



Full Length Article

Photocurable silk fibroin- polyvinylpyrrolidone hydrogel

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ABSTRACT

Photocurable hydrogels enable simple and fast sol-gel transformation and have broad application prospects in biomedical research. In this paper, we report the development of photoinitiated polymerization of N-Vinyl-2-pyrrolidone (NVP) monomer to Polyvinylpyrrolidone (PVP) and interpenetrating network with silk fibroin macromolecular hydrogel. This novel hydrogel material has nearly 100% light transmission and excellent mechanical properties, which opens new possibilities in targeted drug delivery, tissue regeneration, cell encapsulation, bioprinting.

1. Introduction

Hydrogels are extensively used in various biomedical application, where the chemical, physical and biological properties are modulated by a combination of various materials, crosslinking methods, degree, and type of cross-linkers, to meet specific requirements. Traditional methods to induce the sol-to-gel transition of silk is done by controlling the pH, ions, stress, but often those protocols result in gels having inadequate mechanical properties [1]. In order to develop tunable silk hydrogel, a semi-interpenetrating network of fibroin and gelatin hydrogel was developed by blending two components [2,3], which were used for 3D bioprinting [4,5] or organoid culture [6]. Daqi Chen et al. prepared a high strength hydrogel by using shear force to induce orientation of polymer chains and β -sheet crystals of silk fibroin protein [7]. But slow gelation rate is a major limitation for ionically induced self-assembly or enzymatically crosslinking strategy for fibroin protein [8]. Photoinitiated polymerization and light-curing strategy provide a fast and controlled method of in situ formation of a hydrogel. It refers to the chain polymerization of molecules initiated by light (or with a photosensitizer) to activate free radicals, allowing a variety of monomers to polymerize rapidly under ultraviolet light [9–11]. Photocrosslinkable interpenetrating network hydrogel was developed by combining gelatin methacrylate (gelMA) and fibroin, in which crystallization of fibroin was not affected by the presence of gelMA, but the proliferation rate of encapsulated cells decreased with increasing fibroin content [12]. Applegate et al. introduced covalent bonds between tyrosine groups of fibroin, in the presence of riboflavin, under visible light [13]. Often such crosslinking strategies cause precipitation during the treatment, so it may not be suitable for sensitive biomedical application such as bioprinting, or cell delivery via injectable hydrogel. To address the limitation, Kim et al.

first hydrolyzed fibroin protein to methacrylate fibroin, followed by addition of photoinitiator Lithium phenyl-2,4,6-trimethyl benzoylphosphinate and UV light irradiation [14]. Polyvinylpyrrolidone (PVP) is often used as a binder in the pharmaceutical industry, or as a lubricant in ophthalmology applications. In this paper, SF/PVP hydrogel was prepared by UV-curing method using regenerated silk fibroin as raw material and NVP as a monomer with a continuous supply of oxygen gas.

In the present study our strategy focused on the mechanism that, during photolysis disruption of oxygen atoms/ radicals (O) are created, which are highly unstable and attach with any individual oxygen gas molecules (O₂) and create ozone (O₃), which is likely to decompose to O₂ and O [15]. Under UV light, atomic trapping of hydrogen ion (H⁺) by O₂ leads to the formation of hydroperoxyl radicals (HO₂^{*}). This radical further reacts with another free hydroperoxyl radicals that lead to the formation of hydrogen peroxide [16]. Hydrogen peroxide produced in this step reacts with horseradish peroxidase and forms the reactive enzyme intermediate and hydroxyl radicals under UV light which catalyzes the polymerization of N-vinylpyrrolidone, and the resulting polymer can then be entangled with silk fibroin macromolecules to obtain the interpenetrating network hydrogels. This novel highly tunable, near-transparent hydrogels open up new possibilities in the field of silk-based biomaterial, bioprinting, and regenerative medicine applications.

2. Materials and methods

2.1. Materials

Fresh silk cocoons from domestic mulberry silkworm *Bombyx mori* were purchased from Suzhou Seruibar Biological Technology Co., Ltd. NVP (1-vinyl-2-pyrrolidone, C₆H₉NO) (Zhongguo Group Chemical

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Reagent Co., Ltd.), Peroxidase from horseradish (HRP) (Aladdin Industrial Corporation, Shanghai, China), anhydrous sodium carbonate, sodium bicarbonate, sodium chloride and all other chemicals (Sigma Chemicals) used in this experiment were purchased.

