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COMMUNICATION

Fabrication of highly elastic Resilin/Silk fibroin based hydrogel by rapid photo-crosslinking reaction

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A new type of hydrogel combining the highly elastic soft phase of Rec1-resilin and the mechanically strong hard phase *Bombyx mori* Silk fibroin has been reported using a rapid photo-crosslinking method. The improved elasticity and strength through the use of a resilin-based material and silk fibroin has been shown for the first time.

Protein-based hydrogels are crosslinked polypeptide/protein-based networks that have been of interest in biomaterials research due to their high water capacity and resemblance to the extracellular matrix (ECM).¹⁻³ The advantage of using natural protein-based materials is their ability to support cellular interactions, low toxicity and biocompatibility, which is not the case with many synthetic polymers.^{3, 4} In particular, resilin-like proteins (RLPs) have gained increasing research attention in recent years as a result of their remarkable elasticity and resilience properties.⁵ Previously our group has reported a rapid (~60 seconds) Ruthenium-mediated photo-crosslinking method for Rec1-resilin (Rec1), a recombinant resilin protein, that exploits the tyrosine residues (~6.9 mol%) in the protein structure to form three-dimensional hydrogels.^{5, 6} Whilst, the hydrophilic Rec1 hydrogel showed impressive resilience (~92%) and water uptake capacity, the softness of the material was indicated by the low storage modulus (~0.002 MPa).⁶ In addition, a number of RLP-based hydrogels that also display storage moduli in the tens of kilopascals range have been developed to target a range of soft tissue engineering applications.⁷⁻⁹ However, in order to exploit the remarkable resilience of Rec1-resilin for tissue engineering applications where higher modulus values are required, it is necessary to improve its strength. Intervertebral disc regeneration is one such application where there is a need to develop materials with high water uptake capacity and mechanical strength.¹⁰ We have recently shown that the Ruthenium-mediated

photo-crosslinking method can also be applied to *Bombyx mori* silk fibroin from freeze dried and regenerated silk solutions, which contains 4.9 mol% tyrosine.^{11, 12} The regenerated silk fibroin (RSF) hydrogel displayed impressive modulus values (~71 MPa) when fully hydrated and was shown to be not cytotoxic when cultured with a mouse pre-chondrocyte cell line.¹² Therefore, in order to target a range of tissue engineering applications where improved modulus values are required, we report on a co-crosslinked Rec1/RSF hybrid hydrogel. We have shown that by combining the water uptake ability and elasticity of Rec1 with the strength and favourable biocompatibility of RSF we are able to develop hydrogels with tuneable properties. To our knowledge this is the first work to combine a resilin-based material with the abundantly available silk fibroin.

Rec1 was synthesised as reported in our previous work, and RSF aqueous solution was prepared via established procedures (ESI).^{5, 13} The Rec1 and RSF solutions were prepared at concentrations of 20 wt% and 15 wt%, respectively, that correspond to the optimum concentration for this crosslinking reaction as identified for each system.^{6, 12} The formation of dityrosine crosslinks on irradiation of the Rec1/RSF blend was confirmed using Fluorescence spectroscopy. The presence of excitation and emission peaks at 298 and 406 nm, respectively, indicated the presence of dityrosine in the swollen Rec1/RSF hydrogels (Fig. S4).¹⁴⁻¹⁶

An important property to consider when designing biomaterials for use in tissue regeneration is their water uptake capacity, which is related to their ability for supporting cell growth and also the elasticity and mechanical properties of the material. The water uptake capacity (h) of the RSF and Rec1/RSF hydrogels was calculated by measuring the mass of the dry and swollen gels over a period of time until equilibrium was reached (Fig. 1A). The RSF system reached an equilibrium h value of 0.59 ± 0.05 . This was lower than reported for the Rec1 hydrogel, which ranged from 2 to 4.5 depending on the crosslink density.⁶ The decreased ability for RSF to uptake water is attributed to the lower amount of polar residues (19%) present in the structure of silk compared to Rec1 (46%). Therefore, by introducing the more hydrophilic Rec1 it was our aim to improve the water uptake ability of the hydrogel, while

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maintaining the advantageous properties of RSF, in particular its strength. The Rec1/RSF hydrogel displayed an equilibrium h value of 0.85 ± 0.02 . This increase in water uptake is related not only to the increased hydrophilicity through incorporation of Rec1, but is also a result of changes in crosslink density and network structure, which will be investigated further. It also has implications for the elasticity of the hydrogels as water acts as a plasticiser. One-way ANOVA analysis was conducted at a number of time points to analyse the statistical significance of the differences in the water uptake of the RSF and Rec1/RSF hydrogels. It was determined that there were statistically significant differences between the RSF and Rec1/RSF h values at all time points of the water uptake curve as $p < 0.05$ for all time points (ESI). This confirms that the Rec1/RSF hydrogel had a significant increase in water uptake than the RSF hydrogel, which can be attributed to differences in hydrophilicity. Also, changes in the network microstructure are expected to contribute to changes in the water uptake capacity and an investigation into this is in progress.

