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Resilin-Based Hybrid Hydrogels for Cardiovascular Tissue Engineering

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Abstract

The outstanding elastomeric properties of natural resilin, an insect protein, have motivated the engineering of resilin-like polypeptides (RLPs) as a potential material for cardiovascular tissue engineering. The RLPs, which incorporate biofunctional domains for cell-matrix interactions, are cross-linked into RLP-PEG hybrid hydrogels via a Michael-type addition of cysteine residues on the RLP with vinyl sulfones of an end-functionalized multi-arm star PEG. Oscillatory rheology indicated the useful mechanical properties of these materials. Assessments of cell viability via con-focal microscopy clearly show the successful encapsulation of human aortic adventitial fibroblasts in the three-dimensional matrices and the adoption of a spread morphology following 7 days of culture.

Keywords

biomaterials; elastomers; hydrogels; mechanical properties; tissue engineering

1. Introduction

The development of materials for regenerative medicine can be especially challenging when the target tissues or organs undergo periodic loading forces as a part of proper function. Materials designed for these mechanically active environments must be able to withstand these forces without a loss in performance while additionally serving as a guide for tissue repair and regeneration. Cardiovascular tissue, in particular, has distinct elastomeric properties^[1] where elastic fibers, such as collagen and elastin, serve as critical structural components.^[2–5] Therefore, it is important that cardiovascular biomaterials incorporate elasticity into their design to ensure proper function.^[2,3,5]

Biosynthetic approaches to the design of biomaterials for regenerative medicine have been widely studied owing to their exceptional control over the properties of the resulting recombinant polypeptides.^[6–8] Biomimetic domains or biological recognition sequences derived from components of the extracellular matrix (ECM) may be directly engineered into these polymers in an attempt to confer cell-instructive signals that guide tissue repair and regeneration.^[9–11] Utilizing sequences from elastomeric proteins as the structural domains in these recombinant polypeptides offers a particularly exciting strategy given the outstanding mechanical properties of the protein polymers when cross-linked into gels.^[12,13] Elastin-like polypeptides (ELP) in particular have garnered much focus as potential materials for tissue engineering,^[14,15] and cross-linked ELPs have shown promise as injectable scaffolds for tissue repair.^[16–19] In addition, polypeptides based on the insect structural protein resilin have also emerged as potential candidates for material-based regenerative therapies for mechanically demanding applications.^[20,21] Resilin is an elastomeric protein found in specialized compartments of arthropods where it provides the rubber elasticity necessary for flight,^[22] sound production,^[23] and feeding.^[24] This highly disordered, cross-linked protein is rich in proline and glycine residues that may work mutually to confer chain disorder by providing conformation-restricted rigidity and chain flexibility, respectively.^[25–28] The resulting randomly coiled, isotropic three-dimensional network has been shown to behave as an ideal rubber with near-perfect reversible long-range elasticity.^[25,26]

Following the identification of a resilin-like motif sequence by Ardell and Andersen,^[29] there have been numerous recent reports of recombinant resilin-like polypeptides (RLPs), but these studies have focused largely on the physiochemical properties or the structure-function properties of the protein.^[30–43] After our initial reports describing the excellent elastomeric properties of RLPs explicitly designed for regenerative medicine applications,^[20] there have been a handful of additional reports exploring engineered RLPs for biomedical applications.^[20,21,44,45] These RLP-based materials have also inspired the development of highly resilient synthetic hydrogels composed of poly(ethylene glycol) PEG and PDMS.^[46] The novel RLP (RLP12) designed and reported by our group has a modular sequence that incorporates 12 repeats of the resilin-like consensus sequence in addition to domains for cell adhesion, cell-directed degradation, and heparin binding. Our previous work has demonstrated that, when cross-linked into hydrogels by reaction with an amine-reactive phosphine-based cross-linker, the hydrogels were highly resilient (> 90%) and recovered reversibly following deformation,^[21] a characteristic trait of natural resilin.^[21,26,27,47]