2.2. Preparation of silk fibroin solution

Silkworm cocoons are boiled in 0.1 M buffer (pH=9.5) mixed with sodium carbonate and sodium bicarbonate for 30 min, repeat three times to remove the sericin. The degummed silk was dried in an oven at 60 °C to obtain pure silk fibroin fibers. The silk fibroin fiber was dissolved in a 9.3 mol/L lithium bromide solution at a bath ratio of 15:100 at 65 °C, dialyzed for 3 days and filtered to obtain a silk fibroin solution [17–19].

2.3. Preparation of other solutions

NVP was formulated into an aqueous solution of 50 mg/mL. HRP aqueous solution was made of 300 U/mL concentration.

2.4. Preparation of photocured SF/PVP hydrogel

Two groups of hydrogels were prepared to investigate the effect of solid ratio and SF/NVP mass ratio on the structure and properties of the hydrogel. In one group, the fixed SF/NVP mass ratio was 8:2, and the HRP concentration was 10 U/mL, and the adjusted solid content was 10 mg/mL, 20 mg/mL, 30 mg/mL, 40 mg/mL and 50 mg/mL respectively. In the other group, the fixed solid content was 30 mg/mL, and the HRP concentration was 10 U/mL, and the SF/NVP mass ratio was changed, which were 9:1, 8:2, 7:3, 6:4, 5:5.

In order to prepare photocured interpenetrating network hydrogel, oxygen gas was continuously supplied to the prepared mixed solution by an oxygen generator, and the light was irradiated for 30 min under a UV surface irradiation apparatus. As a control group, the silk fibroin solution was placed in a 37 °C incubator for 3 weeks to form pure silk hydrogel. The irradiation distance was 15 mm, solution thickness was 2 mm, UV wavelength was 550 nm.

2.5. Cross-sectional morphology of the hydrogels

The lyophilized gel was prepared according to the above-mentioned method having a solid content of 30 mg/mL and an SF/NVP mass ratio of 8:2. The pure silk fibroin hydrogel was cut into small pieces and rapidly frozen in liquid nitrogen. After vacuum drying, the cross-section of the gel was cut with a slicer, and the surface was sprayed with gold for 90 s, and then taken out, and the morphology was observed by a Hitachi S-4800 scanning electron microscope (SEM) [20–22].

2.6. Structure of SF/NVP hydrogel

2.6.1. Fourier infrared absorption spectroscopy (FTIR)

For samples preparation, the lyophilized hydrogels were grinded as powders. The average diameter of the powders was less than 80 µm (screened with an 80-mesh sieve). Pure silk fibroin was used as a control group. FTIR spectrum of prepared samples was collected using Nicolet 5700 smart type in the range 400–4000 cm⁻¹ by KBr pellet technique at room temperature. Pellets of prepared powdered samples were prepared with KBr in the ratio (1:100) on a hydraulic press.

2.6.2. Wide-angle X-ray diffraction (XRD)

The lyophilized samples were grinded as powders and then pressed into a sample holder. Wide-angle X-ray diffraction (XRD) pattern of the samples was analyzed by a fully automatic X'PERT PRO MPD X-ray diffractometer (Panaco, The Netherlands) equipped with Cu K α (λ =1.54 nm). The target was set to tube current of 35 mA, tube voltage 40 kV, scanning speed 8°/min, in the angle range between 5° and 45°

2.7. Light transmission performance

100 µL prepared SF/NVP mixed solution and the pure silk fibroin solution (control group) were placed in a 96-well plate and irradiated with ultraviolet light for 30 min. The absorbance value in the 550 nm light wave range was measured by a multi-function microplate reader, and the temperature was maintained at 37 °C [23].

2.8. Mechanical properties of the hydrogel

2.8.1. Compressive mechanical properties of photocurable SF/PVP gels

In order to investigate the effects of solid content and SF/NVP mass ratio on the mechanical properties of photocured hydrogels, hydrogels with different solid ratios and different mass ratios were prepared. The control group was pure silk fibroin. Twelve parallel samples for each group were taken. The two sets of hydrogels were made into cylindrical gel samples of size 10 mm (diameter) $\times$ 8 mm (height) using a punching die and compression mechanical tests were performed on a TMS-PRO type texture analyzer. Test conditions: initiation force was 0.02 N, compression speed was 10 mm/min, and compression deformation was 90%.

