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Yang Feng

Biosynthesis and Characterization of Biodegradable Materials Based on Elastin Mimetic Polypeptide

By

Yang Feng

Department of Chemistry

Vincent P. Conticello
Advisor

Elliott L. Chaikof
Committee Member

David Lynn
Committee Member

Accepted:

Dean of Graduate School

Date

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B.S., Huazhong University of Science and Technology, 2000

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An Abstract of

A thesis submitted to the Faculty of the Graduate School of

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2007

Abstract

Genetically directed synthesis provides a method to produce proteins with specific sequence and molecular size. Functional groups such as cell binding ligands, biodegradable substrates, and cell growth factors could be specifically incorporated into the target protein to make materials with novel properties.

The work described herein is focused on biosynthesis and characterization of biodegradable materials based on elastin mimetic polypeptide. Elastin mimetic tri-block proteins undergo a sol-gel transition at a lower critical solution temperature T_t, above which the protein solution is turned into virtually cross-linked hydrogel consisting of endblock aggregates bridged by hydrated midblocks. In order to make the hydrogel biodegradable, plasmin-cleavable sites were incorporated into the midblock peptide sequence.

Proteolytic cleavage experiments showed that the tri-block protein with plasmin-cleavable sites could be degraded by plasmin (0.5 U/mL) both in solution (1 mg/mL) and in the hydrogel state (5 wt%). The degradation rate of the hydrogel was affected by the hydrogel concentration. A 10 wt% hydrogel was not degraded by plasmin when tested under the same conditions. Differential scanning calorimetry (DSC) experiments indicated the sol-gel transition temperature of the tri-block protein was 13.0 ^{o}C, which is lower than a previously reported tri-block protein with shorter endblocks. The results indicated that the properties of the tri-block protein such as the degradation rate and

phase transition temperature could be conveniently adjusted by varying the hydrogel concentration or the sequence and size of the building blocks, which makes it a promising biomaterial as an erodible matrix in tissue engineering applications.

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Table of Contents

Introduction 1

Experimental Methods 13

Results and Discussion 25

Conclusion 34

References 36

List of Figures

Figure 1.	Degradation of GGLGPAGGK-derivatized PEG hydrogels in solutions containing collagenase. ● 2 mg/mL collagenase; ○ 0.2 mg/mL collagenase; ▲ no collagenase.	4
Figure 2.	Scheme for preparing crosslinked hydrogel incorporated with both integrin- binding substrates and MMP-sensitive sequences.	7
Figure 3.	(*A*) Fibroblasts radially invaded the adhesive and MMP-sensitive synthetic hydrogel matrix (bar = 250 μm). (*B*) Migration of spindle-like-shaped fibroblasts occurred in a cohort manner (bar = 150 μm).	9
Figure 4.	Cryo-HRSEM images of vitrified specimens of concentrated aqueous (25 wt %) solution of proteins **1** (A) and **2** (B) prepared via rapid cryo- immobilization from gel specimens equilibrated at 25 ˚C.	12
Figure 5.	(A) Amino acid sequence of the S1VRN monomer. (B) Sequence of the DNA encoding the S1VRN monomer.	16
Figure 6.	Plasmid map of vector pZErO^{TM}-2. (http://www.invitrogen.com)	16
Figure 7.	Scheme for gene construction for the tri-block polypeptide with plasmin cleavable sites incorporated into the central block.	18
Figure 8.	Sequence of the DNA cassette encoding the decahistidine tag adaptor.	20
Figure 9.	Plasmid map of vector pET-24a-d. (http://www.novagen.com)	21
Figure 10.	S1VRN DNA monomer (78 bp) on 4% NuSieveGTG agarose gel.	25
Figure 11.	S1VRN DNA multimers on 1% agarose gel.	26
Figure 12.	Double digestion of the recombinant plasmid containing a 800 bp S1VRN multimer with *Hind* III and *Bam*H I.	27

Figure 13. Double digestion of the recombinant plasmid containing a 1600 bp S1VRN multimer with *Hind* III and *Bam*H I. 27

Figure 14. SDS-PAGE analysis of the small-scale expression of the tri-block protein. From left to right: Protein marker, Before IPTG induction, t = 1 hr w/o induction, t = 2 hrs w/o induction, t = 1 hr with induction, t = 2 hrs with induction. 28

Figure 15. Degradation of S3-S1VRN1600-S3 by plasmin in solution at 18 ^{o}C analyzed by Western blot. No plasmin was added to control solution. 30

Figure 16. Degradation of 10 wt% hydrogel made from S3-S1VRN1600-S3 by plasmin at 37 ^{o}C. 32

Figure 17. Degradation of 5 wt% hydrogel made from S3-S1VRN1600-S3 by plasmin at 37 ^{o}C. 32

Figure 18. DSC plot for S1VRN1600-S3 (0.75 mg/mL) in lysis buffer. 34

Biosynthesis and Characterization of Biodegradable Materials Based on Elastin Mimetic Polypeptide

Introduction

Significant developments in biomaterial science and engineering have been made during the past several decades. Biomaterials have been used in clinical practice and have profoundly improved people's health and life quality. The goal of early biomaterials research was to achieve appropriate physical properties to replace host tissues.[1] A common feature among these materials was that they were biologically inert to minimize the immune response in the host.

In 1980s, bioactive materials reached clinical use. They can elicit a controlled reaction in the physiological environment. For example, bioactive glasses can induce a series of reactions and provide nucleation sites for the growth of new bone with a mechanically strong bond to the implant surface.[1] Another advance was resorbable biomaterials. One example is the copolymer of polylactic acid (PLA) and polyglycolic acid (PGA). These polymers can hydrolyze and decompose into CO_2 and H_2O. They have successfully been used as degradable sutures and proved to be safe in medical applications.[2, 3]

Now, the concepts of bioactive materials and resorbable materials have converged. New generations of biomaterials have been designed to stimulate specific cellular

response at the molecular level.[1] This requires the precise control on the molecular architecture of the materials. Genetically directed synthesis of proteins provides a powerful method to meet this requirement.[4-6] Proteins with precisely defined sequence and molecular size can be produced in selected bacterial expression systems using this method. Unnatural amino acids can also be incorporated into proteins to produce materials with desired structure and function.[7, 8]

The extracellular matrix (ECM) plays an important role in cell migration and tissue regeneration.[9] It is a network consisting of many different types of macromolecules that are mainly produced, secreted and assembled locally by cells in the matrix. Each cell has particular ECM receptors through which the cell migration and differentiation are regulated by ECM molecules.[10] The ECM network is not only a pathway for migrating cells, but also a barrier, which must be degraded and then replaced with new ECM molecules. During the process such as wound healing, migrating cells secrete a number of enzymes to degrade and remodel the matrix macromolecules in their pathway. As for the degradation of collagen, the migrating cells manipulate the balance of collagenase and inhibitors of collagenase to maintain a proteolytic environment that is localized near the cell surface but is inhibited farther away.[10] Enzymes involved in ECM degradation include matrix metalloproteinase (MMP), collagenase, and plasmin.[11-13]

Being structurally similar to the macromolecule-based extracellular matrix, hydrogels made from natural and synthetic polymers have been used as ECM analogues

in the studies of tissue engineering. One example for the natural polymer is collagen. Collagen is a main component of extracellular matrices of mammalian tissues. It has been used as a tissue culture scaffold due to the ready attachment of many different cell types and its cell-induced degradation.[2] However, there are limitations for gels made from natural polymeric sources, such as lack of physical strength, and potential for contamination by viruses or prions when separated from the organism. These limitations have motivated approaches to modify these polymers and to use various synthetic polymers.

Creation of Synthetic Erodible Materials

Synthetic polymers with degradable sequences incorporated in the backbone were also used to make hydrogels. A collagenase-sensitive sequence GGLGPAGGK (B) was incorporated into the backbone of PEG (A) to make an ABA block copolymer.[11] The molecular weight of each PEG block is 6,000. The acryloyl groups were added on both ends. When dissolved in HEPES-buffered saline (HBS, pH 7.4) and exposed to UV light in the presence of initiator, these macromolecules formed a cross-linked hydrogel by photopolymerization. Degradation of the hydrogels by collagenase was examined by placing the hydrogels in HBS containing collagenase and tracking their mass over time. As shown in Fig. 1, the mass of the hydrogels initially increased as some of the LGPA sequences were cleaved, loosening the hydrogel network and more water was able to

enter the hydrogels. The mass of the hydrogels then decreased as more sequences were cleaved, indicating the breaking down of the hydrogel network. The rate of degradation was increased at higher concentration of collagenase. In the absence of collagenase, the hydrogel was not degraded.