In order to further expand the versatility of these RLP materials, we have investigated the production of RLP-PEG hybrid hydrogels that are cross-linked through a Michael-type addition reaction between cysteine residues on the polypeptide and a vinyl sulfone-terminated four-arm star PEG cross-linker (Figure 1). The reaction between the cysteine thiol and vinyl sulfone has been shown to be highly selective at relatively mild pH [48,49] and has been widely applied as a cross-linking mechanism for biomaterial hydrogels.[48,50–66] The use of multicomponent PEG hydrogels has been widely employed owing to the fact that PEG macromers are nonimmunogenic, resist protein adsorption and may utilize a variety of cross-linking chemistries and chain architectures.[67] The development of protein-PEG hybrid hydrogels combines the advantages of PEG macromers with those of biosynthetic polypeptides, namely the cross-linking chemistry and chain architecture flexibility of the PEG macromers and the specificity and inherent bioactivity of the polypeptide component.[48,56] In early reports by the Hubbell and co-workers, [48,56] PEG macromers were used to cross-link recombinant polypeptides through either vinyl sulfone based Michael-type addition [48,56] or photoinitiated polymerization of acrylate groups.[68] Sequences derived from fibrinogen, collagen and anti-thrombin III were utilized in the recombinant polypeptide component and provided cell-matrix interactivity.[48,56,68] In addition, Ehrbar and co-workers [69–72] have demonstrated that enzymatically cross-linking hydrogels via the activity of transglutaminase factor XIIIa offers a promising strategy for mild and specific cross-linking of multicomponent PEG hydrogels. Other strategies include the photoinitiated thiol-ene chemical cross-linking of peptides and PEG macromers developed by Anseth and co-workers.[73,74]

The production of RLP-PEG hydrogels is motivated by these previous reports with the incorporation of resilin-like sequences intended to confer elastomeric properties to hybrid matrices containing cell-instructive domains. Reversible elasticity would be particularly advantageous in cardiovascular applications where the biomaterial would be subjected to hemodynamic cyclic loading forces.[3] A compliant, cell-instructive biomaterial could serve as a beneficial therapeutic material in vascular grafts, angioplasties, or other cardiovascular surgeries. Adventitial fibroblasts, which are located in the tunica adventitia of blood vessels, [75] have emerged as an important regulator of vessel health and thus serve as an appropriate model for assessing the viability of RLP-PEG hydrogels as potential three-dimensional matrices for cardiovascular applications. This work reports the development of higher molecular weight RLPs with increasing numbers of cysteines to permit their incorporation into PEG-based hybrid materials. The cross-linking and mechanical properties of these RLP-PEG hydrogels were assessed via dynamic oscillatory rheology; homogeneous, stable networks are rapidly formed upon mixing of the two components. In addition, the encapsulation of adventitial fibroblasts demonstrates the cytocompatibility of these matrices for primary cells derived from human cardiovascular tissue. These RLP-PEG hydrogels are thus exciting biomaterials with potential as an injectable tissue engineering scaffold for cardiovascular applications.

2. Experimental Section

2.1. Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Waltham, MA) and used as received unless otherwise noted. Ni-NTA agarose resin was purchased from Qiagen (Valencia, CA). Water was deionized and filtered through a ThermoFisher Barnstead NANOpure Diamond water purification system. The RLP12 gene [20,21] in a pUC57 plasmid was obtained from Genscript Corporation (Piscataway, NJ) and the cloning plasmid pGEM-3z was purchased from Promega (Madison, WI). The expression plasmid, pET28a, was obtained from Novagen (EMD Chemicals, Gibbstown NJ). Restriction enzymes, phosphatases, and ligases were purchased from New England

Biolabs (Ipswich, MA). Electroporation was performed using a Gene Pulser Xcell Microbial System from Bio-Rad Laboratories (Hercules, CA). Three *Escherichia coli* strains utilized for cloning included DH5 α chemically competent cells and ElectroMax DH10B electrocompetent cells purchased from Life Technologies (Carlsbad, CA) as well as *dam*-/*dcm*- SCS110 cells purchased from Agilent Technologies (Santa Clara, CA). BL21StarTM (DE3) cells from Life Technologies served as the expression strain, as previously reported.^[20,21]

2.2. Recursive Ligation and Mutagenesis

Recursive ligation methods were employed to construct the higher molecular weight RLPs (RLP24, RLP36 & RLP48; Figure S1, Supporting Information) through the use of the flanking restrictions sites BglII and BclI. Due to the methylation sensitivity of the BclI enzyme, *dam*-/*dcm*- SCS110 cells had to be utilized to produce plasmid DNA from which the RLP12 gene could be wholly digested from the pGEM3z plasmid. This RLP12 gene fragment was then ligated into a BglII-linearized plasmid containing the RLP12, RLP24, or RLP36 gene depending on the stage of recursive ligation. The final genes were then removed by digestion of the flanking BamHI/HindIII restriction sites and were cloned into a pET28a plasmid. Standard techniques were used to complete the recursive ligation of the RLPs.^[76] Gene sequencing (Delaware Biotechnology Institute, Newark, DE) and DNA gel electrophoresis confirmed each stage of cloning as well as the final recombinant genes: RLP24, RLP36 & RLP48. The Ser19 residue of the RLPs was mutated to a cysteine using site-directed mutagenesis via the QuikChange II XL Site-Directed Mutagenesis Kit from Agilent Technologies (Santa Clara, CA). Gene sequencing confirmed the successful mutation. Chemically competent BL21StarTM(DE3) cells were transformed with the mutated pET28aRLP plasmids to yield the host strains used for expression.