2.8.2. Resilience test of photocurable SF/PVP gels

A photocurable hydrogel of different solid content and SF/NVP mass ratio were made into a cylindrical gel sample having a size of 10 mm (diameter) $\times$ 8 mm (height) by a punching die, and two samples were subjected to the sample. The secondary compression, the two compression displacements were all 80% of the original height of the sample, and the variation curve was analyzed in the system software to obtain the values of the parameters, and the variation law was analyzed [24]. Test conditions: Initiating force was 0.02 N, compression speed was 50 mm/min, and compression deformation was 80%.

2.9. Degradation performance

The hydrogel sample after equilibrating for 24 h in a constant temperature incubator at 37 °C, was prepared into a gel block having a size of 5 $\pm$ 2 mm (diameter) $\times$ 5 mm (height) as an *in vitro* simulated degradation test sample. All samples were weighed W_1 as the original wet weight. Then, the prepared gels were placed in a 24-well plate, and 10 mL of PBS solution (pH=7.4) and 2 U/mL of type I collagenase solution was added in a bath ratio of 1:10 and placed in a constant temperature incubator at 37 °C to start degradation. At specific time points 2 h, 4 h, 6 h, 24 h, the corresponding sample was taken out, and the weight was recorded as W_2 after being dried. Another set of pure silk fibroin was used as a control group. Three parallel samples were taken for each sample and the average value was taken.

3. Results

3.1. Cross-sectional morphology of hydrogel

Fig. 1 shows the cross-sectional morphology of pure silk fibroin (SF) hydrogel and photocured SF/PVP hydrogel. The pure silk fibroin has a large number of lamellar structures inside, with a small dimension of irregular pores (Fig. 1F). In contrast, the photocured hydrogel exhibits a three-dimensional vein structure inside, and a large number of random pores, which are intertwined and closely connected. With the increase of solid content, the pore diameter of hydrogel decreases gradually. The hydrogel having a solid content of 40 mg/mL has the most uniform pore diameter (Fig. 1D).

3.2. Structure of SF/PVP hydrogel

In order to study the microstructure changes of the SF/PVP hydrogel, Fig. 2 shows the infrared absorption spectrum and the X-ray diffraction patterns of the prepared samples. It can be seen from Fig. 2 (A and

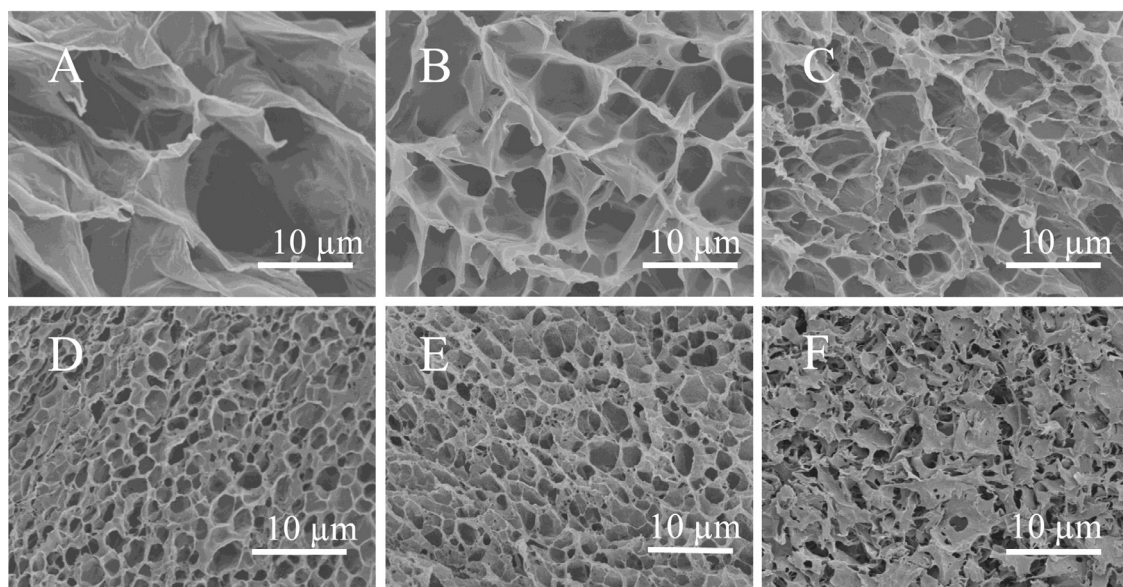


Fig. 1. SEM images of SF/PVP hydrogels samples with different solid content (SF: NVP=8:2). Solid content of (A) 10 mg/mL, (B) 20 mg/mL, (C) 30 mg/mL, (D) 40 mg/mL, (E) 50 mg/mL, and (F) Pure SF hydrogel 30 mg/mL.

