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Robust and Tunable Hybrid Hydrogels from Photocrosslinked Soy Protein Isolate and Regenerated Silk Fibroin

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ABSTRACT

Soy protein isolate (SPI), a plant derived protein is emerging as a potential material for biomedical applications. Herein we report the development and structure-property relationship of photocrosslinked SPI and SPI/silk fibroin (SF) hybrid hydrogels for the first time. The pristine SPI hydrogels were crosslinked at two different structural configurations (i.e. at pH 7 and 12), and SPI/SF hybrid hydrogels were co-crosslinked at pH 7 in three different weight ratios (3:1, 1:1 and 1:3). The fabricated hydrogels were characterized using electron microscopy, X-ray diffraction, Raman and infrared spectroscopy, thermal analysis, small and ultra-small angle neutron scattering, rheology, water uptake and *in vitro* degradation studies. The equilibrium water swollen SPI hydrogel photocrosslinked at pH 7 exhibited a particulate microstructure, controlled degradation in phosphate buffered saline, and a shear storage modulus of ~ 7.7 kPa, which is in the range of human lumbar nucleus pulposus, and significantly higher than soy hydrogels reported by thermal treatment, pressure treatment, salt-induced cold-setting and enzymatic crosslinking. Conversely, the SPI hydrogel photocrosslinked at pH 12 exhibited ordered porous microstructure, higher water uptake of $\sim 1946\%$, and poor water-resistant and mechanical properties. Increase in SF content of the crosslinked SPI/SF hybrid hydrogels demonstrated improved porosity, swelling, molecular chain mobility, elastic and water-resistant properties. An in-depth understanding of the effect of pH and composition on the hierarchical structure and physicochemical properties of the fabricated hydrogels was established. Moreover, the SPI and SPI/SF hybrid inks used for photocrosslinking exhibited flow properties suitable for printing tissue engineering applications. The presented results contribute to a facile fabrication and fundamental understanding of the structure–property relationship of SPI-based hybrid hydrogels.

Keywords: *Soy protein isolate, Silk fibroin, Photocrosslinked hydrogels, Structure-property relationship, Hierarchical structure, Physicochemical property*

INTRODUCTION

Protein-based hydrogels are highly water absorbing three-dimensional (3D) network of protein molecules being held together by crosslinks between the constituents, and are emerging as a new class of smart and responsive soft materials.¹ Among proteins of natural origin, soy protein isolated from soybean and widely used in the food industry, has attracted significant interest in recent years for biomedical applications due to its abundance in nature, low cost, ease of processability, biocompatibility, biodegradability, inherent cell adhesion and growth mediation properties.² Soy protein isolate (SPI) is a highly refined form of soy protein with a minimum protein content of 90% on a moisture-free basis, and has a complex molten globule structure with two major globulins, namely glycinin (11S with molecular weight, M_w of 360 kDa) and β -conglycinin (7S with M_w of 175 kDa).³ The molecular structure and solubility of SPI is sensitive to pH, where SPI have been reported to show increased solubility (up to 2.5-fold) at pH 12 compared to pH 7.³ Several methods including thermal treatment,⁴ pressure treatment,⁵ and cold-set salt-induced gelling⁶ have been reported for fabrication of SPI films and physical gels. However, SPI physical hydrogels developed using such methods exhibit poor mechanical and water-resistance properties, limiting their applications in the field of biomedical engineering including tissue engineering and drug delivery.⁵⁻⁷ Enzymatic and chemical crosslinking of SPI using agents such as transglutaminase,⁸ epichlorohydrin,⁹ glutaraldehyde,¹⁰ and genipin¹¹ have been reported to improve the physical properties of SPI hydrogels. Moreover, a few attempts have been made recently to fabricate SPI-based composite (SPI-polysaccharide,¹² and SPI-ceramic¹³), and hybrid (SPI-synthetic polymer¹⁴) hydrogels with improved mechanical properties. However, the reported hydrogels suffer from long fabrication/gelation time and/or poor mechanical properties which are still inadequate for many targeted tissue engineering applications, such as human lumbar nucleus pulposus (center part of intervertebral disc), which has a shear dynamic modulus in the range of 7.4 to 19.8 KPa, and

confined compression modulus in the range of 0.5 to 1.3 MPa.¹⁵ Moreover, the existing literature gap associated with fabrication of SPI-based hydrogels include lack of a fast and facile fabrication method for chemically crosslinking SPI that can be extended to co-crosslink SPI with other proteins, such as silk fibroin to develop functional protein-based hybrid hydrogels with tunable structure and physicochemical properties. Silk fibroin (SF) is a widely recognized protein for biomedical applications, and we have recently reported the fabrication of pristine SF hydrogels,¹⁶ and SF-based hybrid¹⁷⁻¹⁸ and composite hydrogels¹⁹ via a fast and facile photocrosslinking method.

To optimize the physicochemical and biological properties of SPI-based hydrogels for desired biomedical applications, fundamental understanding of the colloidal structure of SPI dispersions, the hierarchical structure of the crosslinked hydrogels, and how the molecular building blocks transmit properties across the length scales in the macroscopic sample is pivotal. However, the hierarchical structure of SPI-based hydrogels is poorly understood, and there is a paucity of literature addressing this important issue. Small angle neutron scattering (SANS) is a powerful experimental technique for investigating the nanostructure and organization of proteins in concentrated solutions²⁰ and hydrogels.²¹ Moreover, SANS is complementary to electron microscopy and is highly sensitive to hydrogen atoms in the system.¹⁹ Ultra-small angle neutron scattering (USANS) can provide structural information of hydrogels up to a length scale of several micrometers.²² Therefore, a combined SANS and USANS study offers the potential to develop in-depth understanding of the hierarchical structure of hydrogels over a wide length scale. In this paper, we investigate and report the development and structure–property relationship of photocrosslinked SPI and SPI/SF hybrid hydrogel systems for the first time through a combination of an array of complementary experimental techniques including scanning electron microscopy, X-ray diffraction, Raman

and Fourier transform infrared spectroscopy, thermal analysis, SANS, USANS, rheology, water sorption and *in vitro* degradation studies.

MATERIALS AND METHODS

Materials

Soy protein isolate (Pro-Fam[®] 648 with protein content over 90%) was provided by Archer Daniels Midland (ADM), Australia. AAA grade *Bombyx mori* silk fiber top was purchased from Beautiful Silks, Australia. Tris(2,2-bipyridyl)dichlororuthenium(II) hexahydrate [RuII(bpy)₃], ammonium persulfate (APS) and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich, Australia. Deuterium oxide (D₂O) was provided by the Australian Nuclear Science and Technology Organisation (ANSTO). All other chemicals used in this study were of analytical grade and purchased from Chem-Supply, Australia.

Dispersion and solution preparation

A 17.5 wt% SPI stock dispersion was prepared by mixing SPI powder with Milli-Q water. The mixture was stirred gently for 24 hours at 20 °C to ensure complete hydration of the protein and homogeneity of the dispersion. A 17.5 wt% SF stock solution was prepared as described in our previous work.¹⁶ Briefly, raw *Bombyx mori* silk was degummed by boiling in aqueous sodium carbonate solution (0.05 M) for 30 min, washed with Milli-Q water thrice, and air-dried at ambient temperature. The degummed fibroin was then dissolved in aqueous calcium chloride-water-ethanol solution (molar ratio 1:8:2) with a liquor ratio of 1:10 (solute:solvent) at 70 °C for 3 hours. The fibroin solution was then cooled, filtered through 5 µm syringe filter, and dialyzed against Milli-Q water using 3.5K MWCO cellulose dialysis tube. The dialyzed fibroin solution was then centrifuged at 10,000 rpm for 30 min, supernatant filtered through 0.8 µm syringe filter, and concentrated by air-drying. The obtained SF stock solution was

stored at 4 °C to prevent aging/gelation and used within 3 days. The amino acid composition of SPI and SF are given in [Table S1](#) in [Supporting Information](#).