2.3. Expression, Purification, and Characterization of RLPs

As previously reported,^[20,21] protein expression was achieved using Studier auto-induction techniques^[77] using ZYP-5052 media, with cultures grown at 37 °C for 4 h before the temperature was lowered to 24 °C for an additional 24 h of expression. Purification was achieved using metal chelate affinity chromatography according to our previously reported protocols, followed by dialysis, with minor modifications to improve the purity and yield of the protein (50–100 mg protein per liter of cell culture; details in the Supporting Information). Protein expression was confirmed and monitored via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized via Coomassie blue staining. The molecular weight of the RLPs was confirmed by MALDI-TOF analysis conducted at the W.M. Keck Biotechnology Resource Laboratory at Yale University (New Haven, CT). Amino acid analysis confirmed the composition of the polypeptides and was performed by the Molecular Structure Facility at the University of California, Davis (Davis, CA) using a Hitachi L-800 sodium citrate-based amino acid analyzer (Tokyo, Japan).

2.4. Preparation of Reduced Proteins

Pure lyophilized RLP was reduced using tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl) in a 10×10^{-3} M MES 500×10^{-3} M NaCl buffer (pH 5.6) and desalted using a ZebaTM Spin desalting column (7 kDa MWCO, Pierce Rockford, IL). The flow-through was immediately frozen in liquid N₂ and lyophilized. The free thiol content of protein was measured using the Ellman's assay protocol (in 100×10^{-3} M Tris, 2.5×10^{-3} M sodium acetate, 40×10^{-6} M DTNB, pH 8.0);^[78,79] the colorimetric change of the solution upon reduction of the DTNB by the RLPs was monitored using an Agilent 8453 UV-Vis spectrophotometer.

2.5. Synthesis of PEG Vinyl Sulfone Cross-linker

Hydroxyl-terminated four-arm star PEG (10 kDa) was purchased from JenKem Technology USA (Allen, TX) and functionalized with vinyl sulfone following previously established protocols by Lutolf and Hubbell.^[53] ¹H NMR (400 MHz, CDCl₃) confirmed quantitative functionalization (Figure S2, Supporting Information): δ = 3.6 ppm (PEG backbone), 6.1 ppm (CH₂=CH-SO₂, d, 1H), 6.4 ppm (CH₂=CH-SO₂, d, 1H), 6.8 ppm (CH₂=CH-SO₂ dd, 1H).

2.6. Hydrogel Formation and Oscillatory Rheology

RLP-PEG hydrogels were formed by simple mixing of precursor solutions of the PEG and RLP under specific conditions described below. Oscillatory rheology experiments conducted on a stress-controlled AR-G2 rheometer (TA Instruments, New Castle, DE) were used to characterize the cross-linking and subsequent mechanical properties of the RLP-PEG hydrogels. Experiments were conducted at 37 °C using a 20 mm diameter cone-on-plate geometry with 1° cone angle and 25 μ m gap distance. Time and frequency sweeps were performed on all hydrogels. The RLP and PEG precursors were dissolved separately in PBS buffer (pH 7.4); a 0.2 μ L drop of concentrated NaOH was used to adjust the pH of RLP solution to approximately 8.0 and both precursors were kept on ice prior to the experiment. The precursors were then combined, vortexed briefly, and loaded onto the rheometer stage maintained at a temperature of 4 °C. This slowed the cross-linking reaction so that the geometry could be lowered into place prior to gelation. Once the experiment was started, the temperature was quickly increased to and maintained at 37 °C; mineral oil was used to seal the hydrogel and prevent evaporation. The RLP-PEG hydrogels were prepared at concentrations of 20 wt% and were cross-linked at vinyl sulfone to cysteine residue (VS:CYS) ratios of 1:1 and 3:2. Experiments were repeated on three to four samples and representative data presented.

