Supplementary Material (ESI) for Soft Matter

Supplementary Information

Cloning and Expression of RLP12

The general strategy for cloning is shown in Figure 6.2. The DNA sequence for **RLP12** (flanked by BamH I and Hind III) in pUC57 was obtained from Genscript Corporation (Figure 6.3). Subsequent digestion of pUC57-RLP12 with Hind III and BamH I, for 10 hrs each at 37 °C, afforded a DNA cassette for **RLP12**, approximately 770 bp long, which was inserted into the compatible BamH I and Hind III sites of pET28a to generate the expression plasmid, pET28a-RLP12. The authenticity of the plasmid was confirmed by DNA agarose gel electrophoresis and DNA sequencing. The pET28a-RLP12 derived plasmids encode an N-terminal decahistidine tag to permit protein purification via metal chelate affinity chromatography. The recombinant protein, **RLP12**, was expressed in lactose induced cultures, a method suggested by Studier. For expression of polypeptide, the recombinant plasmid, pET28a-RLP12, was transformed into *rne131 E. coli* expression strain BL21Star™(DE3) (Invitrogen). A single colony BL21Star™(DE3) containing pET28a-RLP12 was inoculated in 5 ml sterile LB media containing 30 μg/ml kanamycin and grown overnight. 1 ml of the overnight culture was subsequently used to inoculate 500 ml of ZYP-5052 media (Staudier) for autoinduction of protein expression. The 500 ml cultures were grown in a shaker at 37 °C for first 4-5 hrs till the OD_{600} reached 0.8 and then grown at 25 °C with constant shaking for additional 24 hrs for efficient expression of the target protein. Protein expression was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of samples with normalized OD_{600} and visualized via Coomassie blue staining. Cells were harvested by

centrifugation (10000 *g* for 15 min at 4 °C) and the cell pellet was stored at -80 °C. The frozen cells were lysed by freeze-thaw cycles and the lysed cells were suspended in 8M urea buffer. Lysed cells were discrupted further using a Fisher Scientific model 500 Sonic Dismembrator (10 mm tapered horn) for 30 min with 10 sec bursts and 10 sec cool off time on ice. The cell lysate was centrifuged at 40000 *g* for 60 min, the supernatant was carefully decanted, the pH was adjusted to 8.0 and the supernatant was then incubated with Ni-NTA resin (Qiagen) overnight at 4 °C. The resin was then loaded in a gravitational flow column, washed twice with 8M urea, pH 6.5, containing 1.5 % triton X-100. The column was then washed with native wash buffer (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole, pH 8.0), eluted with 250 mM imidazole and dialyzed (MWCO 3500) against pure water (4X4L). The dialysate was lyophilized to yield a white fluffy solid. The molecular weight of the purified protein was confirmed via SDS-PAGE and MALDI-TOF mass spectrometry; the identity was established via amino acid analysis and the conformational properties of the polypeptides were characterized via circular dichroism spectroscopy and Fourier transform infrared spectroscopy. Amino acid composition analysis was performed by the Molecular Structure Facility at the University of California, Davis (Davis, CA) using a Hitachi L-8800 sodium citrate-based amino acid analyzer (Tokyo, Japan). Protein samples were cleaved by HCl hydrolysis, separated with ion-exchange chromatography, and detected using a ninhydrin reaction.

