The biosynthesis of the additional analogs will allow us to establish the relationship between polymer molecular weight on one hand with hydrogel properties, DNA release, and *in vivo* transfection efficiency on the other.

For another polymeric analog, namely SELP 815K, we have made progress on items (a) and (b). Despite several attempts, the colonies that we have identified so far contain only monomer gene segments. We have not isolated the multimer gene segments encoding for SELP 815K yet. Our strategy is to continue to screen more colonies and if this still fails, to redesign the biosynthetic plan for the cloning, expression and purification of this polymer. This work is under way.

B) Characterization of hydrogels, DNA release, and evaluation of bioactivity:

In year 2, in addition to accomplishing some of the biosynthetic work that was proposed in year 1 as described above, we also accomplished in part the tasks proposed for year 2. The tasks proposed for year 2 were as follows:

Task 1. Formation and characterization of hydrogels:

- a. Formation of hydrogels and determination of degree of swelling and soluble fraction

Task 2. Formation of DNA-containing hydrogels and characterization of DNA release:

- a. Determination of DNA release as a function of polymer structure and concentration
- b. Evaluation of the interaction of DNA with polymer backbone
- c. Evaluation of the bioactivity of the released DNA

Task 3. Concluding year 2 and strategizing for year 3:

- a. Selection of hydrogels with the most appropriate profiles for year 3
- b. Dissemination of data in a conference
- c. Preparation of a manuscript and report for year 2
- d. Preparation of a plan for year 3

We were able to accomplish Task 1 (a), Task 2(a-c) and Task 3 (b-d) for two polymeric analogs (SELP 47K and SELP 415 K). The comparison of the characteristics of the hydrogels made from SELP47K and SELP15K are reported in Appendix 1 (Table 3, Figures 5-7 and related methods, results and discussions). The comparison of DNA release and the interaction of these two polymeric analogs with DNA are reported in Appendix 2. We had previously shown that DNA released from SELP47K remained bioactive until Day 28 (Megeed et al., J Control Rel, 94, 433-445 (2004)). Similar results were obtained with SELP 415K and in addition based on preliminary studies so far we have not seen any significant differences between the soluble fraction of SELP 47K hydrogels vs. SELP 47K hydrogels (unpublished data). Once the third analog namely SELP 815K is biosynthesized, hydrogels made from these polymers can be compared with the other two and hydrogels with the most appropriate profiles can be selected for further studies (i.e, Task 3(a) above).

In addition to progress in Aims 1 and 2 and the additional experiments described above, we also submitted a book chapter related to this project which is currently in press (Appendix 3).

KEY RESEARCH ACCOMPLISHMENTS:

- a) Cloned and expressed three molecular weight analogs of polymer 415K
- b) Fermented in a large scale one 415K analog
- c) Compared the physicochemical characteristics of hydrogels made from 415K and 47K
- d) Compared DNA release as a function of polymer structure and composition for 415K and 47K
- e) Evaluated the interaction of DNA with the polymer backbone of 415K and 47K
- f) Evaluated the bioactivity of DNA release from (d) above.
- g) Designed and synthesized oligonucleotides encoding Polymer 815K
- h) Published, presented and reviewed the results of this research as outlined in the following section.

REPORTABLE OUTCOMES:

A. Manuscripts:

One original research article ([Appendix 1](#)) and one invited book chapter ([Appendix 3](#)) pertaining to research described in this report were submitted during this reporting period and accepted:

M. Haider, V. Leung, F. Ferrari, J. Crissman, J. Cappello, and H. Ghandehari, Molecular Engineering of Silk-Elastinlike Polymers for Matrix-Mediated Gene Delivery: Biosynthesis and Characterization, *Molecular Pharmaceutics*, 2005 Mar-Apr;2(2):139-50. ([Appendix 1](#))

R. Dandu, Z. Megeed, M. Haider, J. Cappello and H. Ghandehari, Silk-Elastinlike Hydrogels: Thermal Characterization and Gene Delivery. In *American Chemical Society Symposium Series on Polymeric Drug Delivery: Science and Application*, S. Svenson (Ed.), ACS, Washington, DC, in press. ([Appendix 3](#))

B) Presentations:

I) Abstracts:

There were total of nine abstracts pertaining to this research during the reporting period. Of these six presentations were made by graduate students and three by the PI:

M. Haider, J. Cappello, and H. Ghandehari, Gene Delivery From Recombinant Silk-Elastinlike Hydrogels: In Vitro Release and Biosynthesis, 31st International Symposium

on Controlled Release of Bioactive Materials, Honolulu, Hawaii, June 13-16, 2004 (Student Poster Presentation).

Z. Megeed, J. Cappello, D. Li, B. O'Malley, H. Ghandehari, Genetically Engineered Biomaterials for Viral & Nonviral Cancer Gene Therapy, 31st International Symposium on Controlled Release of Bioactive Materials, Honolulu, Hawaii, June 13-16, 2004 (Student Podium Presentation).

H. Ghandehari, Z. Megeed, M. Haider, R. Dandu, D. Li, B. W. O'Malley Jr., and J. Cappello, Matrix-Mediated Gene Delivery from Recombinant Polymers, AAPS Conference on Pharmaceuticals and Drug Delivery, Philadelphia, PA, June 7-9, 2004 (Invited Talk by the PI).

M. Haider, J. Cappello, and H. Ghandehari, Biosynthesis of Recombinant Silk-Elastinlike Polymers for Controlled Gene Delivery, Annual Meeting of the American Association of Pharmaceutical Scientists (AAPS), Baltimore, MD, November 7-11, 2004 (Student Poster Presentation).

H. Ghandehari, M. Haider, R. Dandu, and A. Hatefi, Protein-Based Polymers for Cancer Gene Therapy, Annual Meeting of the American Association of Pharmaceutical Scientists (AAPS), Baltimore, MD, November 7-11, 2004 (Invited talk by the PI).

H. Ghandehari, M. Haider, J. Cappello, R. Dandu, and A. Hatefi, Recombinant Polymers for Cancer Gene Therapy, 12th International Symposium on Recent Advances in Drug Delivery Systems, Salt Lake City, Utah, February 21-24, 2005 (Invited talk by the PI).

M. Haider, J. Cappello, and H. Ghandehari, Engineering Polymers for Breast Cancer Gene Therapy, 4th Era of Hope Meeting, Philadelphia, PA, June 8-11, 2005 (Student Presentation).

V. Moolchandani, M. Haider, and H. Ghandehari, In Vitro Release of Plasmid DNA from Structurally Related Silk-Elastinlike Hydrogels, 32nd International Symposium on Controlled Release of Bioactive Materials, Miami, Florida, June 18-22, 2005 (Student Poster Presentation) (**Appendix 2**).

M. Haider, J. Cappello, J. Powell, and H. Ghandehari, Molecular Engineering of Silk-Elastinlike Hydrogels: Influence of Polymer Composition on Rheological Properties and Swelling Behavior, 32nd International Symposium on Controlled Release of Bioactive Materials, Miami, Florida, June 18-22, 2005 (Student Poster Presentation).

II) Invited Talks:

The PI gave invited presentations at the following venues during the period of this report that in part covered the research in this grant:

- Rutgers, The State University of NJ, Department of Pharmaceutics, Piscataway, NJ, April 30, 2004.
- Global Pharmaceutics Education Network Meeting, Kyoto, Japan, May 28, 2004.
- American Association of Pharmaceutical Scientists Meeting on Pharmaceutics and Drug Delivery, Philadelphia, PA, June 8, 2004.
- International Symposium on Controlled Release of Bioactive Materials, Honolulu, Hawaii, June 12, 2004.
- Symposium on Nanomedicine and Drug Delivery, Polytechnic University, Brooklyn, NY, August 19, 2004.
- Annual Meeting of the American Association of Pharmaceutical Scientists, Baltimore, MD, November 10, 2004.
- University of Maryland Baltimore County, Department of Chemistry, Baltimore, MD, December 15, 2004.
- International Symposium on Recent Advances in Drug Delivery Systems, Salt Lake City, Utah, February 21-24, 2005.
- University of Kentucky, Department of Pharmaceutical Sciences, Lexington, KY, March 11, 2005.

C) Cumulative Publications:

During the second year period some time was spent to finalize publications related to the previous reporting period (galley proofs, revisions, finalizing submission, etc.). The following is the cumulative list of publications to-date related to this work pertaining to both the first year and the second year activities:

Z. Megeed, M. Haider, D. Li, B. W. O'Malley Jr., J. Cappello, and H. Ghandehari, In Vitro and In Vivo Evaluation of Recombinant Silk-Elastinlike Hydrogels for Cancer Gene Therapy, *Journal of Controlled Release*, 94, 433-445 (2004).

Z. Megeed, J. Cappello, and H. Ghandehari, Thermal Analysis of Water in Silk-Elastinlike Hydrogels by Differential Scanning Calorimetry, *Biomacromolecules*, 5:793-797 (2004).

M. Haider, Z. Megeed, and H. Ghandehari, Genetically Engineered Polymers: Status and Prospects for Controlled Release, *Journal of Controlled Release*, 95, 1-26 (2004).

Z. Megeed, and H. Ghandehari, Genetically Engineered Protein-Based Polymers: Potential in Gene Delivery. In *Polymeric Gene Delivery: Principles and Applications*, M. Amiji (Ed.), CRC Press, Boca Raton, FL, pp. 489-507 (2005).

M. Haider, V. Leung, F. Ferrari, J. Crissman, J. Cappello, and H. Ghandehari, Molecular Engineering of Silk-Elastinlike Polymers for Matrix-Mediated Gene Delivery: Biosynthesis and Characterization, *Molecular Pharmaceutics*, 2:139-50 (2005).

Z. Megeed, and H. Ghandehari, Recombinant Polymers for Drug Delivery. In *Polymeric Drug Delivery Systems*, G. Kwon (Ed.), Marcel Dekker, Inc. New York, NY, in press.

R. Dandu, Z. Megeed, M. Haider, J. Cappello and H. Ghandehari, Silk-Elastinlike Hydrogels: Thermal Characterization and Gene Delivery. *In American Chemical Society Symposium Series on Polymeric Drug Delivery: Science and Application*, S. Svenson (Ed.), ACS, Washington, DC, in press.

A. A. Dinerman, J. Cappello, M. El-Sayed, S. W. Hoag, H. Ghandehari, Influence of Solute Charge and Hydrophobicity on Partitioning and Diffusion in a Genetically Engineered Silk-Elastinlike Protein Polymer Hydrogel, *Journal of Pharmaceutical Sciences*, submitted.

D) Degrees obtained that are supported by this award:

Mohamed Haider obtained his PhD in Pharmaceutical Sciences in Fall of 2004. Currently he is a joint postdoctoral fellow between our research group and the Department of Biomedical Engineering at Johns Hopkins University. He worked on the biosynthesis, characterization and DNA release from the SELP hydrogels. During his PhD studies he published 5 manuscripts (three as first author) and 13 abstracts.

CONCLUSIONS:

In summary progress was made in the following areas:

A) Finished the biosynthesis of three SELP 415K analogs with incremental increase in molecular weight and started on the biosynthesis of SELP 815K.

B) Compared the physicochemical characteristics of hydrogels made from SELP 415K and SELP 47K.

C) Compared the DNA release characteristics of hydrogels made from SELP 415K and SELP 47K and evaluated the interaction of DNA with these polymers.

The next logical steps are to finish the biosynthesis of the third analog (815K), conduct characterization and release studies using hydrogels made from this analog and evaluate the delivery systems in murine models of breast cancer as proposed in the application.

REFERENCES:

None.

