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Tunable Mechanical Stability and Deformation Response of a Resilin-based Elastomer

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Abstract

Resilin, the highly elastomeric protein found in specialized compartments of most arthropods, possesses superior resilience and excellent high-frequency responsiveness. Enabled by biosynthetic strategies, we have designed and produced a modular, recombinant resilin-like polypeptide bearing both mechanically active and biologically active domains in order to create novel biomaterial microenvironments for engineering mechanically active tissues such as blood vessels, cardiovascular tissues and vocal folds. Preliminary studies revealed that these recombinant materials exhibit promising mechanical properties and support the adhesion of NIH 3T3 fibroblasts. In this report, we detail the characterization of the dynamic mechanical properties of these materials, as assessed via dynamic oscillatory shear rheology at various protein concentrations and cross-linking ratios. Simply by varying the polypeptide concentration and cross-linker ratios, the storage modulus G' can be easily tuned within the range of 500Pa to 10kPa. Strain-stress cycles and resilience measurements were probed via standard tensile testing methods and indicated the excellent resilience (>90%) of these materials, even when the mechanically active domains are intercepted by non-mechanically active biological cassettes. Further evaluation, at high frequencies, of the mechanical properties of these materials were assessed by a custom-designed torsional wave apparatus (TWA) at frequencies close to human phonation, indicated elastic modulus values from 200-2500Pa, which is within the range of experimental data collected on excised porcine and human vocal fold tissues. The results validate the outstanding mechanical properties of the engineered materials, which are highly comparable to the mechanical properties of targeted vocal fold tissues. The ease of production of these biologically active materials, coupled with their outstanding mechanical properties over a range of compositions, suggests their potential in tissue regeneration applications.

Keywords

Resilin; resilin-like polypeptide (RLP); elastomer; resilience; vocal fold; tissue engineering; hydrogel; biomaterial

1. Introduction

Resilin, an elastomeric structural protein, was first observed in the thorax of grasshoppers in the course of an anatomical investigation by La Greca in 1947.¹ In 1960, investigations of other insect organs by Weis-Fogh indicated that resilin behaved as a true physical rubber, providing low stiffness, large strain, efficient energy storage and long-term fatigue resistance.²⁻⁴ The existence of resilin in the specialized compartments of the cuticles among other insect species was quickly discovered, indicating its role in their daily activities where efficient energy storage and repetitive movements are required, such as flight, jumping, leg locomotion and vocalization.^{2, 5-7} Detailed mechanical characterization demonstrated that

the rubber-like protein showed resilience of over 95% under frequencies of 200Hz,⁸ consistent with the function of resilin in insect organs that operate under high frequency conditions; in certain insects, deformations occur at frequencies of nearly 13kHz.^{6, 9} Moreover, it has also been reported that after stretching a dragonfly tendon to over twice its original length for a period of two weeks, the sample recovers its original size without any tearing or fatigue upon the release of stress.²

These excellent mechanical properties have fueled investigations of the structure-property relationships underlying the unique mechanical behavior. Early structural studies of resilin, including X-ray diffraction and microscopy, indicated an amorphous, unoriented protein that lacked any fine secondary structure.^{3, 10} Accordingly, in light of rubber elasticity theory, it was originally suggested by Weis-Fogh that the random-coil, cross-linked three dimensional resilin network resembles an isotropic ideal protein rubber with superior elastomeric features arising from entropic considerations.^{2, 3, 11} Like elastin, resilin has a significant percentage of glycine (31%) in its sequence, which contributes to the large conformational freedom of the polypeptide chain and likely offers at least in part the molecular basis for resilin's outstanding mechanical properties.^{12, 13}

Although the intriguing mechanical properties of resilin were discovered decades ago, not until 2001 did Ardell and Andersen report a gene from *Drosophila melanogaster*, CG15290, as the likely precursor for *Drosophila* resilin; the N-terminal region of this exon is dominated by 18 slightly different copies of a 15-residue motif - GGRPSDSYGAPGGGN.¹⁴ In 2005, Elvin et al. reported the cloning and expression of the first exon of the CG15290 gene as a soluble protein (Rec-1 resilin), which was formed into a solid, rubbery-like biomaterial via Ru(II)-mediated photocross-linking; these materials exhibited higher resilience values and longer fatigue resistance times over those of polymer-based synthetic rubbers.¹⁵ Since then, other resilin-based polypeptide sequences have been reported, including those with the consensus sequences AQTSSQYGAP and GGRPSDSYGAPGGGN, and a sequence containing the chitin-binding domain; the resilin-based polypeptides share similar conformational behavior and excellent mechanical features such as high resilience, large strain and low moduli.¹⁶⁻²⁰ More recently, other modular resilin-based materials have been developed. Lv *et al* utilized folded GB1 domains (from the streptococcal B1 immunoglobulin-binding domain of protein G), alternating with random-coil-like resilin to mimic the complex molecular springs found in the muscle protein titin.²¹