Thermogravimetric analysis (TGA) was employed to examine the composition and stability of the hydrogels (ESI). The derivative of the TGA thermogram of the RSF hydrogel exhibited a single peak (292°C) (Fig. S5). However, the Rec1/RSF hydrogel showed multiple degradation peaks (232°C and 309°C), which is consistent with the behaviour of the Rec1 hydrogel.⁶ This confirms the presence of Rec1 and RSF in the hybrid hydrogel.

The phase behaviour of the hydrogels is important to understand the molecular dynamics of the material in the dry and swollen states, which relates to the mechanical properties exhibited. The glass transition temperatures (T_g) of the RSF and Rec1/RSF hydrogels in the dehydrated and equilibrium swollen states were examined through the use of differential scanning calorimetry (DSC) (Discovery DSC, TA Instruments) (ESI). The T_g s of the dehydrated RSF and Rec1/RSF hydrogels were indicated by a shift in the baseline at 104°C and 75°C , respectively (Fig. 1B). Determination of the T_g was conducted using the TRIOS software (TA Instruments) (ESI). The absence of weight loss observed in the TGA curves at these temperatures confirms that this is the T_g as it indicates that this is indeed a physical process. The T_g for the RSF hydrogel is somewhat lower than for the uncrosslinked silk protein. However, in our previous work we attributed this to the conformation of the hydrogel being predominantly amorphous in nature.¹² The T_g of the Rec1/RSF hydrogel was lower than the RSF hydrogel, which is attributed partly to the presence of the more amorphous Rec1. In addition, crosslink density is proportional to T_g , which indicates that a lower crosslink density of the Rec1/RSF hydrogel compared to RSF also contributes to the lower T_g observed.¹⁷ This will be correlated with crosslink density calculations discussed later in the article.

The behaviour of the hydrogels in the swollen state has implications for their use in biomedical applications, such as tissue engineering. The runs for the equilibrium swollen hydrogels (Figure 1B, c & d) were started at -75°C to cover the largest temperature range possible. The T_g for swollen samples is below zero degrees due to plasticisation in the presence of water and thus we started at a lower temperature than for the dry samples to try and capture this.

Ribeiro et al. and Weska et al. have both shown that freezing and low temperatures do not have an effect on the silk conformation for hydrogels or membranes.^{18, 19} Therefore, we do not expect the use of low temperature will have any effect on the silk structure of our hydrogels. The RSF hydrogel exhibited a shift in the T_g to -24.1°C due to the plasticizing effect of the water and an exothermic peak due to the melting of ice was observed.¹² When analysing the Rec1/RSF hydrogel, a larger exothermic peak was observed. This is attributed to the increased amount of water present in the Rec1/RSF gel compared to the RSF hydrogel. In addition, the T_g was not observed in the temperature range measured as it was shifted to below the range of detection. This indicates that at room temperature in the swollen state, both hydrogels are elastic in nature, which is supported by the decreased storage modulus and $\tan \delta$ values observed by DMA at high water uptake values.

In order to evaluate the viscoelastic properties of the co-crosslinked system, dynamic mechanical analysis (DMA) of the hydrogels was performed using the TA Instruments Q800 DMA. Hydrogel samples were studied using the tension clamp at a range of hydration values (ESI). Fig. 1C and D depict the storage modulus (E') and $\tan \delta$ values for the RSF and Rec1/RSF gels with increasing levels of hydration. The RSF hydrogel exhibited E' values of 1274.55 ± 594.61 MPa ($h = 0.17 \pm 0.04$) to 78.05 ± 15.91 MPa ($h = 0.54 \pm 0.02$). It was observed

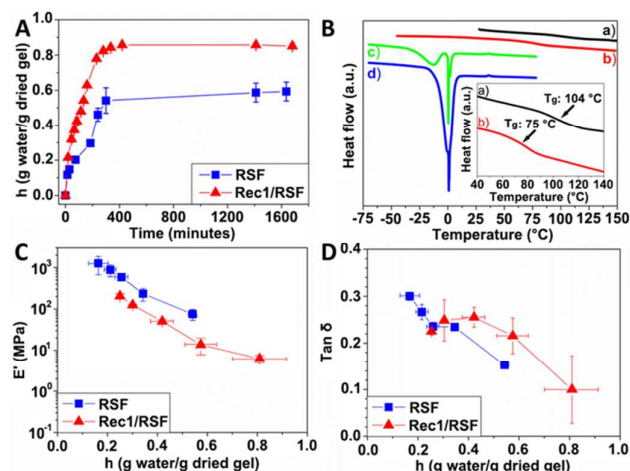


Fig. 1 A) Plot of water uptake (h) as a function of time for the RSF and Rec1/RSF hydrogels. B) DSC thermograms of hydrogels: a) dried RSF, b) dried Rec1/RSF, c) RSF ($h=0.52$) and d) Rec1/RSF ($h=0.85$). C) and D) E' and $\tan \delta$ values obtained from DMA for RSF and Rec1/RSF hydrogels at different hydration levels. Data is presented as mean \pm standard deviation.