Hydrogel fabrication

The list of hydrogel samples prepared, and their code names used in this paper are given in [Table 1](#). For SPI gel pH7 sample fabrication, aqueous dispersion of SPI was mixed with RuII(bpy3), and APS to obtain a final mixture concentration of 15 wt%, 5 mM and 28 mM, respectively. This mixture (ink) was then poured into a Teflon[®] mould and exposed to a 50 W LED white light for 2 min. The gel formed was then turned over and exposed for a further 30 seconds to ensure complete crosslinking of the sample. For SPI gel pH12 sample fabrication, 10% potassium hydroxide solution was added dropwise to the SPI dispersion (to obtain a dispersion pH of 12) and stirred gently for 15 min. RuII(bpy3) and APS was added to the above dispersion, mixed and photocrosslinked as described above. For SPI/SF hybrid hydrogel fabrication, SPI dispersion (at pH 7) and SF solution were first mixed in different weight ratios (1:0, 3:1, 1:1, 1:3 and 0:1) for 3 hours to get a homogeneous mixture, and subsequently mixed with RuII(bpy3) and APS (ink) and photocrosslinked, as described above. The fabricated hydrogels were dialyzed against Milli-Q water to remove any excess material, and subsequently freeze-dried. The water uptake, hydrolytic degradation and viscoelastic properties of samples were measured after dialysis in equilibrium swollen condition, whereas other characterizations were performed using freeze-dried samples.

Water uptake study

The water uptake (h) and cross-link density (v_e) of fabricated hydrogels were calculated using equations 1 to 3.¹⁶

$$h = \frac{W_s - W_d}{W_d} \times 100 \quad \text{----- (1)}$$

$$v_e = \frac{-\ln(1-v_{1,s}) + v_{1,s} + Xv_{1,s}}{V_2[v_{1,s} - \frac{1}{2}v_{1,s}]} \text{ ----- (2)}$$

$$v_{1,s} = \frac{\frac{W_d}{\rho_p}}{\frac{W_s - W_d}{\rho_2} + \frac{W_d}{\rho_p}} \text{ ----- (3)}$$

Where W_s is the equilibrium swollen weight of hydrogel, W_d is the dry weight of hydrogel, $v_{1,s}$ is the volume fraction of the hydrogel at the equilibrium swollen state, ρ_p is the density of the dehydrated hydrogel (taken as 1.43 g/cm³ for SPI and 1.42 g/cm³ for SF),^{16, 23} ρ_2 is the density of water (taken as 0.997 g/cm³), X is the Flory–Huggins interaction parameter between that of the protein and water (taken as 0.50 for SPI and 0.95 for SF),^{16, 23} and V_2 is the molar volume of water (taken as 18 cm³/mol).¹⁶ The W_s of hydrogel was measured after dialysis and removal of excess water using blotting paper, and W_d of hydrogel was measured after air- and vacuum-drying. The experiments were repeated thrice, and the water uptake results are presented as mean values with standard deviation.

***In vitro* degradation study**

To evaluate hydrolytic degradation of fabricated hydrogels *in vitro*, equilibrium swollen hydrogels were submerged in aqueous 10 mM PBS solution (pH 7.4) and equilibrated at 37 °C for one month. The weight of the swollen hydrogel was measured initially (w) and at periodic intervals (w_x), respectively. The percentage of mass loss was calculated using the equation 4. The experiments were repeated thrice, and the results are presented as mean values with standard deviation.

$$\% \text{ Mass Loss} = \frac{w - w_x}{w} \times 100 \text{ ----- (4)}$$

Scanning electron microscopy (SEM)

The microstructure of freeze-dried hydrogel samples was examined using Philips XL30 Field Emission scanning electron microscope operated with an accelerating voltage of 10 kV. The

freeze-dried hydrogels were cut to expose the cross-section and sputter-coated with platinum prior to the examination.

X-ray diffraction (XRD)

The secondary structure of the freeze-dried hydrogel samples was analyzed using Rigaku MiniFlex 600 X-Ray Diffractometer (using Cu k_{α} radiation, $\lambda=1.54$) operating at 40 kV and 15 mA. The scans were performed in continuous scanning mode from 5 to 60° (2θ) with a step size of 0.02° at a rate of 1.2 °/min.

Raman spectroscopy

The secondary structure of freeze-dried SPI hydrogel samples was analyzed using Horiba Instruments LabRam 800HR Raman spectrometer equipped with an Olympus BX 30 fixed-stage microscope. The samples were illuminated by a 633 nm He-Ne laser, generating an intensity of 10 mW at the sample, and the resulting spectra were recorded at ambient temperature using a Peltier-cooled CCD detector. The confocal hole and the entrance slit of the monochromator were fixed at 200 and 100 μm , respectively. Measurements were performed in three replicates with 25 scans for each sample. The intensity and location of the phenylalanine band at 1003 cm^{-1} was used as an internal standard as it is non-sensitive to conformation or microenvironment.²⁴

Fourier transform infrared (FTIR) spectroscopy

The secondary structure of freeze-dried SPI/SF hybrid hydrogel samples was analyzed using Thermo Fisher Nicolet 6700 FTIR spectrometer equipped with attenuated total reflection (ATR) accessory. The spectra were measured in the wavenumber range of 700-4000 cm^{-1} .

Thermal analysis

The thermal transitions in freeze-dried hydrogel samples were investigated by thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC). The thermal

decomposition behavior of fabricated hydrogels was analyzed using Mettler Toledo TGA 1 thermal analyzer, operated under a controlled nitrogen gas flow rate of 25 mL/min. The temperature range of the measurement was 30 to 400 °C with a heating rate of 10 °C/min. The glass transition temperature (T_g) of fabricated hydrogels was analyzed using TA Instrument Q2000 DSC thermal analyzer, operated under a controlled nitrogen gas flow rate of 40 mL/min. The samples were sealed in Tzero hermetic aluminum pans and an empty sealed pan was used as a reference. The temperature range of the measurement was 30 to 250 °C with a heating rate of 10 °C/min.

Rheology

The rheological properties of aqueous SPI dispersions, fabricated hydrogels, and SPI/SF hybrid inks were analyzed using TA instrument HR-2 Discovery hybrid rheometer. To investigate the flow behavior of SPI dispersions and SPI/SF hybrid inks, dynamic viscosity measurement of the SPI dispersions (from 2 to 15 wt%) and SPI/SF hybrid inks (15 wt% protein concentration) were measured at ambient and dark environment, respectively across the shear rates from 0.01 s⁻¹ to 2000 s⁻¹ using 40 mm cone and plate geometry at a constant gap of 52 μm. After loading, the dispersion and ink samples were pre-sheared at 100 s⁻¹ for 60 seconds and allowed to equilibrate for 600 seconds to ensure even distribution of the sample, and to exclude the loading effects. The viscosity recovery (structural recovery) measurements of SPI/SF hybrid inks were investigated using rotational rheology by varying shear rates with time. Briefly, the inks were loaded as described above and a shear rate of 0.1 s⁻¹ was applied for 1 min followed by an increased shear rate of 50 s⁻¹ for 10 seconds and a decreased shear rate of 0.1 s⁻¹ for 1 min. Dynamic oscillation rheology was employed to study the viscoelastic behavior of equilibrium water swollen hydrogels at 10% compression using 20 mm parallel plate geometry. The strain sweep (0.01 to 5%) was performed at a constant frequency of 6.28 rad/s, and the frequency sweep (0.1 to 50 rad/s) was conducted at a constant strain of 0.1%. The data were collected and

processed using TRIOS software. The experiments were repeated thrice, and the results are presented as mean values with standard deviation.