Much attention has been focused on mimicking the excellent mechanical behavior of natural resilin, however, dynamic interactions with cells and subsequent stimulation of explicit cell responses requires more than just a mechanically compatible substrate.²² Our laboratories have thus employed biosynthetic strategies to design a resilin-like polypeptide that is equipped with mechanically active domains as well as with biological cues as a potential scaffold for engineering mechanically active tissues such as those in the blood vessel, heart and particularly vocal folds.²³

Mechanical stresses, deleterious environmental factors and pathological conditions can disrupt the natural pliability of the lamina propria (LP) of vocal folds which controls the production of sound, resulting in a wide spectrum of voice disorders and billions of dollars of lost productivity in the United States alone.^{24, 25} Although surgical techniques have been used to treat vocal fold disorders, they inevitably cause scarring.²⁶⁻²⁸ Tissue engineering (TE) strategies offer compelling alternatives in the treatment of vocal fold diseases, and engineering a comprehensive biomaterial integrated with superior resilience, high-frequency responsiveness, and biological properties would be very promising. Indeed, it is well accepted that the ability to tune the mechanical properties of a substrate is a key design parameter to promote cell-material interactions that stimulate cells to penetrate the implant-

native tissue boundary.^{29, 30} Also, recent studies have indicated that the mechanical strength of a material has a direct impact on cell growth and differentiation.^{31, 32}

The high-frequency responsiveness of resilin recommends the development of resilin-based materials in treatments of vocal fold pathologies. In our previous studies, we demonstrated that a resilin-based hydrogel demonstrated promising mechanical properties and supported the adhesion of NIH 3T3 fibroblasts.²³ Accordingly in the work reported here, we detail a range of mechanical properties feasible with RLP-based materials in both the low- and high-frequency regimes. Purified resilin-like polypeptide (RLP) was cross-linked via a Mannich-type reaction³³ to form a solid, elastomeric rubbery-like biomaterial. RLP-based hydrogels under various conditions yield materials with mechanical properties similar to those of native vocal fold tissues not only in their mechanical strength but also in their resilience, extensibility, and high-frequency responsiveness. Oscillatory rheology, standard tensile testing and torsional wave apparatus experiments have been employed in order to probe gelation kinetics, gel stability, deformation response, resilience and high frequency-responsiveness of RLP-based hydrogels.

2. Materials and Methods

Materials

The DNA sequence for RLP12 (flanked by Bam HI and Hind III) in pUC57 was purchased from Genscript Corporation (Piscataway, NJ). Chemically competent cells of the *rne131 E. coli* expression strain BL21Star™(DE3) were purchased from Invitrogen (Carlsbad, CA). Ni-NTA Agarose resin was purchased from Qiagen (Valencia, CA). THPP (β -[Tris(hydroxymethyl) phosphino] propionic acid (betaine) was purchased from Pierce (Rockford, IL). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) and were used as received unless otherwise noted. Water was deionized and filtered through a NANOpure Diamond water purification system (Dubuque, IO).

Protein expression and purification

Detailed information regarding the genetic construction of the pET28a-RLP12 plasmid has been reported previously;²³ protein expression and purification was also conducted via the methods we have previously reported.²³ The molecular weight and the purity of the RLP were confirmed via SDS-PAGE and MALDI-TOF mass spectrometry; the identity was probed via amino acid analysis, performed by the Molecular Structure Facility at the University of California, Davis (Davis, CA).

Formation of covalently cross-linked RLP films

RLPs were dissolved in pH 7.4 degassed PBS at a final concentration of 200mg/ml. Stock solutions of both the RLP and the cross-linker [tris(hydroxymethyl) phosphino] propionic acid (THPP) were chilled on ice before mixing in order to slow the reaction, preventing cross-linking during handling. Different amounts of THPP were added to the 20wt% RLP stock solution in order to achieve various cross-linking ratios that are indicated as the molar ratio of reactive hydroxymethylphosphine (HMP) groups to lysines present on the RLP chain. To ensure homogenous mixing, the 20wt% mixture was pipetted vigorously after adding the THPP, and followed by careful pipetting of the mixture into 10mm $\times$ 10mm $\times$ 1mm wells in a PTFE mold, uniformly and without bubbles (except for oscillatory rheology measurements, see below). The mixture was allowed to cross-link for 2 hours in a 37°C incubator, with drying of the films. The final products were stored in degassed PBS at 4°C before use. (Similar washing protocols have also been employed for gels used in cell culture experiments, both in our previously reported work²³ and in preliminary data (not shown).) Assessment of possible sol fractions via SDS-PAGE analysis of supernatant buffers