APPENDICES:

Appendix 1) M. Haider, V. Leung, F. Ferrari, J. Crissman, J. Cappello, and H. Ghandehari, Molecular Engineering of Silk-Elastinlike Polymers for Matrix-Mediated

Gene Delivery: Biosynthesis and Characterization, *Molecular Pharmaceutics*, 2:139-150 (2005).

Appendix 2) V. Moolchandani, M. Haider, and H. Ghandehari, In Vitro Release of Plasmid DNA from Structurally Related Silk-Elastinlike Hydrogels, 32nd International Symposium on Controlled Release of Bioactive Materials, Miami, Florida, June 18-22, 2005. Proceedings.

Appendix 3) R. Dandu, Z. Megeed, M. Haider, J. Cappello and **H. Ghandehari**, Silk-Elastinlike Hydrogels: Thermal Characterization and Gene Delivery. In *American Chemical Society Symposium Series on Polymeric Drug Delivery: Science and Application*, S. Svenson (Ed.), ACS, Washington, DC, in press.

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

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 agents¹⁻⁶ 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-

- (1) Haider, M.; Megeed, Z.; Ghandehari, H. Genetically engineered polymers: status and prospects for controlled release. *J. Controlled Release* **2004**, *95*, 1-26.
- (2) Ghandehari, H.; Cappello, J. Genetic engineering of protein-based polymers: potential in controlled drug delivery. *Pharm. Res.* **1998**, *15*, 813-815.
- (3) Nagarsekar, A.; Ghandehari, H. Genetically engineered polymers for drug delivery. *J. Drug Targeting* **1999**, *7*, 11-32.
- (4) Manno, M.; Emanuele, A.; Martorana, V.; San Biagio, P. L.; Bulone, D.; Palma-Vittorelli, M. B.; McPherson, D. T.; Xu, J.; Parker, T. M.; Urry, D. W. Interaction of processes on different length scales in a bioelastomer capable of performing energy conversion. *Biopolymers* **2001**, *59*, 51-64.
- (5) Megeed, Z.; Cappello, J.; Ghandehari, H. Genetically engineered silk-elastinlike protein polymers for controlled drug delivery. *Adv. Drug Delivery Rev.* **2002**, *54*, 1075-1091.
- (6) Wright, E. R.; Conticello, V. P. Self-assembly of block copolymers derived from elastin-mimetic polypeptide sequences. *Adv. Drug Delivery Rev.* **2002**, *54*, 1057-1073.
- (7) Wong Po Foo, C.; Kaplan, D. L. Genetic engineering of fibrous proteins: spider dragline silk and collagen. *Adv. Drug Delivery Rev.* **2002**, *54*, 1131-1143.
- (8) Altman, G. H.; Diaz, F.; Jakuba, C.; Calabro, T.; Horan, R. L.; Chen, J.; Lu, H.; Richmond, J.; Kaplan, D. L. Silk-based biomaterials. *Biomaterials* **2003**, *24*, 401-416.
- (9) Panitch, A.; Yamaoka, T.; Fournier, M. J.; Mason, T. L.; Tirrell, D. A. Design and biosynthesis of elastin-like artificial extracellular matrix proteins containing periodically spaced fibronectin CS5 domains. *Macromolecules* **1999**, *32*, 1701-1703.
- (10) Cappello, J.; Crissman, J. W.; Crissman, M.; Ferrari, F. A.; Textor, G.; Wallis, O.; Whitley, J. R.; Zhou, X.; Burman, D.; Aukerman, L.; Stedronsky, E. R. In-situ self-assembling protein polymer gel systems for administration, delivery, and release of drugs. *J. Controlled Release* **1998**, *53*, 105-117.
- (11) Megeed, Z.; Cappello, J.; Ghandehari, H. Controlled release of plasmid DNA from a genetically engineered silk-elastinlike hydrogel. *Pharm. Res.* **2002**, *19*, 954-959.
- (12) Dinerman, A. A.; Cappello, J.; Ghandehari, H.; Hoag, S. W. Swelling behavior of a genetically engineered silk-elastinlike protein polymer hydrogel. *Biomaterials* **2002**, *23*, 4203-4210.
- (13) Dinerman, A. A.; Cappello, J.; Ghandehari, H.; Hoag, S. W. Solute diffusion in genetically engineered silk-elastinlike protein polymer hydrogels. *J. Controlled Release* **2002**, *82*, 277-287.
- (14) Hyun, J.; Lee, W. K.; Nath, N.; Chilkoti, A.; Zauscher, S. Capture and release of proteins on the nanoscale by stimuli-responsive elastin-like polypeptide "switches". *J. Am. Chem. Soc.* **2004**, *126*, 7330-7335.
- (15) Urry, D. W.; Harris, C. M.; Luan, C. X.; Luan, C.-H.; Gowda, D. C.; Parker, T. M.; Peng, S. Q.; Xu, J. Transductional protein-based polymers as new controlled-release vehicles. In *Controlled Drug Delivery: Challenges and Strategies*; Park, K., Ed.; American Chemical Society: Washington, DC, 1997; pp 405-436.
- (16) Nagarsekar, A.; Crissman, J.; Crissman, M.; Ferrari, F.; Cappello, J.; Ghandehari, H. Genetic synthesis and characterization of pH- and temperature-sensitive silk-elastinlike protein block copolymers. *J. Biomed. Mater. Res.* **2002**, *62*, 195-203.
- (17) Chilkoti, A.; Dreher, M. R.; Meyer, D. E. Design of thermally responsive, recombinant polypeptide carriers for targeted drug delivery. *Adv. Drug Delivery Rev.* **2002**, *54*, 1093-1111.
- (18) Nagarsekar, A.; Crissman, J.; Crissman, M.; Ferrari, F.; Cappello, J.; Ghandehari, H. Genetic engineering of stimuli-sensitive silk-elastinlike protein block copolymers. *Biomacromolecules* **2003**, *4*, 602-607.
- (19) Cappello, J. Genetically Engineered Protein Polymers. In *Handbook of Biodegradable Polymers*; Domb, A. J., Kost, J., Wiseman, D. M., Eds.; Harwood Academic Publisher: Amsterdam, 1997; pp 387-416.
- (20) Cappello, J.; Ferrari, F. Microbial Production of Structural Protein Polymers. In *Plastics from Microbes*; Mobley, D. P., Eds.; Hanser Publishers: New York, 1994; pp 35-92.
- (21) Megeed, Z.; Haider, M.; Li, D.; O'Malley, B. W., Jr.; Cappello, J.; Ghandehari, H. In vitro and in vivo evaluation of recombinant silk-elastinlike hydrogels for cancer gene therapy. *J. Controlled Release* **2004**, *94*, 433-445.

- A.**
MDPVVLQRRDWENPGVTQLNRLAAHPPFASDPMGAGSGAGAGS
[(GVGVVP)₄ GKGVP (GVGVVP)₃ (GAGAGS)₄]₁₂ (GVGVVP)₄ GKGVP
(GVGVVP)₃ (GAGAGS)₂ GAGAMDPGRYQDLRSIIHHHHHH
- B.**
MDPVVLQRRDWENPGVTQLNRLAAHPPFASDPMGAGSGAGAGS
[(GVGVVP)₄ GKGVP (GVGVVP)₁₁ (GAGAGS)₄]₆ (GVGVVP)₄ GKGVP
(GVGVVP)₁₁ (GAGAGS)₂ GAGAMDPGRYQDLRSIIHHHHHH
- C.**
MDPVVLQRRDWENPGVTQLNRLAAHPPFASDPMGAGSGAGAGS
[(GVGVVP)₄ GKGVP (GVGVVP)₁₁ (GAGAGS)₄]₆ (GVGVVP)₄ GKGVP
(GVGVVP)₁₁ (GAGAGS)₂ GAGAMDPGRYQDLRSIIHHHHHH
- D.**
MDPVVLQRRDWENPGVTQLNRLAAHPPFASDPMGAGSGAGAGS
[(GVGVVP)₄ GKGVP (GVGVVP)₁₁ (GAGAGS)₄]₆ (GVGVVP)₄ GKGVP
(GVGVVP)₁₁ (GAGAGS)₂ GAGAMDPGRYQDLRSIIHHHHHH

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 (GVGVVP) 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). pHydration 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.

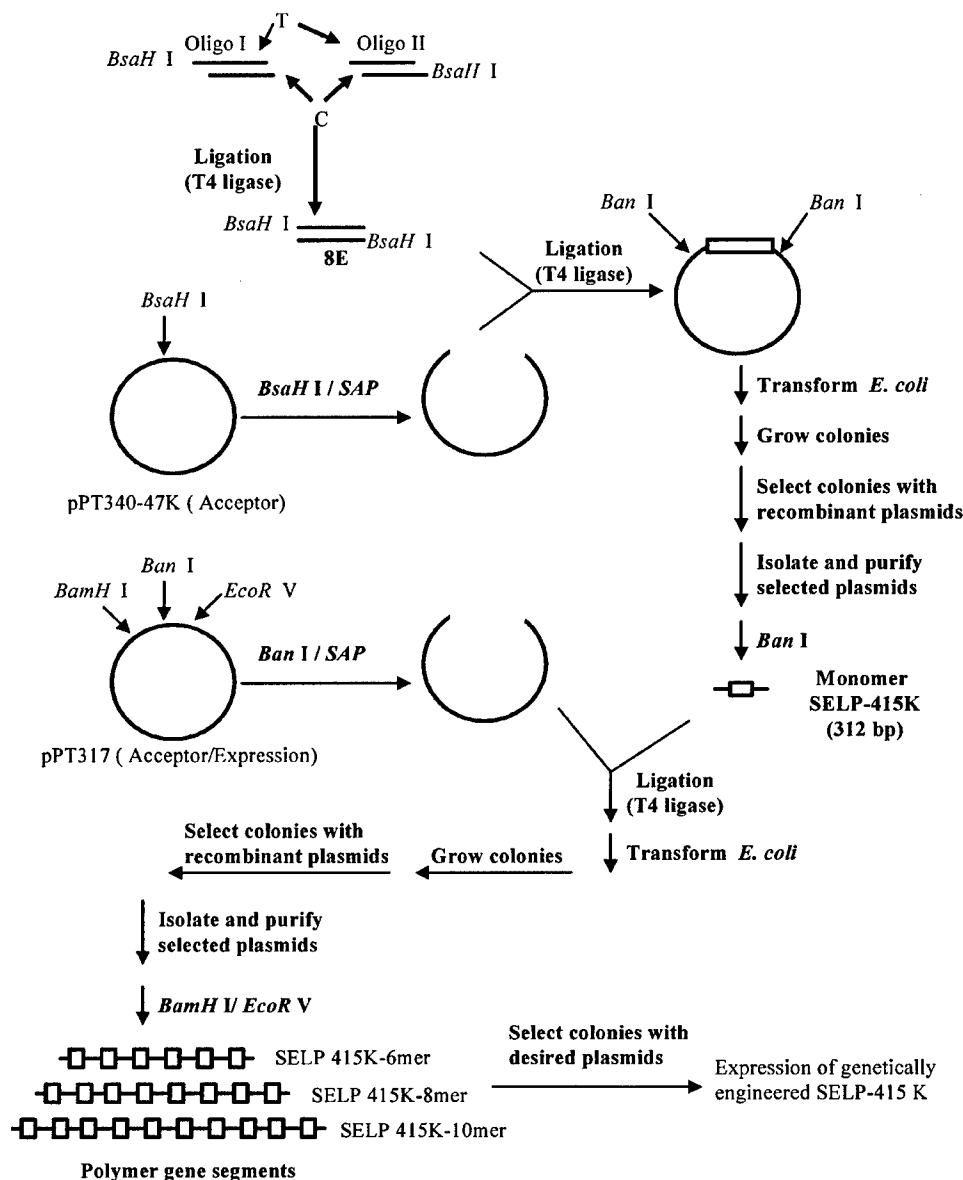
The general biosynthetic methodology is outlined in Scheme 1. The details of the methodology are described below.