Small angle neutron scattering (SANS)

The hierarchical structure of the SPI dispersion (prepared using D₂O) and equilibrium D₂O swollen hydrogels were analyzed using the Bilby and Quokka SANS instruments, respectively at ANSTO.²⁵⁻²⁶ The neutron scattering length density (SLD) of D₂O ($6.36 \times 10^{-6} \text{ \AA}^{-2}$) provides good contrast against protein-based gels and also minimizes the incoherent scattering from hydrogen in the system.²¹ The dispersion samples were loaded into demountable Bilby cell assembly of 20 mm diameter and 1 mm path length. The D₂O swollen hydrogels (approx. 1.5 mm thick) were cut into discs of 16 mm diameter and loaded into demountable Quokka cell assembly of 20 mm diameter and 2 mm path length, along with excess D₂O. The Bilby data were

collected in time-of-flight mode using wavelengths from 2 to 18 Å, in a singular detector setup with the back detector at 18 m, and the curtain detectors at 4 m and 5 m.²⁵ The data were collected in the scattering vector, q (equation 5)²¹ range of 0.001 to 0.3 Å⁻¹:

$$q = \frac{4\pi \sin\theta}{\lambda} \text{----- (5)}$$

where 2θ is the angle of scattering, and λ is the wavelength of neutrons. For the Quokka data, sample-detector distances of 2, 12 and 20 m with neutron wavelength of 5 and 8.1 Å⁻¹ (for lens optics), respectively were employed to cover the q range of 0.0007 to 0.3 Å⁻¹.²⁶ All measurements were performed at 25 °C with a sample aperture diameter of 12 mm. The obtained Bilby and Quokka data were reduced using the Mantid suit, and NCNR SANS reduction macros in the Igor software package, respectively.²⁷ The data were transformed to absolute scale using an attenuated direct beam transmission measurement, and the D₂O scattering was subtracted from the sample data using the PRIMUS computer program.²⁸

Furthermore, the incoherent background scattering from respective data were determined with a high- q power law fit ($0.15 \text{ \AA}^{-1} < q < 0.3 \text{ \AA}^{-1}$) using the SasView computer program (<https://www.sasview.org/>) and subtracted for analysis.²¹ Moreover, structural parameters of samples were determined by fitting desired model functions to SANS data using the SasView computer program.

Ultra-small angle neutron scattering (USANS)

USANS analysis was performed on equilibrium D₂O swollen hydrogels using Kookaburra USANS instrument at ANSTO.²⁹ The USANS instrument was operated with a neutron wavelength of 4.74 Å, and the identical SANS cells loaded with samples (used for SANS measurement) were used for USANS measurements. The scattering data were collected at ambient temperature in the q range 0.00005-0.001 Å⁻¹ using a sample aperture of 12 mm diameter. The sample data were reduced with an empty SANS cell scattering using Python scripts running in Gumtree software based on the standard procedure.²⁹ The data were then de-smearred using the Lake algorithm (incorporated in the NIST USANS macros), and subsequently merged with the SANS data.

RESULTS AND DISCUSSION

Aqueous dispersion of SPI

Flow properties and critical crosslinking concentration of SPI dispersion. To determine the critical crosslinking concentration (concentration required for uniform network structure), rotational rheology was employed to investigate the flow behavior of aqueous SPI dispersion at pH 7. [Figure 1A](#) shows the shear rate dependent apparent viscosity profile of SPI dispersion from 2 to 15 wt% concentration. It can be observed that the apparent viscosity of SPI dispersion showed a progressively increasing trend with increase in the concentration irrespective of the experimental shear rate. The increase in viscosity with increase in SPI concentration can be

ascribed to the increase in the intermolecular non-covalent physical forces of attraction (hydrophobic and electrostatic interactions) between soy globulins due to increased molecular crowding.³⁰ The primary structure of SPI is dominated by two major globulins, namely glycinin (11S) and β -conglycinin (7S) which have different secondary structures and properties.³ The quaternary structure of glycinin is complex and consists of six monomers (hexameric protein), where each subunit is made of an acidic chain (A) and a basic chain (B) linked by disulfide bonds. Based on homology, the five identified subunits of glycinin are divided into two groups. Group-1 members are methionine (Met) rich and have two cysteine (Cys) amino acid residues in basic chain and three Cys in the acidic chain segment. Group-2 members are relatively poor in Met. The quaternary structure of β -conglycinin is comprised of a hetero-trimer made of three subunits (α -68kDa, α' -72kDa, and β -52kDa) associated via hydrophobic interactions.³¹⁻³² The SPI dispersions showed shear thinning behavior (decrease in viscosity with increase in shear rate) for concentrations between 4 and 15 wt%, indicating the presence of a network structure, which breaks down at higher shear rates, and is in general agreement with previous reports.^{4, 30} The degree of shear thinning was observed to increase with increase in SPI concentration. Negligible shear thinning behavior was observed for 2 wt% sample, and the measured viscosity was nearly independent over a range of shear rate (10-1000 s⁻¹). The viscosity of 15 wt% SPI dispersion at a low shear rate of 0.1 s⁻¹ is more than four orders of magnitude higher when compared to viscosity at high shear rate (2000 s⁻¹). This is because at very low shear rates the stable aggregates formed by the glycinin subunits of SPI are stabilized by disulfide bonds and the side chains are entangled with neighboring chains resulting in high viscosity.⁴ With the increase in shear rate, disentanglement of side chains and alignment of the molecules offer less shear resistance thereby decreasing the viscosity.⁴ For shear thinning fluids, the shear rate dependency of viscosity is described by power law model, as given in the equation 6.³³

$$\eta(\dot{\gamma}) = K \dot{\gamma}^{n-1} \text{ ----- (6)}$$

where η (γ) is the viscosity, K is the flow consistency index, γ is the shear rate, and n is the flow behavior index. The shear thinning curves (at concentrations between 4 and 15 wt%) were fitted to equation 6 using power law regression (Figure S1 in Supporting Information), and the corresponding values of K , n and coefficient of determination (R^2) are given in Table S2 in Supporting Information. As the concentration of SPI is increased, the dispersion is found to be thicker (K increases) and more shear thinning (n decreases).³⁴ At 15 wt% concentration, the dispersion becomes much thicker (good network structure) which resulted in drastic increase in viscosity, as shown in Figure 1B. Therefore, a concentration of 15 wt% was considered highly suitable for photocrosslinking SPI at steady state. Moreover, the measured viscosity of 15 wt% SPI dispersion is in the range of $0.83\text{--}4.27 \times 10^4$ Pa.s at low shear rates of $0.1\text{--}0.01$ s⁻¹, a close range ($1.4\text{--}3.7 \times 10^4$ Pa.s) reported for 3D printing polymer hydrogels.³⁵

Figure 1. Rheological properties of SPI dispersion in water: (A) shear rate dependant viscosity profile (at different concentrations), and (B) concentration dependant viscosity profile (at different shear rate).

Effect of pH on the hierarchical structure of SPI dispersions: The hierarchical structure of aqueous SPI dispersion was studied at two different pH (7 and 12), as the molecular structure of SPI is often reported to be sensitive to pH.³ Figure 2A compares the SANS intensity profile of 15 wt% SPI dispersed in D₂O at pH 7 and 12. The plot shows three distinctive scattering