2.7. In Vitro Cell Culture of Aortic Adventitial Fibroblasts

Human aortic adventitial fibroblasts (AoAF) were a generous gift of Dr. Robert Akins (Nemours, Wilmington, DE). The cells were cultured according to protocols provided by Lonza (Basel Switzerland) and using the Lonza SCGM™ BulletKit™ media system. The cells for the encapsulation experiments were between passage numbers 7 and 11. Prior to the encapsulation, the AoAF cells were lifted, counted, and resuspended in stromal cell growth media (SCGM) at a concentration of 5 000 000 cells per mL. Aliquots of 50 000 cells (10 μ L) were prepared in microcentrifuge tubes for each of the gels. A solution of the PEG precursor was prepared in 10 μ L of PBS buffer (pH 7.4) while the RLP24 was dissolved into 30 μ L of PBS buffer (pH 7.4) with phenolphthalein (1×10^{-3} M) included as a pH indicator. The pH of the RLP solution was adjusted using 0.2 μ L drops of 2 N NaOH until a slight pink hue was observed indicating the pH was approximately 8.2. Hydrogels were produced at 20 wt% concentration and cross-linked at a 3:2 ratio (VS:CYS) to ensure sufficient cross-linking in the presence of cells. The precursors were mixed and briefly vortexed before being gently triturated with the 10 μ L of media containing the cells. This solution was then loaded onto a glass-bottom dish (MatTek Corporation Ashland, MA) and placed into a cell culture incubator where the hydrogels were cross-linked at 37 °C; media (3 mL) was added to the gels after 3 min of cross-linking. Hydrogels were lifted from the glass-bottom dish after an hour of incubation to facilitate diffusion of media into the hydrogel; media was exchanged every 3 d. Cell viability and proliferation analysis were performed on three sample hydrogels at days 0, 1, 3, and 7. Hydrogels were costained with Calcein AM and Ethidium homodimer-1 (Live/Dead® Life Technologies Carlsbad, CA) and DRAQ5™ stain (BioStatus Limited Shephshed, United Kingdom) to assess the viability of encapsulated cells and the total number of cell nuclei, respectively. Hydrogels were washed 2-3 $\times$ with approximately 5 mL of PBS and then incubated at 37 °C in a 5% CO₂ incubator for 30 min

in 700 μL of PBS containing: $5 \times 10^{-6} \text{ M}$ DRAQ5, $2 \times 10^{-6} \text{ M}$ Calcein AM, and $4 \times 10^{-6} \text{ M}$ Ethidium homodimer-1. Cell viability and nuclei number were visualized using fluorescence laser scanning confocal microscopy (Zeiss LSM 510 NLO multi-photon, Carl Zeiss, Inc., Thornwood, NY). To ascertain proliferation of the aortic fibroblasts in the PEG–RLP gels, microscopy data were acquired, in each hydrogel, from four adjacent regions approximately 1800 μm in length and width and 400 μm in depth. The numbers of cells in each tile were determined via image analysis (see below). Three hydrogels were imaged in this manner for each time point; the center of the four tiles was chosen to be the middle of the hydrogel and the tiles were independently assigned. In total, approximately 5 mm^3 (5 μL) of each hydrogel (50 μL) was imaged and analyzed. These data were processed using Volocity 3D Image Analysis Software (PerkinElmer, Waltham, MA) to determine numbers of cell nuclei for both the membrane-permeable Draq5 stain as well as the membrane-impermeable ethidium homodimer-1 stain; the number of living cells was calculated as the difference between the two counts.

3. Results and Discussion

3.1. Design of RLPs

The sequences of all the RLPs, with the detailed amino acid sequences of the various domains, are presented in Figure 1. As previously reported,^[20,21] these constructs utilized a slightly modified version of the putative resilin-like sequence (GGRPSDS F GAPGGGN) derived from the first exon of the *Drosophila melanogaster* CG15920 gene.^[29] The tyrosine residue included in the original motif was substituted with a phenylalanine residue (shown in bold) for the potential future incorporation of nonnatural amino acids that would confer photocross-linking properties. Tensile testing and oscillatory rheology have shown that this substitution does not affect the mechanical properties of the RLP12 hydrogels.^[20,21] Additionally, the RLP includes sequences for cell-matrix interactions including an integrin-binding domain for cell adhesion (GRGDSPG),^[80] a matrix metalloproteinase (MMP) sensitive sequence (GPQGIWGQ)^[81] for cell-directed matrix degradation, and a heparin-binding domain (HBD) (KAAKRPKAAKDKQTK)^[82] for the sequestration of heparin and growth factors.

RLPs of higher molecular weight were produced to yield polypeptides with multiple cysteine residues, which would permit cross-linking of these RLP sequences into a network using Michael-type addition reactions. Recursive ligation of the original RLP12 gene yielded RLP24, RLP36, and RLP48 with cysteine functionalities of two, three, and four, respectively (Figure S3, Supporting Information). A restriction digestion using BamHI and HindIII of the four pET28a/RLP genes and a pET28a plasmid, followed by DNA gel electrophoresis, was performed; results are shown in Figure 2A. As illustrated in the figure, bands are present at approximately 800, 1500, 2200, and 2900 bp; these lengths correspond to the expected base-pair length of the four RLP genes. (The band at 5300 bp results from the linearized expression plasmid.) These results confirm the success of the recursive ligation of the RLP genes and their insertion into the pET28a expression plasmid. Further confirmation of these results was provided by gene sequencing (data not shown).