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 $\mu\text{g/mL}$ 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'-CGTACCAGGAGTAGGCGTACCGGGAGTAGGAGTGCCGGGTGTAGGAGTTCCTGGAGTTGGC 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. BsaHI, BanI, BamHI, and EcoRV: 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 BsaHI 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 BsaHI 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 BanI, 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 μ L 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 from the induced cultures by centrifugation at 6000g 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 $\times$ 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 μ m 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-mm³ 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, μ = 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.

$$q = \frac{W_s}{W_d} \quad (1)$$

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 pHDrion capsules, and the ionic strength of each solution was adjusted to 0.16 M with NaCl. Hydrogels were equilibrated for 24 h 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 *BanI* restriction recognition site at each end of the monomer construct.

Agarose gel electrophoresis of *BanI* 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 *BanI* 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 appear-

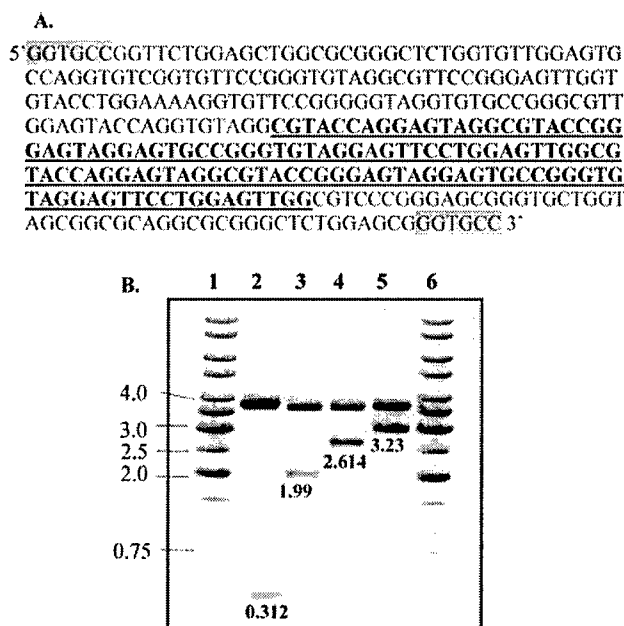


Figure 2. Analysis of monomer gene segments. (A) DNA sequencing data for SELP415K monomer gene segment constructed from insertion of eight elastin units 8E (underlined) inside SELP47K sequence resulting in a 318 bp segment flanked by *BanI* restriction endonuclease recognition sites (highlighted in gray). (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-*BanI* digest. Lanes 3–5: plasmid-*BamHI*/*EcoRV* digest (lane 3, 6-mer; lane 4, 8-mer; lane 5, 10-mer).

ance of high molecular weight products larger than 312 bp following the double digestion of the expression vector pPT317 containing multimer gene segments with *BamHI* and *EcoRV* 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 ($[312 \times 6] + 118$ 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.

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

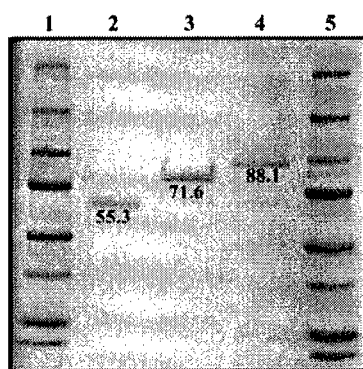


Figure 3. SDS-PAGE analysis of expressed SELP415K polymers of different molecular weights (values shown as molecular weight $\times 10^{-3}$). Lanes 1 and 5: molecular mass markers (20–250 kDa). Lane 2: SELP415K-6mer. Lane 3: SELP415K-8mer. Lane 4: SELP415K-10mer.

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-

Table 1. Amino Acid Composition Analysis of the Polymers

amino acid	415K-6mer		415K-8mer		415K-10mer	
	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.

6mer, 415K-8mer, and 415K-10mer agree with the expected amino acid compositions based on the expected amino acid sequences.

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 function 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

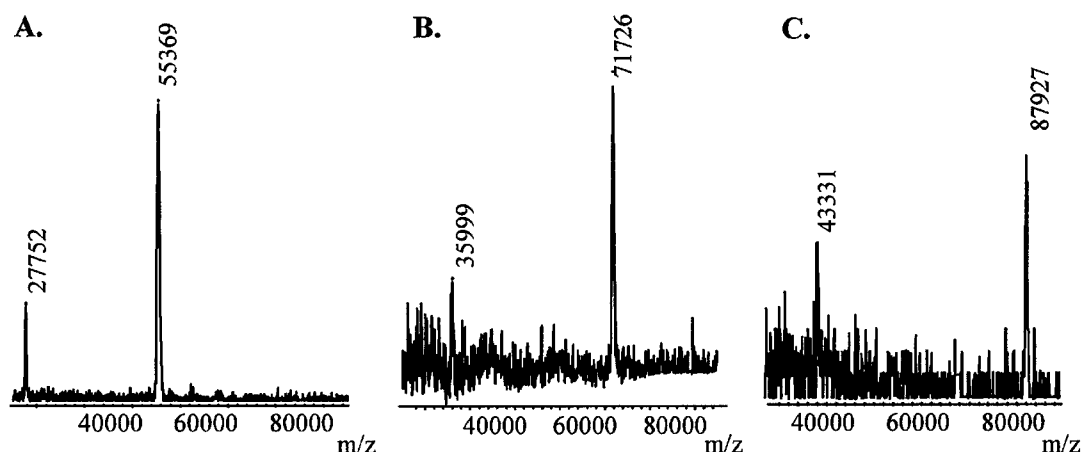


Figure 4. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) spectra: (A) SELP415K-6mer; (B) SELP415K-8mer; (C) SELP415K-10mer.

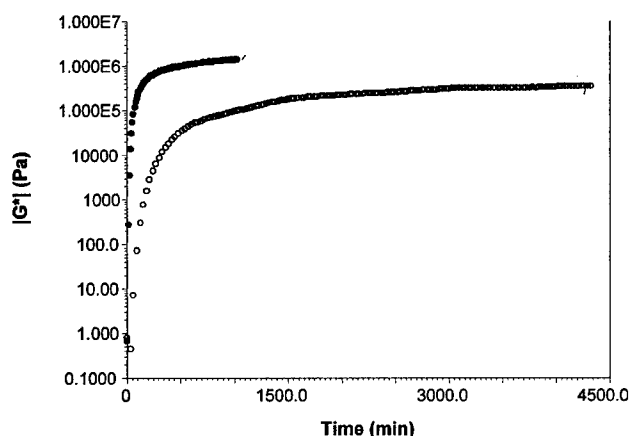


Figure 5. Determination of complex shear modulus versus time at 35 °C: (○) polymer 415K-8mer; (●) polymer 47K.

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.

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

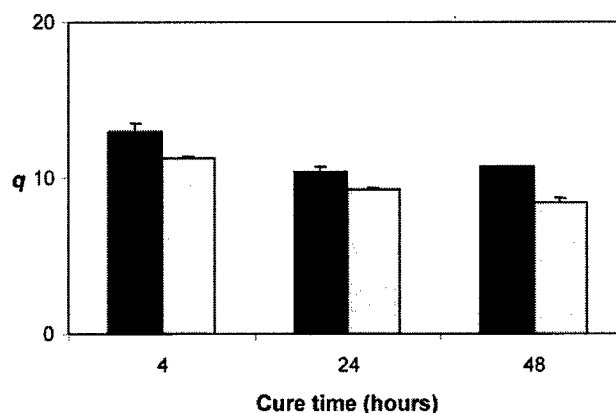


Figure 6. Effect of gelation (cure) time on the weight equilibrium swelling ratio (q) as a function of polymer structure: (black bars) 415K-8mer; (gray bars) 47K. Bars represent mean value $\pm$ 1 standard deviation ($n = 3$).

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

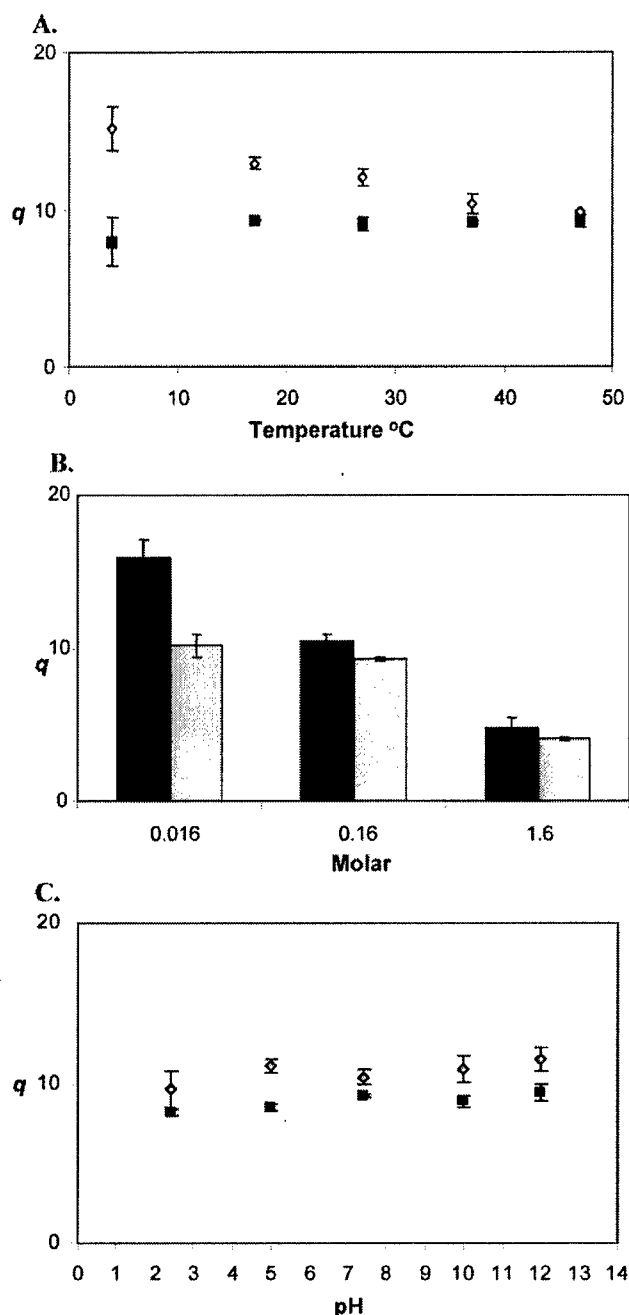


Figure 7. Influence of environmental conditions on the weight equilibrium swelling ratio, q , of 12 wt % polymer hydrogels cured for 24 h at 37 °C: (A) effect of temperature in 1X PBS (pH 7.4, $\mu = 0.16$); (B) effect of ionic strength at 37 °C in phosphate buffer solution (pH 7.4); (C) effect of pH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

(22) Hoffman, A. S. Hydrogels for biomedical applications. *Adv. Drug Delivery Rev.* **2002**, *54*, 3–12.

(23) Peppas, N.; Khare, A. Preparation, structure and diffusional behavior of hydrogels in controlled release. *Adv. Drug Delivery Rev.* **1993**, *11*, 1–35.