regions: a high- q region ($0.09 < q < 0.3 \text{ \AA}^{-1}$), a mid- q region ($0.003 < q < 0.09 \text{ \AA}^{-1}$), and a low- q region ($0.001 < q < 0.003 \text{ \AA}^{-1}$). In general, the slope estimated from high- q region (i.e. Porod region) of the small angle scattering data provides information about the “fractal dimension” of the formed structures (domains); whereas the low- q and mid- q region (i.e. Guinier region) allows for the estimation of fractal size.³⁶ Thus the Porod region corresponds to a probed range smaller than the scattering objects and probing the local structure. The fractal dimension (Porod slope) of SPI was estimated as ~ 2.9 using a Power law fit at high- q region ($0.1 < q < 0.25 \text{ \AA}^{-1}$). Such a slope value represents ‘mass fractal’ in a branched system and gels; a colloidal dispersion with relatively dense internal structure.³⁶ Such structure quality is typical to that of globular proteins in water and has been previously reported for other globular proteins such as bovine serum albumin.³⁷ In SPI, this structural feature can be attributed predominantly to the 11S glycinin.³⁸ Moreover, a measured thermal glass transition (T_g) of $\sim 215 \text{ }^\circ\text{C}$ (Figure S2 in Supporting Information) supports the SPI to be 11S-enriched.³⁹ The SANS data was further assessed by means of the Kratky plot (Figure 2B), which divides out the decay of the scattering at high- q and shows certain other features more qualitatively evident.³⁶ The Kratky plot of SPI at pH 7 clearly showed two broad peaks and a shoulder peak around q value of $\sim 0.007 \text{ \AA}^{-1}$, $\sim 0.02 \text{ \AA}^{-1}$ and $\sim 0.09 \text{ \AA}^{-1}$, respectively. Conversely, increasing pH of SPI dispersion from 7 to 12 showed decrease in intensity or disappearance of the peak at $q \sim 0.007 \text{ \AA}^{-1}$, and the peak at $\sim 0.02 \text{ \AA}^{-1}$ shifted towards higher- q (i.e., smaller structures). Moreover, the zeta potential of SPI dispersion was measured to increase from -15.24 ± 0.40 to -32.15 ± 1.20 for changing pH from 7 to 12. A change in the pH or ionic strength of the dispersion media has been previously reported to degrade or dissociate 11S structure of glycinin due to increased electrostatic repulsion.⁴⁰⁻⁴¹ The observed difference in scattering with change in pH is related to the unfolding/dissociation and/or re-association of 11S and their aggregates resulting in increased solubility at pH 12 (observed with change in dispersion colour from turbid to clear, as seen in

Figure 3A).^{3, 42} To gain further insights into the conformational changes and organization of SPI at different pH in detail, the SANS data were fitted (Figure 2C and 2D) with shape-independent form factor models (Guinier-Porod + power law) to estimate structural parameters. The Guinier-Porod model calculates the scattering for a generalized Guinier/power law object that can be used to determine the size and dimensionality of scattering objects.⁴³ A dimension variable, $S=0$ (for 3D globular structures) was used for Guinier-Porod fitting.⁴³ The fits returned a radius of gyration (R_g) of ~ 26.9 nm, ~ 8.0 nm and ~ 2.0 nm for the peaks, which may be attributed to the glycinin subunits such as $>15S$ (unresolved 11S aggregates), 11S and 3S, respectively.⁴⁴⁻⁴⁵ The 3S structure is the 11S dissociated structure containing one acidic and one basic polypeptides (building block of 11S).^{40, 45} The R_g of two peaks for pH 12 sample was determined to be ~ 5.9 nm and ~ 2.0 nm, which may be attributed to $\overline{7S}$ (glycinin trimer, different to β -conglycinin) and 3S glycinin subunits, respectively.⁴⁴⁻⁴⁵ This observation suggests that, at pH 12, the 11S glycinin of SPI dissociates and/or re-associates into 7S glycinin trimers, and the SANS Porod slope at high- q corresponds to the intrinsic structure of 3S glycinin subunits.^{40, 45} Moreover, the absence of Guinier plateau and the observed increase in intensity at the low- q region signifies intermolecular interactions (large agglomeration trending beyond SANS measurement range) or network structure in the system,⁴⁶ which is in general agreement with rheology results.

Figure 2. (A) SANS intensity profile, and (B) Kratky plot of 15 wt% SPI dispersion in D₂O. (C) and (D) are shape-independent form factor models (Guinier-Porod (GP) + power law (PL)) fit to SANS data of SPI at pH 7 and 12, respectively.

Photocrosslinked SPI hydrogels:

Effect of pH on the water uptake and microstructure of SPI hydrogels. The SPI used in this study has a 38.6 mol% non-polar, 47.6 mol% charged polar, and 13.8 mol% uncharged polar amino acids with a tyrosine content of 3.7 mol% (Table S1 in Supporting information), which can be potentially used for fabrication of photocrosslinked hydrogel via dityrosine crosslinking.¹⁶ Moreover, the ruthenium–persulfate cross-linking system used to fabricate hydrogels in this work has been previously reported (at similar concentration range) to be non-toxic and minimally inflammatory (after 30 days) to mammalian cells, which supports the

applied cross-linking method to be completely biological.⁴⁷ Figure 3A shows the schematic of photocrosslinked SPI hydrogel fabrication (see Figure S3 in Supporting Information for full reaction mechanism), and the optical images of equilibrium water swollen hydrogels. With SPI exhibiting different structural conformations at pH 7 (closed) and pH 12 (open) studied by SANS, SPI hydrogels were photocrosslinked at pH 7 and 12 to study their structure-property differences. The photocrosslinked SPI hydrogels swelled macroscopically when exposed to water and became brittle when dry. Water swelling is an important property of hydrogels to be considered for tissue engineering applications, which directly relates to the diffusive and transport properties of nutrients/gases/regulatory factors, and facilitate cell-biomaterial interactions, cell differentiation and proliferation.⁴⁸ Moreover, the balance between water uptake (h) and mechanical strength is of critical importance for application of hydrogels in the field of tissue engineering, where the strength of the hydrogel is dictated by its crosslink density (v_e), which can be calculated from water uptake results using the equation 2.¹⁹ The h and v_e values of fabricated SPI hydrogels measured from water uptake study are presented in Table 1. The SPI hydrogel photocrosslinked at pH 7 (SPI gel pH7) exhibited a h and v_e value of $\sim 662\%$ and $0.283 \times 10^{-3} \text{ mol/cm}^3$, respectively; where the h value of fabricated hydrogel is five times higher than extruded soy protein sheets,⁴⁹ and ten times higher than amino crosslinked soy protein hydrogels.¹⁰ The SPI gel pH7 exhibited a particulate microstructure (Figure 3B), which is due to the globular (closed) structural configuration of SPI (as seen from SANS results) during crosslinking, and is similar to microstructure of hydrogels crosslinked using glyceraldehyde and/or glutaraldehyde.¹⁰ Conversely, the SPI hydrogel photocrosslinked at pH 12 (SPI gel pH12) exhibited a h and v_e value of $\sim 1946\%$ and $0.279 \times 10^{-3} \text{ mol/cm}^3$, respectively; where the h value of fabricated hydrogel is two times higher than soy protein/poly(N-isopropylacrylamide) interpenetrating polymer network hydrogels.⁵⁰ The significantly higher h value measured for SPI gel pH12 is attributed to the increased hydrophilicity and solubility

the unfolded state of SPI globulins (as seen from SANS results) at photocrosslinking pH of 12 create large (4-6 μm) ordered porous microstructure and channels that can be clearly observed in SEM micrographs (Figure 3B). The porosity of a hydrogel is related to its unbound water accommodation, which directly influences the water uptake. The lower h and higher v_e value of SPI gel pH7 could greatly influence its mechanical properties.

Figure 3. (A) Schematic of photocrosslinked SPI hydrogel fabrication with optical pictures of dispersion and equilibrium water swollen hydrogels. (B) SEM micrographs of respective freeze-dried hydrogels.

Table 1: Water uptake and thermal analysis results of photocrosslinked hydrogels.