that the E' of the Rec1/RSF hydrogel was significantly lower than the RSF hydrogel and the values ranged from 209.65 ± 30.48 MPa ($h = 0.25 \pm 0.002$) to 6.56 ± 1.35 MPa ($h = 0.81 \pm 0.11$). This is several orders of magnitude larger than the E' reported for the Rec1 hydrogel (~ 0.002 MPa) and indicates that by forming a co-crosslinked system with Rec1 and RSF we are able to tune the modulus values.⁶ One-way ANOVA analysis was carried out to

compare the E' values of the RSF and Rec1/RSF hydrogels at the equilibrium swelling water content of each sample (ESI). The E' value for the equilibrium swollen Rec1/RSF hydrogel was statistically significantly lower than the E' of the equilibrium swollen RSF hydrogel as $p < 0.05$. In addition, the E' values for both samples at h values of ~ 0.55 and ~ 0.26 were analysed using the one-way ANOVA to compare the modulus properties of the hydrogels at the same water content. At both of these h values (~ 0.55 and ~ 0.26), the E' of the RSF and Rec1/RSF hydrogels were shown to be statistically significantly different ($p < 0.05$). From this it was concluded that the increased water content of the Rec1/RSF hydrogel was not the only factor that led to the decreased modulus when compared to the RSF hydrogel. It is proposed that differences in the crosslink density and network structure of the hydrogels are responsible for the differences in mechanical properties. An investigation into the network structure of the hydrogels using neutron scattering techniques is underway. The Rec1/RSF hydrogel possessed higher modulus values in comparison with a chemically crosslinked hybrid gel composed of chitosan and silk fibroin that reported E' values in the several tens to hundreds of kilopascals.²⁰ The authors observed that the addition of silk fibroin to chitosan resulted in stiffer materials with increased modulus values.²⁰ This is in agreement with our results that show that the incorporation of RSF to Rec1 produced a stiffer hydrogel than Rec1 alone. In addition, the modulus values for our Rec1/RSF hydrogel are significantly higher than other RLP-based chemically crosslinked hydrogels that have values in the range of tens of kilopascals.^{7, 8} This will allow us to apply this hybrid material to such applications where increased stiffness is required.

The presence of water increased the chain mobility of the RSF and Rec1/RSF hydrogel networks as indicated by decreased T_g values. The decreased stiffness was confirmed by the decreasing E' and $\tan \delta$ values with increasing levels of hydration indicating predominantly elastic behaviour.²¹ However, the viscoelastic behaviour was observed as the water content decreased, evident by the increase in $\tan \delta$ values. The differences in elasticity of the Rec1/RSF and RSF hydrogels is attributed to changes in the microstructure and also the increased hydrophilicity that allows the hydrogel to retain a larger proportion of water.

The crosslink density (n), which quantifies the number of moles of active network chains per unit volume (mol/m^3), was calculated for the swollen hydrogels according to the rubber elasticity theory using the expression: $n = E'/3RT = \rho/M_c$.²²⁻²⁴ M_c represents the molecular weight between crosslinks (g/mol), E' is the tensile storage modulus (MPa) as measured from DMA experiments, R is the universal gas constant ($8.314 \text{ J}/\text{mol}\cdot\text{K}$), T is the absolute temperature (298 K) and ρ is the density for RSF ($1.421 \text{ g}/\text{cm}^3$) and Rec1/RSF ($1.352 \text{ g}/\text{cm}^3$). The n values for the RSF and Rec1/RSF hydrogels in the swollen state were calculated to be $9,545.6 (\pm 3097.6) \text{ mol}/\text{m}^3$ and $840.8 (\pm 240.8) \text{ mol}/\text{m}^3$, respectively. As the RSF hydrogel exhibited a higher storage modulus value than Rec1/RSF, consequently the RSF hydrogel had a significantly higher crosslink density. Using the density, the M_c values for the RSF and Rec1/RSF hydrogels were calculated as $157.1 (\pm 51.0) \text{ g}/\text{mol}$ and