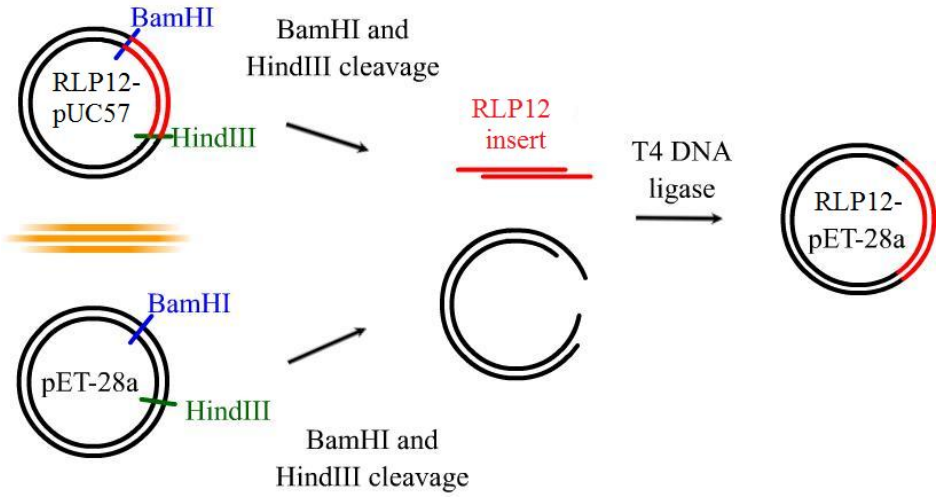

Fig. S1 Cloning schematic of RLP12 gene in pET28a plasmid

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          AarI
          ~~~~~~~~~
BamHI     BspMI                    BclI
~~~~~~~~ ~~~~~~~~                  ~~~~~~~~
 G   S   H   L   R   G   G   G   G   D   Q   K   G   G   R   P   S   D
GGA TCC CAC CTG CGT GGT GGC GGC GGT GAT CAG AAA GGC GGT CGT CCG TCG GAT
CCT AGG GTG GAC GCA CCA CCG CCG CCA CTA GTC TTT CCG CCA GCA GGC AGC CTA
 S   F   G   A   P   G   G   G   N   G   G   R   P   S   D   S   F   G
AGC TTT GGT GCT CCA GGC GGT GGT AAC GGT GGT CGT CCG TCT GAT TCA TTC GGC
TCG AAA CCA CGA GGT CCG CCA CCA TTG CCA CCA GCA GGC AGA CTA AGT AAG CCG
 A   P   G   G   G   N   G   G   R   P   S   D   S   F   G   A   P   G
GCC CCG GGT GGT GGC AAT GGT GGT CGC CCG AGT GAT AGC TTC GGC GCC CCG GGC
CGG GGC CCA CCA CCG TTA CCA CCA GCG GGC TCA CTA TCG AAG CCG CGG GGC CCG
 G   G   N   G   G   K   G   G   R   P   S   D   S   F   G   A   P   G
GGC GGT AAT GGT GGT AAA GGT GGC CGC CCG TCT GAT AGC TTC GGT GCC CCG GGT
CCG CCA TTA CCA CCA TTT CCA CCG GCG GGC AGA CTA TCG AAG CCA CGG GGC CCA
 G   G   N   G   G   R   P   S   D   S   F   G   A   P   G   G   G   N
GGT GGC AAC GGT GGT CGT CCG AGC GAT TCG TTT GGC GCG CCT GGC GGT GGC AAC
CCA CCG TTG CCA CCA GCA GGC TCG CTA AGC AAA CCG CGC GGA CCG CCA CCG TTG
 G   G   R   P   S   D   S   F   G   A   P   G   G   G   N   G   G   K
GGC GGT CGT CCG TCG GAT AGC TTC GGT GCG CCT GGT GGC GGC AAC GGT GGC AAA
CCG CCA GCA GGC AGC CTA TCG AAG CCA CGC GGA CCA CCG CCG TTG CCA CCG TTT
 G   G   G   R   G   D   S   P   G   G   G   R   P   S   D   S   F   G
GGT GGT GGC CGT GGT GAC TCT CCG GGT GGT GGC CGC CCG TCG GAT AGC TTC GGT
CCA CCA CCG GCA CCA CTG AGA GGC CCA CCA CCG GCG GGC AGC CTA TCG AAG CCA
 A   P   G   G   G   N   G   G   R   P   S   D   S   F   G   A   P   G
GCG CCT GGT GGC GGC AAC GGT GGT CGT CCT TCT GAT TCG TTC GGT GCG CCA GGT
CGC GGA CCA CCG CCG TTG CCA CCA GCA GGA AGA CTA AGC AAG CCA CGC GGT CCA
 G   G   N   G   G   R   P   S   D   S   F   G   A   P   G   G   G   N
GGC GGT AAT GGT GGT CGT CCG AGC GAT TCT TTT GGT GCG CCG GGC GGT GGC AAT
CCG CCA TTA CCA CCA GCA GGC TCG CTA AGA AAA CCA CGC GGC CCG CCA CCG TTA
 G   G   K   G   G   R   P   S   D   S   F   G   A   P   G   G   G   N
GGT GGC AAA GGT GGT CGT CCA TCT GAT AGC TTT GGT GCC CCG GGT GGC GGT AAC
CCA CCG TTT CCA CCA GCA GGT AGA CTA TCG AAA CCA CGG GGC CCA CCG CCA TTG
 G   G   R   P   S   D   S   F   G   A   P   G   G   G   N   G   G   R
GGC GGT CGT CCG TCT GAT TCG TTT GGT GCG CCT GGC GGT GGT AAT GGT GGT CGT
CCG CCA GCA GGC AGA CTA AGC AAA CCA CGC GGA CCG CCA CCA TTA CCA CCA GCA
 P   S   D   S   F   G   A   P   G   G   G   N   G   G   K   G   G   G
CCT TCC GAT AGT TTT GGC GCC CCG GGC GGT GGC AAC GGT GGT AAA GGT GGT GGC