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 swelling¹² 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 reported¹⁵ 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-

- (24) Jeong, B.; Kim, S. W.; Bae, Y. H. Thermosensitive sol-gel reversible hydrogels. *Adv. Drug Delivery Rev.* **2002**, *54*, 37–51.
- (25) Lee, J.; Macosko, C. W.; Urry, D. W. Elastomeric polypentapeptides cross-linked into matrixes and fibers. *Biomacromolecules* **2001**, *2*, 170–179.
- (26) Lee, J.; Macosko, C. W.; Urry, D. W. Swelling behavior of γ -irradiation cross-linked elastomeric polypentapeptide-based hydrogels. *Macromolecules* **2001**, *34*, 4114–4123.
- (27) McMillan, R. A.; Conticello, V. P. Synthesis and characterization of elastin-mimetic protein gels derived from a well-defined polypeptide precursor. *Macromolecules* **2000**, *33*, 4809–4821.

eters such as equilibrium degree of swelling,^{28,29} phase transition in swollen polymer networks,³⁰ and solute diffusion in hydrogels³¹ 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.

- (28) Canal, T.; Peppas, N. A. Correlation between mesh size and equilibrium degree of swelling of polymeric networks. *J. Biomed. Mater. Res.* **1989**, *23*, 1183–1193.
- (29) Brannon-Peppas, L.; Peppas, N. A. Dynamic and equilibrium swelling behaviour of pH-sensitive hydrogels containing 2-hydroxyethyl methacrylate. *Biomaterials* **1990**, *11*, 635–644.
- (30) Dusek, K.; Patterson, D. Transition in swollen polymer networks induced by intramolecular condensation. *J. Polym. Sci., Polym. Phys. Ed.* **1968**, *6*, 1209–1216.
- (31) Amsden, B. Solute diffusion within hydrogels. Mechanisms and models. *Macromolecules* **1998**, *31*, 8382–8395.

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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In Vitro Release of Plasmid DNA from Structurally Related Silk-Elastinlike Hydrogels

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ABSTRACT SUMMARY

The objectives of this study were to examine the influences of polymer structure, ionic strength of the media and gelation time on DNA release from hydrogels made of recombinant silk-elastinlike protein polymers (SELPs).

INTRODUCTION

Recent research in our laboratory involves the use of SELPs for matrix-mediated gene delivery^{1,2}. These block copolymers are made of silk-like (GAGAGS) and elastin-like (GVGVVP) repeats using genetic engineering techniques. SELPs of appropriate sequence and composition are liquid at room temperature. They can be mixed with plasmid DNA and form gene-laden hydrogels upon injection at body temperature due to the hydrogen bonding of silk units. Previously we reported DNA release and prolonged *in vitro* and *in vivo* transfection efficiency from one SELP analog, namely SELP 47K (Structure shown in Fig. 1A)². Factors that influenced DNA release and transfection efficiency from SELP 47K hydrogels included polymer concentration, ionic strength of the media and the hydrogel cure time. Our goal in this study is to examine the influence of *polymer structure* on DNA release under various conditions. The hypothesis is that an increase in the length of elastin repeating units in the polymer backbone, while maintaining the length of silk repeating units constant, will result in an increase in the rate of release of plasmid DNA. DNA release characteristics from hydrogels made of SELP 415K (Fig. 1B) and SELP 47K (Fig. 1A) are compared.

A. AGSGAGAGS [(GVGVVP)₄ GKGVVP (GVGVVP)₃ (GAGAGS)₄]₁₂
(GVGVVP)₄ GKGVVP (GVGVVP)₃ (GAGAGS)₂ GAG
B. AGSGAGAGS [(GVGVVP)₄ GKGVVP (GVGVVP)₁₁ (GAGAGS)₄]₇
(GVGVVP)₄ GKGVVP (GVGVVP)₁₁ (GAGAGS)₂ GAG

Fig. 1. Amino acid sequences of: A) SELP 47K; B) SELP 415K. Lys (K) residues underlined. Head and tail sequences not shown.

SELP 415K has 8 more elastin units in the monomer repeat than SELP 47K. The influences of polymer structure, hydrogel cure time and ionic strength of the media on DNA release from the hydrogels are evaluated.

EXPERIMENTAL METHODS

Preparation of DNA-containing hydrogels. SELP 415K polymers were biosynthesized and scaled up by procedures described previously³. SELP 47K was provided by Protein Polymer Technologies, Inc. (San Diego, CA). Plasmid pRL-CMV-*luc* 4.08 kbp (Promega, Madison, WI) was propagated in Novablue Singles Competent Cells (Novagen, San Diego, CA), purified using an EndoFree Giga Kit (QIAGEN Sciences, Germantown, MD) according to manufacturer's

instructions. Syringes containing frozen 12 wt% polymer solutions were thawed in a beaker containing 500 ml of water for 5 min at room temperature. Polymer solutions and plasmid DNA were gently mixed. The volume of the mixture was adjusted by addition of PBS and MilliQ water to yield 250 µg/ml plasmid DNA in 11.5 wt% polymer. The mixtures were then transferred to disposable syringes (1ml), incubated at 37 °C for 4 h and 48 h respectively. After the appropriate cure time the DNA-containing hydrogels were cut into 50 mm³ cylindrical discs using razor blade for further release studies.

Evaluation of DNA release. DNA-containing hydrogel discs were placed in 4 ml glass vials containing 3 ml of the release buffer. PBS (10mM, pH 7.4, 0.01% w/v NaN₃) buffers with total ionic strengths (µ), adjusted with NaCl of 0.016 M, 0.16 M, and 1.6 M respectively were used as release media. Vials containing hydrogels submerged in buffer were incubated at 37 °C in a shaking (120 rpm) incubator for the duration of the experiments. At predetermined time points, the buffer was sampled and replaced with fresh buffer. The amount of DNA in the release media was determined by using Picogreen DNA Quantitation Kit (Molecular Probes, Eugene, OR) and cumulative released DNA was calculated by procedure described previously¹. Results are reported as average of duplicate or triplicate hydrogels ± standard deviation.

Interaction of SELP copolymers with plasmid DNA. The interaction of both polymers containing one lysine residue per monomer repeat (Fig. 1) with plasmid DNA at various ionic strengths was evaluated by a turbidity assay. DNA: polymer complexes were formed at the charge ratios of 2:1, 1:1, and 1:2 assuming full ionization of the lysine residues and DNA phosphates at pH 7.4. The upper limit conc. of polymer used in the complexes was 0.0094 µg/µL. Polymer solutions were added drop wise to solutions containing 50 µg of plasmid pRL-CMV-*luc* in PBS with ionic strengths adjusted to 0.016 M and 0.16 M to a final volume of 137 µL. After incubation for 30 min the absorbance at 400 nm was determined by spectrophotometric techniques. Results are reported as average of triplicate ± standard deviation.

RESULTS AND DISCUSSION

Effect of polymer structure and cure time on DNA release. Fig. 2 shows the release profile of plasmid DNA from both polymer constructs at 4 h and 48 h cure times respectively. At 4 h cure time cumulative release from 415K hydrogels was higher than 47K up to day 21. This is due to the lower crosslinking density of 415K since there are an overall lower number of silk units in the polymer backbone than 47K polymers (Fig. 1). Beyond day 21 there was no significant difference in cumulative amount

of DNA released from these two hydrogels cured for 4 h suggesting that the remainder of DNA is trapped in the matrices.

An increase in cure time from 4 h to 48 h resulted in lower cumulative release for both polymers. This difference in release was more pronounced for hydrogels made from SELP 47K compared to SELP 415K.

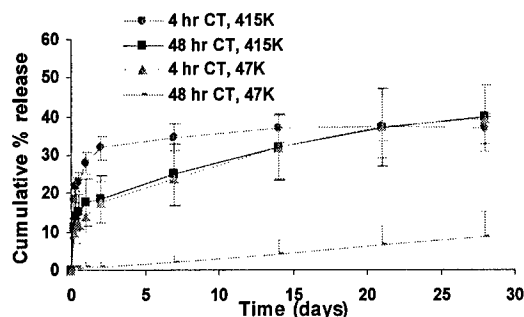


Fig. 2. Influence of polymer structure and cure time (CT) on DNA release from hydrogels at pH=7.4 and $\mu=0.16$ M.

Previously we had shown that an increase in cure time for both polymers results in a decrease in their degree of swelling^{3,4}. These physically crosslinked hydrogels are known to have a considerable amount of soluble polymers that do not participate in the hydrogel network⁴. Increase in cure time allows the interaction of more polymer chains with each other and therefore increased crosslinking density, resulting in decreased release. The reason for the pronounced effect of cure time for 47K is the higher number of silk units in the polymer backbone that allows a higher probability of inter-polymer interaction compared to 415K.

Influence of ionic strength on DNA release. The release of DNA from hydrogels made of SELP 415K showed a strong dependence on the ionic strength of the medium with the highest release observed at 0.16 M (Fig. 3).

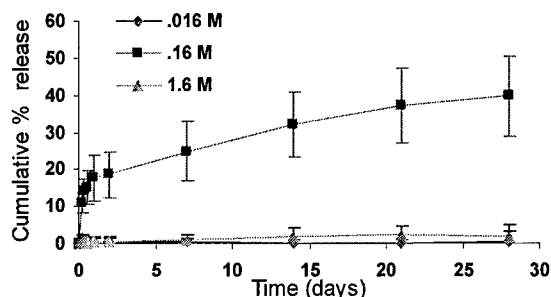


Fig. 3. Influence of ionic strength on DNA release from SELP 415K hydrogels at pH=7.4.

Previously we had shown that an increase in ionic strength for SELP 415K hydrogels results in a decrease in their degree of swelling⁴. The lower degree of swelling of SELP 415K at high ionic strength (1.6 M) and potential interaction of this polymer with plasmid DNA at low ionic strength (0.016 M) can explain the lower release of

plasmid DNA at these ionic concentrations compared to the physiologically relevant ionic strength of 0.16 M.

Influence of polymer structure and ionic strength on interaction with plasmid DNA. Recombinant techniques allow the introduction of functional amino acid residues at precise locations in the polymer backbone. SELP 415K was designed such that it has a similar molecular weight as SELP 47K but with longer elastin-like units. However there are five more lysine residues in 47K compared to 415K. This reduces the interaction of negatively charged plasmid DNA with the 415K polymer backbone. To evaluate this phenomenon we measured the absorbance of DNA/polymer complexes at low non-gel forming concentrations. The absorbance of polymer 47K mixture with plasmid DNA showed a substantial increase when prepared at low ionic strength compared to high ionic strength (Fig. 4). Mixtures of SELP 415K and plasmid DNA showed lower turbidity at both ionic strengths compared to their SELP 47K counterparts in part due to the presence of lower number of lysine residues. However other factors such as the self assembly of the polymers in response to the environmental conditions observed previously⁵ should not be ruled out.

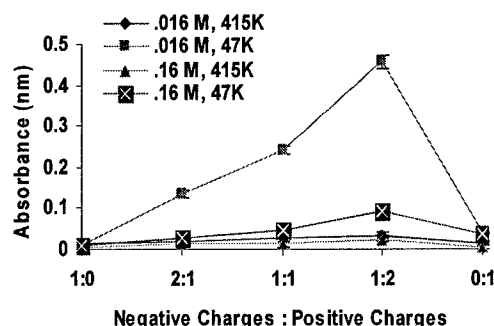


Fig. 4. Influence of polymer structure and ionic strength on the turbidity of DNA: Polymer complexes at room temperature.