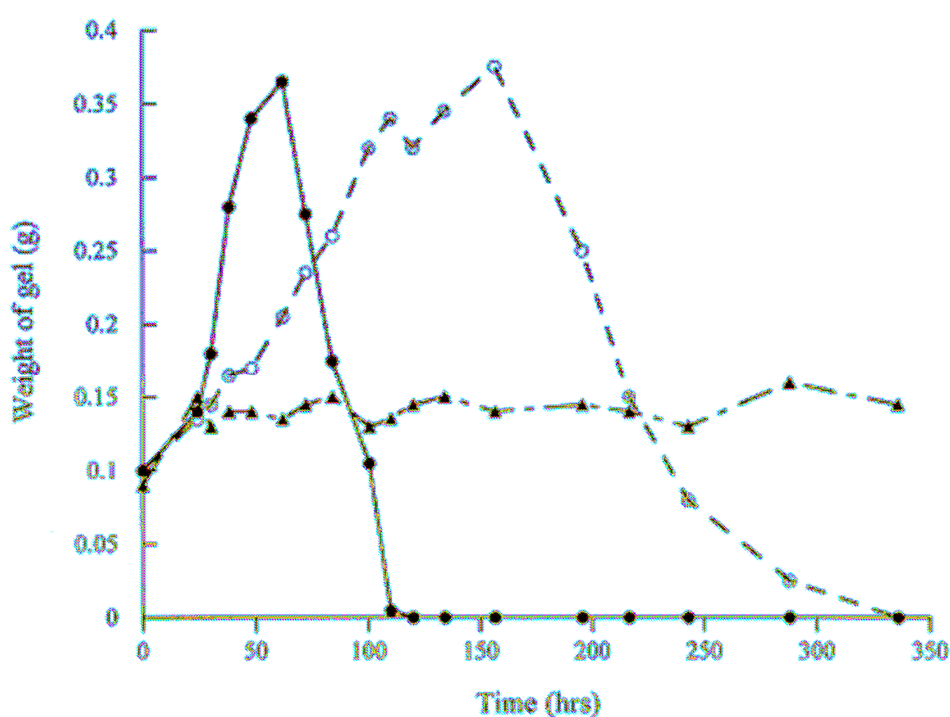


Fig. 1. Degradation of GGLGPAGGK-derivatized PEG hydrogels in solutions containing collagenase. ● 2 mg/mL collagenase; ○ 0.2 mg/mL collagenase; ▲ no collagenase.[11]

An oligopeptide GPQGIAGQ and its mutated version GPQGIWGQ were selected as substrates for MMP-1, a member of the matrix metalloproteinase (MMP) family.[12] These substrates can be cleaved at the Gly-Ile bond. Charged amino acids (Arg and Asp) were added to flank the sequences in order to increase water solubility. Substrate hydrolysis

was followed by fluorescamine reaction and kinetic parameters for MMP-1 hydrolysis of these oligopeptides were determined by Michaelis-Menten analysis. The results showed that the double-reciprocal plots (1/V~1/[S]) were linear over the entire range of substrate concentrations studied, indicating that Michaelis-Menten kinetics was obeyed. The K_m values were determined to be 0.3-0.9 mM. When cross-linked into the hydrogel network, because the substrate concentration within the gel was much higher than K_m, the zero-order hydrolysis rate should be observed. This hypothesis was confirmed by the linear increase of the amount of degraded substrate with time measured by a quantitative fluorescamine assay.

Though not so specific as intact collagen, oligopeptide Ala-Pro-Gly-Leu was selected as a collagenase substrate.[13] The expected cleavage site is between Gly and Leu, with a Gly residue in the immediately N-terminal to the cleaved peptide bond and a bulky hydrophobic Leu residue immediately C-terminal to the cleaved peptide bond. Similarly, oligopeptide Val-Arg-Asn was selected as a plasmin substrate, with a cleavage site between the Arg and Asn residues. The oligopeptides were linked to the both ends of poly(ethylene glycol) (MW 6000) by the reaction between the PEG end hydroxy groups and the oligopeptide C-terminal carboxyl groups. Acryloyl groups were introduced to the both ends of the BAB block (A=PEG, B=oligopeptide) to form the macromer Acr-oligopeptide-PEG-oligopeptide-Acr. Then the macromer was pipetted into molds and exposed to ultraviolet to form hydrogel disks by photopolymerization. The initial wet

weight of each disk was measured. Then they were placed into HBS and weighed again after swelling for 24 h. Then the disks were placed into buffers with or without collagenase. The wet weights of the disks were measured and the buffer solutions were replaced at predetermined time points. For collagenase-sensitive hydrogels containing APGL sequence, the weight increased from an initial value of 0.07 g to about 0.37 g at the equilibrium swelling level. After that, the weight decreased dramatically at the presence of collagenase; the higher the collagenase concentration, the faster the weight decreased. While in the absence of protease and in the presence of plasmin, the weight of the collagenase sensitive hydrogel remained unchanged as time passed, indicating that the hydrogel was specifically degraded by the targeted protease. Similar results were observed for plasmin sensitive hydrogel.

Cell adhesive ligands are also required for cell adhesion and migration. Studies have shown that cells are able to adhere to and migrate through artificial hydrogels that contain both the appropriate adhesive and degradable ligands. If either the adhesive or the degradable sequence were removed from the hydrogel structure, no cell migration through the material can be observed.[12] In a previous paper,[14] it was shown that cell adhesion on the PEG-based hydrogels was significantly retarded compared to a control surface of glass. This resistance of adsorption was attributed to the hydrophilicity and high segmental mobility of PEG in water. The oligopeptide sequence Arg-Gly-Asp (RGD), which is present in many adhesive proteins, has been shown to be a key

attachment site for a variety of integrin cell surface receptors.[15-17] Cell adhesion experiments showed that incorporating RGD sequence into the backbone of poly(2-hydroxyethyl methacrylate) promoted the adhesion of mouse fibroblast on the surface of the polymer film.[18]

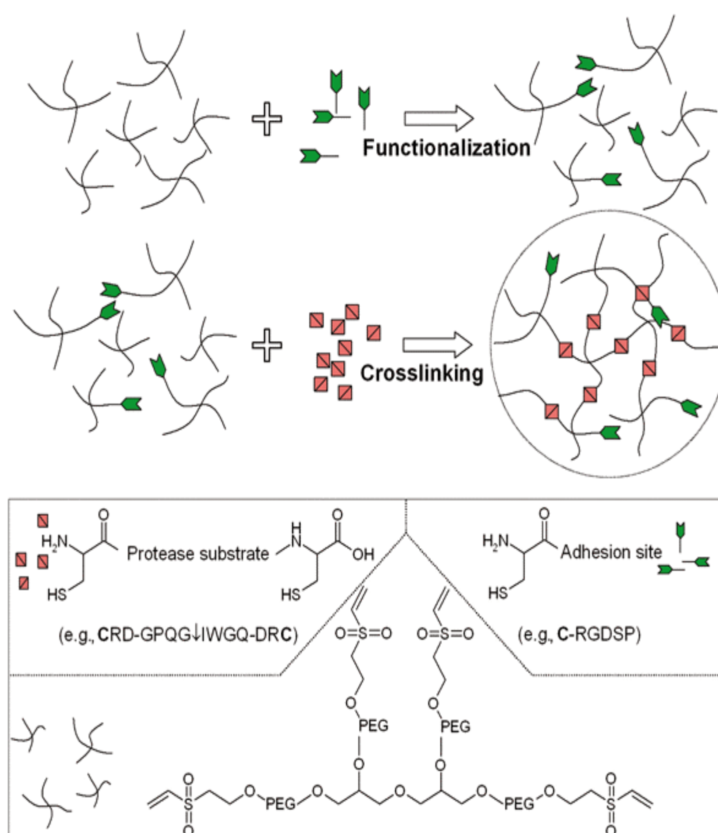


Fig. 2. Scheme for preparing crosslinked hydrogel incorporated with both integrin- binding substrates and MMP-sensitive sequences.[12]

To make synthetic hydrogel networks degradable and invasive by cells, a combination of integrin-binding sites and substrates for MMP have been incorporated

into the hydrogel by molecular engineering.[12] When preparing a cross-linked hydrogel, 4-arm-PEG-tetravinyl sulfone (20 kg/mol) was dissolved in buffer to a final concentration of 10% (wt/vol). Then, the integrin-binding peptide containing a RGD sequence and a Cys residue was added into this solution. After 10 min, a buffered solution containing a bis-cysteine peptide with an MMP-sensitive sequence was added. The adhesion peptides were grafted into the polymer backbone by the Michael-type addition reaction between the –SH groups in the Cys residues and the vinyl sulfone-functionalized multiarm PEG. The cross-linked hydrogel was formed by the Michael-type addition reaction between the bis-cysteine peptide and the multiarm PEG (Fig. 2).

Human foreskin fibroblasts (HFFs) were used to study the three-dimensional cell invasion in the hydrogel prepared as above.[12] Cell-loaded fibrin clots (3×10^7 cells per ml) were placed into the precursor solution before gelation to embed the cells in the gel. Samples were cultured in serum-containing DMEM for up to 20 days. Cells were imaged by inverted phase-contrast microscopy and confocal scanning laser microscopy. The results showed that HFF clusters grew out radially into the matrix (Fig. 3A) by three-dimensional invasion in a spindle-like pattern. Confocal laser microscopy revealed that the migration of these fibroblasts occurred in a cohort manner.[12] The cells moved by keeping cell-cell contacts and grew into cord-like structures (Fig. 3B). The cell invasion distance increased approximately linearly with culture time. The total distance exceeded 1mm in 7 days. Cell invasion was largely influenced by the MMP sensitivity of the

degradable peptide. The higher the MMP sensitivity, the faster the invasion rate. Meanwhile, RGD ligand concentration was important. A concentration of 10 μM of RGD peptide was the minimal concentration necessary for HFF invasion. An increased network crosslink density also decreased the invasion rate dramatically, even completely impaired cell invasion.