indicated that essentially all of the RLPs were crosslinked into the films. Cross-linking efficiencies of RLP12 films were assessed via amino acid analysis and consideration of the total number of lysines in a RLP chain (11 lysines) as follows. For 100% efficient cross-linking at a cross-linking ratio of 1:0.5 (lysine to THPP), 5.5 lysines would be reacted, while at all other cross-linking ratios (1:1, 1:2, 1:3, 1:4 and 1:5) all of the lysines would be reacted. However, due to the facile oxidation of THPP, the reaction efficiency is low, so the use of increasing amounts of THPP yielded a higher fraction of reacted lysines. Due to the stability of the alkyamine linkage (formed upon the Mannich reaction of THPP with lysines) to acid hydrolysis, amino acid analysis was employed to detect how many unreacted lysines were left in the cross-linked hydrogels (based on a reduction of lysine content versus the unreacted RLP); this was thus used as a method to determine the cross-linking efficiency. The average number of reacted lysines for RLP-based hydrogels at 1:0.5, 1:1, 1:2, 1:4 and 1:5 cross-linking ratio are 1.1, 2.4, 3.2, 4.3 and 5.4, respectively, which correspond to cross-linking efficiencies (percentage of lysines reacted) given in Table 4.

Swelling ratio and water content measurements

RLP films prepared as described above were lyophilized for two days before measuring the dry weight. After the dry weights were measured, samples were immersed in phosphate buffer saline (PBS) for 24 and 48 hours at 37°C. The wet weights were measured after blotting the excess water using filter paper. The water content of these hydrogels was evaluated in terms of the percentage water content (WC) and swelling ratio (q) at equilibrium. $WC = (W_s - W_d)/W_s \times 100$, $q = W_s/W_d$, where W_s and W_d are the weights of swollen and dry hydrogels. Data reported are an average 8 measurement of 3 different samples, with error reported as the standard deviation.

Oscillatory rheology experiments

Bulk oscillatory rheology characterization of RLP12 hydrogels was conducted at 25 or 37°C on a stress-controlled rheometer (AR2000, TA Instruments, New Castle, DE) with a 20-mm diameter cone-on-plate geometry, 1.56 degree cone angle and at a 33µm gap distance. Dynamic oscillatory time, frequency and strain sweeps were performed. The samples were prepared by addition of a desired amount of [tris(hydroxymethyl)phosphino] propionic acid (THPP) to RLP12 solutions in PBS (pH 7.4) in a micro-centrifuge tube and were kept on ice before loading onto the rheometer bottom plate, to suppress the crosslinking reaction. The temperature was maintained at 25 or 37°C prior to the experiment in order to minimize the thermal expansion of the instrument. The top plate was lowered to the desired gap distance; the solution was applied via pipette, covered with mineral oil at the edge in order to prevent water evaporation, and then allowed to crosslink *in situ*. Strain sweeps were performed on samples from 0.01% to a maximum strain of 1000% to determine the limit of the linear viscoelastic regime (LVE). Dynamic oscillatory time sweeps were collected at angular frequencies of 6 rad/s and 1% strain chosen from the LVE and it was observed that the storage modulus (G') and the loss modulus (G'') reached a plateau in less than 20 mins. Rheological properties were examined by frequency sweep experiments ($\omega = 0.1$ -100rad/s) at a fixed strain amplitude of 1%. Experiments were repeated on 3 to 4 samples and representative data are presented.

Tensile testing

Films for tensile tests were prepared in contact lens molds by addition of desired amounts of THPP to 20wt% RLP12 solutions in saline buffer (pH 7.2). The films were cross-linked at 37°C for 2 hrs, after which the films dried. Before the measurements, the films were hydrated in PBS to equilibrium and cut into dogbone specimens with a stainless steel die (width 2mm; length 6mm). The test samples were mounted on an Instron 4502 mechanical tester equipped with a 500 gram load cell and were tested at room temperature under

hydrated conditions utilizing a tank containing saline buffer around the grips. Stress-strain data were recorded at a uniform strain rate of 5mm per minute and the films were subjected to three cycles each to strains of 30%, 60%, and 100%, and then to failure. Resilience was calculated from the areas under the loading and unloading curves.

