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

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Supporting Information

Materials and methods

1. Material preparation

Rec1 was synthesised by cloning, expression, and purification of exon 1 of the *Melanogaster* gene CG15920, as a water soluble protein as described elsewhere[1, 2]. Rec1 has a molecular weight of 28.4 kDa with 310 amino acid residues as previously characterised.[1, 2]

Bombyx mori silk was purchased as raw silk from Beautiful Silks, Australia. The raw silk was degummed by boiling in aqueous 0.02 M Na_2CO_3 for 30 minutes. The silk was rinsed in distilled water (DW) three times and allowed to dry at room temperature overnight. The fibroin was then dissolved in a calcium chloride, water, ethanol solution ($CaCl_2$/H_2O/EtOH 1/8/2 molar ratio), with a liquor ratio of 1:20, at 70°C for 3 hours. Following this, the solution was dialysed using SnakeSkin dialysis tubing (3.5 kDa molecular weight cut-off) (ThermoFisher Scientific) for several days and centrifuged at 10,000 rpm and 20°C for 20

minutes. The solution was diluted using DW to obtain the concentrations required for the experiments. The main amino acid constituents of the regenerated silk fibroin (RSF) have been confirmed through nuclear magnetic resonance (NMR) (Figure S2). In addition, the molecular weight range was measured through SDS-page experiments and was found to be from approximately 37 to 200 kDa, which is common for regenerated silk solutions (Figure S3).[3, 4]

2. Hydrogel Synthesis

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. The RSF solution was added to the Rec1 solution in a 1:1 volumetric ratio giving a mass ratio of 1:0.75 (Rec1:RSF). The catalyst (tris(2,2-bipyridyl) dichloro ruthenium(II) hexahydrate) and electron acceptor (ammonium persulphate) were employed at concentrations of 5 mM and 28 mM, respectively, as established in our previous work.[5] The combined Rec1/RSF solution was exposed to a 250 W light source for 120 seconds, and the gels were turned over and exposed for a further 60 seconds to ensure full crosslinking. Samples were leached in distilled water to remove unreacted material and characterised for water uptake capacity, thermal behaviour, mechanical properties, and secondary structure content.

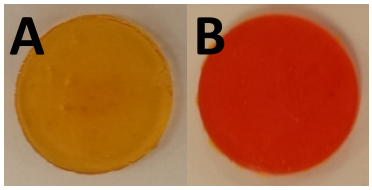

Figure S1: Photograph of leached A) RSF hydrogel and B) Rec1/RSF hydrogel

3. Material characterization

RSF was analysed with nuclear magnetic resonance (NMR) in D_2O. ^1H NMR spectra were obtained on a 300 MHz Bruker Avance spectrometer operating at 300.138 MHz. Reference relative to 0 ppm for TMS. The predominant presence of glycine (Gly) and alanine (Ala) that makes up the structure of silk fibroin has been confirmed.[6] In addition, the presence of the tyrosine groups required for crosslinking has also been confirmed through the peaks in the spectra, which were correlated with literature values.[6] Molecular weight range was determined using SDS-PAGE and was found to be from approximately 37 to 200 kDa (Figure S3).