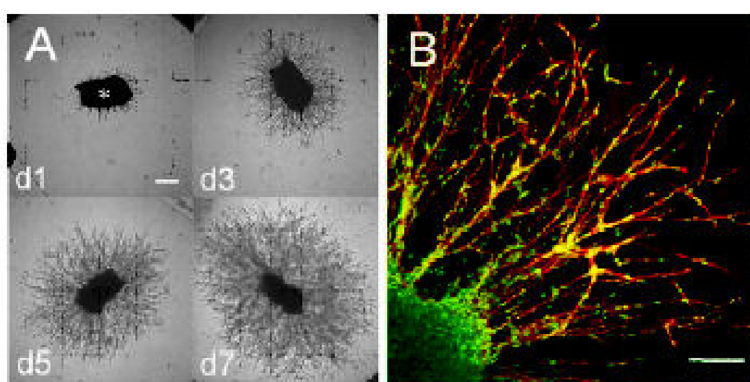


Fig. 3. (*A*) Fibroblasts radially invaded the adhesive and MMP-sensitive synthetic hydrogel matrix (bar = 250 μm). (*B*) Migration of spindle-like-shaped fibroblasts occurred in a cohort manner (bar = 150 μm).[12]

Hydrogels with homogeneously seeded cells were prepared and the cell viability was studied.[11] A suspension of human aortic smooth muscle cells (HASMCs) was mixed with PEG-diacrylate derivative containing degradable sequences and acryloyl-PEG-KQAGDV (The peptide KQAGDV is a potent adhesion ligand for SMCs). Then the mixture was added into a disk-shaped mold (20 mm diameter × 2 mm thick) and photopolymerized. After one week of culture, the hydrogels were incubated with serum-free MEEM containing chloromethylfluorescein diacetate (CMFDA) at 37 ºC for 4 h. CMFDA is a

nonfluorescent fluorescein derivative that is taken up by viable cells, where the diacetate group is cleaved to perform fluorescence. The 50 μm sections of these hydrogels were taken on a cryostat and the sections were evaluated under phase-contrast microscopy and fluorescence microscopy to examine cell viability. Viable cells, indicated by fluorescence upon CMFDA staining, were evident 24 h after photopolymerization and after one week of culture. At both time points, approximately 95% viability was observed.

Elastin-Mimetic Protein-Based Biomaterials

Elastin is a natural protein in ECM that provides elasticity for many tissues due to its highly dynamic domains in the relaxed state. Its hydrophobic domain, which is responsible for the elasticity, is best described as a compact amorphous structure with distorted β-strands and turns.[19] Since native elastin is large and insoluble, many studies have focused on elastin-based peptides.

Elastin-mimetic proteins have a pentapeptide repeat unit [(Val/Ile)-Pro-Xaa-Yaa-Gly] in common.[20] They usually undergo a phase transition at a lower critical solution temperature, T_t. Changing the residues in the repeat sequence will affect the phase behavior and the mechanical properties of the material. Substitution of Ala for Gly in the third (Xaa) position results in a change in property from elastomeric to plastic. A decrease in the polarity of the side chain of the fourth residue (Yaa) will lower the phase transition temperature T_t, above which a coacervater phase is formed. Proteins **1** and **2** were

synthesized to study the gelation of the elastin-mimetic proteins, as the sequences shown below. Protein **1** has an elastic and hydrophilic central block rich in glutamic acid as well as the plastic and hydrophobic end blocks. While protein **2** only has a short elastic linker between the two plastic end blocks.

{VPAVG[(IPAVG)$_4$(VPAVG)]$_{16}$IPAVG}-[X]-{VPAVG[(IPAVG)$_4$(VPAVG)]$_{16}$IPAVG}

1; [X] = VPGVG[(VPGVG)2VPGEG(VPGVG)2]30VPGVG

2; [X] = VPGVGVPGVG

Differential scanning calorimetry (DSC) studies showed that proteins **1** and **2** displayed endothermic transitions at 23 ºC and 21 ºC, respectively, that were virtually independent of the pH of the solutions. The rescan experiment, shown in dotted line, indicated that the transition was reversible upon cooling and rescan.[20] Cryo-HRSEM studies showed a micro-phase separated morphology for both proteins 1 (Fig. 4A) and 2 (Fig. 4B) prepared via rapid cryo-immobilization from gel specimens equilibrated at 25 ºC, which is above T_t. It's proposed that the tri-block elastin-mimetic polypeptides can potentially self-assemble into networks through virtual cross-links formed from micro-phase separation of the identical end block domains. In this state, the central block remains hydrated as it is below its respective transition temperature, imparting elastomeric behavior to the matrix formed from self-assembly of the tri-block polypeptide.

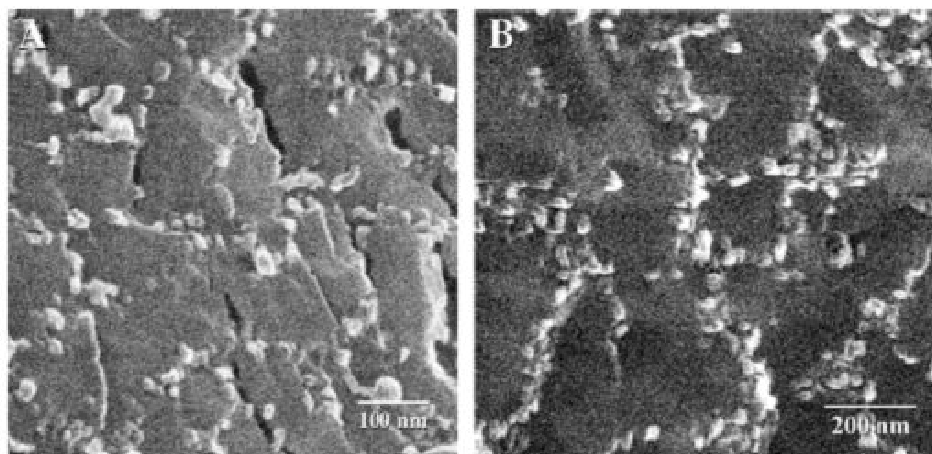


Fig. 4. Cryo-HRSEM images of vitrified specimens of concentrated aqueous (25 wt %) solution of proteins **1** (A) and **2** (B) prepared via rapid cryo-immobilization from gel specimens equilibrated at 25 °C.[20]

Mechanical analysis on a similar tri-block protein $[(IPAVG)_4(VPAVG)]_{16}$-$[(VPGVG)_4(VPGEG)]_{48}$-$[(IPAVG)_4(VPAVG)]_{16}$ showed that these materials displayed a wide range of mechanical properties tunable by varying the processing conditions, such as solvent, pH, and temperature.[21, 22]

These properties described above indicated that hydrogels made from elastin-mimetic block polypeptide could be potential biomaterials for tissue engineering applications. We describe herein the biosynthesis and characterization of a recombinant polypeptide with plasmin-degradable sites incorporated into the central elastomeric block. Studies on this polypeptide showed that it is readily degradable by plasmin both in buffer solution and in the hydrogel state. By varying the sequence and/or the size of the building blocks, one can adjust the mechanical properties and the degradation rate of the materials and make them suitable for use as potentially biocompatible hydrogels.

Experimental Methods

Materials

All chemical reagents were purchased from either Fisher Scientific (Pittsburgh, PA) or Sigma Chemical Corporation (St. Louis, MO) unless otherwise specified. All restriction endonucleases were obtained from New England Biolabs, Inc. (Beverly, MA). Plasmid pZErO^{TM}-2 and *E. coli* strain TOP10F' were purchased from Invitrogen Corporation (Carlsbad, CA). Plasmid pET-24d and *E. coli* strain BL21(DE3) were obtained from Novagen (Madison, WI). QIAGEN Plasmid Mini kits and QIAfilter Plasmid Maxi kits were purchased from QIAGEN Inc. (Chatsworth, CA). Synthetic oligonucleotides were purchased from Sigma/Genosys Biotechnologies, Inc. (The Woodlands, TX). His-tag AP Western Reagents were purchased from Novagen (Madison, WI).

General Methods

Procedures for the manipulation of DNA, the transformation of competent cells, and the growth and induction of bacterial cultures were adapted from the published literature or instructions supplied by manufactures. Reagents for the manipulation of DNA and bacterial culture were sterilized by either autoclave or passage through a 0.22 μm filter. Reactions with restriction endonucleases were performed in the reagent buffers supplied by the manufacture. DNA sequence analyses were performed at the Emory University

Center for Fundamental and Applied Molecular Evolution on a Hitachi 3100 Genetic Analyzer. Protein electrophoresis was performed on 10-15% gradient discontinuous SDS poly- acrylamide gels on a PhastSystem from Amersham Pharmacia Biotech and visualized via a silver staining procedure. DSC experiments were carried out on a VP-DSC Micro- Calorimeter from MicroCal, LLC.