The original RLP12 gene encoded for a single cysteine residue that was located near the C-terminus of the polypeptide; recursive ligation introduced polypeptides with higher functionality, but with cysteine residues that were located only toward the C-terminal end of the domains (Figure S3, Supporting Information) rather than throughout the full polypeptide, which caused a significant fraction of the N-terminal end of the RLP to not be crosslinked into the network. Initial rheological characterization of RLP–PEG hydrogels using these original RLP constructs yielded materials with moduli far below predicted values and too low for practical use in clinical application (data not shown). In order to permit effective

crosslinking of the N-terminal end of each polypeptide chain and minimize any deleterious effect of dangling chain ends,^[83] the Ser19 residue at the N-terminus of each polypeptide was mutated to a cysteine via site-directed mutagenesis (data not shown). The polypeptides with this S19C mutation have been used for all further characterization and application.

3.2. Protein Expression, Purification, Characterization, and Reduction

Each of the RLPs was expressed using Studier auto-induction techniques and purified under native conditions using Ni-NTA affinity chromatography. SDS-PAGE analysis was conducted on the purified RLP fractions (Figure 2B). The bands on the gel are present at 30, 52, 76, and approximately 100 kDa, consistent with the expected molecular weights of the RLP12, RLP24, RLP36, and RLP48 proteins, respectively. The lack of any other significant bands in the SDS-PAGE analysis indicates the purity of the RLPs. In this analysis, the RLPs migrate slightly higher than their theoretical values, but this is consistent with our previous results for RLP12^[20] as well as with results from RLPs expressed by other laboratories.^[30,32] Atypical SDS-binding has been described as cause for this discrepancy and has been noted for proteins with unusual amino acid composition.^[32,84] MALDI-TOF analysis of intact RLPs was performed to confirm the exact molecular weights of the proteins; analysis shows that the major peaks fall within 1% of the theoretical molecular weights (Figure S4, Supporting Information). Amino acid analysis of the RLPs demonstrated that the composition of the purified proteins was within 10% of the expected values (Table S1, Supporting Information). Given that the composition of the protein is consistent with that expected theoretically, the existence, in low percentages, of a slightly lower molecular weight product likely results from truncation or degradation as has been described in other reports of RLP expression.^[31,37,45] Isolation of products that lack these lower molecular weight components can be achieved by lowering the temperature of the expression culture and by harvesting during the exponential phase of the expression.^[45] Given the small overall percentage of sample comprising the truncated product, such optimization was not conducted in these studies and the polypeptides were used without further purification.

With molecular weights of 71.1 kDa and 92.9 kDa, the RLP36 and RLP48 proteins represent the highest molecular weight RLPs reported to date; additionally, these RLPs represent some of the highest yielding RLPs expressed outside of high-cell density fermentation.^[21,31,44,45] As shown in Table 1, each of the RLPs could be expressed and purified in significant quantities ranging from 50 to 100 mg L⁻¹ culture depending on the specific polypeptide. RLP24 yielded the greatest quantity per liter of expression (100 mg L⁻¹ culture), which although less than values reported for elastin-like polypeptides (1.6 g L⁻¹ of expression media),^[85] is greater than yields often reported for other protein polymers.^[48,68] The high yields under native conditions for purification suggest that these RLPs are highly soluble and easily expressed by their bacterial hosts. Natural resilin and RLPs have been shown to be relatively heat stable^[27,31,32] and this property has proven useful in the purification of RLPs including the RLPs described in this work.^[31,32] Purification of RLPs was improved through the inclusion of β -mercaptoethanol to the purification buffers to prevent disulfide formation and through the addition of a heating step to selectively precipitate nonresilin bacterial proteins from the cell-free lysate. RLPs with free thiol were prepared by first reducing the cysteine residues of the RLP via treatment with TCEP-HCl in an acidic buffer, followed by desalting, freezing and lyophilization. Using this method, RLPs with approximately 90%–100% free thiol content could routinely be produced.

3.3. Oscillatory Rheology Experiments

The gelation and mechanical properties of the RLP-PEG hydrogels play an important role in determining whether the materials could be potentially useful in a desired clinical capacity.