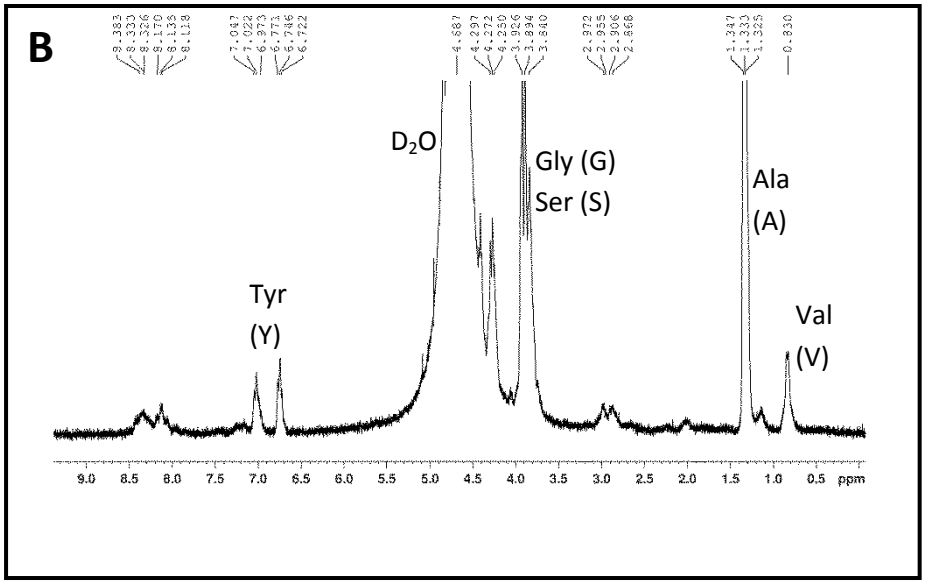

Figure S2: NMR of RSF in D_2O.

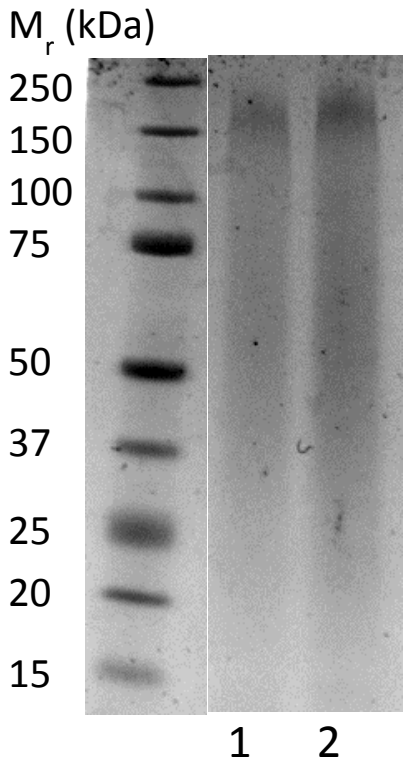

Figure S3: SDS-PAGE of RSF at 20 µg/mL (Lane 1) and 40 µg/mL (Lane 2).

Fluorescence measurements using the Cary Eclipse Fluorescence Spectrometer were undertaken to confirm the formation of dityrosine crosslinks in the Rec1/RSF hydrogel. A sample gel was prepared with a Rec1 concentration of 67 mg/mL, RSF concentration of 50 mg/ml, Ru(II)$(bpy)_3^{2+}$ concentration of 0.2 mM, and an APS concentration of 20 mM. Lower concentrations of Rec1, RSF, and Ru(II)$(bpy)_3^{2+}$ were used to prepare a gel that was transparent in order to be suitable for fluorescence measurements using the Cary Eclipse Fluorescence Spectrometer. However, the same ratio of Rec1 to RSF was employed. A corresponding solution of Rec1/RSF at the same concentrations, without Ru(II)$(bpy)_3^{2+}$ and APS was prepared for comparison. Figure S4 shows the fluorescence spectra of the hydrogel and solution. The peak at 406 nm in the emission spectra of the Rec1/RSF hydrogel confirms the presence of dityrosine crosslinks in the gel structure that is not exhibited for the Rec1/RSF solution.[1, 7, 8]