CONCLUSION

Results of this study demonstrate that by controlling SELP structure at the molecular level using recombinant techniques it is possible to control plasmid DNA release profiles. This information is useful for the design and development of biodegradable and biocompatible SELP-based matrix-mediated delivery systems for breast cancer and other gene therapy applications.

REFERENCES

1. Megeed et al, *Pharm Res*, 19:954,2002.
2. Megeed & Haider et al, *J Control Rel*, 94:433,2004.
3. Haider et al, *Mol Pharm*, in press.
4. Dinerman et al, *Biomaterials*, 23:4203,2002.
5. Nagarsekar et al, *Biomacromolecules*, 4:602,2003.

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Silk-Elastinlike Hydrogels: Thermal Characterization and Gene Delivery

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Silk-elastinlike protein polymers (SELPs) are a class of genetically engineered block copolymers composed of tandemly repeated silk-like (GAGAGS) and elastin-like (GVGVP) peptide blocks. One of these polymers, SELP-47K, undergoes self-assembly in aqueous medium and has been extensively characterized for controlled drug and gene delivery applications requiring *in situ* gelation. This chapter provides a review of the thermal characterization of SELP-47K hydrogels, *in vitro* and *in vivo* delivery of plasmid DNA, and potential of controlled adenoviral gene delivery from these systems.

Introduction

The significant interest and resources that the scientific community has

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dedicated to gene therapy research have rapidly led to an interesting and somewhat conflicting state of development. Specifically, the *potential* of gene therapy in treating diseases and the significant *challenges* facing the field now occupy almost equal status in the minds of many scientists. The resources dedicated to gene therapy have been justified by the fact that it has the potential to treat a number of diseases, including those of congenital origin, such as cystic fibrosis, and those that arise from genetics gone awry, like cancer. More recently researchers have recognized the potential of gene therapy to treat infectious diseases, adding additional emphasis to the "potential" side of the equation, and providing even more motivation for addressing the "challenges." One of the greatest challenges facing the field of gene therapy is the effective delivery of genes. This fact has stimulated tremendous interest in understanding the barriers to gene delivery, and in developing delivery systems (vectors) that overcome them. Gene delivery vectors can be broadly categorized as viral or nonviral (1). Nonviral vectors, predominantly composed of cationic polymers, lipids, or peptides are generally considered to be relatively safe, but their effectiveness is limited by low transfection efficiencies (2-4). Viral vectors generally exhibit higher transfection efficiencies (5), but their usage has been limited by their actual and potential toxicities, and immunogenicity (6).

In cancer gene therapy, two primary methods have been used for the administration of viral and nonviral vectors: *systemic* - usually intravenous, or *localized* - often intratumoral. The major obstacles facing therapeutic application of systemically administered gene vectors to solid tumors are the degradation of nucleic acids in the circulation, transient transgene expression after transfection, low transfection efficiency, a large volume of distribution, and inefficient penetration of most vectors beyond the tumor periphery (1,2,7,8).

Intratumorally administered, matrix-mediated controlled delivery of genes and viruses, can potentially address some of these challenges. Encapsulating naked DNA, nonviral vectors, or viral vectors in polymeric matrices or hydrogels provides several advantages. These include (i) protection of the vector from enzymatic degradation, (ii) prolonged vector delivery, potentially increasing the duration of transgene expression and decreasing the frequency of administration, and (iii) efficient localization of transgene expression to the tumor, possibly decreasing the systemic toxicity of some transgenes (e.g., interferons, interleukins) (8,9). Various chemically synthesized and natural polymers (e.g., poly(D,L-lactide-co-glycolide)(PLGA), gelatin-alginate coacervates) have been used as matrices for controlled gene delivery (9-11). While some degree of success has been achieved, the limitations of these polymers must also be considered. Chemically synthesized polymers, generally produced by random copolymerization, have a distribution of molecular weights (polydisperse). There is little or no control over the sequence of the monomer

repeats in the polymer backbone making the prediction of their biological fate(s) such as biodistribution and biodegradation complex from a drug delivery perspective. Moreover, residual organic solvents from polymer synthesis and/or matrix fabrication may negatively impact the stability of bioactive agents incorporated in the matrix (12). Due to their complex and sometimes variable structure, most matrices composed of natural polymers are not easily customized for optimizing gene delivery for specific clinical needs.

Recombinant DNA technology has enabled the synthesis of *genetically engineered protein-based polymers* incorporating peptide blocks from naturally occurring proteins such as elastin, silk, and collagen, to produce materials not found in nature, such as silk-elastin and silk-collagen (13,14). The sequence of these polymers is encoded at the DNA level which is subsequently translated to protein polymer. DNA directed polymer synthesis can be carried out with unprecedented fidelity using the cellular machinery. This allows exquisite control over the sequence and molecular weights of these polymers, enabling the optimization of the macromolecular architecture and thereby their physicochemical properties. These polymers are stereoregular, monodisperse, biocompatible, biodegradable and their synthesis does not require the use of organic solvents. The bioprocessing technologies used for the scale-up and purification of conventional recombinant proteins have been adapted for protein-based polymers to produce large quantities of endotoxin-free materials for use *in vivo*. A number of classes of recombinant polymers have now been synthesized, including the elastin-like polymers (ELPs) (15,16), silk-like polymers (SLPs) (17,18), silk-elastinlike polymers (SELPs) (13,19), poly(glutamic acid)s (20), silk-collagen polymers (14), and others (21). Several of these polymers have been characterized for biomedical applications including controlled release (22,23), tissue repair (24), and targeted drug delivery (25). In our laboratory, we use genetically engineered protein polymers both as a research tool, to understand structure/property relationships, and as a therapeutic tool, to deliver DNA to solid tumors. This chapter reviews our efforts to use thermal methods to understand polymer properties of SELPs as they relate to drug delivery as well as their evaluation as matrices for controlled delivery of plasmid DNA and adenoviral vectors for cancer gene therapy.

Silk-Elastinlike Protein Polymers

Silk-elastinlike polymers (SELPs) are a class of genetically engineered block copolymers composed of tandemly repeated silk-like (with the amino acid sequence - GAGAGS) and elastin-like (with the amino acid sequence - GVGVP) peptide blocks. Incorporation of silk-like blocks imparts crystallinity

and enhances the cross-linking density while the incorporation of elastin-like blocks improves their aqueous solubility. The structural and mechanical properties of these polymers can be optimized by carefully altering the *ratio(s)* and *sequence(s)* of the silk-like or elastin-like units present in the monomer repeat and/or the number of monomer repeats in the polymer. This allows the use of SELPs for a variety of biomedical applications including dermal augmentation, structural materials for control of urinary incontinence and drug delivery. Recombinant techniques enable customization of the physicochemical properties of SELPs by incorporation of peptide motifs that induce gel formation, control biodegradation, and enable stimuli-sensitivity or biorecognition to suit specific drug delivery applications (26, 27). The synthesis, characterization, biocompatibility and biodegradation of SELPs have been well-established (26-29). SELP-47K, a copolymer with four silk-like blocks and seven elastin-like blocks in its primary repetitive sequence (Figure 1, bold) is soluble in aqueous medium, and undergoes an irreversible sol-to-gel transition to form hydrogels. This transition is slow at room temperature (several hours) yet occurs rapidly (within minutes) at 37 °C, making SELP-47K potentially useful for controlled delivery applications requiring *in situ* gelation.

MDPVVLQRRDWENPGVTQLNRLAAHPPFASDPMGAGSGAG
 AGS[(GVGV**P**)₄GKG**V**P(GVG**V**P)₃(GAGAGS)₄]₁₂(GVGV**P**)₄GK
 GVP(GVG**V**P)₃(GAGAGS)₂GAGAMDPGRYQDLRSHHHHHH

Figure 1. SELP-47K amino acid sequence. (*M*-methionine, *D*-aspartate, *P*-proline, *V*-valine, *L*-leucine, *Q*-glutamine, *R*-arginine, *W*-tryptophan, *E*-glutamate, *N*-asparagine, *G*-glycine, *T*-threonine, *H*-histidine, *F*-phenylalanine, *K*-lysine, *Y*-tyrosine, *S*-serine).

Analysis of SELP-47K Hydrogels by Differential Scanning Calorimetry

Hydrogel swelling, the release of solutes, phase transitions, and the chemical stability of solutes incorporated into hydrogels are potentially influenced by the nature and extent of water imbibed by the hydrogel and its interaction with the polymer matrix (30-34). For many years, a discrepancy has been noted between the (total) amount of water present in a hydrogel (determined gravimetrically) and the amount detectable by calorimetric methods during freezing. This "non-freezable" fraction of water has often been attributed to hydrogen bonding between polymeric chains and water in their vicinity

(35,36). However, recent reports indicate that other factors play a role, including polymer hydrophobicity, polymer glass transition temperature, hydrogel pore size, and the diffusivity of water in the hydrogel matrix (37,30). A certain fraction of the non-freezable water could be “bound”, with an inherently restricted molecular mobility, hindering solute diffusion through the hydrogel network (30). In an effort to better understand the interaction of SELP-47K with water, we used DSC to probe the nature and the existence of non-freezable water and its influence on solute diffusion in these hydrogel systems (38).

Amount of Non-freezable Water in SELP-47K Hydrogels. Calorimetric measurements indicated that SELP-47K hydrogels contain up to 27 wt% of non-freezable water (Figure 2A). This trend was qualitatively similar to that observed with poly(methylmethacrylate) (PMMA) hydrogels, though SELP-47K hydrogels contained 4-5 times as much non-freezable water at equivalent levels of hydration.

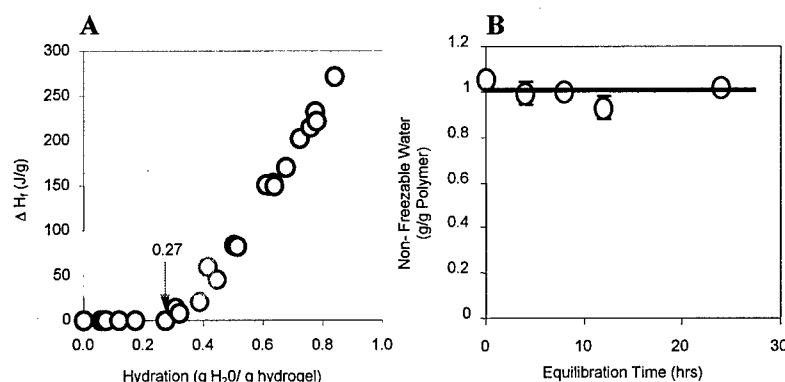


Figure 2. A. Heat of fusion of water in hydrogels as a function of hydration (g H₂O/g hydrogel). DSC did not detect any water in the hydrogels until approximately 27 wt%. Above this water content, the heat of fusion increased linearly ($r^2=0.9831$). (Adapted from *Biomacromolecules*. 2004, 5, 793-797. Copyright 2004 Am. Chem. Soc) B. Effect of equilibration time at -15 °C on the amount of non-freezable water in hydrogels with 87 wt% water. Each data point is the mean $\pm$ SD of triplicates. (Reproduced from *Biomacromolecules*. 2004, 5, 793-797. Copyright 2004 Am. Chem. Soc)

This discrepancy may be attributed to the relatively high hydrophobicity of SELP-47K (22.4% valine, 12.2% alanine). Previous studies have shown that increasing the fraction of hydrophobic polymer in an interpenetrating polymer network resulted in an increase in the non-freezable water content (37). Equilibration of PMMA hydrogels at -15 °C for up to 14 hours resulted in a

significant decrease in the amount of non-freezable water (39). This observation was explained by considering the restricted diffusion of water molecules during freezing and the accompanying transition from a rubbery to a glassy state. By contrast, equilibration of SELP-47K hydrogels for up to 24 hours did not decrease the amount of non-freezable water (Figure 2B), indicating that the intrinsic properties of the polymer (i.e., hydrophobicity) may be more important than kinetic factors.