Torsional wave experiments

The mechanical properties of RLP-based hydrogels at high frequencies (>10Hz) were evaluated using a custom-designed torsional wave apparatus (TWA), as previously described.^{34, 35} Preparation of hydrogel films was conducted as described above. Hydrogel samples were immersed in pH 7.4 PBS at ambient temperature and then shipped overnight to Brown University (Providence, RI) for mechanical testing. Just prior to testing (via TWA) samples were cut from the films using a dermal punch (3, 4, 5 or 6mm diameter, depending on the desired frequency range of the measurement). The thin, cylindrical cross-linked samples were sandwiched between two vertically aligned rigid hexagonal plates. The sample was enclosed in an environmental chamber with controlled temperature (34–37°C) and humidity (>94%). The bottom plate was driven sinusoidally by a galvanometer with frequencies varying from 10Hz up to 400Hz. As the bottom plate oscillates (at a rotational angle of <0.2°), a torsional wave propagates through the sample, driving the top plate into oscillation. The amplitude of rotation of both plates was monitored by an optical lever technique and the signals were captured by photodiode detectors. The experimentally determined amplification factor, defined as the ratio of the amplitude of rotation of the top plate to that of the bottom plate, reaches a peak value at the resonance frequency of the sample. A test on a sample includes a frequency sweep that includes the resonance frequency of the sample. For each test, a constant modulus and loss angle (with respect to frequency) were chosen that provided the best fit, in the least squares sense, between the linear viscoelastic model of the amplification factor and the experimental results. Further information on data reduction and the principles behind the TWA can be found in Ref 34. Good agreement between the predicted linear viscoelastic response and experimental data was observed for all samples tested.

3. Results and Discussion

Protein expression and purification

The sequence of the resilin-like polypeptide with 12 repeats designated as RLP12 is showed in Table 1.

We employ 12 repeats of pro-resilin putative consensus sequence (GGRPSDSFGAPGGGN) derived from the first exon of *Drosophila melanogaster* CG15920 in our resilin-like polypeptide. We replaced tyrosine with phenylalanine to permit incorporation of non-natural amino acids that would facilitate photo-crosslinking. The integrin binding domain GRGDSPG is included to promote cell adhesion.³² The MMP-sensitive substrate domain GPQGIWGQG was chosen to control the degradation of the matrix in order to promote cell migration and proliferation.³³ The heparin-binding domain KAAKRPKAAKDKQTK, based on its previously demonstrated heparin-binding affinity, is also included to control release of growth factors.³⁴ Facilitated via biosynthetic strategies, we designed this modular resilin-like polypeptide bearing both mechanically active, repetitive domains and necessary biological cassettes in order to create a novel biomaterial. Such a material may mimic natural extracellular matrix (ECM) microenvironments for engineering mechanically active tissues such as blood vessel, cardiovascular tissues and vocal folds.

The expression of RLP12 via the Studier auto induction method, instead of traditional IPTG induction, yields higher cell masses and higher yields.^{23, 39} Cell pellets were first lysed

under denaturing conditions, followed by buffer exchange and elution from a Ni-NTA column in native buffer, followed by dialysis to remove salts. High yields (70-75mg/L) and purity (>95%) were attained via this Ni-NTA immobilized affinity chromatography purification method. The composition and purity of the RLP12 was confirmed via SDS-PAGE, MALDI-TOF and amino acid analysis; the composition and purity of the samples reported here are identical to those previously reported.²³

Hydrogel formation and swelling ratio and water content measurements

Hydrogels were formed via covalent cross-linking of soluble RLPs with the cross-linker THPP, via a Mannich-type condensation reaction at desired concentrations and different cross-linking ratios. A schematic of the cross-linked resilin-based hydrogel network and images of the gels are shown in Figure 1. Variations in the opacity and color of the gels are observed depending on the cross-linker content, polymer concentration, and rate of gelation.

Hydrogels were soaked in water to remove unreacted THPP (suggested via the constant mass of the samples) and subsequently quenched in liquid nitrogen, and lyophilized for two days before measuring the dry weight. Various samples, crosslinked under different conditions, were immersed in pH 7.4 PBS for 24 hours in a 37°C incubator. Surface moisture was removed using filter papers and swollen weights were measured using a microbalance. The swollen weight was measured again after an additional 24 hours of immersion; no significant change was observed, suggesting that samples reached equilibrium after 24 hours of soaking. Water content and swelling ratios were then calculated as described in the experimental methods section. Representative data from these measurements are shown in Figure 2; the left panel shows the equilibrium swelling ratio while the right panel shows the equilibrium water content observed, for 20wt% RLP hydrogels at 1:1, 1:2 and 1:4 (lysine : HMP) cross-linking ratios.