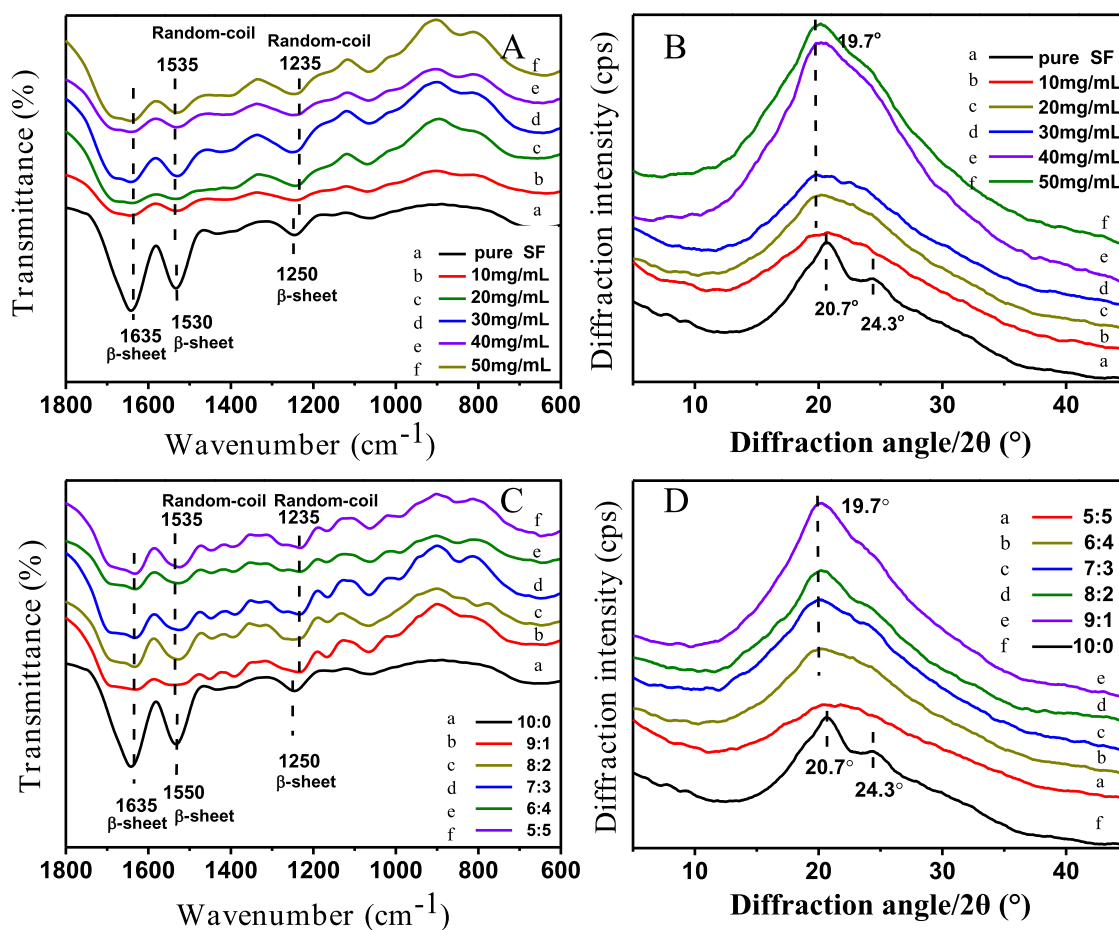


Fig. 2. Structure test of SF/PVP hydrogel. (A, B, SF: NVP=8: 2, C, D, solid content: 30 mg/mL).