Heat Capacity of Non-Freezable Water. Truly bound water can be considered to be in a different thermodynamic state than bulk water, with inherent restriction in its molecular mobility due to hydrogen bonding between water molecules themselves and/or between water molecules and polymer chains (40,41).

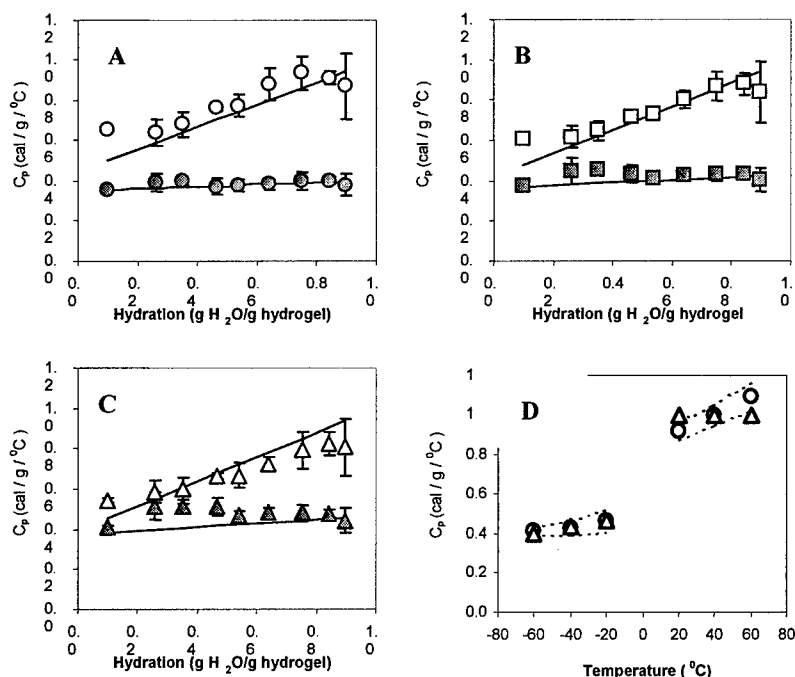


Figure 3. Effect of hydrogel hydration (g H₂O/ g polymer) on C_p at A. 60 °C (open circle) and -60 °C (filled circle) B. 40 °C (open box) and -40 °C (filled box) C. 20 °C (open triangle) and -20 °C (filled triangle) D. Calculated (O) partial heat capacity of water in SELP-47K hydrogels, at various temperatures, versus accepted values in the literature (Δ). Dotted lines are 95% confidence interval of data set with 45 data points for each temperature. (Reproduced from *Biomacromolecules*. 2004, 5, 793-797. Copyright 2004 Am. Chem. Soc)

To evaluate the molecular mobility of non-freezable water, the heat capacities of SELP-47K hydrogels were measured at different temperatures and levels of hydration, and compared to those calculated from Equation 1,

$$\text{Equation(1)} \quad C_{p(\text{hydrogel})} = f_{\text{water}} C_{p(\text{water})} + f_{\text{polymer}} C_{p(\text{polymer})}$$

where C_p is the heat capacity of the hydrogel, water, or polymer, and f is the fraction (g/g hydrogel) of the water or polymer. Equation 1 is applicable to a binary system composed of a water phase and a polymer phase. The measured hydrogel heat capacities fit well with the predictions of Equation 1 (Figures 3A-C). Deviations from the predictions of Equation 1 were observed at levels of hydration between 0.2 and 0.4 and were attributed to a previously observed elastin glass transition (42). Calculated values for the heat capacity of water in the hydrogels closely correspond with literature values for bulk water (Figure 3D), indicating that it is unlikely that all of the non-freezable water in SELP-47K hydrogels is "bound."

The DSC studies demonstrate the presence of up to 27 wt% non-freezable water in SELP-47K hydrogels, which is not substantially affected by equilibration of the hydrogels at -15 °C. The presence of non-freezable water in SELP-47K hydrogels may be influenced by the hydrophobicity of the polymer and/or the presence of nanocavities in the hydrogel network (36,37), as observed in other protein-based systems. However, the indirect nature of calorimetric measurements precludes conclusions about the primary origins of the non-freezable water and its influence on the macromolecular diffusion in these hydrogels. Further structural characterization by pulse field gradient nuclear magnetic resonance (NMR) experiments could provide insight into the nature of water in these systems. This could aid in the understanding of the diffusion of solutes (genes) through their matrices, since non-freezable water is not available for solute diffusion through the pores of the hydrogel.

Gene Delivery from SELP-47K Hydrogels

As previously described, SELP-47K undergoes spontaneous self assembly in aqueous solution to form hydrogels. This self-assembly occurs through physical crosslinking of the silk-like units by hydrogen bonding, and it is accelerated when the polymer is transferred from room temperature to body temperature. The kinetics of this sol-to-gel transition allow solutions of polymer and bioactive agents with aqueous solubility to be prepared at room temperature and injected through a small gauge needle, with hydrogel matrices formed *in*

situ within minutes. The hydrogels release the incorporated bioactive agents through diffusion and/or matrix degradation (22,23). While the applications of SELP-mediated controlled gene delivery are numerous, we have focused our efforts on the controlled delivery of plasmid DNA and adenoviral vectors to solid tumors.

Controlled Delivery of Plasmid DNA from SELP-47K

As mentioned previously, the ability to obtain sufficient transfection *efficiency* and *duration* are two major challenges facing viral and nonviral gene delivery. Matrix-mediated gene delivery can address these issues by controlling the delivery of nucleic acids or gene vectors over prolonged periods of time. An additional benefit that arises from this approach is the precise spatial localization of the delivered vectors. The matrix also has the potential to protect nucleic acids or gene vectors from degradation. In an effort to evaluate the potential of SELPs as matrices for the delivery of plasmid DNA we have evaluated the release of DNA from SELP-47K matrices *in vitro*, and the delivery of DNA from SELP-47K matrices to solid tumors *in vivo* (43).

***In vitro* Release of Plasmid DNA from SELP-47K Hydrogels**

SELP hydrogel systems undergo *in situ* gelation and can be molded to a variety of shapes including cylinders, disks and films. We chose to use a cylindrical shape for our hydrogel systems for *in vitro* evaluation(s), as our experience with these polymers has been that cylindrical hydrogels could be made with minimal inter-sample variability (size and shape) and waste. This also allowed us to adapt established diffusion models from literature for the evaluation of their release properties. The influence of plasmid DNA conformation and size, polymer concentration, hydrogel geometry and cure time, and DNA concentration, on DNA release from cylindrical SELP-47K hydrogels were evaluated over 28 days (23,43). Hydrogels were prepared by mixing aqueous solutions of DNA and SELP-47K at room temperature, and gel formation was induced by incubation at 37 °C. Hydrogel were cut and the release studies were carried out. The diffusivity of DNA from these hydrogels occurring in the two dimensions (axial and radial) was calculated using Equation 2 (44,45),

Equation(2)

$$\frac{M_t}{M_\infty} = 1 - \frac{32}{\pi^2} \sum_{i=1}^{\infty} \frac{1}{\alpha_i^2} \exp\left(-\frac{\alpha_i^2}{r^2} D_e t\right) \sum_{j=0}^{\infty} \frac{1}{(2j+1)^2} \exp\left(-\frac{(2j+1)^2 \pi^2}{h^2} D_e t\right)$$

where r is the radius of a cylinder, h is the height of a cylinder, M_t is the cumulative amount of solute released at time t , M_∞ is the amount released as $t \rightarrow \infty$, D_e is the average effective intra-gel diffusivity of the solute, and α_i are the roots of the zero-order Bessel function, $J_0(\alpha_i) = 0$. D_e was estimated from a nonlinear fit of Equation 2 to the experimental release data, using *Mathematica* software.

Effect of Plasmid Conformation and Size on In Vitro Release. Restriction enzymes were employed to produce DNA predominantly in the linear, open-circular, or supercoiled forms. The cumulative percentage of each form of DNA released from SELP-47K hydrogels was in the order of linear, supercoiled, and open-circular. The open-circular form was practically not released, probably due to its impalement on the polymer chains (Table 1) (43). Plasmids were released in a size-dependent manner, from 2.6 to 11 kilobases (kb) (Table 1) (43).

Table 1. Influence of size and conformation of plasmid DNA and geometry of hydrogel on release

Plasmid	Geometry	Plasmid size (kbp) ^a	Hydrogel surface area (cm ²)	De ^b (cm ² /sec)
pUC18	Disc	2.60	0.74	2.55±0.51 × 10 ⁻¹⁰
<i>l</i> -pRL-CMV ^c	Disc	4.08	0.74	1.94 ±0.27 × 10 ⁻¹⁰
<i>sc</i> -pRL-CMV ^d	Disc	4.08	0.74	8.90±0.12 × 10 ⁻¹¹
<i>oc</i> -pRL-CMV ^e	Disc	4.08	0.74	1.96±0.83 × 10 ⁻¹³
pRL-CMV	Large Disc	4.08	1.70	1.76±0.28 × 10 ⁻¹⁰
pRL-CMV	Cylinder	4.08	1.07	9.23±0.15 × 10 ⁻¹¹
pCFB-EGSH-Luc	Disc	8.50	0.74	3.09±0.43 × 10 ⁻¹¹
pFB-ERV	Disc	11.00	0.74	1.70±0.52 × 10 ⁻¹²

^a *kbp* = kilobase pairs, ^b D_e = average effective diffusivity of plasmid DNA in hydrogel determined by nonlinear fit of release data to Equation 2, ^c *l* = linearized, ^d *sc* = supercoiled, ^e *oc* = open-circular. Samples in which the conformation is not denoted were a mixture of the three conformations, primarily supercoiled (Reproduced with permission from J. Cont. Rel. 2004, 94, 433-445. Copyright 2004 Elsevier Science).

Previous studies have showed that increasing the polymer concentration and hydrogel cure time decreases the rate of DNA release, while increasing the DNA concentration from 50 to 250 $\mu\text{g/ml}$ did not influence the rate of release (23)

Effect of Hydrogel Geometry on In Vitro Release. The analysis of the influence of hydrogel geometry on release may be useful in determining where and how to inject SELP matrices. The effect of hydrogel geometry on DNA release was studied by preparing cylinder-like and disc-like hydrogels both having equivalent volumes. Disc-like hydrogels released DNA faster than their cylindrical analogues (Table 1) (43). This was attributed to their larger surface to volume ratio and was accurately described by fitting the release data to Equation 2.

In Vitro Bioactivity of Encapsulated Plasmid DNA. Plasmid DNA incorporated in SELP-47K hydrogels retained bioactivity, even after incubation in PBS, at 37 °C, for 28 days (43). Effective diffusivities of plasmid DNA described above can be correlated with the amount released from SELP-47K hydrogels. These results demonstrate that release is dependent on the *size* and *conformation* of plasmid DNA, the *geometry* and *cure time* of the hydrogel, and the *concentration* of the polymer.

From a clinical perspective, optimal delivery rate(s) for different cancer gene therapy applications vary dependent on factors such as tumor stage, its aggressiveness (metastatic or non-metastatic), the type and location of the tumor, among others. The *in vitro* studies described above demonstrate the potential of *optimizing* the release kinetics of plasmid DNA of different sizes from SELP-based systems by carefully altering their polymer concentration and cure time to suit specific gene delivery applications.