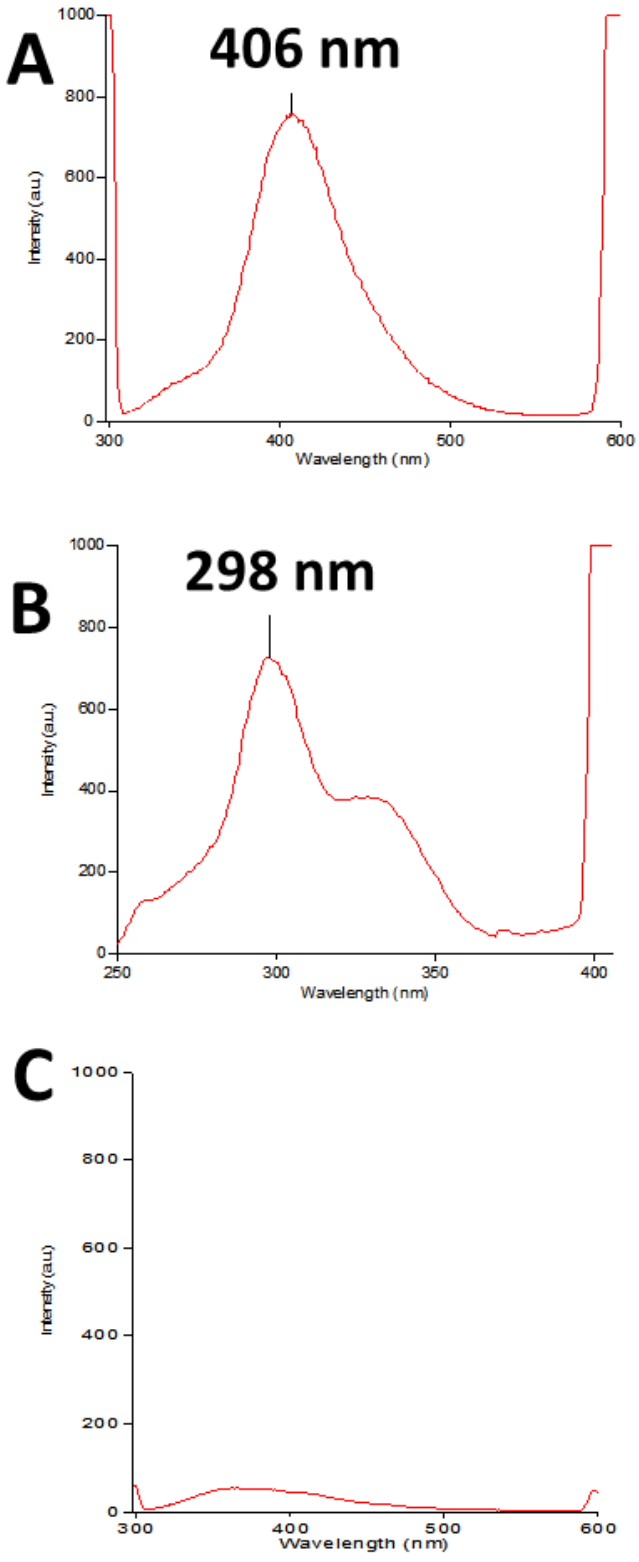

Figure S4: A) Fluorescence emission spectra of Rec1/RSF hydrogel (Ru: 0.2 mM and APS: 20 mM), excited at 298 nm. B) Fluorescence excitation spectra of Rec1/RSF hydrogel (Ru: 0.2

mM and APS: 20 mM) measuring with emission at 406 nm. C) Fluorescence emission spectra of corresponding Rec1/RSF solution, excited at 298 nm.

To measure the water uptake capacity of the hydrogels they were first vacuum dried to constant weight, which was measured using a microbalance capable of measuring four decimal places. Then the gels were immersed in DW and the weight was monitored at different time intervals. The water uptake (h) was calculated using equation 1, where w_s and w_d refers to the mass of the swollen and dried gels, respectively.

$$h = \frac{w_s - w_d}{w_d}$$ Equation 1

Thermogravimetric analysis of the vacuum dried RSF and Rec1/RSF hydrogels was conducted using the TA Instruments Discovery TGA. The experiments were run in a nitrogen atmosphere under a heating rate of 10°C/min.