In situ oscillatory rheology offers a practical approach to elucidating the gelation kinetics and the ultimate mechanical properties of the RLP-PEG hydrogels, and also provides direct correlation to our previous work on PEG-based hydrogels and their impact on cardiovascular cell phenotypes. Thorough evaluation of the tensile properties of these materials, to permit comparison of the elastomeric behavior of these RLP-PEG hydrogels versus the highly elastomeric behavior of RLP-only hydrogels, is currently underway. For the oscillatory rheology experiments, RLP-PEG hydrogels prepared in PBS at a 20 wt% concentration were characterized via dynamic oscillatory rheology using a cone-on-plate geometry at either a 1:1 or 3:2 ratio of vinyl sulfone groups to cysteine residues. Figure 3A shows representative time sweeps for the cross-linking of RLP24-PEG hydrogels at the two cross-linking ratios investigated. These data demonstrate that the 3:2 cross-linking ratio leads to higher moduli and more rapid cross-linking than the 1:1 cross-linking ratio; the final moduli of the 3:2 hydrogels were consistently in the 12–13 kPa range while the 1:1 hydrogels had final moduli in the 8–9 kPa range. As the inset in Figure 3A shows, gelation took place in under a minute [as indicated here by the crossover of the storage modulus (G') and loss modulus (G'')], although the storage modulus did not reach a plateau until much later, which may suggest that the reaction of the thiol and vinyl sulfone groups is incomplete at shorter timescales during gelation. While we did not explicitly evaluate the extent of reaction in these gels in this initial report, such evaluation will be a part of future studies. *In situ* oscillatory rheology for RLP-PEG hydrogels cross-linked with RLP12, RLP36, or RLP48 indicated similar results (data not shown). These data are consistent with the gelation profiles of other multicomponent-PEG hydrogel networks cross-linked via Michael-type additions,^[48,53] although are in contrast to the gelation profiles of RLP12 hydrogels (25 wt %) cross-linked with β -[tris(hydroxymethyl)phosphino]propionic acid (THPP) (which reached plateaus within 10 min).^[21] This disparity is likely due to the difference in the cross-linker; the diffusion of small molecule cross-linkers such as THPP is not as restricted as macromolecules such as the PEG-VS.

To assess the stability of the RLP-PEG hydrogels across a range of shear rates, dynamic oscillatory frequency sweeps ranging from 0.1 to 100 rad s⁻¹ were employed; Figure 3B provides representative data from experiments with the RLP24-PEG hydrogels. The values of the storage modulus were insensitive to frequency over the frequencies investigated, indicating that the hydrogels behave as elastic solid-like materials expected of permanently cross-linked networks. Similar insensitivity to angular frequency was observed for the RLP-PEG hydrogels cross-linked with RLP12, RLP36, and RLP48 (data not shown) and for the previously reported RLP hydrogels cross-linked with THPP.^[20,21] Interestingly, at higher angular frequencies the RLP-PEG networks behaved more elastically than other recombinant protein-PEG hydrogels cross-linked by the same chemistry;^[48] the recombinant protein-PEG hydrogels reported by Rizzi et al. demonstrated increasing phase angle and loss modulus starting at angular frequencies beginning in the 2–5 Hz range, which was attributed to network structure relaxations at those higher frequencies. The relative insensitivity of the RLP-PEG hydrogels at high angular frequencies indicates a robust network with a potential wider range of clinical applications.

Table 2 provides a summary of the observed final elastic moduli for hydrogels cross-linked with all four of the RLPs. With the exception of RLP12, there was little change in the final elastic moduli of the RLP-PEG hydrogels regardless of the number of cysteines on the RLP, owing to the fact that the molecular weight between cross-links does not change between the various RLP constructs.^[20,83] Hydrogels cross-linked using RLP24, RLP36, and RLP48 had elastic moduli in the 7–9 kPa range when cross-linked in a 1:1 ratio and of approximately 11–12 kPa when cross-linked in a 3:2 ratio of vinyl sulfone to cysteine. The reason that RLP12, which contains only two cysteine residues, does not follow this trend is likely due to its propensity to form network defects; the failure of one cysteine to react with a load

bearing part of the network would result in the formation of a dangling chain end.^[53,83,86] Nevertheless, the final mechanical properties of these hydrogels fall within 5–10 kPa, a suitable range for cardiovascular applications.^[87,88]

3.4. Encapsulated Cell Viability and Proliferation Assays

Human aortic adventitial fibroblasts (AoAF) were mixed at room temperature into a solution containing RLP24 and PEG-VS at a 3:2 ratio of vinyl sulfone to cysteine; the resulting hydrogels were cultured at 37 °C in a 5% CO₂ incubator in SCGM growth media. Cell viability was analyzed several hours following encapsulation, out to 7 d of culture, using fluorescent laser scanning confocal imaging via live/dead staining. Representative images shown in Figure 4 illustrate the great excess of the calcein-AM stain (green) and relative absence of ethidium homodimer-1 (red), indicating that the majority of cells survive encapsulation and remain viable out to 7 d; indeed, the AoAFs not only survive encapsulation but also begin to spread out and adopt a spindle-shaped morphology by day 7. Figure 4A presents day 0 data for the AoAFs a few hours following encapsulation and shows viable cells with a rounded morphology that are small in size. Twenty-four hours following encapsulation (Figure 4B), the majority of the AoAFs remain rounded, but a few appear to begin spreading in the hydrogel. By day 3 (Figure 4C), these extensions become more clearly defined and more cells adopt this phenotype; additionally, the cells have also increased in size. By day 7 (Figure 4D), a majority of the AoAFs has adopted a spread phenotype and the cells have also grown considerably larger in comparison to those analyzed immediately following encapsulation (Day 0). The data clearly show that the RLP–PEG matrices can support encapsulated fibroblasts, but it also suggests that the cells may be interacting with the biological cues engineered into the RLP. The transition from a rounded to extended morphology over the course of a week suggests that these cells may be locally degrading the RLP–PEG matrix and perhaps are forming focal adhesions to the integrin binding domains.^[56,58,69] It is well documented the inclusion of matrix degradation sequences and cell adhesion domains facilitates cell spreading in 3D^[56,58,73,74] and that the absence of these domains results in cells that remain rounded.^[16,18] Therefore, due to the clear morphological change of the AoAFs and the fact that it took several days for this transformation to occur, it is reasonable to suggest that the cells are remodeling and adhering to the RLP–PEG matrix. RLPs that lack the cell-binding domains and MMP-sensitive sequences are currently under investigation.