Synthetic Gene Construction

We designed an elastic peptide monomer sequence (denoted as S1VRN) incorporated with a plasmin-degradable site: VRN (Fig. 5A). Purified oligonucleotides corresponding to the sense and antisense strands of the DNA monomer were dissolved in EB buffer (10 mM Tris-HCl, pH 8.5) to a final concentration of 0.5 μg/μL. The oligonucleotides (20 μL each) were mixed with 5M NaCl (4 μL), 1M $MgSO_4$ (4 μL), and sterile water (152 μL), and heated to 99 ˚C to melt residual secondary structure. The mixture was gradually cooled to 30 ˚C over a period of 4 hrs and incubated at 4 ˚C to anneal the two strands together. The mixture was analysed by preparative agarose gel electrophoresis (4% NuSieveGTG agarose, 1X TBE buffer), which indicated a single band at the correct position versus the DNA standards. The synthetic duplex was isolated by ethanol precipitation and dried *in vacuo*. Then the duplex was phosphorylated by T4 polynucleotide kinase (50 U) in 1X T4 DNA ligase buffer (150 μL) at 37 ˚C for 1 hr. The reaction mixture was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) to

remove the enzyme, and the phosphorylated duplex was isolated by ethanol precipitation, dried *in vacuo*, and dissolved in 50 μL EB buffer.

Cohesive end ligation was performed between the synthetic DNA cassette and the pZErO-2 (Fig. 6) vector double-digested by *Hind* III and *Bam*H I in the presence of T4 DNA ligase in 1X ligase buffer at 16 ˚C for 16 hrs. An aliquot of this ligation mixture (2 μL) was used to transform competent cells of *E. coli* strain TOP10F' (40 μL). The transformed cells were resuspended in 1 mL SOC medium and incubated at 37 ˚C with agitation for 1 hr. The transformation mixture (100 μL) was cultured on LB/Kanamycin plates (LB: 1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.5; 1.5% Agar; 50 μg/mL kanamycin) at 37 ˚C for 16 hrs. Six transformants were used to inoculate separate cultures of LB medium in the presence of kanamycin (50 μg/mL). The cultures were incubated at 37 ˚C with agitation for 16 hrs. Plasmid DNA was isolated from cultures by QIAGEN Plasmid Mini kit. The clones were screened by double digestion with *Hind* III and *Bam*H I. Recombinant plasmids were identified by analysis of the digested products with 4% NuSieveGTG agarose gel electrophoresis and confirmed by DNA sequencing.

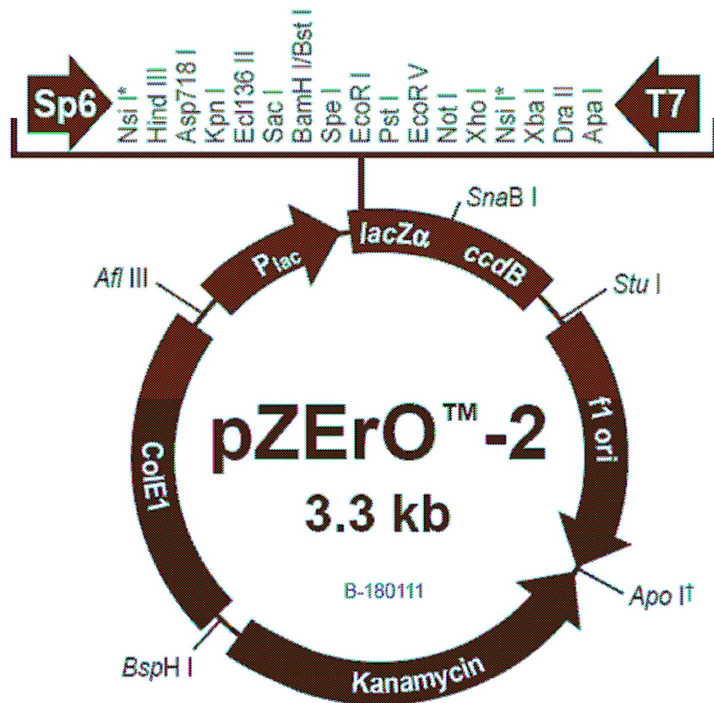
A. (VPGAG)$_2$GVRNGG(VPGAG)$_2$

B.

```
                 V   P   G   A   G   V   P   G   A   G   G   V   R   N   G   G   V   P   G
     AG CTT GAA GAC GTT CCA GGT GCA GGC GTA CCG GGT GCT GGC GGT GTT CGT AAC GGT GGT GTT CCA GGC
          A CTT CTG CAA GGT CCA CGT CCG CAT GGC CCA CGA CCG CCA CAA GCA TTG CCA CCA CAA GGT CCG
HinD III      Bbs I

 A   G   V   P   G   A   G
GCA GGT GTA CCG GGT GCG GGT GTT CCA AGA GAC GG
CGT CCA CAT GGC CCA CGC CCA CAA GGT TCT CTG CCC TAG
                                   BsmB I   BamH I
```

Fig. 5. (A) Amino acid sequence of the S1VRN monomer. (B) Sequence of the DNA encoding the S1VRN monomer.



* The two *Nsi* I sites in the MCS are the only sites in the vector.

† There are two tandem *Apo* I sites at this location. *Apo* I also recognizes the *EcoR* I site.

Fig. 6. Plasmid map of vector pZErO^{TM}-2. (http://www.invitrogen.com)

A single colony containing the correct recombinant plasmid was used to inoculate 500 mL of LB medium in the presence of kanamycin (50 μg/mL). The plasmid was isolated from cell cultures via QIAfilter Plasmid Maxi kit and was double digested by *Bbs* I and *Bsm*B I to release the S1VRN monomers, which were separated from other fragments by preparative agarose gel electrophoresis (4% NuSieveGTG agarose, 1X TBE buffer). A 78 bp band, which corresponded to the expected size of the DNA monomer, was excised from the gel, macerated, and extracted with EB buffer. The gel fragments were removed by filtration through a 0.45 μm microcentrifuge filter, and the DNA monomer was recovered from the filtrate via ethanol precipitation.

The S1VRN DNA monomer (5 μg) was mixed with T4 DNA ligase in 1X ligase buffer and incubated at 16 ˚C for 16 h. The reaction mixture was loaded on 1% agarose gel and separated by electrophoresis. The 500-1000 bp, 1-2 kbp, and 2-4 kbp fragments were excised from the gel, respectively, and recovered via Zymoclean DNA recovery kit. The DNA multimers were ligated with pZErO-2 vector and transformed into TOP10F'. Plasmids were isolated from screened transformants and sent for sequencing. A multimer with correct sequence and a size of 800 bp was identified. The plasmid containing the 800 bp multimer (2 μg) was double digested with *Bsm*B I and *Nco* I and a 2800 bp segment was recovered from agarose gel. Another aliquot of the plasmid (2 μg) was double digested with *Bbs* I and *Nco* I and a 2100 bp segment was recovered from agarose gel. These two segments were ligated together with T4 DNA ligase. A multimer with a

size of 1600 bp was created and confirmed by DNA sequencing (Fig. 7).