$1,676.7 (\pm 480.2) \text{ g}/\text{mol}$, respectively. As the M_c is inversely proportional to the E' , RSF has a lower molecular weight between crosslinks when compared to the Rec1/RSF hydrogel, which accounts for the higher modulus values for the RSF hydrogel. Elvin et al. measured the modulus of ruthenium-crosslinked Rec1 to be 2.5 kPa in the swollen state and thus calculated the M_c of crosslinked Rec1 to be $14,161 \text{ g}/\text{mol}$.¹⁴ This was lower than the predicted value ($8,500 \text{ g}/\text{mol}$) that was calculated based on the amount of tyrosine present in the chains and relied on the assumption that all di-tyrosine crosslinks are intermolecular. However it is possible that many of the crosslinks formed are intramolecular, which do not contribute to mechanical strength.¹⁴ Sando et al. also used this method for crosslinking α -keratose extracted from wool with a tyrosine content of $3.8 \text{ mol}\%$.²⁵ The modulus values for the di-tyrosine crosslinked α -keratose gels ranged from 9.5 to 53.5 kPa , depending on the solvent conditions and reactant concentrations.²⁶ This was reflected in M_c values ranging from $12,200$ to $21,400 \text{ g}/\text{mol}$, which is in agreement with the estimated value ($17,800 \text{ g}/\text{mol}$) based on the number of tyrosine residues and indicates that the majority of di-tyrosine crosslinks are intermolecular.²⁶ In comparison with the Rec1 and α -keratose hydrogels, the RSF and Rec1/RSF hydrogels exhibited lower M_c values. This is because the hydrogels containing silk fibroin possess higher modulus values. Furthermore, a higher M_c for the Rec1/RSF system compared to the RSF hydrogel was also reflected in the lower T_g observed for the Rec1/RSF hydrogel. In order to gain a deeper understanding of the microstructure of the hydrogels and how this correlates to the observed behaviour, neutron scattering studies are currently being undertaken.

The secondary structure of the hydrogels was measured using Photoacoustic Fourier Transform Infrared Spectroscopy (PA-FTIR) (Nicolet Magna-IR Spectrometer 750). The Magic Plot software was used to fit the Amide I region to determine the secondary structure content (Fig. S7).²⁷ It was observed that the secondary structure of the Rec1/RSF hydrogels was very similar to that of the measured RSF hydrogel conformations (Table 1). The dominant structure for both hydrogels was β -turn, with smaller contributions from random coil and β -sheet structures. Other RLP hydrogels have also been shown to be dominated by random and β -turn conformations upon covalent crosslinking.⁷ It has been shown that β -sheet contents of $\sim 55\%$ were observed for physically gelled silk compared to $\sim 20\%$ for silk fibroin solutions, which is much higher than the values reported here.^{20, 28} Therefore, this method of photo-crosslinking does not induce the random coil to beta-sheet transition as has been observed in silk fibroin hydrogels that form due to random coil to beta-sheet transitions. Also, from the FTIR analysis it is clear that the RSF and Rec1/RSF hydrogels have no significant differences in terms of secondary protein structure.

Table 1. Secondary structure content of RSF and Rec1/RSF hydrogels obtained from Amide I region of PA-FTIR spectra.

Wavenumber range (cm ⁻¹)	Secondary structure	RSF (%)	Rec1/RSF (%)
1616-1621	Aggregate β -strand/ β -sheets (weak, intermolecular)	15.9 \pm 0.2	13.3 \pm 0.8
1628-1637	β -sheets (strong, intramolecular)	16.7 \pm 1.8	16.6 \pm 1.2
1638-1655	Random coils	17.7 \pm 0.6	20.8 \pm 4.0
1663-1685	β -turns	35.2 \pm 0.1	35.5 \pm 5.8
1697-1703	β -sheets (weak, intermolecular)	14.5 \pm 1.5	13.7 \pm 2.2

Conclusions

In this work we have successfully established a rapid ruthenium-mediated photo-crosslinking method to form hybrid hydrogel materials using Rec1 and RSF for the first time. The Rec1/RSF hydrogel showed improved water uptake capacity compared to the RSF hydrogel, which is important for regenerative medicine applications. In addition, we have shown that by combining the hard and soft phases of RSF and Rec1 we are able to tune the mechanical properties and achieve an E' value of \sim 6.56 MPa when swollen, which is significantly higher than the Rec1 hydrogel (0.002 MPa). This rapid photo-crosslinking method is a simple way to tune biomaterial properties by taking advantage of the remarkable elastic properties of resilin with the biocompatible and strength properties of silk fibroin. This work serves as a platform for designing biomaterials using resilin and silk based materials with tuneable properties that can potentially be applied to a range of tissue engineering applications. Future work is underway to gain an understanding of the level of tuneability that is possible by varying the ratios of Rec1 and RSF.

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A new type of hydrogel combining the highly elastic soft phase of Rec1-resilin and the mechanically strong hard phase of *Bombyx mori* silk fibroin has been reported using a rapid photo-crosslinking method.