Sample*	Mass ratio (SPI:SF)	Water uptake, h (%)	Crosslink density, v_e ($\times 10^{-3}$ mol cm^{-3})	Glass transition temperature, T_g ($^{\circ}\text{C}$)
SPI gel pH12	1:0	1946 ± 98	0.279	-
SPI gel pH7	1:0	662 ± 33	0.283	207.57
SPI/SF (3:1) gel	3:1	1497 ± 74	0.293	181.16
SPI/SF (1:1) gel	1:1	1895 ± 94	0.304	179.73

SPI/SF (1:3) gel	1:3	2864 ± 143	0.317	178.17
SF gel	0:1	2362 ± 118	0.330	168.85

**The SPI gel pH12 sample was photocrosslinked at pH 12, whereas all other samples at pH 7.*

Effect of pH on the secondary structure of SPI hydrogels. To determine the secondary structural differences of photocrosslinked SPI hydrogels, SPI powder and hydrogels were qualitatively and quantitatively characterized using XRD and Raman spectroscopy. The XRD pattern of SPI powder and hydrogels (Figure 4A) exhibited semi crystalline nature with major 2 θ peaks observed at 9.8° and 19.8° which corresponds to α -helix and β -sheet structures, respectively.⁵¹⁻⁵² Several broad/shoulder peaks around 2 θ value of 16°, 25° and 42° were also observed. However, there remains paucity of information on designation of these peaks to SPI. On the other hand, secondary structures of other semi crystalline protein such as SF have been extensively studied using XRD, and several other peaks corresponding to α -helix, β -sheets, β -strands, turns and unordered structures have been reported.^{19, 53} We attempted to estimate the quantitative secondary structural information of SPI powder and hydrogels by deconvoluting the XRD data (Figure S4 in Supporting Information) with 2 θ peaks reported for α -helix (9.3°, 12.3° and 15.8°), β -sheets/strands (19.8°, 22.8° and 24.7°) and turns/unordered (28.2°, 32.4°, 40.0° and 49.4°) structures of SF.^{19, 53} The data were deconvoluted using the MagicPlot software (<https://magicplot.com/>) with Gaussian curve fitting, and the estimated secondary structural content of SPI is presented in Figure 4B. The estimated secondary structures for SPI powder (α -helix ~22%, β -sheets/strands ~44%, and turns/unordered ~34%) is in general agreement with values reported previously.²⁴ It can be observed that the SPI powder and SPI gel pH7 exhibited similar secondary structure contents, whereas, SPI gel pH12 showed decrease in the β -content and subsequent increase in turns/unordered structure. Figure 4C shows the Raman spectra of SPI powder and hydrogels with characteristic frequency peaks

observed for amide I ($1620\text{--}1720\text{ cm}^{-1}$), aromatic amino acids ($1590\text{--}1620\text{ cm}^{-1}$ and $1160\text{--}1250\text{ cm}^{-1}$), amide II ($1520\text{--}1590\text{ cm}^{-1}$), C α -H deformation (1450 cm^{-1}), amide III ($1250\text{--}1360\text{ cm}^{-1}$), phenylalanine doublet ($970\text{--}1055\text{ cm}^{-1}$), C α -C stretch (935 cm^{-1}), tyrosine doublet ($830\text{--}850\text{ cm}^{-1}$) and tryptophan (756 cm^{-1}).⁵⁴⁻⁵⁵ The amide I band of Raman spectra is related to changes in protein backbone conformation (involving mainly C=O stretching vibrations, and partly N-H in-plane bending of peptide groups) and is widely employed in determining secondary structure of proteins.²⁴ The amide I band of Raman spectra were deconvoluted (Figure S5 in Supporting Information) with frequencies corresponding to α -helix (1656 cm^{-1}), β -sheets/strands (1633 cm^{-1} and 1669 cm^{-1}), turns/random coil (1643 cm^{-1} , 1685 cm^{-1} , 1698 cm^{-1} and 1715 cm^{-1}) structures,⁵⁴⁻⁵⁵ and the estimated secondary structural content of SPI is presented in Figure 4D. The secondary structural information obtained by Raman analysis is in good agreement with XRD results. The polymorph α -form (type II β -turn and may be less extended helix) is hydrophilic in nature, whereas β -form (antiparallel β -pleated sheets and aggregated strands) is hydrophobic.¹⁹ Therefore, the higher h value exhibited by SPI gel pH12 is due to its relatively higher content of α -form and unordered structures (due to dissociated state of glycinin 11S during crosslinking, as seen from SANS results), which could greatly influence its hydrophilicity and degradation behavior. This is to note that h value is also dependent on the nature of the pores and pore volume.

Figure 4. (A) X-ray diffractogram, (B) quantitative secondary structure estimates from XRD data, (C) Raman spectra, and (D) quantitative secondary structure estimates from Raman spectroscopy data of photocrosslinked SPI hydrogels compared with SPI powder.

Effect of pH on the viscoelastic properties of SPI hydrogels. Protein-based hydrogels exhibit complex viscoelastic properties, which strongly correlate with their hierarchical structure, h , v_e and degree of crystallinity. The viscoelastic properties of photocrosslinked SPI hydrogels were measured using dynamic oscillatory rheology in both strain and frequency sweep modes to compare its suitability for human lumbar nucleus pulposus tissue engineering, reported at similar experimental conditions.¹⁵ The strain sweep was performed to determine the linear viscoelastic region of SPI hydrogels with respect to strain, whereas frequency sweep to

determine the linear equilibrium modulus plateau.⁵⁶ Figure 5 shows the measured storage modulus (G') and loss modulus (G'') of equilibrium water swollen SPI hydrogels in the linear viscoelastic regime; where G' is observed to be greater than G'' in the whole measurement range, and therefore the elastic response of SPI hydrogels is greater than viscous nature. In both modes, the G' and G'' of SPI gel pH7 was observed to be an order of magnitude higher than SPI gel pH12. Moreover, G' was observed to be nearly independent of strain over a range of 0.01-0.1 strain%. However, with increase in strain beyond 1%, the G' begins to drop indicating network fracture.⁵⁶⁻⁵⁷ Therefore, a strain of 0.1% was used to evaluate the frequency dependency of the hydrogels. At low frequencies the samples showed nearly frequency independent behavior. However, at a higher frequency (i.e. low relaxation time), the marginal increase in modulus indicates diminishing of the sample flexibility and increase in mechanical rigidity. At 0.1% strain, the SPI gel pH7 exhibited a G' value of ~ 7.7 kPa, whereas SPI gel pH12 exhibited ~ 0.8 kPa, respectively. The obtained modulus values of SPI gel pH7 are significantly higher than that reported for thermal-set,⁴ enzyme⁸ and glutaraldehyde¹⁰ cross-linked soy protein hydrogels, and is in the range of human lumbar nucleus pulposus.¹⁵ The higher G' magnitude and tan delta (~ 0.17) observed for SPI gel pH7 directly implies that it is much stiffer than SPI gel pH12 (tan delta ~ 0.13),⁵⁸ which can be attributed to its structural organization, higher v_e and lower h values. The SPI gel pH12 is extremely soft that may deform under its own weight, and make manual handling of the sample a rather difficult experimental task, which may be the reason for observed fluctuations in dynamic frequency and strain sweeps measurements.

Figure 5. Dynamic viscoelastic properties of photocrosslinked SPI hydrogels. (A) and (B) are strain and frequency sweep, respectively.

Effect of pH on the hydrolytic degradation of SPI hydrogels. The *in vivo* application of protein-based hydrogels for tissue engineering requires that the hydrogel degrades in a controlled rate under physiological conditions. Therefore, the photocrosslinked SPI hydrogels were investigated for *in vitro* hydrolytic degradation in 10 mM PBS (pH 7.4), which match the osmolarity and ion concentrations of the human body. Figure 6 shows the *in vitro* degradation behavior of photocrosslinked SPI hydrogels in PBS over a period of 18 days. It was observed that the SPI gel pH12 degraded faster, showing around 52% mass loss in 7 days after which it lost its structural integrity; whereas, the SPI gel pH7 showed structural integrity up to 18 days retaining ~62% of mass, which is higher than soy hydrogels reported by thermal treatment,⁵⁹ and enzyme crosslinking.⁶⁰ The observed higher water-resistance or stability of SPI gel pH7 could be attributed to its globular nature.

Figure 6. *In vitro* degradation behavior of photocrosslinked SPI hydrogels in phosphate buffered saline (pH 7.4).