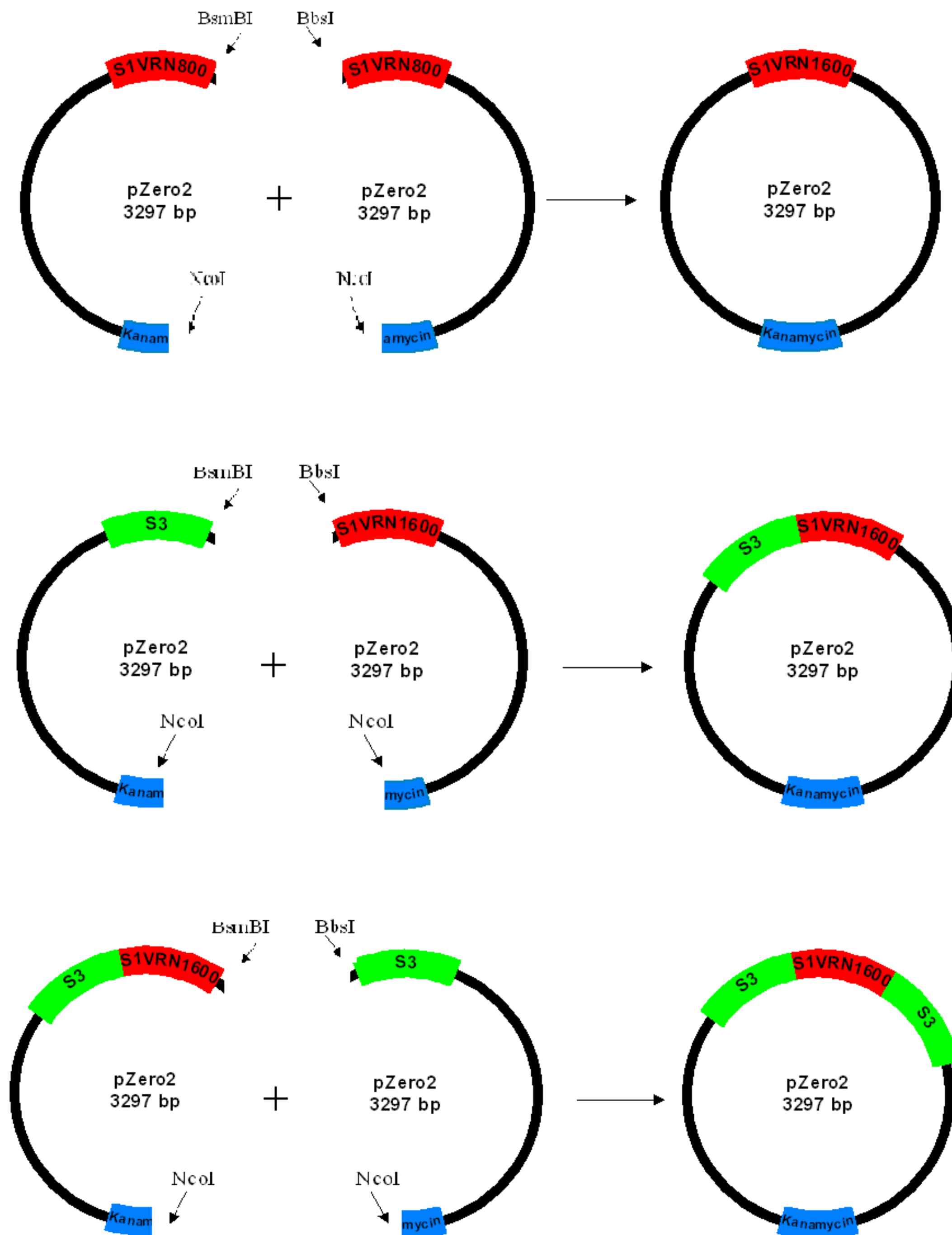


Fig. 7. Scheme for gene construction for the tri-block polypeptide with plasmin cleavable sites incorporated into the central block.

The plasmid containing the DNA segment (about 2000 bp) encoding a plastic polypeptide (S3) with a repeat sequence of $(IPAVG)_5$ was used to construct a tri-block DNA cassette S3-S1VRN1600-S3 using similar methods described above (Fig. 7). The tri-block cassette was double digested with *Bbs* I and *Bsm*B I, recovered from agarose gel, and stored at –20 ˚C for later use.

Construction of the Expression Vector

The sense and anti-sense oligonucleotides for the decahistidine adaptor sequence (Fig. 8) were annealed, phosphorylated, and purified as described above for the S1VRN DNA monomer. The *Nco* I and *Xho* I digested plasmid pET-24d (Fig. 9) was mixed with the adaptor DNA cassette, T4 DNA ligase, and ligase buffer and incubated at 16 ˚C for 16 hrs. Competent cells of *E. coli* strain TOP10F' were transformed with the ligation mixture and cultured on LB/kanamycin plates at 37 ˚C overnight. Six transformants were used to inoculate separate cultures of sterile LB medium (6 mL) under kanamycin (50 μg/mL) selection at 37 ˚C for 16 h. Plasmids were isolated from the bacterial cell cultures using QIAGEN Plasmid Mini Kit. The clones were screened for the presence of the insert by double digestion with *Nco* I and *Xho* I. After electrophoresis on 1% agarose gel, positive clones were sent for sequencing from the T7 promotor primer (Novagen, Inc.).

```
        V   P   R   D   R   R   R   S   R   P   G   V   G   G   S   D   D   D   D   K   G
  C ATG GTT CCA AGA GAC CGT CGA CGG TCT CGT CCA GGT GTT GGC GGA TCC GAC GAC GAC GAC AAG GGC
        CAA GGT TCT CTG GCA GCT GCC AGA GCA GGT CCA CAA CCG CCT AGG CTG CTG CTG CTG TTC CCG
Nco I                  Bsa I          Bsa I

 H   H   H   H   H   H   H   H   H   H
CAT CAT CAT CAT CAT CAT CAT CAT CAT CAC TAA TC
GTA GTA GTA GTA GTA GTA GTA GTA GTA GTG ATT AGA GCT
                                               Xho I
```

Fig. 8. Sequence of the DNA cassette encoding the decahistidine tag adaptor.

A recombinant plasmid (4 μg) containing the correct adaptor sequence was double digested with *Bsa* I (40 U) in 1X NEBuffer 3 (total volume is 50 μL) at 50 ˚C for 2 h. The linear plasmid was recovered from 1% agarose gel and dephosphorylated with Shrimp Alkaline Phosphatase at 37 ˚C for 30 min. The reaction mixture was heated to 65 ˚C and kept for 15 min to inactivate the enzyme.

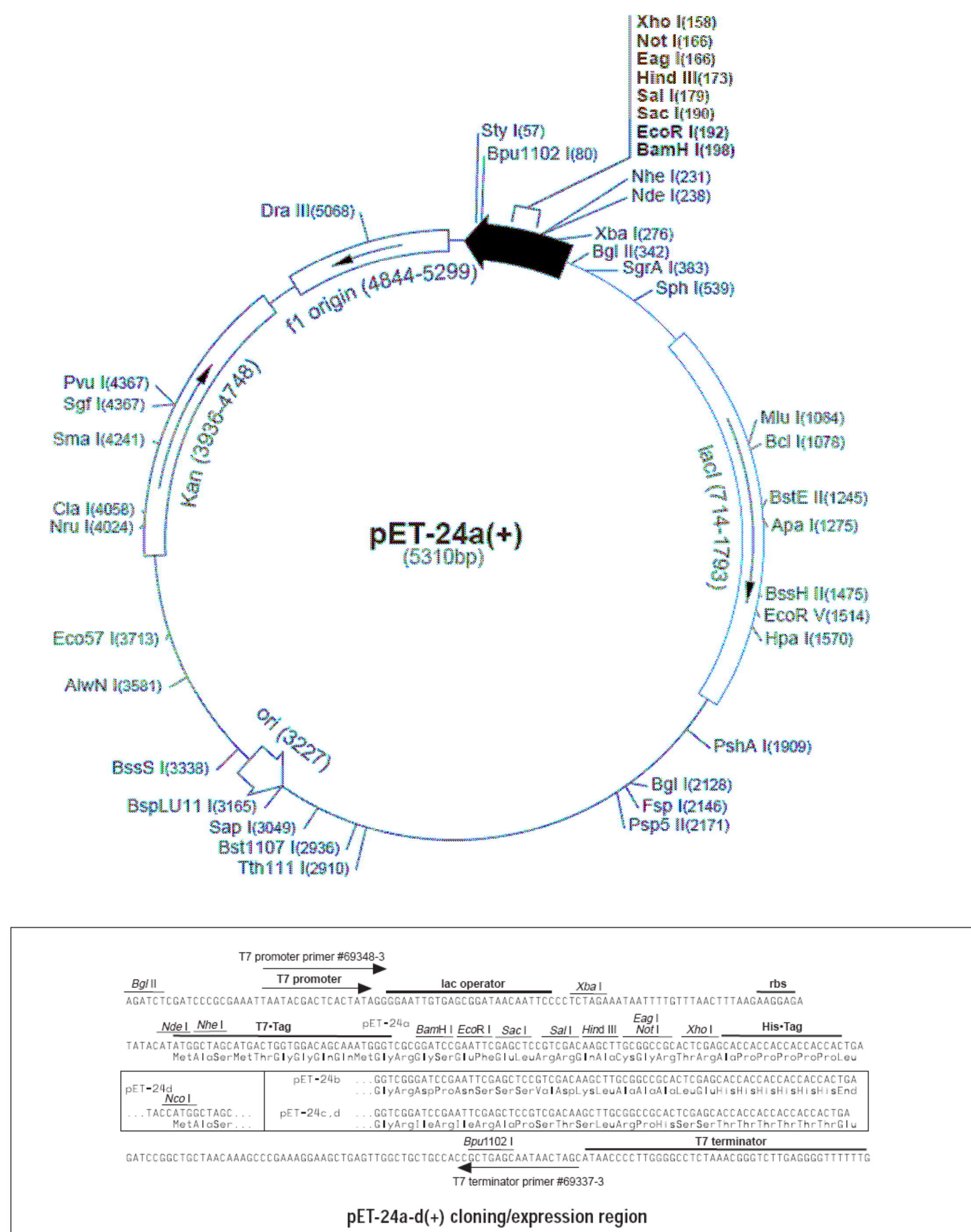


Fig. 9. Plasmid map of vector pET-24a-d. (http://www.novagen.com)

Previously constructed tri-block DNA cassette S3-S1VRN1600-S3 (140 ng) was mixed with the *Bsa* I-digested and dephosphorylated His-tag adaptor/pET24-d (113 ng), T4 DNA ligase, ligase buffer, and sterile water to a total volume of 10 μL. The reaction mixture was incubated at 16 ˚C for 16 h, transformed into TOP10F', and cultured on LB/Kanamycin plates at 37 ˚C overnight. Six transformants were used to inoculate separate cultures of LB medium with kanamycin (50 μg/mL), which were incubated at 37 ˚C overnight. Plasmid DNA was isolated from the cell cultures via QIAGEN Plasmid Mini kit and screened for the presence of the tri-block cassette by double digestion with *Nco* I and *Xho* I. The digested fragments were analyzed by 1% agarose gel electrophoresis.