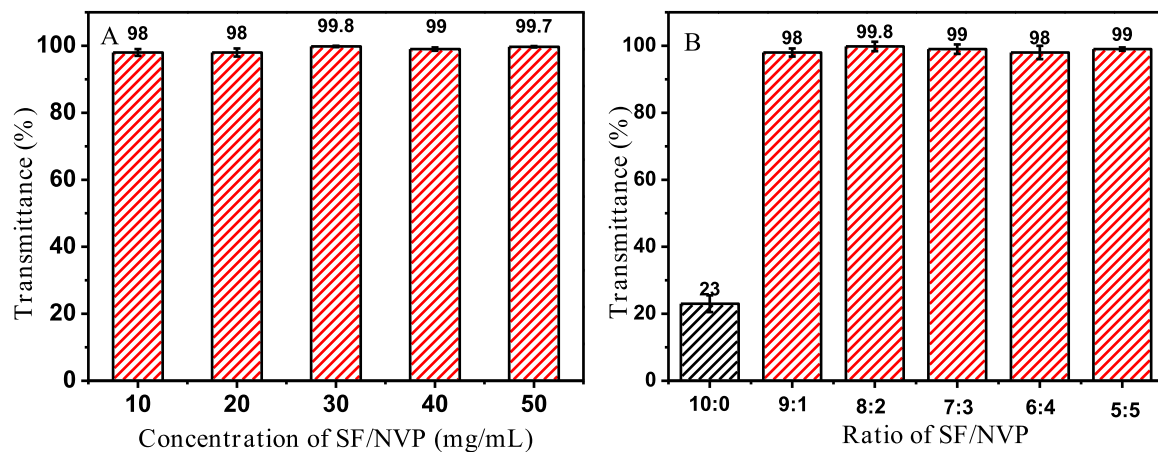


Fig. 3. The light transmittance of the hydrogel at 550 nm visible light. (A) Different solid content: SF: NVP=8:2. (B) Different mass ratio: solid content: 30 mg/mL.

C) that the pure SF gel has obvious absorption peaks at 1635 cm^{-1} , 1530 cm^{-1} , 1250 cm^{-1} , corresponding to the characteristic peaks of the β -sheet structure of amide I, amide II and amide III. The absorption peaks of SF/PVP hydrogels at 1635 cm^{-1} , 1535 cm^{-1} , and 1235 cm^{-1} are characteristic peaks of predominantly β -sheet and random coils [25,26]. XRD is an effective tool for identifying the crystalline and amorphous nature of materials [27,28]. The X-ray diffraction curve of the photocurable hydrogel at a solid content of less than 30 mg/mL is a large taro peak (Fig. 2B), exhibiting a typical amorphous structure. When the solid content reached 40 mg/mL and 50 mg/mL, the absorption peak at 19.7° gradually became sharper. A similar trend occurred when the silk fibroin ratio was increased (Fig. 2D), indicating that the arrangement of the silk fibroin molecular chains in the hydrogel was gradually regularized. While the pure silk fibroin gel is sharp at 20.7° , and a similar absorption peak appears near 24.3° , which indicates a silk II crystal structure.

3.3. The transparency of hydrogel

The absorption of light in matter follows the Lambert-Beer law:

$$T = \frac{I}{I_0} = 10^{-ab} \quad (1)$$

Where T is light transmittance. I and I_0 have transmitted light and incident light intensity. 'a' is absorption coefficient, related to the nature of the material and the wavelength of light. 'b' is the incident optical path.

Since the thickness of the artificial cornea and contact lens is generally about 0.5 mm, the hydrogel transmittance T of 0.5 mm thick is corrected according to the actual thickness of the hydrogel (4.4 mm), so:

$$T = 100 - \frac{0.5(100 - T_0)}{d} \quad (2)$$

Where T is corrected gel transmittance, T_0 is tested gel transmittance value.

Fig. 3 shows the results of light transmission test at 550 nm after equilibration of photocured SF/PVP hydrogel with pure silk fibroin for 24 h. It can be seen from Fig. that the light-curing hydrogel is more transparent. The transmittance is over 98%, which is much higher than the light transmittance of pure SF hydrogel ($23 \pm 1.9\%$). This is because photocuring SF/PVP hydrogels contain less β -sheet crystals than pure silk gel. Secondly, these hydrogels are formed by the formation of covalent bonds, and the use of ultraviolet to generate free radicals induces the polymerization of monomers to obtain a hydrogel with predominantly random coil structure. When the mass ratio is 8:2, the reaction in the whole system is relatively complete, the permeability is close to 100%. It might be used as an ideal contact lens material or can be used for easy imaging of morphological changes of encapsulated cells in the hydrogel.

3.5. Mechanical properties of the hydrogel

3.5.1. Compressive strength of the hydrogel

Compressive strength of pure SF hydrogel increases with increasing strain rates. Then pure SF hydrogel breaks down at 50% strain, indicating its fragile nature, while the photocured SF/PVP hydrogels could withstand strains up to 80% (Fig. 4A). Cracking occurred at about 90%, indicating that the excellent resilience of SF/PVP hydrogel. Fig. 4B shows that the same solid content SF/PVP hydrogel and pure SF hydrogel at room temperature. In terms of compressive strength, the strength of SF/PVP hydrogel material can reach 60.9 kPa, which is much higher than 9.94 kPa of pure silk fibroin hydrogel. This is due to the fact that the silk fibroin macromolecules entangled with the PVP chain producing a dense interpenetrating network structure that gives the hydrogel a higher compressive strength.