As can be deduced from the figure, the swelling ratios of 20wt% at 1:1, 1:2 and 1:4 are approximately 9, 7 and 6. Accordingly, the water contents are approximately 90%, 87% and 82% respectively, which is comparable to values observed for natural resilin (water content 60%) and other polypeptide-based hydrogels (45-85%).^{2, 33, 40-42} A decrease in the swelling ratio is consistent with the increase in cross-linking efficiency as is expected, however, even at a relatively high cross-linking ratio of 1:4, the percentage water content at equilibrium is still higher than 80%, which suggests the hydrophilicity of RLP-based hydrogels. This hydrophilicity is promising for the use of these materials in tissue engineering applications, and may also offer advantages in immobilization of hydrophilic drugs or loading active growth factor over their hydrophobic counterparts.^{38, 43-46}

Oscillatory Rheology Experiments

The introduction of implantable biomaterials into tissue or organs may alter the mechanical properties of the tissue, and in an organ such as the vocal fold, for example, would alter phonation. This would be particularly true of biomaterial-based interventions involving the vocal fold mucosa, which is the major vibratory portion of the vocal fold in small-amplitude oscillations such as phonation onset and offset.^{47, 48} Sound production requires the propagation of a surface mucosal wave,⁴⁷ and thus the shear properties are important in the evaluation of candidate biomaterials. Accordingly, in situ oscillatory shear rheology experiments are relevant in the characterization of hydrogels, and provide an understanding of the impact of cross-linking conditions on gelation kinetics and gel stability.^{49, 50} The shear mechanical moduli of the cross-linked RLPs were characterized via dynamic oscillatory shear mode rheometry using a cone-on-plate geometry, after cross-linking the hydrogels under physiological conditions. Various compositions all showed fast gelation upon mixing with cross-linker. As shown in Figure 3, the elastic modulus (G') for a 25wt%

hydrogel with a 1:1 cross-linking ratio exceeds the loss modulus (G'') in less than 2 minutes at 25 °C.

The gelation times can be tuned via variations in RLP concentration, cross-linker ratio, and the cross-linking temperature. Higher protein concentrations, larger cross-linking ratios, and higher cross-linking temperature all result in a shorter gelation time (data not shown). For example, the gelation time is less than 15 seconds when 25wt% RLP12 is cross-linked at 37°C. As the data in Figure 3 also show, both G' and G'' reach a plateau within 10 minutes, with a 50-fold difference between their values, indicating the formation of an elastic hydrogel. The loss tangent (δ), which provides a relative measure of the viscous to elastic properties of a material, remains constant at a very low value (less than 0.05) during the entire time sweep (1 hour), suggesting the high elasticity and energy-storing features of cross-linked RLP hydrogels. The fast gelation suggests potential applications employing *in vivo* injection of RLP-based biomaterials.^{40, 46} In order to evaluate the stability of RLP-based hydrogels across various frequencies, 20wt% RLP hydrogel samples (with various cross-linking contents) were characterized via frequency sweeps in dynamic oscillatory shear mode. As shown in Figure 4, the insensitivity of G' over the low frequency range (0.1 to 100rad/s) demonstrated the stability and elastic-solid like behavior of these permanently cross-linked RLP-based hydrogels. A slight decrease of G' and increase of G'' (data not shown) at lower frequency (0.1rad/s) was observed, may suggest the activity of some physical interactions or the unraveling of entangled RLP12 chains at longer relaxation times.

A summary of the elastic shear moduli observed for various hydrogels is presented in Table 2. As shown in the table, by varying the polypeptide concentration and cross-linker ratios, G' can be easily tuned to values ranging from 500Pa to 10kPa. The increase in moduli observed with increasing THPP concentration reflects the low reaction efficiency of the cross-linking (see experimental section above); the increase in THPP thus permits further cross-linking rather than simple modification of lysine side-chains. This facile manipulation of shear moduli offers versatility in the choice of specific material compositions for a range of soft tissue engineering applications, for example soft vocal fold tissues from 500-5000Pa and cardiovascular tissues from 5000-10000Pa.^{46, 51, 52}

Tensile testing and resilience measurements

Repeated loading and unloading strain cycles in standard tensile testing format were employed in order to probe the deformation response, analyze hysteresis, determine the longitudinal elasticity and calculate the resilience of hydrated RLP-based hydrogel films. Hydrogels at 20wt%, with 1:1, 1:2 and 1:4 cross-linking ratios, were analyzed based on their range of elastic shear moduli; multiple samples at each condition were tested. For each film, 3 consecutive strain loading and unloading cycles were applied, first up to 30%, then to 60%, and then to 100% strain before straining to break. Representative data from these experiments are shown in Figure 5.