Photocrosslinked SPI/SF hybrid hydrogels

Water uptake and microstructure of hybrid hydrogels. The SPI photocrosslinked at pH 7 exhibited particulate microstructure, and higher mechanical and water-resistant properties compared to SPI photocrosslinked at pH 12. Therefore, pH 7 was selected to blend and co-crosslink SPI and SF to study the effect of SF content on the structure and properties of photocrosslinked SPI/SF hybrid hydrogels. The equilibrium swelling and microstructure of SPI/SF hybrid hydrogels was evaluated using water uptake and SEM (Figure 7) study. The h and v_e values of SPI/SF hybrid hydrogels obtained from water uptake results are provided in Table 1. The pristine SF gel exhibited relatively higher h and v_e values compared to SPI gel pH7. The obtained v_e value for SF gel ($0.330 \times 10^{-3} \text{ mol/cm}^3$) is in general agreement with previous reports.^{16, 19} The relatively higher h value of SF gel can be attributed to its highly ordered porous microstructure, whereas the higher v_e value can be attributed to its higher tyrosine content (5.6 mol%; Table S1 in Supporting Information). The particulate structure of SPI gel pH7 is due to the globular (closed) structural configuration of SPI (largely 11S glycinin and aggregates, as seen from SANS results) during crosslinking, whereas the ordered porous

microstructure of SF gel is attributed to the random coil configuration of silk molecules during crosslinking.²¹ It can be observed that the macroscopic size of the equilibrium water swollen SPI/SF hybrid hydrogels (Figure 7), and their porous microstructure and order increases with increase in SF content. The SPI/SF (1:1) and SPI/SF (1:3) hybrid hydrogels exhibited ordered, interconnected and interpenetrated 3D porous microstructure network. The order and average pore size of SPI/SF (3:1) hybrid hydrogel is random and small in nature. The h and v_e values along with porosity of SPI/SF hybrid hydrogels also increased with increase in SF content. Interestingly, the SPI/SF (1:3) gel exhibited the highest h value amongst the fabricated hydrogels, whereas the SF gel exhibited the highest v_e . In co-crosslinked SPI/SF hybrid hydrogels, the di-tyrosine crosslinks can occur between SPI-SPI, SPI-SF, and SF-SF chains. It is also reported that the order or arrangement of secondary structure also influences the h value of SF gel.²¹ Therefore, given the complexity of the system, the difference in v_e of fabricated hydrogels may be attributed to tyrosine content in the sample; whereas, the difference in h value of hydrogels may be due to the difference in hierarchical structure, secondary structures and molecular chain mobility.²¹ The obtained h values of photocrosslinked SPI/SF hybrid hydrogels are comparable to those of photocrosslinked SF/polyvinyl alcohol hydrogels.⁶¹ The difference in h and v_e values of SPI/SF hybrid hydrogels are expected to greatly influence their mechanical and degradation properties.

Figure 7. Optical pictures and SEM micrographs of freeze-dried SPI/SF hybrid hydrogels compared to pristine SPI and SF hydrogels: (A) SPI gel pH7, (B) SPI/SF (3:1), (C) SPI/SF (1:1), (D) SPI/SF (1:3), and (E) SF gel.

Secondary structure and molecular chain mobility of hybrid hydrogels. The effect of SF content on the secondary structure of SPI/SF hybrid hydrogels was characterized both qualitatively and quantitatively using XRD and FTIR spectroscopy. The XRD pattern of SPI/SF hybrid hydrogels is compared to pristine SPI and SF hydrogels in [Figure 8A](#). The SPI gel pH7 exhibited semi crystalline nature with two major 2θ peaks observed at 9.8° and 19.8° respectively, which corresponds to α -helix and β -sheet structures (as discussed earlier). Conversely, the SF gel exhibited semi crystalline nature with one major broad peak around 21.9° , indicating the presence of both polymorphs of silk: silk I (α -form–type II β -turn and may be less extended helix) and silk II (β -form–antiparallel β -pleated sheets) structures.^{16, 53} For SPI/SF hybrid hydrogels, the intensity of α -helix peak were observed to gradually decrease

with increase in SF content. The observed decrease in intensity of α -helix peak with the increase in SF content may be due to the increased glycine amino acid content (from SF) in the system, which may act as a α -helix breaker for SPI structures.⁶² In order to delve deeper understanding of the secondary structure of SPI/SF hybrid hydrogels and their associated properties, the XRD spectrum of fabricated hydrogels were deconvoluted ([Figure S6 in supporting information](#)) with 2 θ peaks corresponding to α -helix (9.3°, 12.3° and 15.8°), β -sheets/strands (19.8°, 22.8° and 24.7°) and turns/unordered (28.2°, 32.4°, 40.0° and 49.4°) structures.^{19, 53} The estimated secondary structural contents are presented in [Figure 8B](#). It can be observed that the secondary structure of SPI gel pH7 and SF gel samples are dominated by β -sheet/strand structures. However, with increase in SF content, SPI/SF hybrid hydrogels exhibited a decrease trend in α -helix and β -sheet/strand structures, and subsequently increase in turns/unordered structure. The increase in unordered structure of SPI/SF hybrid hydrogels with increase in SF content is in general agreement with water uptake results. [Figure 8C](#) compares the FTIR spectra of SPI/SF hybrid hydrogels to pristine SPI and SF hydrogels. The spectra exhibited two major peaks between 1600-1700 cm^{-1} and 1500-1600 cm^{-1} , corresponding to amide I and amide II conformational bands of proteins. The secondary structural estimates of fabricated hydrogels were obtained by deconvoluting ([Figure S7 in Supporting Information](#)) the FTIR amide I band with frequencies corresponding to α -helix (1656-1662 cm^{-1}), β -sheets/strands (1605-1615 cm^{-1} , 1616-1621 cm^{-1} , 1622-1627 cm^{-1} , and 1628-1637 cm^{-1}) and turns/unordered (1638-1646 cm^{-1} , 1647-1655 cm^{-1} , 1671-1685 cm^{-1} , and 1686-1696 cm^{-1}) secondary structures.⁶³⁻⁶⁴ The estimated secondary structural contents are presented in [Figure 8D](#). The secondary structures obtained from FTIR results are in general agreement with XRD results. The decrease in crystallinity of SPI/SF hybrid hydrogels with increase in SF content suggests change in secondary structure and solubility of SPI in the

presence of SF (due to SPI-SF molecular interactions), which could greatly influence their mechanical and degradation properties.

Figure 8. (A) X-ray diffractogram, (B) quantitative secondary structure estimates from XRD data, (C) FTIR spectra, and (D) quantitative secondary structure estimates from FTIR data of SPI/SF hybrid hydrogels compared to pristine SPI and SF hydrogels.

The effect of SF content on the molecular chain mobility of SPI/SF hybrid hydrogels was studied by DSC technique. The measured T_g values of fabricated hydrogels (Figure 9) are given in Table 1, where the T_g values for pristine SPI and SF hydrogels are in general agreement with literature reports.^{19, 39} The SF gel exhibited the lowest T_g (~ 168 °C), whereas the SPI gel pH7 exhibited the highest (~ 216 °C) amongst the fabricated hydrogels, and therefore has the

lowest chain mobility. The measured T_g of SPI gel pH7 is similar to that of SPI powder (Figure S2 in Supporting Information), and therefore indicate the SPI gel pH7 to contain glycinin globules.³⁹ The SPI/SF hybrid hydrogels showed T_g between that of SPI gel pH7 and SF gel, which supports miscibility/molecular interaction of macromolecules and co-crosslinking between SPI and SF.

Figure 9. DSC thermogram showing glass transition of SPI/SF hybrid hydrogels compared to pristine SPI and SF hydrogels.