GGA AGG CTA TCA AAA CCG CGG GGC CCG CCA CCG TTG CCA CCA TTT CCA CCA CCG
R   G   D   S   P   G   G   G   P   Q   G   I   W   G   Q   G   G   R
CGT GGC GAT AGT CCG GGC GGT GGC CCG CAG GGT ATT TGG GGC CAG GGC GGC CGT
GCA CCG CTA TCA GGC CCG CCA CCG GGC GTC CCA TAA ACC CCG GTC CCG CCG GCA
 G   G   C   K   A   A   K   R   P   K   A   A   K   D   K   Q   T   K
GGC GGT TGC AAA GCG GCA AAA CGT CCG AAA GCG GCA AAA GAT AAA CAG ACG AAG
CCG CCA ACG TTT CGC CGT TTT GCA GGC TTT CGC CGT TTT CTA TTT GTC TGC TTC
       BglII
       ~~~~~~~~
 G   E   D   L   G   D   P   M   A   S   M   T   G   G   Q   Q   M   G
GGC GAA GAT CTG GGT GAC CCG ATG GCG TCG ATG ACC GGT GGT CAG CAG ATG GGC
CCG CTT CTA GAC CCA CTG GGC TAC CGC AGC TAC TGG CCA CCA GTC GTC TAC CCG
      HindIII
      ~~~~~~~~
 *   P   S   L
TGA CCA AGC TTG
ACT GGT TCG AAC
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Fig. S2 DNA sequence of RLP12

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Circular Dichroic (CD) and Fourier-Transform Infrared Spectroscopy (FTIR)

Circular dichroic spectra were recorded on Jasco-810 spectropolarimeter (Jasco, Inc., Easton, MD, USA) using a 0.1 cm path length cell, with a 1 nm bandwidth and a scan rate of 20 nm/min. The spectra reported are averages of 3 scans with a standard deviation of 3%. CD spectra were recorded for uncrosslinked and crosslinked **RLP12** in PBS (pH 7.4) at concentration of 50 μM from 190 to 250 nm. The CD spectrum of the crosslinked RLP12 was recorded by loading RLP12 (330 μM) and THPP in a demountable window cell comprising two precision-made quartz plates mounted in a holder to yield a cell with a path length of 0.1 mm. Mean residue ellipiticity values were determined by employing standard equations; concentrations of peptides in the CD samples were determined gravimetrically.

FTIR experiments were performed using a Nexus 670 FTIR spectrometer (Thermo Nicolet, Madison, Wisconsin) with unpolarized light and a MCT detector. Spectra taken at a resolution of 4 cm^{-1} from 400 to 4000 cm^{-1} were obtained by signal averaging 1000 scans. Samples were loaded into a liquid cell with a 15 μm Teflon spacer and quartz windows. Samples were prepared by the dissolution of **RLP12** in pH 7.4, 10 mM phosphate buffer in D_2O at concentrations of 3.63 mM. A background of pH 7.4, 10 mM phosphate buffer in D_2O was subtracted from all sample spectra. The amide-I region (1600 cm^{-1} to 1700 cm^{-1}) was deconvoluted into Gaussian peaks using the multiple-peak fitting function in Origin Data Analysis software (OriginLab, Northampton, MA). Three peaks were employed for fitting the data, which was determined to be the optimal number of peaks for the fit based on assessment of R^2 values.