Figure 5A presents three repeats, for RLP hydrogels of various cross-linking ratios, of loading and unloading cycles of up to 30% strain, which is the approximate average strain sustained by the lamina propria (LP) during the production of sound.²⁴ Figures 5B-D show the third loading and unloading cycles, for RLP hydrogels (20wt%, at cross-linking ratio 1:1, 1:2 and 1:4 respectively), for strain values up to 30%, 60% and 100%; only the third cycles are shown for simplicity. The fact that the curves in Figure 5B are significantly less smooth compare with those in Figure 5C and 5D results from the sensitivity of the instrument, at the lower tensile loads, to environmental disturbances during measurement. The overlap of the stress-strain curves during loading and unloading suggests the negligible hysteresis, fast recovery and excellent elasticity of the RLP-based hydrogels, even up to 100% strain, despite the alteration in the amino acid sequence of resilin and the introduction

of the biologically active domains. The resilience of these hydrated RLP-based gels was calculated by dividing the area from the unloading curve by that of the loading curve, and the values of each cycle at different strains are listed in Table 3.

As shown in Table 3, the RLP-based hydrogels displayed high resilience values ranging from around 90% to 97.7% over repeated strain cycles, which is consistent with natural resilin and previously reported resilin-based polypeptides, and improved over available data reported for ELP-based hydrogels.^{15, 18, 20, 21, 41, 53, 54} Although novel synthetic polymer-based hydrogels have also demonstrated high resilience,⁵⁵ these RLP-based hydrogels, endowed with biological properties, high water content and high resilience at elevated frequencies, may serve as superior candidates for vocal fold regeneration. Hydrogels of the above compositions maintain high resilience even up to 200% strain (data not shown), recovering approximately 90% energy upon unloading. Although the resilience decreased for a given sample with initial increases in applied strain, after the first cycle the resilience recovered and stabilized throughout the remaining cycles. This observation is likely due to stabilization of load-induced changes in microstructure, such as the dissociation of entanglements, after initial stretching and is also consistent with observations reported for other recombinantly synthesized elastin-like polypeptides (ELPs).⁴¹ Thus, appropriate preconditioning might be a reasonable route to optimize the resilience of these materials in use.

Strain-to-break experiments on these RLP hydrogels were also conducted in order to determine the longitudinal elasticity and the Young's modulus (from the linear region (5-15%) of the stress-strain curve). As shown in Figure 6, the highest strain-to-break values observed for the RLP hydrogels at 20wt% 1:1, 1:2 and 1:4 cross-linking ratio were approximately 410%, 320%, and 250%, respectively.

The average strain-to-break and other general mechanical properties are listed in Table 4.

With increasing amounts of cross-linking, the strength increases while the strain-to-break decreases, as expected, owing to the suppression of the chain movement and increased stiffness of the materials at higher cross-linking ratios. The average strain-to-break for the RLP hydrogel at 1:1 cross-linking ratio reaches 335% with a cross-linking efficiency of approximately 21.4% (characterized via amino acid analysis, see above), which is consistent with the properties reported for natural resilin (200% strain-to-break, 20% cross-linking efficiency) and other recombinantly synthesized RLPs (310% strain-to-break, 18.8% cross-linking efficiency) under the same strain rates.^{2, 15, 18} The nuances and heterogeneity in strain-to-break values, as well as resilience values, might arise from sample-specific local defects, micro-cracks, different cross-linkers, methods, sample gripping, loading, and variations of sample thickness. Encouragingly, the Young's modulus of RLP-based hydrogel films in our experiments, which ranges from 15-35kPa, compares favorably with those of human vocal fold tissue (20-40kPa) and other RLPs (25.5kPa).^{15, 18, 56} A summary of the mechanical properties for elastin, resilin, ELPs, RLPs, and vocal folds are listed in Table 5.

High frequency responsiveness of RLP hydrogels

Our motivation in creating RLP-based hydrogels stems from their potential application in regenerating highly mechanically active vocal fold tissues. Hence, it is important to gain a fundamental understanding of hydrogel viscoelasticity at human phonation frequencies (75-1000Hz). A direct measurement at frequencies close to human phonation was evaluated using a custom-designed TWA. In these experiments, the hydrogel samples were cast in a thin, rectangular film. Cylindrical samples were then cut out from this film and were mounted between two vertically aligned acrylic plates, enclosed in an environmental chamber with temperature (34-37°C) and humidity (>94%), which targeted conditions

consistent with their potential use in the physiological environment. RLP hydrogels with a concentration of 20wt% and different cross-linking ratios were evaluated, as the general range of moduli of vocal fold is represented with this group of samples. The measurements were performed within the linear viscoelastic region, achieved by an imposed rotational angle of less than 0.2° . Representative data are shown in Figure 7, including an optical image of a sample (the thin disc) sandwiched between the plates. The best fit to the data is shown as a curve, while the experimental results are shown as open-square symbols. It is obvious that the linear viscoelastic wave analysis provides an excellent fit to the observed frequency dependence of the amplification factor over a range of frequencies spanning the peak.