Hierarchical structure of hybrid hydrogels. The effect of SF content on the hierarchical structure (from 2 nm to 12.5 μm) of the equilibrium D_2O swollen SPI/SF hybrid hydrogels

were investigated using SANS and USANS. The combined SANS and USANS curves of fabricated hydrogels (Figure 10A) showed similar distinctive regions as the SPI dispersion at pH 7 (Figure 2A), but now the neutron scattering data extended to even lower- q : starting at the high- q end, there is a high- q Porod region ($0.1 < q < 0.3 \text{ \AA}^{-1}$), a mid- q Guinier-like region (about $0.003 < q < 0.1 \text{ \AA}^{-1}$), a low- q Porod-like region ($0.0004 < q < 0.003 \text{ \AA}^{-1}$) and a low- q Guinier region ($0.000035 < q < 0.0004 \text{ \AA}^{-1}$). The Porod slope at high- q of the SPI gel pH7 (~ 2.9) obtained from power law fit in the high- q region ($0.12 < q < 0.25 \text{ \AA}^{-1}$) is the same as that of the SPI dispersion (discussed previously), which signifies that the globular structures in SPI dispersion is conserved in the hydrogel. Conversely, the SF gel showed a Porod slope of ~ 4 , which can be attributed to hydrophobic domains containing β -sheet structures (formed by photocrosslinking) that exhibit a sharp interface with the surrounding environment (amorphous protein matrix and/or D₂O).^{19, 21} The SPI/SF hybrid hydrogels showed a progressive increase in Porod slope with increase in SF content, which is attributed to an increased contribution from the hydrophobic domains of SF. The mid- q region of the SPI gel exhibits similar features to that of the SPI dispersion (see Kratky plot in Figure 10B), with broad feature at about 0.007 and $0.02 \text{ \AA}^{-1}$, supporting SPI globulins and their aggregates to be present in the photocrosslinked hydrogel. With increasing SF content, these features become less pronounced, even for the SPI/SF (3:1) hybrid hydrogel, and instead appearance of a shoulder at about $0.085 \text{ \AA}^{-1}$ becomes more dominant. This may be due to a disruption and decrease in the volume fraction of SPI globulins aggregates (observed in SANS curves of SPI dispersion) and increase in the volume fraction of SF hydrophobic domains.^{19, 21} Conversely, the mid- q scattering intensity of SF hydrogel is also contributed by hydrophilic domains containing unordered secondary structures.^{19, 21} The observed trends in SANS intensity can be clearly seen from the Kratky plot (Figure 10B). The USANS region (low- q) shows a strong upturn with power law behavior of $\sim q^{-3.5}$ (low- q Porod-like region) that plateaus at very low- q (low- q

Guinier region). This feature is characteristic of porous structure (clearly seen in Kratky plot), and is likely to stem from micropores and channels observed in the SEM image of the hydrogels.³⁶ Therefore, the observed differences in water uptake of the fabricated hydrogels may be predominantly due to porous microstructure, and their inhomogeneities.

Figure 10. (A) SANS and USANS intensity profile, and (B) Kratky plot of SPI/SF hybrid hydrogels compared to pristine SPI and SF hydrogels.

Viscoelastic properties of hybrid hydrogels. The effect of SF content on the viscoelastic properties of SPI/SF hybrid hydrogels were measured using dynamic rheological experiment in both strain and frequency sweep modes. Figure 11A and Figure 11B show the strain dependency of storage modulus (G') and loss modulus (G'') of the fabricated hydrogels, respectively; whereas, Figure 11C and Figure 11D shows the frequency dependent rheological characteristics. The marginal decrease in the modulus of the hydrogels at higher strain ($>> 1\%$) indicates breakage of the network structure, whereas increase in the modulus at higher frequency indicates increase in mechanical rigidity.⁵⁶⁻⁵⁷ The higher value of G' measured at a strain of 0.1% for SPI gel pH7 directly implies it to be the stiffest amongst the fabricated hydrogels (Figure 11E).⁵⁸ The SPI/SF hybrid hydrogels demonstrated G' in the order of SPI/SF (3:1) $>$ SPI/SF (1:1) $>$ SPI/SF (1:3). The decrease in stiffness of SPI/SF hybrid hydrogels with

increase in SF content can be attributed to increase in pore size and order of porous microstructure (Figure 7), increase in water uptake (water acts as a plasticizer) and disruption in the globular structure of SPI in the presence of SF (Figure 10). In SPI/SF (1:1) and SPI/SF (1:3) hybrid hydrogels, SF appears to be the continuous phase with SPI as the dispersed phase with significant role as pore modifiers. Moreover, the observed decrease in tan delta or loss factor (Figure 11F) of these SPI/SF hybrid hydrogels with increase in SF content demonstrates increased elasticity and resilience.⁶⁵ The measured G' value of SPI/SF hybrid hydrogels in the range of 0.2-1.4 kPa is comparable to that of microtubule,⁶⁶ and peptide-heparin⁶⁷ supramolecular hydrogels applied for regenerative medicine.

Figure 11. Dynamic viscoelastic properties of SPI/SF hybrid hydrogels compared to pristine SPI and SF gels: (A) and (B) are strain sweep measurements, (C) and (D) are frequency sweep measurements, and (E) and (F) are compares storage modulus and tan delta from both sweeps, respectively.

Hydrolytic degradation of hybrid hydrogels. Figure 12 shows the effect of SF content on the *in vitro* hydrolytic degradation behavior of SPI/SF hybrid hydrogels in 10 mM PBS (pH 7.4) over a period of 28 days. The SF gel showed the highest water-resistivity, whereas SPI gel pH7 showed the least amongst the fabricated hydrogels. The higher stability of SF gel in PBS compared to SPI gel pH7 may be attributed to its higher glycine and alanine amino acid contents and their repeat sequence in primary structure.⁶⁸ The SPI/SF hybrid hydrogels showed improved water-resistivity with increase in SF content. The SPI/SF (3:1), SPI/SF (1:1) and SPI/SF (1:3) hybrid hydrogels showed mass recovery values of ~84%, ~87% and ~90%, respectively after 28 days.

Figure 12. *In vitro* degradation behavior of SPI/SF hybrid hydrogels compared to pristine SPI and SF gels.

Flow properties of SPI/SF hybrid inks for printability

To examine the likelihood of printing developed hydrogel systems for tissue engineering application, pristine SPI, pristine SF, and SPI/SF hybrid inks at same protein concentration (15 wt%) used for hydrogel fabrication was assessed using rotational rheology. Figure 13A shows the shear rate dependent apparent viscosity profile of SPI/SF hybrid inks compared to pristine protein inks. It is observed that the pristine SPI ink behaves as a shear thinning fluid (decrease

in viscosity with increase in shear rate) throughout the experimental condition, whereas the pristine SF ink show shear thinning behavior up to a shear rate of 50 s^{-1} , above which exhibits Newtonian behavior. The plateau region (Newtonian region) of SF ink observed at high shear rates can be attributed to the formation of more ordered structural re-organization of silk fibroin molecules under shear, which restricts the network breakage.⁶⁹ Blending SF with SPI systematically decreased the viscosity of the SPI ink at low shear rates, and initiated the onset of Newtonian behavior (observed for SF ink) at higher shear rates, which shifts to lower shear rates with increase in SF content. The structure of SPI is composed of two major globulins, namely glycinin (11S) and β -conglycinin (7S) which have different secondary structures including α -helix.³ On the other hand, SF is dominated by Glycine (43.4 mol%), known as a helix breaker which could disrupt the SPI structure,⁶² thereby decreasing the viscosity of blends. Therefore, the decrease in viscosity of SPI/SF ink at low shear rates (Shear thinning region) with the increase in SF content can be attributed to the decrease in helix structures, whereas the observed higher viscosity of SPI/SF inks at higher shear rates (Newtonian region) indicate formation of strong interacting (intermolecular interactions between SPI and SF molecules) network which cannot be breached by shear in the maximum tested range. All the inks tested showed shear thinning behavior between a shear rate of 0.01 and 1.0 s^{-1} , which is an important parameter in extrusion-based printing as the viscous ink deforms more slowly which provide good resolution to the final printed gel structure.⁷⁰ Among the tested inks, the pristine SPI exhibited the highest viscosity (at 0.1 s^{-1}) followed by SPI/SF (3:1), pristine SF, SPI/SF (1:1) and SPI/SF (1:3), respectively. All the inks exhibited viscosity in the range of $0.06\text{--}8.0 \times 10^3 \text{ Pa.s}$ at low shear rates of $0.01\text{--}0.1 \text{ s}^{-1}$, which is in good agreement with values previous reported for bioinks used for 3D printing.⁷¹ Moreover, the lower viscosity of SPI/SF hybrid inks, and the non-toxic and minimally inflammatory nature of ruthenium–persulfate cross-linking system used in this study could be potentially used as bioinks for printing cells.⁴⁷