Delivery of Plasmid DNA from SELP-47K Hydrogels to Solid Tumors

In an effort to establish the feasibility of using SELP-47K hydrogels to deliver plasmid DNA to solid tumors, we investigated the intratumoral delivery of a reporter plasmid (*Renilla* luciferase) to subcutaneous tumors in a murine (athymic *nu/nu*) model of human breast cancer (MDA-MB-435 cell line) (43). SELP-47K / DNA solutions containing 4, 8, or 12 wt% polymer and 70 μg DNA per 100 μl were injected directly into the tumors. At predetermined time points, animals were euthanized, tumors were resected and homogenized, and luciferase expression was assayed. To evaluate the potential of SELP-47K to

localize delivery to the tumor, we also evaluated the transfection of the skin approximately 1 cm around each tumor.

Intratumoral injection of 4 or 8 wt% hydrogels resulted in significantly enhanced tumor transfection for up to 21 days when compared to naked DNA, while 12 wt% matrices enhanced transfection up to 3 days (Figure 4A). The levels of tumor transfection mediated by the three concentrations of polymer were statistically equivalent until 7 days, when the 4 and 8 wt% matrices were both more effective than 12 wt% and naked DNA (Figure 4A). The high levels of tumor transfection observed initially (up to 7 days) in case of 12 wt% hydrogels is probably due to the burst release upon administration. The irregular (atypical) pore distribution in the perimeter of the hydrogel could lead to a faster release of the “loosely entrapped” DNA from their surfaces irrespective of polymer concentration and potentially obscure any actual differences in their release rates occurring initially.

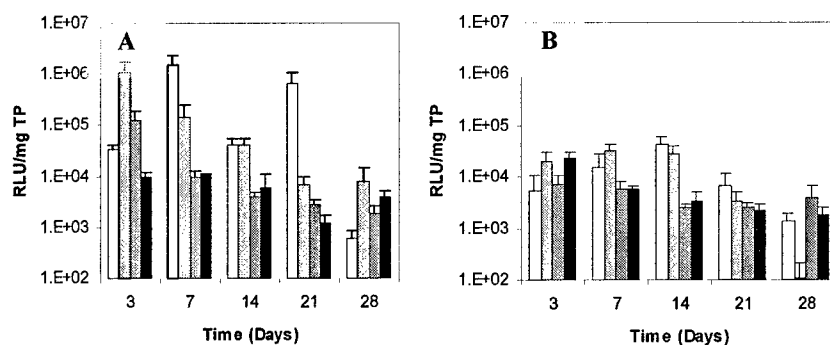


Figure 4. Expression of *Renilla luciferase* in tumors (A) and in skin directly surrounding the tumors (B), after intratumoral injection. Bars represent 4 wt% polymer (white), 8 wt% polymer (light gray), 12 wt% polymer (dark gray), and naked DNA without polymer (black). Each bar represents the mean $\pm$ SEM for $n=4$ or $n=5$ samples. (Reproduced with permission from J. Cont. Rel. 2004, 94, 433-445, Copyright 2004 Elsevier Science)

The higher transfection observed in case of the 4 and 8 wt% hydrogels up to day 21 is consistent with *in vitro* release data (23) indicating lower release rates at higher polymer concentrations, due the entrapment of DNA within the matrices. The delivery of DNA from 4, 8, and 12 wt% polymer hydrogels resulted in a mean 142.4-fold, 28.7-fold, and 3.5-fold increase in tumor transfection, respectively, compared with naked DNA over the entire 28 day period.

Overall, the mean tumor transfection was 42.0, 27.2, and 4.6 times greater than skin transfection for 4, 8, and 12 wt% hydrogels, respectively, over the entire 28 day period, compared to a 1.3 fold difference between tumor and skin transfection for naked DNA (Figure 4A and 4B). This indicates that the enhancement of transfection was largely restricted to the tumor, suggesting a spatial localization benefit from the matrix. The decrease in transfection over time can be attributed to a corresponding decrease in the release of plasmid due to entrapment, or depletion due to release and/or degradation.

Controlled Delivery of Adenoviral Vectors from SELP-47K

As previously mentioned, viral vectors are generally associated with relatively high transfection efficiencies, but their clinical application is limited by safety concerns (1,6). To reduce the risk of oncogene disruption by integrating viral vectors such as retroviruses, non-integrating viral vectors, such as adenoviruses have been widely explored (5). However, non-integrating viral vectors are similar to nonviral vectors in that they suffer from limitations in the duration of transgene expression, which may be exacerbated by their rapid clearance. Furthermore, viral vectors face the additional challenge of escaping, or minimizing, the body's immune response, which can increase the rate of clearance, further reducing the duration of transgene expression, and lead to serious systemic or local inflammation.

Encapsulation of viral vectors in hydrogels may begin to address some of these challenges. The controlled release of small amounts of viral vectors from a matrix reservoir may increase the duration of transfection while simultaneously reducing the amount of antigen available for sampling and detection by the immune system. Furthermore, encapsulation of a viral vector in a matrix may protect it from degradation though the potential to include stabilizers in the matrix remains to be explored. Based on the promising results obtained when delivering plasmid DNA from SELP-47K, we sought to investigate the potential of using this polymer as a matrix for the controlled delivery of adenoviral vectors.

***In vitro* Release of Adenoviral Vectors from SELP-47K Hydrogels**

Adenoviral DNA is encapsulated in a protein coat that is essential for its protection. These viral coat proteins may potentially interact with the amino-acid backbone of the SELP-47K polymer, leading to an altered viability and transfection efficiency. In an effort to evaluate the long-term viability of

adenoviruses incorporated in SELP-47K hydrogels we have carried out an *in vitro* release and bioactivity study with an adenovirus containing the gene for green fluorescent protein (AdGFP) (43). SELP-47K/AdGFP solutions were prepared at 4, 8, and 11.3 wt% polymer. The mixtures were allowed to gel and hydrogel discs were placed in a phosphate buffered saline (PBS) release medium. At predetermined time points, release medium was collected and used to transfect HEK-293 cells. Transfection was observed up to 22 days with AdGFP released from the 4 wt% hydrogels, indicating that viable AdGFP continued to be released in this time frame. The number of transfected cells obtained with the virus released from the 8 wt% hydrogel was less than that obtained from the 4 wt% hydrogel, and the 11.3 wt% hydrogel did not release any detectable adenovirus after the first day. These results demonstrate that adenoviral release can be controlled over a continuum by controlling polymer composition and that the bioactivity of the released adenoviruses decreases with time.

Influence of Adenovirus on the Swelling Ratio of SELP-47K Hydrogels

Adenoviral vectors are typically delivered in large amounts (about 10^6 to 10^{11} pfu) in the clinic to produce therapeutic effects. Given the protein nature of adenoviral coats they could potentially interact with the polymeric chains and influence the degree of swelling of the hydrogel system. The equilibrium swelling ratio (q = weight of wet hydrogel / weight of dry hydrogel) is an indication of the pore size and mechanical properties of a hydrogel, and can often be used to predict the rate of release of solutes. Swelling studies were performed to evaluate the influence of adenoviral incorporation and polymer concentrations on the swelling properties of SELP-47K hydrogels over time (46). Adenovirus-loaded hydrogels were fabricated (Figure 5) and the swelling ratio was measured up to 15 days by previously described methods (47).

Results indicate that the incorporation of adenovirus does not significantly alter the swelling properties over a period of 15 days (Figure 6A-B). Consistent with previous observations (in the absence of adenovirus), the swelling ratio of the hydrogels with and without adenovirus decreased as the polymer concentration increased (Figure 6A) (46). At given polymer concentrations (4, 8, and 11.7 wt%), the degrees of swelling of the hydrogels were not influenced by the incubation time (Figure 6B). In the range studied (10^4 , 10^6 and 10^8 pfu / 50 μ l hydrogels), an increase in the amount of adenoviruses did not significantly change the degree of swelling of the hydrogels up to 15 days (Figure 6B).

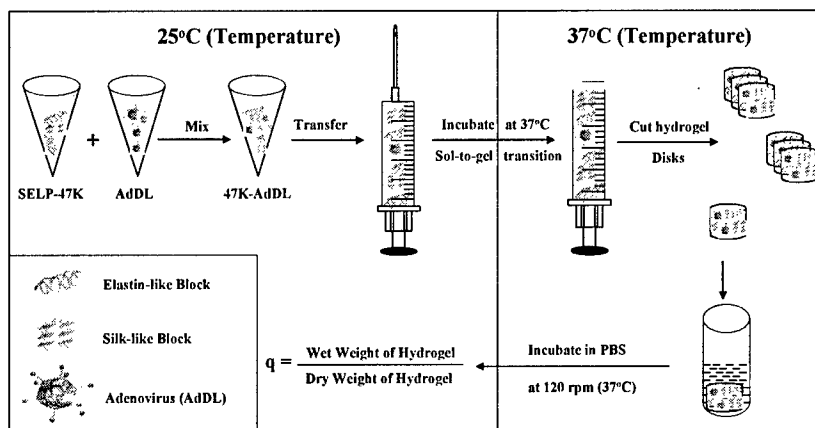


Figure 5. SELP-47K and the adenovirus DL312 (AdDL) were mixed to obtain desired compositions, and were drawn in 1 ml syringes at room temperature (25 °C). Syringes were incubated at 37 °C for 4 hours to induce gel formation. Hydrogel discs were cut using a sterile razor blade, placed in 1 ml PBS media, and mildly agitated at 120 rpm at 37 °C. Hydrogels were retrieved and the swelling ratio (q) was determined in triplicate at predetermined time points.

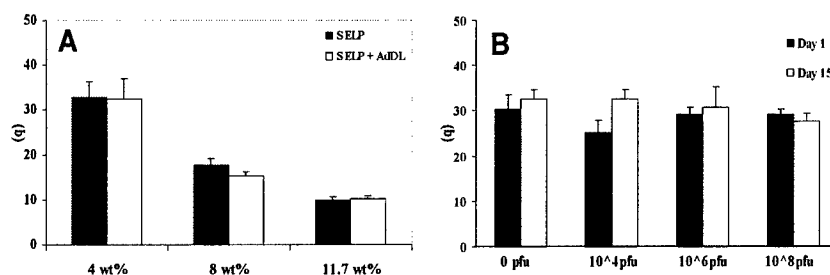


Figure 6 A. Influence of polymer concentration (wt%) and the presence of adenovirus (10^6 pfu/50 μ l hydrogel) on degree of swelling (q , mean $\pm$ SD) at day 15. B. Influence of adenoviral concentration on the degree of swelling (q , mean $\pm$ SD) of 4 wt% hydrogels over time (Reproduced from reference 46).

While the potential interactions of SELP copolymers with adenoviral particles need to be further characterized, these studies indicate that the network properties of SELP-47K are largely unaffected by the presence of adenovirus.

Release and bioactivity studies demonstrate that bioactive adenovirus is released from SELP-47K hydrogels for up to 22 days. The swelling studies suggest a predictable release of adenovirus from these hydrogels, due to an absence of substantial changes in network properties. The next logical steps are quantifying the amount of adenovirus released, the proportion that is bioactive, and the feasibility of using SELP-47K hydrogels for adenoviral gene delivery *in vivo*.