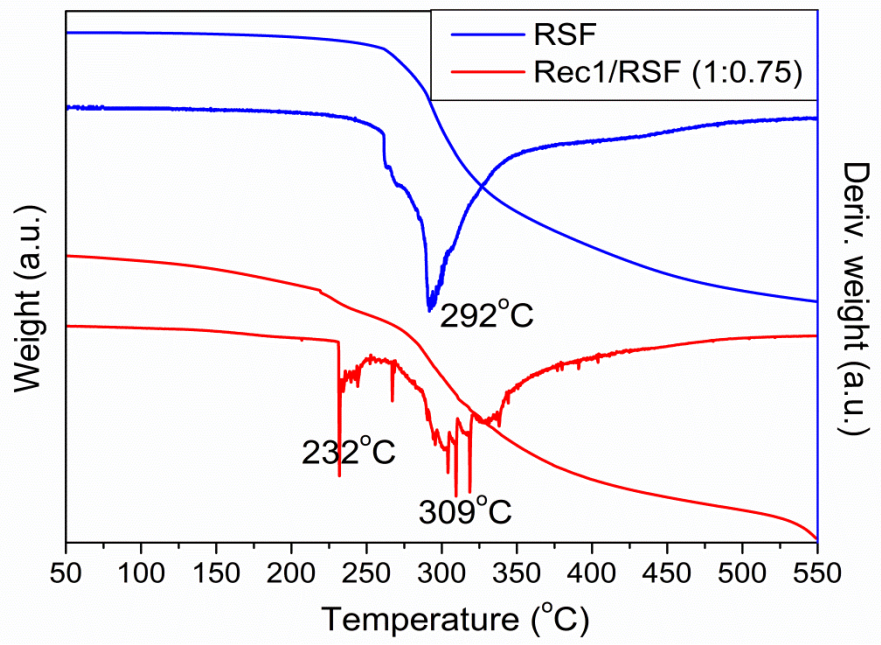

Figure S5: TGA thermogram of RSF and Rec1/RSF hydrogels.

The TA Instruments Discovery DSC was used to measure the thermal behaviour of the hydrogels in the dry and swollen states. The instrument was calibrated for baseline and cell constant prior to conducting the experiments. Samples were weighed and sealed in hermetic aluminium pans for measurement with an empty pan used as a reference. Samples were run at a heating rate of 10°C/min under a nitrogen atmosphere (50 mL/min). The TRIOS software was used to determine the T_g of the hydrogels as shown in Figure S6.