To determine whether the fibroblasts were proliferating, cell nuclei were stained with Draq 5 in addition to Live/Dead stains and imaged using fluorescent laser scanning confocal microscopy; the number of nuclei was counted using Volocity 3D Image Analysis Software imaging software. Three individual hydrogels were imaged per time point and a significant area of each hydrogel was imaged ($\approx 5 \mu\text{L}$) in an effort to prevent bias. It is assumed that all nuclei would be stained with the membrane permeable Draq 5 and that only the membrane-compromised cells would be stained with the ethidium homodimer, therefore the difference between the two counts represents the number of living cells. Figure 5 reports the average number of living cells at each time point in approximately a 5 μL volume of RLP–PEG hydrogel. In the figure, the bars represent the average of three gels with the error bars indicating one standard deviation; the open circles provide the actual numbers of living cells recorded for an individual hydrogel. The general trend of the data indicates that the number of living cells drops initially, as has been reported for other 3D cell constructs,^[73,89] but recovers by the final time point at day 7. Unfortunately, there is a large spread in the data (particularly for days 0 and 7), which most likely results from an initial lack of homogeneity of cell dispersion in these gels during gelation. Despite this experimental error, the number of living cells remains stable, and perhaps increases with time, providing further evidence that the RLP–PEG matrices can successfully support encapsulated fibroblasts.

Hybrid hydrogels consisting of protein and PEG offer a strategy to designing tissue engineering materials that combine the inherent bioactivity of proteins with the versatility of synthetic systems. Seliktar and co-workers^[90] have employed PEGylated-fibrinogen/fibronectin copolymers that could be cross-linked into hybrid hydrogels through the photoinitiated polymerization of acrylate moieties on the PEG chains. These hydrogels demonstrated utility in the three-dimensional cell culture of cardiomyocytes,^[91] neonatal human foreskin fibroblasts,^[92] and smooth muscle cells.^[93] Although the fibrinogen/fibronectin conferred the hydrogels with cell adhesive and cell-mediated degradation properties,^[90–93] there was still little to no control over the size and sequence of the protein component of these hydrogels and therefore cell-matrix interactions could not be adapted to specific biological applications.^[48] Halstenberg et al.^[68] and Rizzi et al.^[48,56] demonstrated that protein polymers, in which size and amino acid sequence are exactly controlled, could be cross-linked using PEG cross-linkers into elastic hybrid matrices using photopolymerization and Michael-type addition, respectively. The protein polymers contained sequences derived from extracellular matrix (ECM) proteins that conferred enzyme-degradable and cell adhesive properties. Encapsulated neonatal foreskin fibroblasts demonstrated the ability to migrate through the matrices which contained the degradable sequences suggesting that the cells were actively remodeling and interacting with their microenvironment.^[48,56,68]

The results presented here indicate the potential of RLP-PEG matrices as tissue engineering materials for cardiovascular tissue engineering. Future work will investigate the role of the MMP-sensitive domain and the presence of integrin-binding domains in the development of the spindle-like AoAF morphology. Additionally, matrix stiffness has previously been indicated by our group as having an impact on the proliferation and phenotypes of AoAFs,^[87,94] and the use of RLP-PEG hydrogels for 3D encapsulation of these cells may provide a more physiologically relevant analysis of the effects of matrix elasticity on these cells.

4. Conclusions

This study reports the development and production of three high-molecular-weight RLPs, which can be cross-linked into elastic hybrid hydrogels via a Michael-type addition reaction with a PEG-vinyl sulfone cross-linker. These hydrogels can be cross-linked under benign conditions and successfully encapsulate human aortic adventitial fibroblast cells. The encapsulated cells remained viable over 7 d of culture and began to adopt a spread morphology consistent with the natural fibroblast phenotype. These hybrid hydrogels present a promising strategy to the development of tissue engineering materials for cardiovascular applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Scheme of the hydrogel formation and details of the cross-linking chemistry are presented alongside the amino acid sequence of the RLPs. Additionally, a legend indicating the resilin-like and biological domains is presented beneath the amino acid sequence. The parentheses indicate repeated sequences; in total, there are 12 repeats of the resilin sequence and two repeats of the integrin-binding domain within the larger brackets. The brackets indicate the repeated sequences of the higher molecular weight RLPs (RLP12 $\times$ = 1, RLP24 $\times$ = 2, RLP36 $\times$ = 3, RLP48 $\times$ = 4).