As predicted by theory, the reduced data clearly show that the resonance frequency is directly related to the thickness and area of the samples, as well as the material properties. More specifically, an increase in resonance frequency of RLP-based hydrogels can be achieved by decreasing the thickness and increasing the area of the samples. Sample sizes varied, with radii between 1.64mm to 3.09mm, and thicknesses between 0.22mm to 0.60mm. By changing the geometry of tested samples, the viscoelastic properties could be measured over a wide range of frequencies.

Figure 8 summarizes the frequency-dependent viscoelastic properties of RLPs at 20wt% resilin-like polypeptide concentration and a 1:1 (lysine : HMP) cross-linking ratio over a higher frequency range that includes the typical physiological frequency range of male voice production. Storage moduli are shown as solid squares and $\tan(\delta)$ values are shown as open symbols. As shown in Figure 8, the cross-linked RLPs have low $\tan(\delta)$ values generally within 0.1-0.4, with elastic modulus values ranging from 200-2500Pa. These high frequency data clearly differ from the results presented for the dynamic oscillatory rheology measurements above, almost certainly owing to unavoidable differences in sample conditions between the two experiments. In the oscillatory rheology experiments, gels were formed in situ on the rheometer stage, while in the TWA experiments, gels were measured as swollen free-standing gels. Due to the different characterization conditions, no correlations between these two sets experiments can be made. More importantly, however, the viscoelastic behavior observed from these samples at high frequencies is within the range of experimental data collected on excised porcine and human vocal fold tissues with G' ranging from 500-5000Pa and $\tan(\delta)$ within 0.2-0.4 (data not shown),³⁵ rendering our materials potential candidates for vocal fold regeneration applications; studies detailing the impact of these materials on cell behavior are underway.

Conclusions

Given the excellent mechanical properties of naturally occurring resilin, the modular RLP12 described herein offers opportunities for the engineering of mechanically active tissues and organs. Bacterial expression of the RLP12 provided good yields of protein of high purity. Rheological characterization indicates that cross-linking of the polypeptide with THPP creates a hydrogel with fast gelation kinetics and tunable mechanical properties ranging from 500 to 10000Pa. Tensile testing of hydrated RLP12 films demonstrated their negligible hysteresis, outstanding elastomeric properties and high resilience. Torsional wave experiments showed the mechanical properties at high frequencies, with moduli and $\tan(\delta)$ close to those of excised porcine and human vocal fold tissues. The data validate the outstanding mechanical properties of our materials, highly comparable to targeted vocal fold tissues and thus suggest their promising potential in this type of tissue regeneration application. Future studies will detail the impact of the mechanical properties of these materials on the activities of vocal-fold-relevant cell types.

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Figure 1. Schematic cross-linked resilin-based hydrogel network and images of gels

The left panel is an uncross-linked 20wt% RLP solution; the middle panel is a 20wt% RLP hydrogel with a 1:1 cross-linking ratio (amine : HMP); the right panel is a film of a 20wt% RLP hydrogel with 1:1 cross-linking ratio 20wt%.

Figure 2. Swelling ratio and water content of RLP-based hydrogels

Equilibrium swelling ratio (q) and percentage water content (%) for 20wt% RLP-based hydrogels were tested on an average of 8-repeat measurements at 1:1, 1:2 and 1:4 (lysine : HMP) cross-linking ratios, with error reported as the standard deviation. The equilibrium swelling ratios and water content values are statistically different from one another ($p < 0.05$).

Figure 3. Time sweep for *in situ* cross-linking of RLP12

Oscillatory rheology time sweep of 25wt% RLP12 1:1 (lysine : HMP) cross-linking ratio was conducted at 25°C with frequency at 6 rad/s and 1% strain for an hour. Viscoelastic properties of RLP-based hydrogels are represented via storage modulus G' (solid phase, solid squares) and loss modulus G'' (liquid phase, open squares).

Figure 4. Oscillatory rheology frequency sweep experiments for RLP-based hydrogels
Frequency sweeps over a range of 0.1 to 100rad/s were conducted on 20wt% RLP-based hydrogels at 1:0.5, 1:1, 1:2, 1:4 and 1:5 (lysine : HMP) cross-linking ratios at 37 °C and 1% strain.

Figure 5. Tensile testing experiments on RLP-based hydrogels

Three repeated strain-loading and unloading cycles were employed for hydrated 20wt% RLP12 films at various cross-linking ratios. A, three repeats up to 30% strain at various cross-linking ratios; B, third cycle of loading and unloading (up to 30%, 60% and 100% strain) for RLP hydrogels with a 1:1 cross-linking ratio; C, third cycle of loading and unloading (up to 30%, 60% and 100% strain) for RLP hydrogels with a 1:2 cross-linking ratio; D, third cycle of loading and unloading (up to 30%, 60% and 100% strain) for RLP hydrogels of a 1:4 cross-linking ratio.