Expression, Purification, and Characterization of the Tri-block Protein

The plasmid containing the tri-block cassette and the decahistidine-tag adaptor in pET24-d vector was used to transform competent cells of *E. coli* strain BL21(DE3). The transformation reaction mixture was cultured on LB/kanamycin plates at 37 ˚C overnight. Small-scale expression was carried out in 25 mL LB/kanamycin (50 μg/mL) with or without IPTG (1mM) induction. The whole cell lysates at t = 0, 1 hr, and 2 hrs were analyzed by SDS-PAGE and visualized by silver staining.

Large-scale expression was performed in 1 L TB medium. A single colony was used to inoculate 50 mL LB medium containing 50 μg/mL of kanamycin. After incubated at 37

˚C overnight, this culture was used to inoculate 1 L fresh TB medium containing 50 μg/mL of kanamycin. The expression culture was incubated at 37 ˚C with agitation at 175 rpm for 48 h. The cells were harvested by centrifugation at 4000xg, 4 ˚C for 20 min and resuspended in 100 mL lysis buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7.0). The cell resuspension was frozen/thawed for three cycles at –80 ˚C and room temperature, respectively. After the third thaw, lysozyme (100 mg in 2 mL sterile water) and protease inhibitor cocktail (430 mg in 2 mL DMSO and 8 mL sterile water) were added and shaken at 225 rpm, room temperature for 30 min. $MgCl_2$ (100 μL, 1M) and endonuclease (aka benzonase, 14 μL) were added to the lysate. The mixture was shaken at 225 rpm, 4 ˚C overnight.

The lysate was transferred into a 250 mL centrifuge bottle and centrifuged at 14500 rpm, 4 ˚C for 30 min. The supernatant was decanted into eight 40 mL centrifuge tubes and mixed with 7 mL of NaCl solution (5 M) in each tube. The tubes were shaken at 225 rpm, 37 ˚C for 1 hr and centrifuged at 20,000 rpm, 37 ˚C for 30 min to collect the protein pellets. The supernatant was decanted and lysis buffer (20 mL) was added to each centrifuge tube. The tubes were shaken at 225 rpm, 4 ˚C overnight to resuspend the protein pellets. The cold/hot centrifuge was performed for three cycles to purify the protein. Then the protein solution was filtered through a 5 μm pore size membrane and dialyzed against deionized water (4 L ×6) with the water changed every 12 h. Then the purified protein was lyophilized. The degradation of the tri-block protein by plasmin was

tested both in solution and in hydrogel state. The degradation products were visualized by Western blot.

DSC experiments were performed on the tri-block protein dissolved in lysis buffer (0.75 mg/mL). The lysis buffer was used as a reference. Both the sample solution and the reference solution were degassed under vacuum for 5 min to prevent the formation of bubbles when heating. The scan range of temperature was set between 1 ˚C and 30 ˚C and the scan rate was 40 ˚C/hr. Scan up (heating) and scan down (cooling) were repeated five times to test the reversibility of the phase transition.

Results and Discussion

Synthetic Gene Construction

The elastic peptide monomer sequence S1VRN was successfully inserted into pZErO-2 vector and the recombinant plasmid was extracted using QIAfilter Plasmid Maxi kit. The plasmid was double digested with *Bbs* I and *Bsm*B I to release the S1VRN monomers, which were separated by electrophoresis on 4% NuSieveGTG agarose gel (Fig. 10). The 78 bp band was cut from the gel and the monomer was isolated from gel fragments by filtration through a 0.45 μm microcentrifuge filter followed by ethanol precipitation.

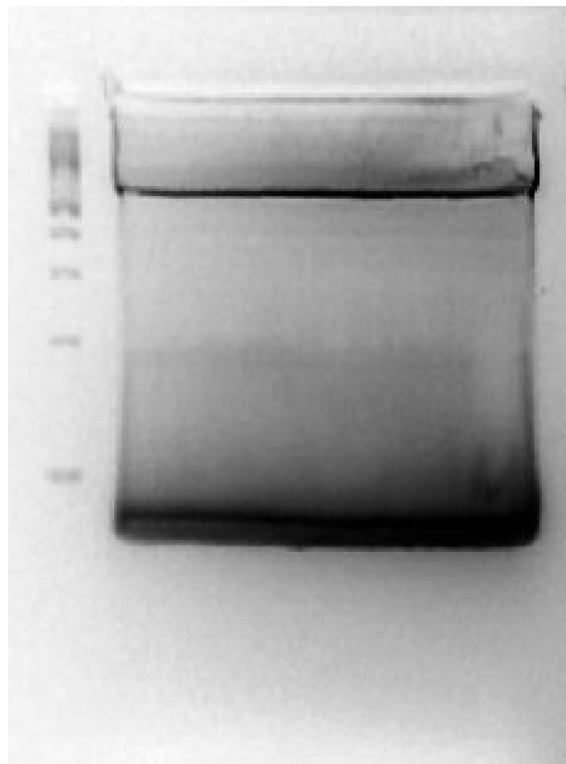


Fig. 10. S1VRN DNA monomer (78 bp) on 4% NuSieveGTG agarose gel.

The monomer was multimerized by self-ligation in the presence of T4 DNA ligase at 16 ˚C for 16 hrs. The reaction mixture was loaded on 1% agarose gel and separated by electrophoresis. The multimers displayed a ladder with size range from 500 bp to 5000 bp (Fig. 11). The 500-1000 bp, 1-2 kbp, and 2-4 kbp fragments were separated and purified individually. The multimers were ligated with acceptor plasmid (S1VRN/pZErO-2 recombinant plasmid digested by *Bbs* I and then dephosphorylated by Antarctic Phosphatase). The ligation mixtures were transformed into TOP10F'. Recombinant plasmids were isolated from screened transformants and double digested with *Hind* III and *Bam*H I (Fig. 12). A multimer with a size of 800 bp was identified and confirmed by DNA sequencing. This multimer was dimerized to make a 1600 bp multimer (Fig. 13), which was also confirmed by DNA sequencing.

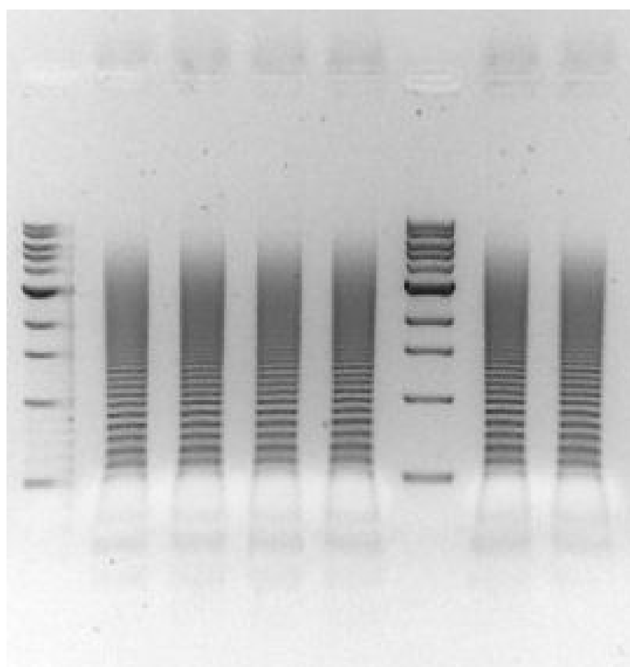


Fig. 11. S1VRN DNA multimers on 1% agarose gel.

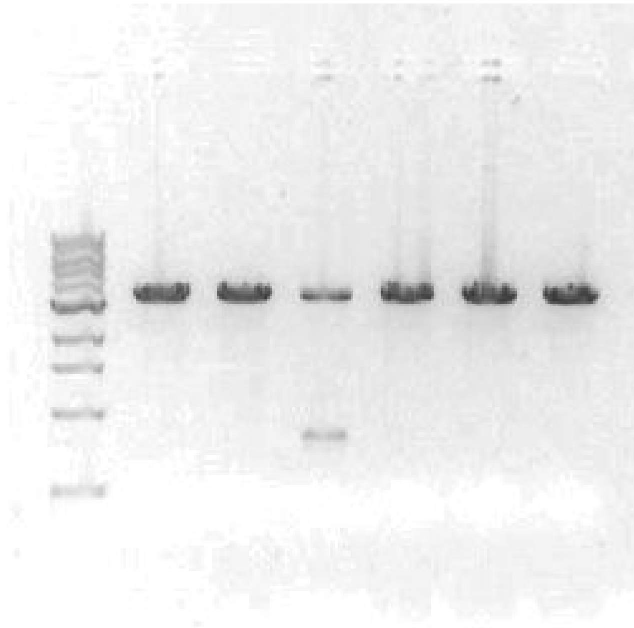


Fig. 12. Double digestion of the recombinant plasmid containing a 800 bp S1VRN multimer with *Hind* III and *Bam*H I.