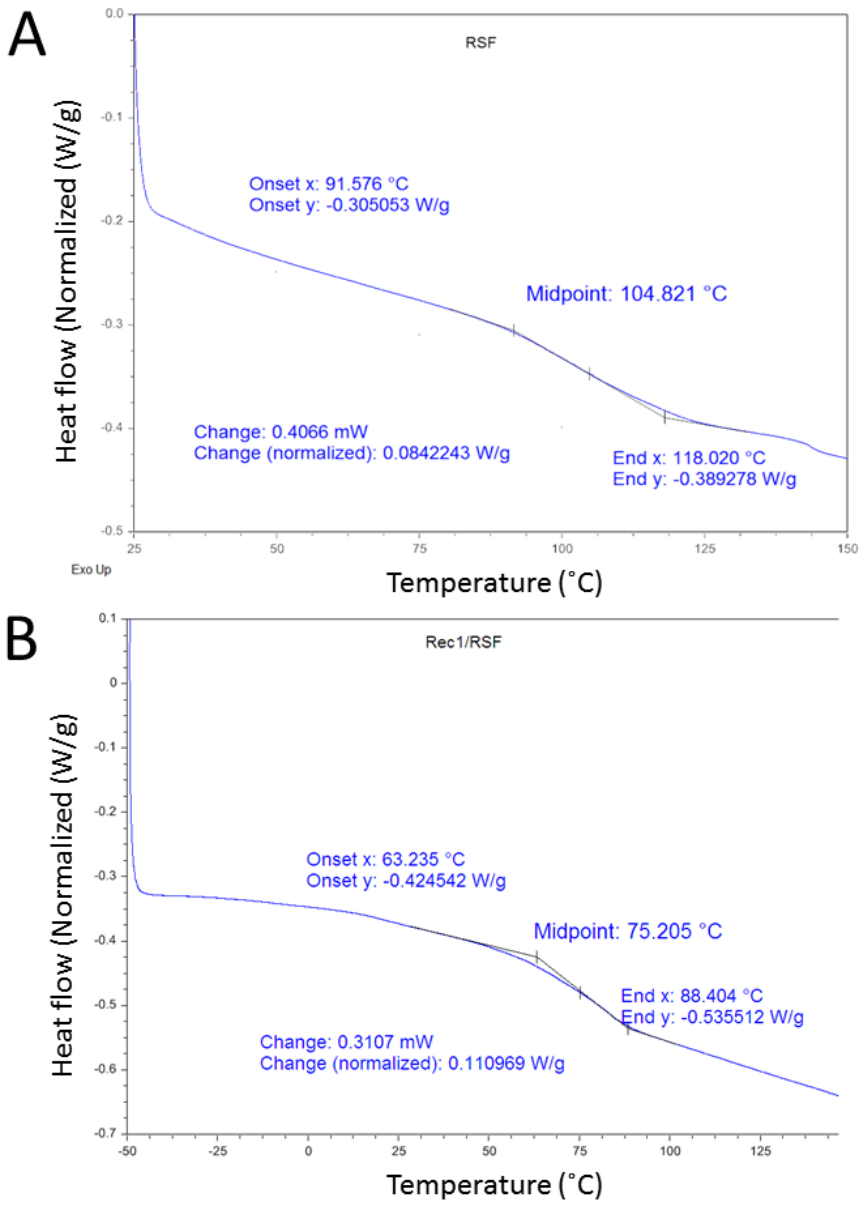

Figure S6: Representative DSC thermograms of dried A) RSF and B) Rec1/RSF hydrogels with determination of T_g shown.

The mechanical properties of the hydrogels were measured using the TA Instruments Q800 DMA equipped with humidity accessory. Samples of rectangular geometry were immersed in DW for 24 hours to reach equilibrium water uptake. Samples were mounted using the tension clamp and run at ambient temperature with an amplitude of 10 µm and a preload force of 0.010 N. DMA runs were conducted with humidity ramping to 50% and held for the one hour at isothermal temperature conditions. The samples were run in consecutive runs with the moisture content measured before and after each run to correlate the values with the moisture level, which decreased with time.

Infrared spectra of the RSF and Rec1/RSF hydrogels were acquired using the Nicolet Magna-IR Spectrometer 750 in photo-acoustic mode with a carbon black reference. The range used was 400 to 4000 cm^{-1} and the data was processed with the Omnic computer program. The amide I region was Gaussian curve fitted to determine the secondary structure components of the hydrogels. This was undertaken using the Magic Plot software (Figure S7).