Supplementary Material (ESI) for Soft Matter

Rheology

Dynamic oscillatory time, frequency and strain sweeps were performed using an AR2000 stress-controlled rheometer (TA Instruments, New Castle, DE) with 20 mm diameter cone and plate geometry, 1.56 degree cone angle, at a 33 μm gap distance. The samples were prepared by addition of a desired amount of [tris(hydroxymethyl)phosphino]propionic acid (THPP) to 25 wt% **RLP12** solutions in PBS (pH 7.4) in a micro-centrifuge tube and were kept on ice before loading onto the rheometer to suppress the crosslinking reaction. The top plate was lowered to the desired gap distance and the solutions were applied via pipette, the temperature was raised to 25 °C and the solution was allowed to crosslink. Dynamic oscillatory time sweeps were collected at angular frequencies of 6 rad/s and 1% strain and it was observed that the storage modulus (G') and the loss modulus (G") reached a plateau in 2 hrs. An initial strain amplitude sweep was performed 25 °C at different frequencies to determine the linear viscoelastic range for the protein polymer. Rheological properties were examined by frequency sweep experiments (ω = 0.1 - 100 rad/s) at fixed strain amplitude of 1% followed by a strain sweep at a fixed frequency of 6 rad/s. Experiments were repeated on 3 to 4 samples and representative data presented.

Tensile Tests

Films for tensile tests were prepared in a teflon mold (10mm X 10mm) by addition of an equimolar ratio of reactive hydroxymethylphosphine (HMP) groups in THPP and lysine residues to 25wt% **RLP12** solution in PBS (pH 7.4). The films were crosslinked at 37 °C for 2 hrs until the films became dry. Before the measurement, the films were hydrated in PBS for 24 hrs 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 250 gram load cell and were tested under hydrated conditions utilizing a tank containing phosphate buffered saline around the grips. Stress-strain data were recorded at a uniform strain rate of 10% gauge length per minute and the films were stretched until they failed. Molecular weight between crosslinks was calculated using the stress-strain plot and the theory of rubber elasticity. If the material is assumed to behave as an ideal network, the shear modulus (G) can be related to the crosslinking density through the expression

$$G = \frac{\rho \mathrm{RT}}{M_c}\left(1 - \frac{2M_c}{M}\right)$$

The shear modulus is equal to one-third of the elastic modulus for an incompressible material (v = 0.5), a good approximation for rubbery hydrated protein films. The chain mass density ρ is calculated by multiplying the density of resilin (1.33 g/cm3) by the measured polymer volume fraction in the films, M_c is the average molecular weight between cross-links, and the term (1 - $2M_c/M$) represents the fraction of the chain ends that do not contribute to the load bearing capacity of the material, where M is the molecular weight of the **RLP12** (27.52 kDa).

Cell Spreading and alamarBlue® Assay

Adhesion and proliferation of mouse fibroblast NIH 3T3 cells to the **RLP12** scaffold were investigated by the microscopic observation of cell morphology and the alamarBlue assay (Invitrogen). NIH 3T3 cells were maintained in tissue culture flasks at 37 °C in a humidified incubator with 5% CO_2. **RLP12** solution containing THPP was adsorbed on a 24 well tissue culture plates and crosslinked for 2 hrs. The surface was then rinsed with

100% ethanol, sterilized for 30 min under a UV germicidal lamp and finally rinsed with sterile PBS. Cells were seeded onto the crosslinked **RLP12** surfaces at 5000 cells/cm^2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% calf serum, 1% penicillin/streptomycin, and 1% sodium bicarbonate (Gibco). The alamarBlue® assay was used in the evaluation of cellular metabolic activity. 10% alamarBlue® reagent containing media was added to each well and incubated for 4 hours prior to 100 uL aliquots being removed to a 96 well plate. Fluorescence emission was measured using a microplate fluorescence reader at 590 nm. Fresh, non-alamarBlue containing media was applied to each well after the reading was complete. Cells seeded on **RLP12** matrices were visualized using fluorescent microscopy. The samples were fixed with 4% paraformaldahyde and stained for cell nuclei (DAPI, 10 μg/mL) and actin stress fibers (FITC-phalloidin, 0.66 μg/mL) at days 1 through 3. Statistical analysis was performed with two-tailed Student's t-Test, assuming unequal variances for evaluation of data. A *p*-value of less than 0.05 was considered statistically significant.