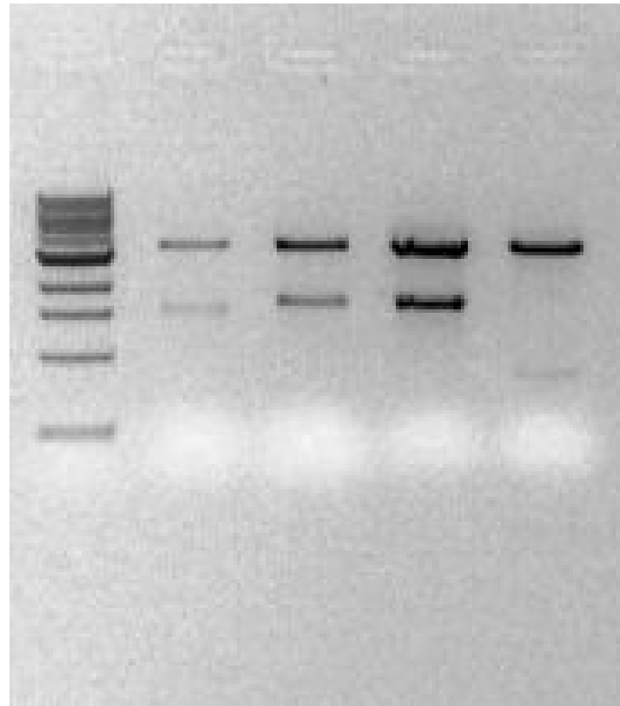


Fig. 13. Double digestion of the recombinant plasmid containing a 1600 bp S1VRN multimer with *Hind* III and *Bam*H I.

Synthesis of the tri-block protein

Genetically directed synthesis provides a method to produce proteins with specific sequence and considerable yield. The tri-block protein S3-S1VRN1600-S3 with two identical plastic end blocks and an elastic central block was successfully expressed and purified from *E. coli* strain BL21(DE3) with a yield of 300 mg in 1 L of TB medium.

For the SDS-PAGE analysis of the small-scale expression (Fig. 14), the target protein displayed a band at around 150 kDa, which is consistent with the theoretical molar mass (158 kDa).

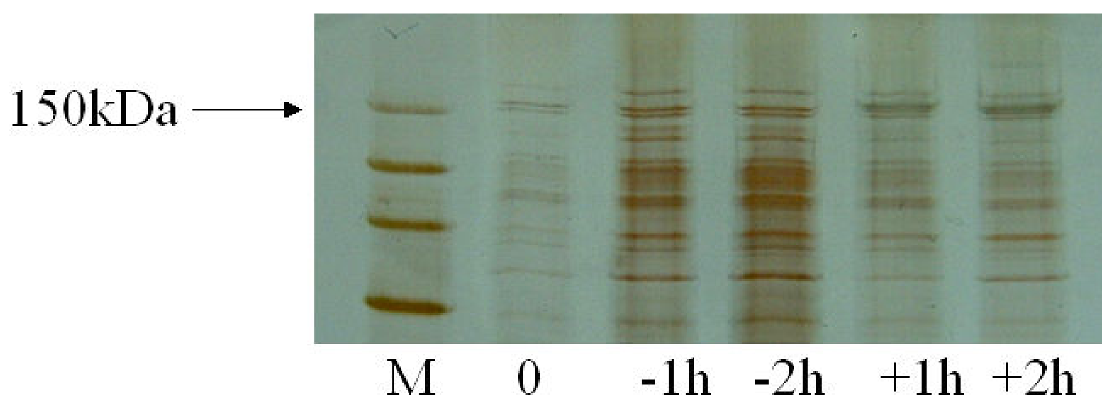


Fig. 14. SDS-PAGE analysis of the small-scale expression of the tri-block protein. From left to right: Protein marker, Before IPTG induction, t = 1 hr w/o induction, t = 2 hrs w/o induction, t = 1 hr with induction, t = 2 hrs with induction.

The tri-block protein undergoes a sol-gel transition at a lower critical temperature T_t. The protein is soluble in lysis buffer when the temperature is below T_t, while it becomes insoluble when the temperature is increased above T_t. The protein was purified through

three cycles of cold and hot centrifugation due to the phase transition property. The final product is a white spongy solid.

Degradation by plasmin in solution

To produce an elastin-mimetic protein that is biodegradable by plasmin, we designed a monomeric peptide sequence: $(VPGAG)_4$GVRNGG with a plasmin cleavage site between Arg and Asn residues. In order to make the peptide chain more flexible and accessible to the enzyme, Gly residues were added to flank both ends of the cleavage site. We manipulated the DNA cassette encoding this peptide monomer and obtained multimers with a size of 800 and 1600 base pairs, corresponding to 10 and 20 repeats of the monomer, respectively.

To test the degradation of S3-S1VRN1600-S3 by plasmin in solution, we mixed the tri-block protein (1 mg/mL) and plasmin (0.5 U/mL) in HBS (pH 7.4) /NaN_3 (0.2 mg/mL). NaN_3 was added to prevent bacteria growth. Reaction mixture was incubated at 18 ^{o}C. An aliquot of 10 μL of the reaction mixture was transferred into a clean vial at 24 and 48 hours. The aliquots were analyzed by Western blot. The results (Fig. 15) indicated that the tri-block protein was degraded by plasmin, showing a band at 62kDa corresponding to the size of S3 block (theoretically 58kDa).

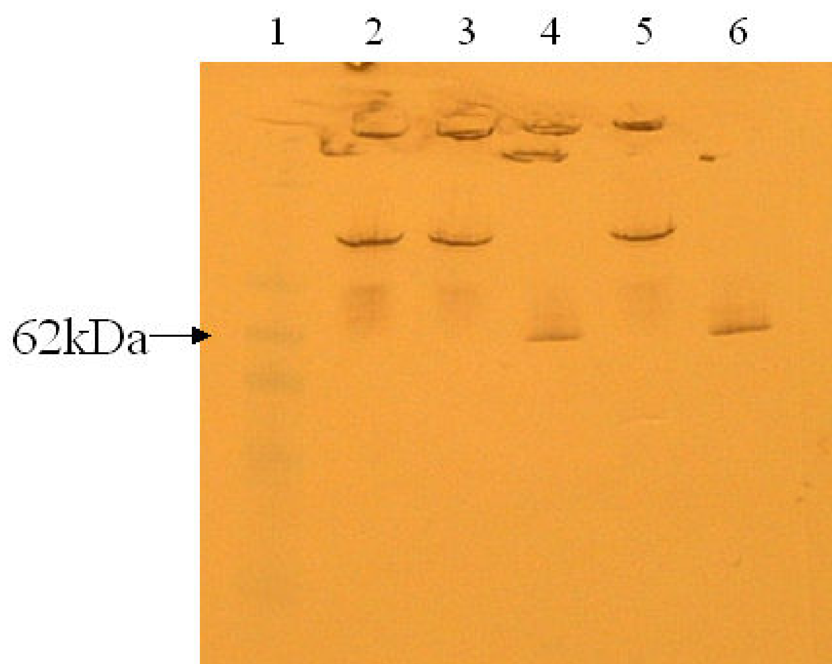


Fig. 15. Degradation of S3-S1VRN1600-S3 by plasmin in solution at 18 °C analyzed by Western blot. Lane 1, Molecular weight standards; Lane 2, t = 0; Lane 3, t = 24 h control; Lane 4, t = 24 h with plasmin; Lane 5, t = 48 h control; Lane 6, t = 48 h with plasmin. No plasmin was added to control solution.

Degradation by plasmin in hydrogel state

To test the degradation of hydrogel by plasmin, a 10 wt% hydrogel was made by dissolving S3-S1VRN1600-S3 in sterile, double-distilled H_2O at 4 °C and incubating it at 37 °C for 30min. The hydrogel was incubated in 1 mL HBS/NaN_3 at 37 °C for 24 h to let it swell to an equilibrium level. Then the hydrogel was incubated in 1 mL HBS/ NaN_3/plasmin (0.5 U/mL) at 37 °C. Another hydrogel was incubated in 1 mL HBS/NaN_3 (no plasmin) at 37 °C as a control. At preset time points, the hydrogel was removed from the solution, dried by filter paper, and weighed on an electric balance. After examining

the data (Fig. 16), we found the mass of the hydrogels decreased gradually over time in almost the same trend in the presence and absence of plasmin, indicating the 10 wt% hydrogel was not degraded by plasmin.

We did the same experiment with a 5 wt% hydrogel made from S3-S1VRN1600-S3 (Fig. 17). In the presence of plasmin (0.5 U/mL), the weight of the hydrogel decreased significantly compared with the control, indicating the 5 wt% hydrogel was degradable by plasmin. The difference in degradation results at different concentrations of hydrogel implies that at lower concentration there is more space in the hydrogel structure, making the cleavage sites more accessible to the enzyme. Therefore, the hydrogel at lower concentration can be degraded by plasmin more easily. The degradation rate could be conveniently adjusted by varying the hydrogel concentration, presumably as a consequence of greater porosity at lower polypeptide concentration.