As the solid content increases, the compressive strength of the photocured SF/PVP hydrogel material also increases (Fig. 4C). This is because the increase in solid content leads to an increase in the number of crosslinking points in the entire photocuring system, thereby increasing the degree of cross-linking of the hydrogel so that the compressive strength is further improved. Fig. B shows that at the mass ratio of 8:2, the hydrogel material has the greatest compressive strength.

3.5.2. Resilience of hydrogel

All the photocured hydrogels have higher rebound rates than the rebound rate ($13.9 \pm 0.52\%$) of pure silk fibroin hydrogel (Fig. 5). Due to the interpenetrating structure of the polymer network inside the material, the physical crosslink density of the molecular chain is greatly improved, so that the SF/PVP hydrogel material undergoes elastic deformation without being damaged when subjected to compression.

As shown in Fig. 5, in terms of resilience, the hydrogel material with a ratio of (SF: NVP) of 8:2 has the highest resilience ($93.9 \pm 2\%$), which is much larger than that of pure SF hydrogel. As the content of NVP monomer increases, the resilience of the hydrogel material gradually becomes smaller, and the hydrogel resilience at a ratio of (SF: NVP) of 5:5 is $79 \pm 3\%$. This indicates that as the NVP content increases, the interpenetrating network structure entangled by the radical polymerization becomes less, the physical crosslink density decreases, and the resilience also decreases.

3.6. Degradation performance of hydrogel

In the PBS solution without collagenase, the wet weight of the SF/PVP gel and the pure SF gel sample decreased slightly (Fig. 6). However, in collagenase solution, the SF/PVP hydrogel degraded $76 \pm 1.3\%$ on the first day, and the degradation rate was significantly faster than