Figure 2.

(A) Agarose gel showing the BamHI/HindIII digestion of pET28aRLP48, pET28aRLP36, pET28aRLP24, pET28aRLP12, & pET28a (left to right). The linearized pET28a plasmid is clearly resolved between 5000 and 6000 bp while the RLP genes are represented by the bands migrating at approximately 800 bp to 3200 bp. (B) Coomassie stained SDS-PAGE gel (12%) showing the purified RLP proteins, RLP48, RLP36, RLP24, & RLP12 (left to right).

Figure 3.

(A) Oscillatory rheology time sweep of a 20 wt% RLP24-PEG hydrogel cross-linked at 1:1 (gray) or 3:2 (black) molar ratios of vinyl sulfone to cysteine, at 37 °C with an angular frequency of 6 rad s⁻¹ and a strain of 1%. The inset demonstrates that the storage modulus (G' , solid squares) exceeds the loss modulus (G'' , open squares) within a minute. (B) Oscillatory rheology frequency sweep experiments for RLP24-PEG hydrogels. Frequency sweeps were conducted over a range from 0.1 to 100 rad s⁻¹ for 20 wt% hydrogels cross-linked at 1:1 (gray) or 3:2 (black) vinyl sulfone to cysteine at 37 °C. The closed symbols indicate the storage modulus (G') and the open symbols indicate the loss modulus (G'').

Figure 4.

Fluorescent laser scanning confocal microscopy of human aortic adventitial fibroblasts (AoAF) encapsulated in 20 wt% RLP24-PEG hydrogel cross-linked at a 3:2 vinyl sulfone to cysteine ratio, stained using Live/Dead stains. Calcein AM (Live) stain is shown in green and ethidium homodimer (Dead) stain is shown in red. Images are z-stacks of hydrogels (200 μm thick) taken at 10× magnification using a water lens. Representative images from (A) day 0, (B) day 1, (C) day 3, and (D) day 7 are presented; the AoAFs remain viable throughout the entire experiment. Initially, the cells are rounded but as the experiment progresses they begin to adopt a spread morphology.

Figure 5.

Proliferation data for encapsulated human aortic adventitial fibroblasts (AoAF) in RLP24-PEG hydrogels over 7 d of cell culture. The bars represent the average number of living nuclei counted at a given time point; the error bars represent one standard deviation from the mean and the open circles represent the counts for each hydrogel analyzed ($n = 3$). The number of living cells was determined by finding the difference between the number of nuclei stained by membrane-permeable Draq5 and the number of nuclei stained by membrane-impermeable ethidium homodimer. Fluorescent laser scanning confocal microscopy was used to analyze four adjacent z-stacks that were $1800\ \mu\text{m} \times 1800\ \mu\text{m} \times 400\ \mu\text{m}$ (length $\times$ width $\times$ depth) for three different hydrogels at each time point. Volocity was used to count the cell nuclei for both the Draq5 and the ethidium homodimer channels. The results show that the number of nuclei remains relatively stable and that by day 7 the cells may be beginning to proliferate.

Table 1

Resilin-like polypeptides molecular weights and yields.

RLP	Theoretical molecular weight [Da]	Protein yield ^{a)} [mg L ⁻¹ expression]	Specific yield ^{a)} [mg g ⁻¹ cell pellet mass]
RLP12	27590	77.2	4.0
RLP24	49390	99.6	5.2
RLP36	71189	79.0	4.0
RLP48	92989	53.2	2.7

^{a)} values are mean of at least two separate purifications.

Table 2

Summary of mechanical properties for RLP – PEG hydrogels.

RLP	Number of cysteines ^{a)}	Cross-linking ratio[VS:CYS] ^{b)}	
		1:1	3:2
		Storage modulus [Pa] ^{c)}	Storage modulus [Pa] ^{c)}
RLP12	2	2630 ± 620	7190 ± 380
RLP24	3	8040 ± 1385	12300 ± 1601
RLP36	4	7770 ± 395	11930 ± 1630
RLP48	5	8950 ± 730	11480 ± 360

^{a)} Number of cysteines contained in the polypeptide;^{b)} the ratio of vinyl sulfone groups to cysteine residues in a 20 wt% hydrogel;^{c)} value for storage modulus is an average of at least three samples and the (±) error is equal to one standard deviation.