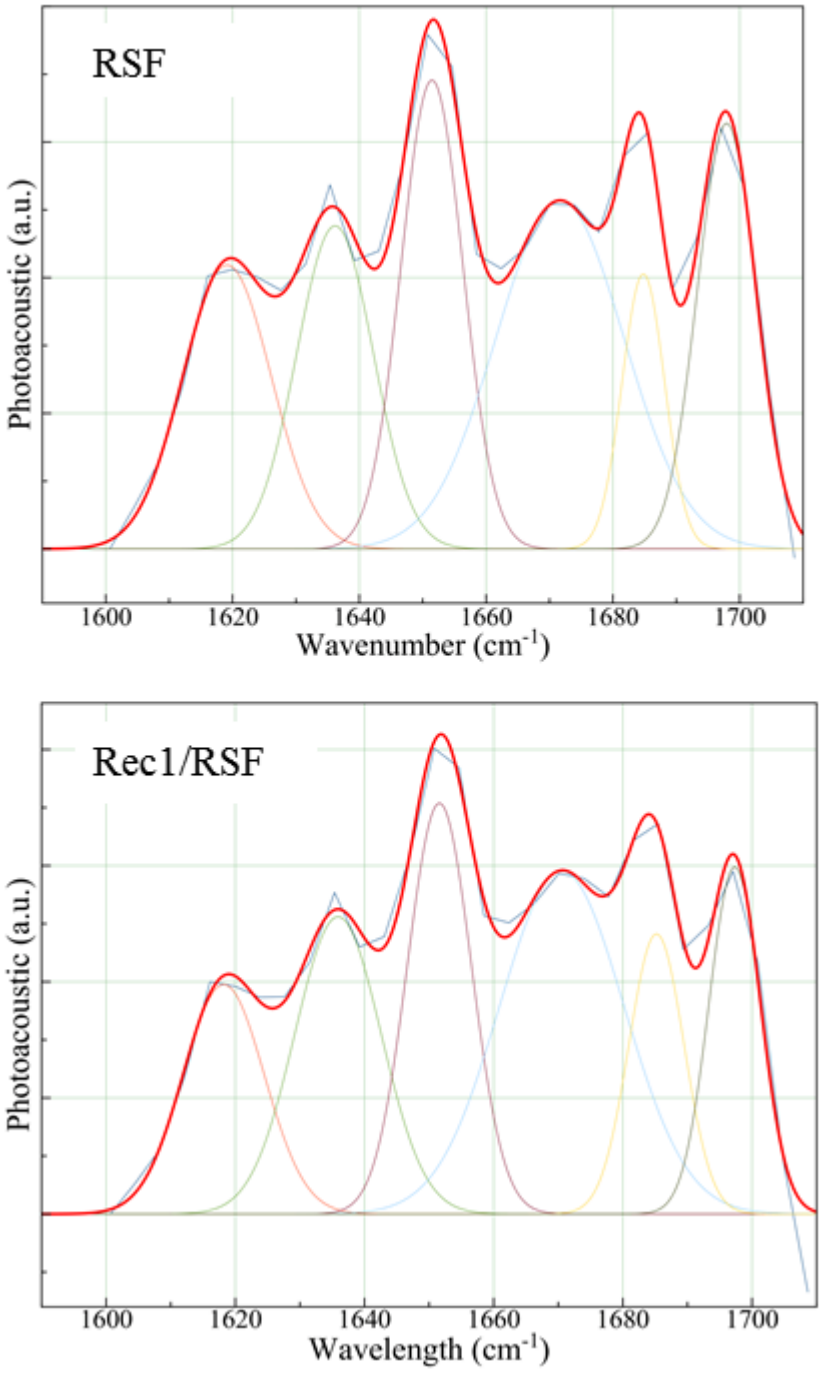

Figure S7: PA-FTIR spectra of RSF and Rec1/RSF hydrogels in the amide I region, showing Gaussian fitting to determine the secondary structure content.

4. Statistical analysis – One-way ANOVA

One-way ANOVA analysis of the water uptake (h) data and E' data was carried out using Microsoft Excel to determine the whether there are statistically significant differences between the properties of the RSF and Rec1/RSF hydrogels. The differences between the h

values of the RSF and Rec1/RSF hydrogels at time points of 15, 30, 75, 300, 1410 and 1635 minutes was analysed. For all time points the $p<0.05$, which meant that there was statistically significant differences between the water uptake of the RSF and Rec1/RSF hydrogels throughout the water uptake process. An example of the one-way ANOVA analysis at 1635 minutes is shown in Figure S8A.

For the E' values, one-way ANOVA analysis was employed to compare between the E' of RSF and Rec1/RSF gels at the equilibrium swelling content of each respective gel (i.e. h=~0.55 for RSF and h=~0.81 for Rec1/RSF). The $p<0.05$ and thus the results were statistically different. We also compared the E' values at the same water contents (h=~0.55 and ~0.26). Also, at h values of 0.55 and 0.26, the $p<0.05$, which indicates that even at the same water content the E' values of the RSF and Rec1/RSF hydrogels are statistically significantly different. An example of the one-way ANOVA analysis for the E' values of the equilibrium swollen RSF and Rec1/RSF hydrogels is shown in Figure S8B.

A

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
RSF h (time=1635 mins)	2	1.186578	0.593289	0.002882
Rec1/RSF h (time=1635 mins)	2	1.701418	0.850709	0.000288

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.066265	1	0.066265	41.80687	0.023094	18.51282
Within Groups	0.00317	2	0.001585			
Total	0.069435	3				

B

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
RSF E' equilibrium swollen	4	312.19	78.0475	253.2083
Rec1/RSF E' equilibrium swollen	3	18.106	6.035333	1.739092

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	8889.861	1	8889.861	58.2481	0.000614	6.607891
Within Groups	763.1031	5	152.6206			
Total	9652.964	6				

Figure S8: One-way ANOVA analysis of the: A) water uptake (h) data for the RSF and Rec1/RSF hydrogels at time = 1635 minutes; and B) storage modulus (E') data for the equilibrium swollen RSF and Rec1/RSF hydrogels.

References

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