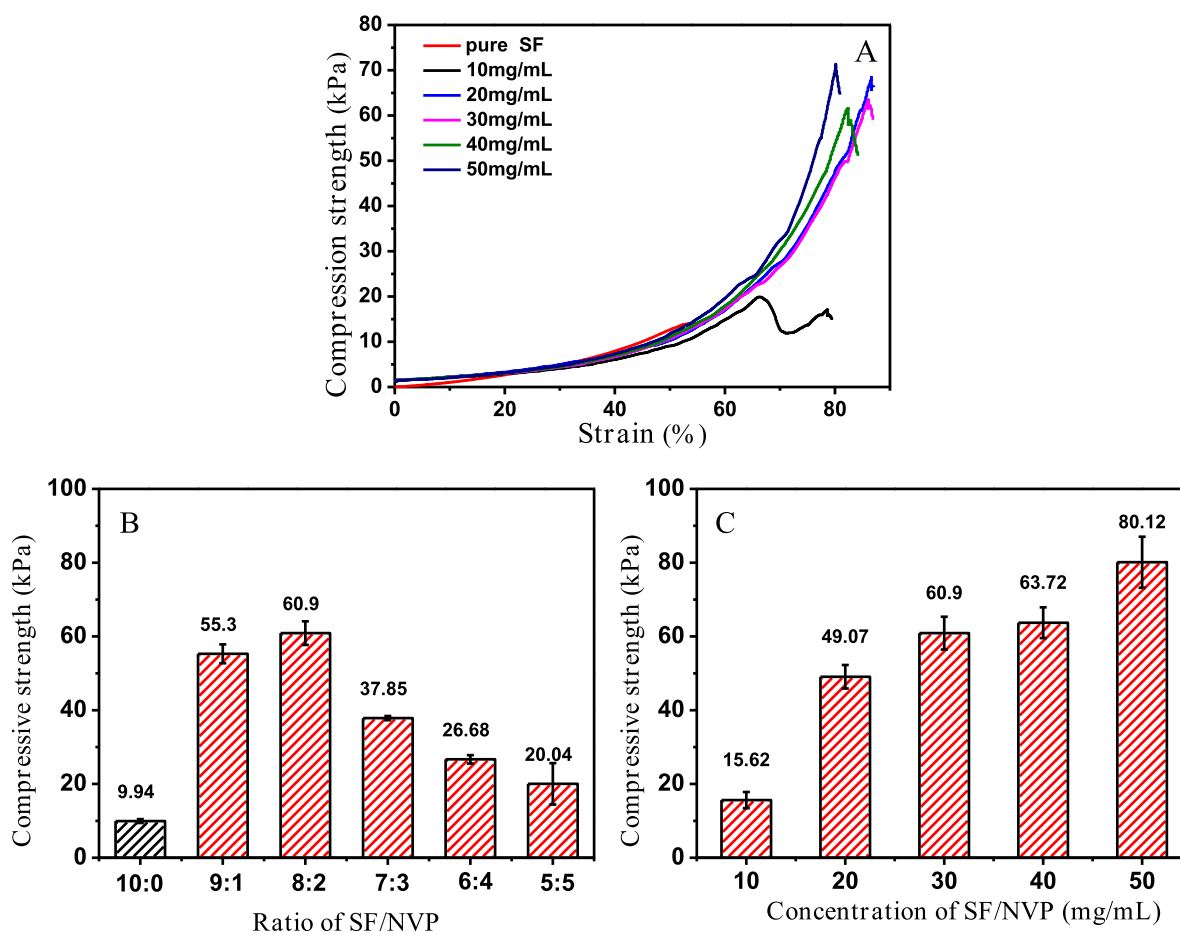


Fig. 4. Compressive strength of SF/PVP hydrogel. (Initiation force: 0.02 N, compression speed: 10 mm/min, compression deformation: 90%). (A) Stress-strain curve: SF: NVP=8:2; solid content: 30 mg/mL. (B) Different mass ratio: solid content: 30 mg/mL. (C) Different solid content: SF: NVP=8:2.

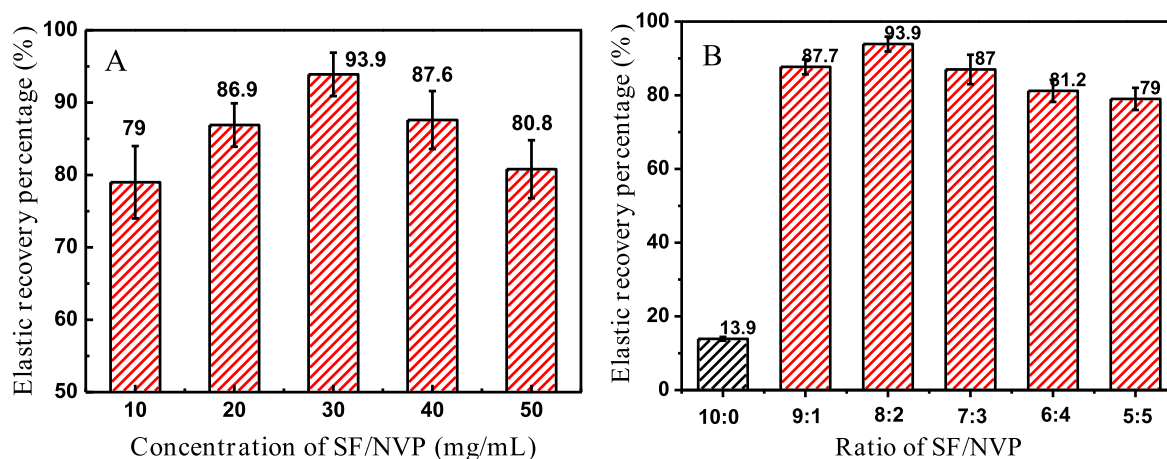


Fig. 5. The resilience of hydrogel material. (Initiating force: 0.02 N, compression speed: 50 mm/min, compression deformation: 80%). (A) Different mass ratio: solid content: 30 mg/mL. (B) Different solid content: SF: NVP= 8:2.

that of the pure SF hydrogel ($39.3 \pm 1.1\%$). This is because after collagenase degrades SF, the remaining PVP is dissolved in water due to the lack of cross-linking points of the interpenetrating network. This causes disintegration of the hydrogel.