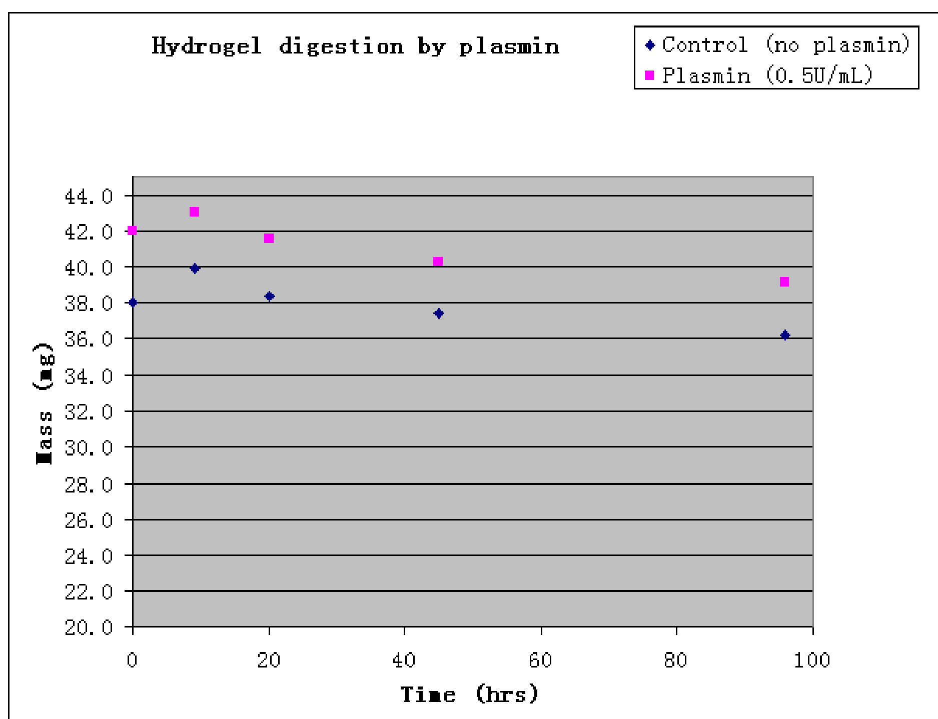


Fig. 16. Degradation of 10 wt% hydrogel made from S3-S1VRN1600-S3 by plasmin at 37 °C.

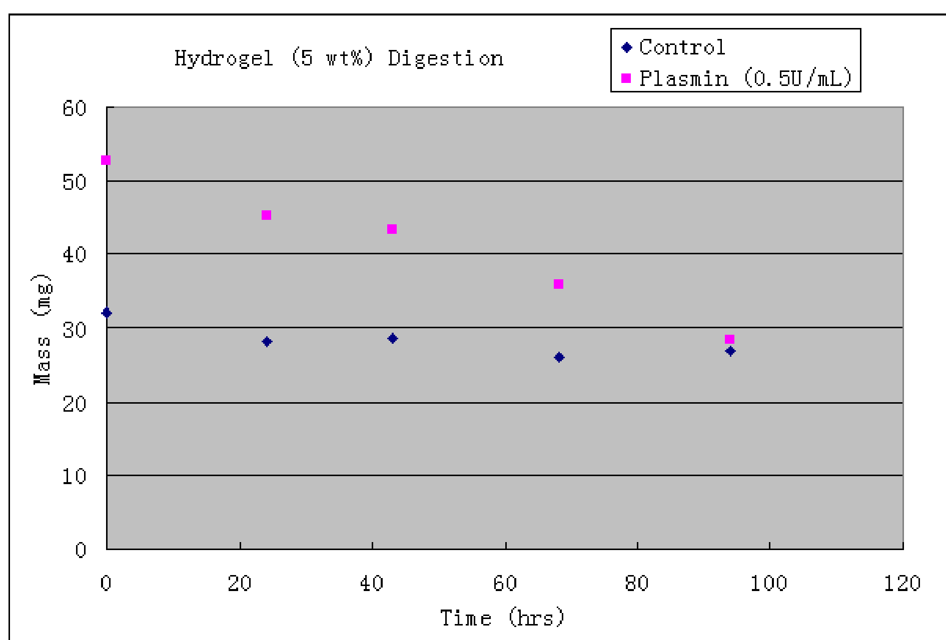


Fig. 17. Degradation of 5 wt% hydrogel made from S3-S1VRN1600-S3 by plasmin at 37 °C.

DSC experiments

Differential scanning calorimetry (DSC) studies on the tri-block protein S3-S1VRN1600-S3 (0.75 mg/mL in lysis buffer) showed that the protein undergoes a sharp endothermic transition at 13.0 ^{o}C (Fig. 18). The transition is reversible upon cooling and rescan since the rescan curves overlapped one another.

The transition temperature of S3-S1VRN1600-S3 is lower than the tri-block protein reported previously.[20] The reason might be that the size of the end block (S3) is much bigger than that of the tri-block in literature. In S3, there are about 27 repeats of peptide sequence $(IPAVG)_5$, which contains 675 amino acid residues. For the previously reported tri-block protein with a transition temperature at 23.1 ^{o}C, the end block VPAVG $[(IPAVG)_4(VPAVG)]_{16}IPAVG$ contains 410 amino acid residues. The results indicated that the transition temperature could be increased by reducing the length of the hydrophobic end blocks.

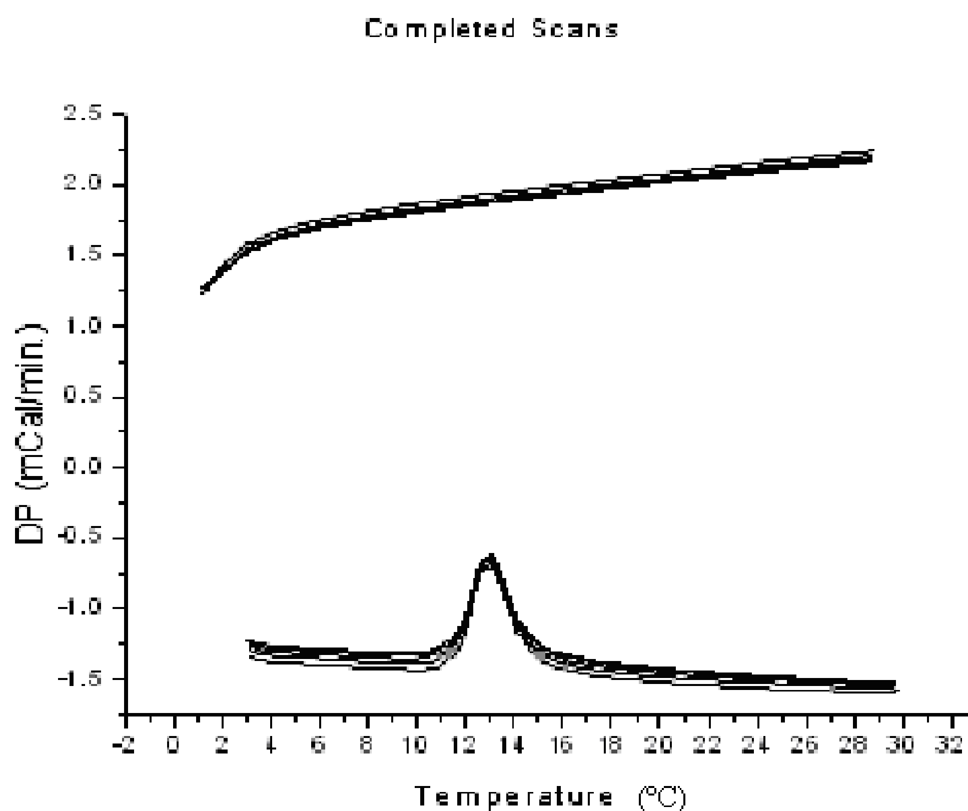


Fig. 18. DSC plot for S1VRN1600-S3 (0.75 mg/mL) in lysis buffer.

Conclusion

Plasmid cleavage sites were successfully incorporated into the midblock of an elastin mimetic tri-block protein. The protein was expressed in *E. coli* strain BL21(DE3) with a C-terminal decahistidine tag. A single band at a mass of 150 kDa was observed on SDS-PAGE gel, corresponding to the theoretical size of 158 kDa. The protein was purified by three cycles of cold/hot centrifuge and filtration through a 5 μm pore size membrane. Degradation experiments showed that the tri-block protein could be degraded

by plasmin both in solution (1 mg/mL) and in hydrogel state (5 wt%), while a 10 wt% hydrogel was not degraded by plasmin when tested in the same condition. DSC experiment indicated the sol-gel transition temperature of the tri-block protein was 13.0 ^{o}C， which is lower than a previously reported tri-block protein with shorter endblocks.

In summary, the properties of the tri-block protein S3-S1VRN1600-S3 such as the degradation rate and phase transition temperature can be conveniently adjusted by varying the hydrogel concentration or the sequence and size of the building blocks, which makes it a biomaterial suitable for applications in tissue engineering.

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