Synthesis and Characterization of Short Resilin-like Peptides

Resilin-like peptides containing *p*-azido-phenylalanine (AzF) were produced via solid-phase methods, , owing to the reported incorporation of AzF into proteins in vivo and the utility of AzF in photochemical crosslinking of elastin-like polypeptides. A short resilin-like peptide with the sequence H2N-AzF-GAPGGGN(GGRPSDS-AzF-GAPGGGN)$_2$GGRPSDS-AzF-COOH was synthesized on a 2-chlorotrityl chloride resin (Novabiochem San Diego, CA) using an automated solid phase peptide synthesizer (PS3,

Protein Technologies, Inc, Tucson, AZ). The amino acid residues were activated for coupling with HBTU in the presence of 0.4M methyl morpholine in DMF and deprotection was carried out in 20% piperidine in DMF for approximately 30 min. Standard coupling cycles of 60 min were used for coupling. Cleavage of the peptide from the resin was performed in 95:2.5:2.5 Trifluoroacetic acid (TFA): Triisopropylsilane (TIPS): water for 3-4 h. TFA was evaporated and cleavage products were dissolved in ether. The water-soluble peptides were extracted with water and lyophilized. Peptides were purified by reversed phase HPLC (Waters, MA, USA), using a Symmetry C-18 column. The identity of each peptide was confirmed by ESI-MS (AutospecQ, VG Analytical Manchester, UK).

The CD spectrum of resilin-like peptides was recorded on a Jasco-810 spectropolarimeter (Jasco, Inc., Easton, MD, USA) using a 0.1 cm path length cell, with a 1 nm bandwidth and a scan rate of 20 nm/min. CD spectra were recorded in 10 mM PBS (pH 7.4) at concentration of 50 μM from 190 to 250 nm. The resilin-like peptides were reacted photochemically to both polymerize and crosslink the peptides produced above. Analysis of the peptides and crosslinked peptides via circular dichroic spectroscopy (at low concentrations in both cases) show the presence of a weak negative band at 230nm, a weak positive band at ca. 215nm, and a strong negative band at approximately 198nm (Fig S8), indicative of random coil and β-turn formation and consistent with the CD spectra of the RLP12 at low concentrations (Fig S5). (IR analysis of the peptide before and after photocrosslinking indicates, via evaluation of the azide asymmetric stretch at

$2130cm^{-1}$, successful photoreaction of the azide moieties in the peptide. After photochemical reaction, this azide stretch is completely absent from the IR spectrum.)

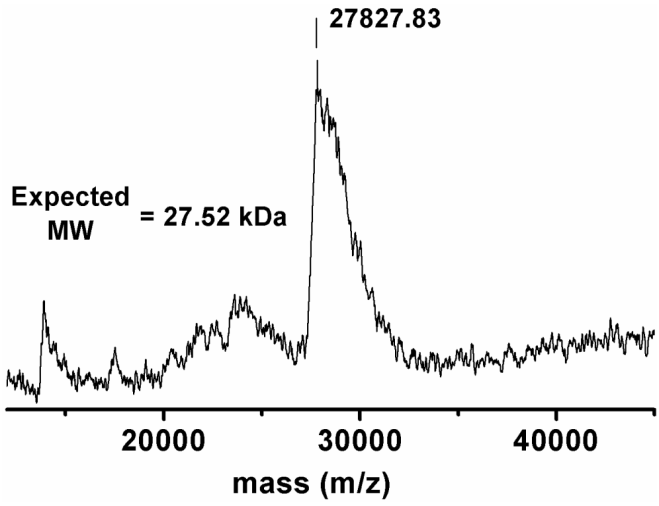

Fig. S3 MALDI of RLP12. The difference between their calculated (27.52 kDa) and experimentally measured (27.82 kDa) molecular weight was 1%.