Summary and Conclusion

Thermal characterization of SELP-47K hydrogels indicates that a fraction of the water in the hydrogel is non-freezable in nature, but with a heat capacity that is similar to bulk water. The nature of this water and the pore sizes of the hydrogels need to be further investigated to allow correlation of these parameters with DNA release. *In vitro* and *in vivo* studies of the release of plasmid DNA suggest that by carefully altering the polymer concentration and hydrogel cure time, one may optimize the DNA release kinetics. The *in vitro* bioactivity and swelling studies with adenoviruses demonstrate the feasibility of using SELP-47K hydrogels for controlled adenoviral delivery. The biodegradability, and biocompatibility of the SELPs, and the ability to control their structure by genetic engineering techniques shows promise for the design and development of novel matrices for localized and controlled nonviral and viral gene delivery.

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References

1. Verma, I.M.; Somia, N. Gene therapy-promises, problems and prospects. *Nature*. 1997, 389, 239-242.
2. Luo, D.; Saltzman, W.M. Synthetic DNA delivery systems. *Nat. Biotechnol.* 2000, 18, 33-37.
3. Liu, F.; Huang, L. Development of non-viral vectors for systemic gene delivery. *J. Controlled Rel.* 2002, 78, 259-266.

4. Guo, X.; Szoka Jr., F.C. Chemical approaches to triggerable lipid vesicles for drug and gene delivery. *Acc. Chem. Res.* 2003, 36, 335-341.
5. Rots, M.G.; Curiel, D.T.; Gerritsen, W.R.; Haisma, H.J. Targeted cancer gene therapy: the flexibility of adenoviral gene therapy vectors. *J. Controlled Rel.* 2003, 87, 159-165.
6. Worgall, S.; Wolff, G.; Falck-Pedersen, E.; Crystal, R.G. Innate immune mechanisms dominate elimination of adenoviral vectors following in vivo administration. *Hum. Gene Ther.* 1997, 8, 37-44.
7. Davidson, B.L.; Hilfinger, J.M.; Beer, S.J. Extended release of adenovirus from polymer microspheres: potential use in gene therapy for brain tumors. *Adv. Drug Deliv. Rev.* 1997, 27, 59-66.
8. Jain, R. K. Delivery of molecular and cellular medicine to tumors. *Adv. Drug Deliv. Rev.* 1997, 26, 79-90.
9. Doukas, J.; Chandler, L.A.; Gonzalez, A.M.; Gu, D.; Hoganson, D.K.; Ma, C.; Nguyen, T.; Printz, M.A.; Nesbit, M.; Herlyn, M.; Crombleholme, T.M.; Aukerman, S.L.; Sosnowski, B.A.; Pierce, G.F. Matrix immobilization enhances the tissue repair activity of growth factor gene therapy vectors. *Hum. Gene Ther.* 2001, 12, 783-798.
10. Beer, S.J.; Matthews, C.B.; Stein, C.S.; Ross, B.D.; Hilfinger, J.M.; Davidson, B.L. Poly (lactic-glycolic) acid copolymer encapsulation of recombinant adenovirus reduces immunogenicity in vivo. *Gene Ther.* 1998, 5, 740-746.
11. Kalyanasundaram, S.; Feisnstein, S.; Nicholson, J.P.; Leong, K.; Garver Jr., R.I. Coacervate microspheres as carriers of recombinant adenovirus. *Cancer Gene Therapy* 1999, 6, 107-112.
12. van de Weert, M.; Hennink, W. E.; Jiskoot, W. Protein instability in poly(lactic-co-glycolic acid) microparticles. *Pharm. Res.* 2000, 17, 1159-1167.
13. Cappello, J.; Crissman, J.; Dorman, M.; Mikolajczak, M.; Textor, G.; Marquet, M.; Ferrari, F. Genetic engineering of structural protein polymers. *Biotechnol. Prog.* 1990, 6, 198-202.
14. Teule, F.; Aube, C.; Abbott, A.G; Ellison, M.S. Production of customized novel fiber proteins in yeast (*Pichia pastoris*) for specialized applications: in Proceedings of the 3rd International Conference on Silk, Montreal, Canada, June 16-19, 2003.
15. McPherson, D.T.; Morrow, C; Minehan, D.S.; Wu, J; Hunter, E; Urry, D.W. Production and purification of a recombinant elastomeric polypeptide, G-(VPGVG)19-VPGV, from *Escherichia coli*. *Biotechnol. Prog.* 1992, 8, 347-52.
16. Meyer, D.E.; Chilkoti, A. Genetically encoded synthesis of protein-based polymers with precisely specified molecular weight and

- sequence by recursive directional ligation: Examples from the elastin-like polypeptide system. *Biomacromolecules*. 2002, 3, 357-367.
17. Arcidiacono, S.; Mello, C.; Kaplan, D.; Cheley, S.; Bayley, H. Purification and characterization of recombinant spider silk expressed in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* 1998, 49, 31-38.
 18. J.T. Prince, K.P. McGrath, C.M. DiGirolamo, D.L. Kaplan, Construction, cloning, and expression of synthetic genes encoding spider dragline silk. *Biochemist.* 1995, 34, 10879-10885.
 19. Nagarsekar, A.; Crissman, J.; Crissman, M.; Ferrari, F.; Cappello, J.; Ghandehari, H. Genetic engineering of stimuli-sensitive silk-elastinlike protein block copolymers. *Biomacromolecules*. 2003, 4, 602-607.
 20. Zhang, G.; Fournier, M.J.; Mason, T.L.; Tirrell, D.A. Biological synthesis of monodisperse derivatives of poly(α ,L-glutamic acid): model rodlike polymers. *Macromolecules*. 1992, 25, 3601-3603.
 21. Haider, M.; Megeed, Z.; Ghandehari, H. Genetically engineered polymers: status and prospects for controlled delivery. *J. Controlled Rel.* 2004, 95, 1-26.
 22. Dinerman, A.A.; Cappello, J.; Ghandehari, H.; Hoag, S.W. Solute diffusion in genetically engineered silk-elastinlike protein polymer hydrogels. *J. Controlled Rel.* 2002, 82, 277-287.
 23. Megeed, Z.E.; Cappello, J.; Ghandehari, H. Controlled release of plasmid DNA from a genetically engineered silk-elastinlike hydrogel. *Pharm. Res.* 2002, 19, 954-959.
 24. Betre, H.; Setton, L.A.; Meyer, D.E.; Chilkoti, A. Characterization of a genetically engineered elastin-like polypeptide for cartilaginous tissue repair. *Biomacromolecules*. 2002, 3, 910-916.
 25. Meyer, D.E.; Shin, B.C.; Kong, G.A.; Dewhirst, M.W.; Chilkoti, A. Drug targeting using thermally responsive polymers and local hyperthermia. *J. Controlled Rel.* 2001, 74, 213-224.
 26. Megeed, Z.; Cappello, J.; Ghandehari, H. Genetically engineered silk-elastinlike protein polymers for controlled drug delivery. *Adv. Drug Deliv. Rev.* 2002, 54, 1075-1091.
 27. Ferrari, F.; Richardson, C.; Chambers, J.; Causey, S.C.; Pollock, T.J.; Cappello, J.; Crissman, J.W. U.S. Patent 5,243,038, USA, Protein Polymer Technologies, Inc. 1993.
 28. Nagarsekar, A.; Crissman, J.; Crissman, M.; Ferrari, F.; Cappello, J.; Ghandehari, H. Genetic synthesis and characterization of pH- and temperature-sensitive silk-elastinlike protein block copolymers. *J. Biomed. Mater. Res.* 2002, 62, 195-203.
 29. Cappello, J.; Crissman, J.W.; Crissman, M.; Ferrari, F.A.; Textor, G.; Wallis, O.; Whitledge, J.R.; Zhou, X.; Burman, D.; Aukerman, L.; Stedronsky, E.R. In-situ self-assembling protein polymer gel systems

- for administration, delivery, and release of drugs. *J. Controlled Rel.* 1998, 53, 105-117.
30. Hoffman, A.S. Hydrogels for biomedical applications. *Adv. Drug Deliv. Rev.* 2002, 43, 3-12.
 31. Lele, A.K.; Hirve, M.M.; Badiger, M.V.; Mashelkar, R.A. Predictions of bound water content in poly(*N*-isopropylacrylamide) gel. *Macromolecules.* 1997, 30, 157-159.
 32. Netz, P.A.; Dorfmueller, T. Computer simulation studies of anomalous diffusion in gels: Structural properties and probe-size dependence *J. Chem. Phys.* 1995, 103, 9074-9082.
 33. Yoshioka, S.; Aso, Y.; Terao, T. Effect of water mobility on drug hydrolysis rates in gelatin gels. *Pharm. Res.* 1992, 9, 607-612.
 34. Shibukawa, M.; Aoyagi, K.; Sakamoto, R.; Oguma, K. Liquid chromatography and differential scanning calorimetry studies on the states of water in hydrophilic polymer gel packings in relation to retention selectivity *J. Chromatography.* 1999, 832, 17-27.
 35. Higuchi, A.; Iijima, T. D.s.c. investigation of the states of water in poly(vinyl alcohol-co-itaconic acid) membranes. *Polymer* 1985, 26, 1833-1837.
 36. Liu, W.G.; De Yao, K. What causes the unfrozen water in polymers: hydrogen bonds between water and polymer chains? *Polymer* 2001, 42, 3943-3947.
 37. Tian, Q.; Zhao, X.; Tang, X.; Zhang, Y. Fluorocarbon-containing hydrophobically modified poly(acrylic acid) gels: Gel structure and water state. *J. Appl. Polym. Sci.* 2003, 89, 1258.
 38. Megeed, Z.; Cappello, J.; Ghandehari, H. Thermal analysis of water in silk-elastinlike hydrogels by differential scanning calorimetry. *Biomacromolecules.* 2004, 5, 793-797.
 39. Bouwstra, J.A.; Salomonsdevries, M.A.; Vanmilttenburg, J.C. The thermal behavior of water in hydrogels. *Thermochim Acta.* 1995, 248, 319-327.
 40. Ishikiriya, K.; Todoki, M. Heat capacity of water in poly(methyl methacrylate) hydrogel membrane for an artificial kidney. *J. Polym. Sci. Pol Phys.* 1995, 33, 791-800.
 41. Hoeve, C.A.; Tata, A.S. The structure of water absorbed in collagen. *J. Phys. Chem.* 1978, 82, 1660-1663.
 42. Kakivaya, S.R.; Hoeve, C.A. The glass point of elastin. *Proc. Natl. Acad. Sci.* 1975, 72, 3505-3507.
 43. Megeed, Z.; Haider, M.; Li, D.; O'Malley Jr., B.W.; Cappello, J.; Ghandehari, H. In vitro and in vivo evaluation of recombinant silk-elastinlike hydrogels for cancer gene therapy. *J. Controlled Rel.* 2004, 94, 433-445.

44. Fu, J. C.; Hagemer, C.; Moyer, D. L. A unified mathematical model for diffusion from drug-polymer composite tablets. *J. Biomed. Mater. Res.* 1976, 10, 743-758.
45. Siepmann, J.; Ainaoui, A.; Vergnaud, J. M.; Bodmeier, R. Calculation of the dimensions of drug-polymer devices based on diffusion parameters. *J. Pharm. Sci.* 1998, 87, 827-832.
46. Dandu, R.; Li, D.; O'Malley Jr., B.W.; Cappello, J.; Ghandehari, H. Matrix-mediated adenoviral gene delivery with recombinant silk-elastinlike hydrogels: in Proceedings of the 31st Annual Meeting and Exposition of the Controlled Release Society, Honolulu, Hawaii, June 12-16, 2004.
47. Dinerman, A.; Cappello, J.; Ghandehari, H.; Hoag, S.W. Swelling characteristics of silk-elastinlike hydrogels. *Biomaterials*. 2002, 23, 4203-4210.