⁷⁰ In addition to shear thinning, structure recovery is also an important factor to be considered for 3D printing to determine the quality/resolution of printed construct. Therefore, we studied the structural recovery of protein inks by varying the shear rates (low-high-low) with respect to time, as shown in Figure 13B. It was observed that all the inks have an excellent recovering ability (in a few seconds), and the amount of recovery varied with the change in SF content. The percentage recovery was calculated by the ratio of the stabilized (plateau) viscosity after and before shear rate of 50 s^{-1} , and the prepared inks demonstrated the percentage recovery of 75.34%, 83.16%, 88.57% and 94.41% for SPI, SPI/SF (3:1), SPI/SF (1:1) and SPI/SF (1:3), respectively. The calculated values elucidate that the structural recovery of SPI/SF ink increases with the increase in SF content, favoring 3D printing applications. Therefore, the observed shear thinning and recovering behavior support printing potential of complex structures using the prepared pristine and hybrid protein inks. Overall, it is clearly observed that the presence of SF strongly influences the SPI by disrupting and making network with shear as a variable. An in depth investigation of 3D printing of different types of hydrogel structures using the prepared inks and *in vitro* cell viability/proliferation of fabricated hydrogels will be reported in the future for tissue engineering applications, and is beyond the scope of this article.

Figure 13. Rheological properties of SPI/SF hybrid inks compared to pristine SPI and SF inks (sol): (A) shear rate dependent viscosity profile (at different weight ratios), and (B) viscosity recovery profile.

CONCLUSIONS

In summary, pristine SPI hydrogels and co-crosslinked SPI/SF hybrid hydrogels of tunable morphology, porosity and viscoelastic characteristics have been successfully fabricated by a fast and facile photocrosslinking method. The SPI hydrogel photocrosslinked at pH 7 exhibited a particulate microstructure, higher crosslink density, crystallinity, shear storage modulus and tan delta, and water-resistivity compared to SPI hydrogel photocrosslinked at pH 12. Conversely, the SPI hydrogel photocrosslinked at pH 12 exhibited well-ordered porous microstructure and higher water uptake. The difference in observed microstructure of SPI hydrogels is attributed to the difference in their secondary structure (11S glycinin and aggregates at pH 7, whereas 7S glycinin at pH 12) due to pH and its perpetuity during crosslinking. The obtained mechanical and water-resistant properties of the photocrosslinked SPI hydrogels are superior to those reported by thermal treatment, pressure treatment, salt-induced cold-setting and enzymatic crosslinking. Moreover, it is evident that the addition of SF to SPI disrupts the secondary structural organization of SPI. Progressive increase in the SF content of the SPI/SF hybrid hydrogels increased the crosslink density, porosity, molecular chain mobility, water uptake and resistivity, whereas decreased the crystallinity, shear storage modulus and tan delta. The optimized SPI/SF hybrid hydrogels are sustainable, robust and biocompatible with tunable mechanical, rheological and bio-degradation properties, and hence have great potential as a multifunctional material for various biomedical applications. Moreover, the shear thinning and recovery nature of pristine SPI ink and SPI/SF hybrid inks along with rapid gelation kinetics of the crosslinking mechanism have the potential to facilitate

a noble platform to develop 3D printable photocrosslinked SPI-based hybrid hydrogel systems for tissue engineering applications.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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SUPPORTING INFORMATION

Table S1. Amino acid composition of soy protein isolate (SPI) and silk fibroin (SF).

Amino acid residue	Amino acid	Symbol	Mol %	
			<i>SPI</i> *	<i>SF</i> ¹
Non-polar side chain	Glycine	Gly/G	4.1	43.4
	Alanine	Ala/A	4.1	27.6
	Valine	Val/V	4.7	2.37
	Leucine	Leu/L	7.8	0.7
	Isoleucine	Ile/I	4.8	0.8
	Methonine	Met/M	1.3	0.2
	Proline	Pro/P	5.1	0.8
	Phenylalaline	Phe/F	5.2	0.1
	Tryptophan	Try/W	1.4	0.7
Total nonpolar			38.5	76.7
Uncharged polar side chain	Serine	Ser/S	5.1	10.4
	Threonine	Thr/T	3.6	0.9
	Asparagine	Asn/N	-	1.1
	Glutamine	Gln/Q	-	-
	Tyrosine	Tyr/Y	3.7	5.6
	Cysteine	Cys/C	1.3	0.1
Total uncharged polar			13.7	18.1
Charge polar side chain	Lysine	Lys/K	6.2	0.2
	Arginine	Arg/R	7.7	1.1
	Histidine	His/H	2.5	0.1
	Aspartic acid	Asp/D	11.6	1.6
	Glutamic acid	Glu/E	19.2	1.8
Total Charged Polar Side Chain			47.2	4.8
Total			100.0	100.0

*Supplied by ADM Australia.

Estimation of SPI dispersion flow parameters:

The flow parameters of SPI dispersions were obtained by fitting power law function to apparent viscosity vs shear rate data using the curve expert software,² as shown in Figure S1.

Figure S1. Experimental and power law fit of apparent viscosity vs shear rate data of SPI dispersion at (A) 15 wt%, (B) 12.5 wt%, (C) 10 wt%, (D) 8 wt%, (E) 6 wt%, and (F) 4 wt% concentration.

Table S2: Estimated SPI dispersion flow parameters.

SPI conc. (wt%)	K	n	R ²
4	0.04 ± 0.001	0.63 ± 0.016	0.9689
6	0.70 ± 0.004	0.19 ± 0.008	0.9985
8	2.91 ± 0.025	0.20 ± 0.009	0.9985
10	12.36 ± 0.146	0.13 ± 0.016	0.9961
12.5	29.81 ± 0.55	0.056 ± 0.023	0.9938
15	896.50 ± 11.70	0.0262 ± 0.02	0.9952

Thermal analysis of SPI powder:

Figure S2. (A) Thermogravimetric analysis (TGA), and (B) Differential scanning calorimetry (DSC) thermograms of SPI powder.

Mechanism of photocrosslinking:

Figure S3. Photocrosslinking reaction schematic.

Estimation of fabricated hydrogel secondary structure:

The secondary structure of fabricated hydrogels were obtained by deconvoluting X-ray diffractogram, Raman and Fourier transform infrared (FTIR) spectra using MagicPlot software (<https://magicplot.com/>) with Gaussian curve fitting,³⁻⁴ as shown in Figure S4-S7.

Figure S4. Deconvoluted X-ray diffractogram of (A) SPI, (B) SPI gel pH7, and (C) SPI gel pH12.

Figure S5. Deconvoluted Raman spectrum of (A) SPI, (B) SPI gel pH7, and (C) SPI gel pH12.

Figure S6. Deconvoluted X-ray diffractogram of (A) SPI gel pH7, (B) SPI/SF (3:1) gel, (C) SPI/SF (1:1) gel, (D) SPI/SF (1: 3) gel and (E) SF gel.

Figure S7. Deconvoluted FTIR spectrum of (A) SPI gel pH7, (B) SPI/SF (3:1) gel, (C) SPI/SF (1:1) gel, (D) SPI/SF (1: 3) gel and (E) SF gel.

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