	calc mol %	obs mol %
Asx	10.000	10.538
Ser	11.333	10.612
Glx	3.000	2.761
Pro	10.000	10.400
Gly	38.000	38.625
Ala	6.000	6.001
Met	2.333	1.101
Phe	4.000	4.317
Lys	3.667	3.661
Arg	6.333	6.818

Table S4 Amino acid analysis of RLP12

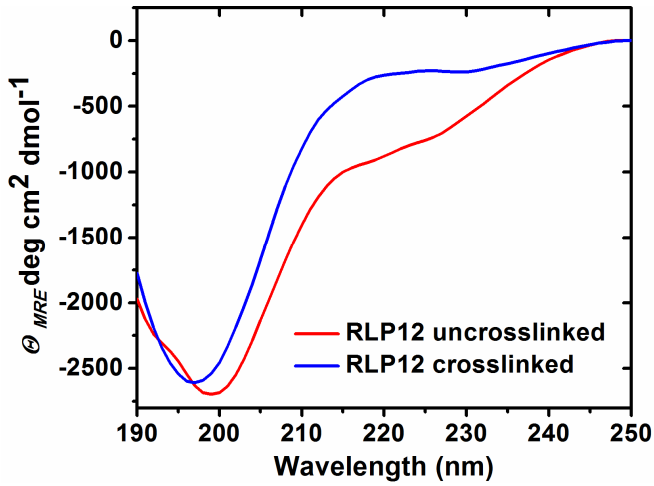

Fig. S5 CD spectrum of crosslinked and uncrosslinked RLP12 in 10 mM PBS, pH 7.4 (25 °C); the CD value is normalized for concentration. The increased contribution from features indicative of β–turns (ca. 230nm) likely results from the increased concentration of RLP12 probed in the crosslinked sample, and is consistent with the increased β–sheet contribution observed in the FTIR results for samples at higher concentrations (Fig. S6 below).

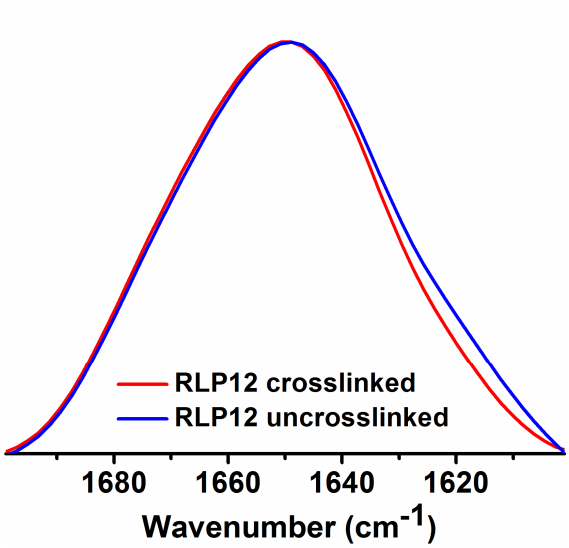