Figure 6. Strain-to-break tensile testing experiments on RLP-based hydrogels
Strain-to-break experiments were employed for hydrated 20wt% RLP12 films at 1:1, 1:2 and 1:4 cross-linking ratios.

Figure 7. Frequency dependence of the amplification factor for RLP-based hydrogels
20wt% 1:1 (lysine : HMP) cross-linking ratio RLP hydrogel disc sample was sandwiched between the plates, fit model is shown as curve and experimental results are shown as open square symbols.

Figure 8. Rheological properties of RLP-based hydrogels at elevated frequencies

Samples at 20wt% RLP12 concentration 1:1 cross-linking ratio with various dimensions are tested over a 30-150Hz frequency range. Elastic moduli are represented in solid symbols while $\tan(\delta)$ values are shown as open symbols.

Table 1
Amino acid sequence of resilin-like polypeptide (RLP)

MGSSHHHHHSSGLVPRGSHMASMTGGQQMGRGSHLRGGGGDQK[(GGRPSDSF
GAPGGGNGGRPSDSFGAPGGGNGGRPSDSFGAPGGGNGGK)₂GGGRGDSPG]₂GPQGIWGQ
IWGQGGRGGCKAAKRPKAAKDKQTKGEDLGDPMASMTGGQQMG

Resilin-based sequence with Y replaced by F	GGRPSDSFGAPGGGN
Cell adhesion domain *	GRGDSPG
Proteolytic degradation domain **	GPQGIWGQ
Polysaccharide-binding domain ***	KAAKRPKAAKDKQTK

* GRGDSPG: cell adhesion domain from fibronectin binds to cell through $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins³⁶

** GPQGIWGQ: proteolytic degradation domain from human $\alpha(I)$ collagen³⁷

*** CKAAKRPKAAKDKQTK: polysaccharide binding domain for heparin sequestration and controlled delivery and release of growth factors³⁸

Table 2
Elastic shear moduli of RLPs at various RLP12 concentrations and cross-linking ratios

-NH ₂ :-HMP/[RLP12]	10wt%	15wt%	20wt%	25wt%
1:0.5			600±100 Pa	
1:1	1275±120 Pa	1600±200 Pa	2800±500 Pa	4450±430 Pa
1:2		1850±320 Pa	4600±225 Pa	9300±630 Pa
1:4			6850±220 Pa	
1:5			10600±170 Pa	

Errors reported as the standard deviation from a minimum of 5 measurements.

Table 3
Resilience values on 20wt% RLP-based films with various cross-linking ratios at 30%, 60% and 100% strains

Strain Cycles/Strain	30%	60%	100%
1:1-C1	92.15±0.10%	90.57±0.71%	89.95±0.96%
1:1-C2	95.31±0.04%	93.18±0.36%	92.75±1.22%
1:1-C3	95.42±0.14%	93.52±0.24%	93.1±0.98%
1:2-C1	94.7±1.08%	93.54±0.95%	91.89±1.23%
1:2-C2	96.9±0.26%	94.42±1.71%	93.88±1.23%
1:2-C3	97.67±1.0%	94.72±1.68%	94.40±1.16%
1:4-C1	93.93±1.19%	91.84±0.40%	91.15±0.31%
1:4-C2	96.18±0.26%	93.55±0.59%	92.96±0.47%
1:4-C3	96.90±0.31%	93.89±0.62%	93.52±0.48%

Error is reported as the standard deviation from a minimum of 3 measurements. C refers to the cycle number of the measurement.

Table 4
General mechanical properties for 20wt% RLP-based hydrogels at 1:1, 1:2 and 1:4 cross-linking ratios

Cross-linking Ratio	Cross-linking Efficiency (%)	Young's Modulus (kPa)	Strength $\sigma_{\max}$ (kPa)	Strain-to-break (%)
1:1	21±1% (2.4)	13.9±3.8	40.8±8.0	335±42%
1:2	29± 1% (3.2)	24.0±0.7	68.9±18.4	277±40%
1:4	39±1% (4.3)	33.8±2.5	72.5±8.7	246±30%

Error reported as the standard deviation from a minimum of 3 measurements

Table 5
Summary of mechanical properties of elastin, resilin, ELPs, RLPs and vocal folds.⁵⁷

	Elastin	Resilin	ELPs	RLPs	Vocal
Modulus E_{int} (MPa)	1.1	0.6-0.9	0.2-1.0	0.01-0.03	0.02-0.04
Extensibility (%)	90	200	90-500	250-400	30-200
Resilience (%)	90	93	70-80	90-100	NR

NR = Not Reported