Fig. S6 **Amide I region of the FTIR spectrum of crosslinked and uncrosslinked RLP12 in deuterated PBS, 25 °C**

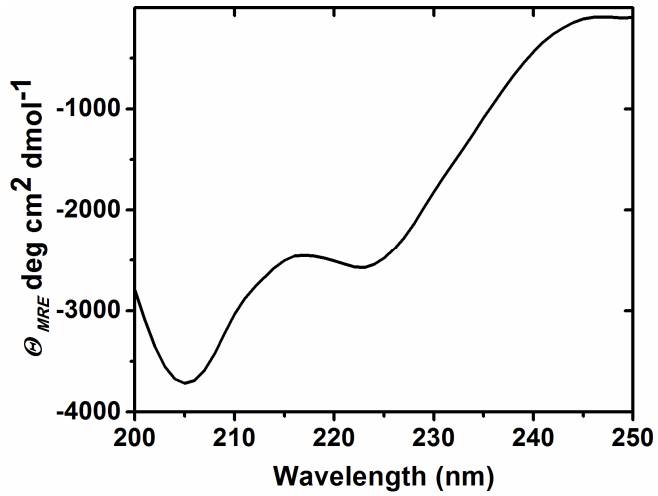

Fig. S7 **CD of RLP12 in TFE (10 µM, 25 °C)**

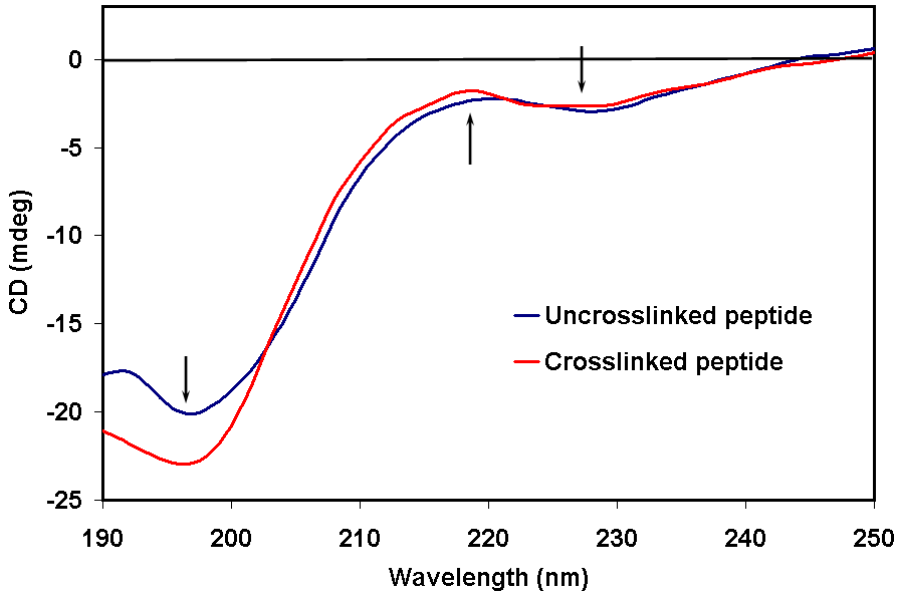

Fig. S8 CD spectrum of resilin-like peptide in 10 µM PBS, pH 7.4 (50 µM, 25 °C; gravimetric determination of protein concentration in the crosslinked peptide was difficult, so the ellipticity in millidegrees is reported here.)

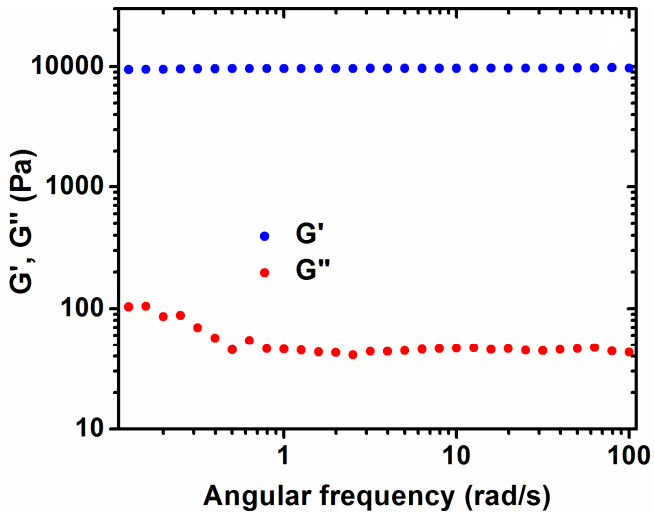

Fig. S9 Frequency sweep of RLP12

	RLP12 unx	RLP12-1X
actual mole %	3.57	2.50
estimated mole %	3.67	3.67
% lys residues reacted	2.73	31.96
# lys residues reacted	0.29	3.51

Table S10 **Amino acid analysis of crosslinked and un-crosslinked RLP12 to determine the extent of lysine residues reacted**