4. Discussion

The interpenetrating polymer network (IPN) structure allows the development of unique hydrogel formed by two or more polymers inter-

twined by molecular chains [29]. The most important feature is the ability to mix thermodynamically incompatible polymers in order to have kinetically stable alloy properties, thereby achieving performance complementation between components [30]. At the same time, special structural morphological features such as interfacial structure, interpenetrating and two-phase continuous shape make them have special synergistic effects on unique performance or function [31]. In this work, we envisage that the silk fibroin molecular chain would provide excellent mechanical properties as a skeleton chain, and the PVP molecular chain

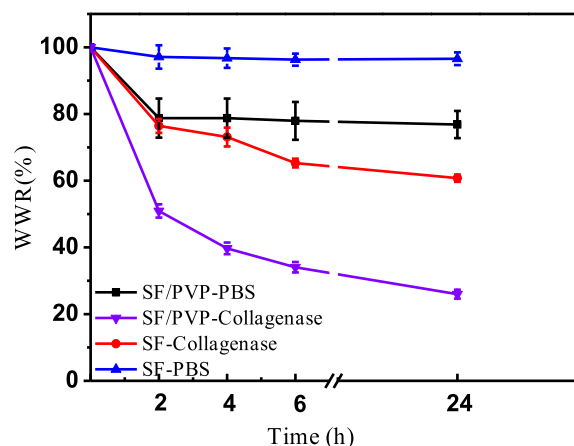


Fig. 6. Degradation of SF/PVP hydrogel materials. solid content: 30 mg/mL, SF: NVP= 8:2, collagenase concentration: 2 U/mL, PBS solution: pH=7.4.

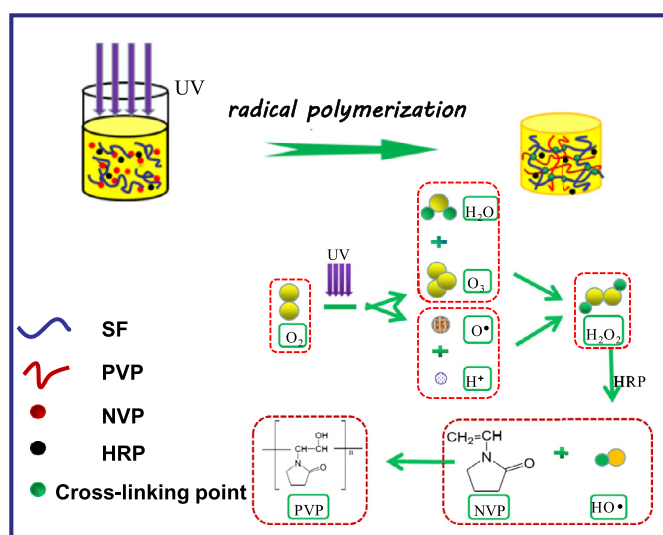


Fig. 7. Scheme of photocuring SF/PVP hydrogel.

would be physically entangled to form an IPN. Thereby this strategy might enable us to effectively improve the elasticity and transparency of the gel.

Poly(N-vinyl pyrrolidone) (PVP) is a synthetic polymer with good biocompatibility and can be used as main component of temporary skin covers and wound dressings [32]. Depending on the method of polymerization, PVP preparation methods may include radiation polymerization, photopolymerization, and chemical polymerization [33]. In this paper, in order to develop tunable hydrogels we combined photopolymerization and chemical cross-linking, that offers a simple and quick sol-to-gel transition. Advancements in tissue engineering are dependent on the development of inexpensive, easily prepared, biodegradable and cytocompatible materials that can match the durability, resilience and mechanical properties of native tissues. The UV irradiated hydrogels presented here results in the formation of robust hydrogel network to achieve high elasticity, degradability and excellent resilience with polymerization of NVP under UV and supply of oxygen. Moreover, UV irradiation was found to be highly efficient for the decomposition of the molecular oxygen for the production of hydrogen peroxide (Fig. 7).

The PVP was generated by polymerization of N-vinylpyrrolidone, which requires very little free radicals to induced polymerization. Since the ultraviolet rays can satisfy the radicals required for the entire reaction, there is no need to add a photosensitizer. The HRP is added to cat-

alyze some O_3 formed by UV changed into free radical. HRP that acts as an important heme-containing enzyme have two metal centers one iron heme group and two calcium atoms [11]. H_2O_2 bonds to the vacant octahedral positions on the iron atom to imitate the reaction for the production of hydroxyl radicals [12]. N-vinylpyrrolidone (NVP) contains an unsaturated functional group $-C=C-$ and the initiator H_2O_2 generates free hydroxyl radicals under the catalysis of horseradish peroxidase (HRP). Further, polymerization of the monomer is initiated, and the resulting polyvinylpyrrolidone is entangled with the dityrosine bridges of silk fibroin macromolecule to obtain an SF/PVP hydrogels. As reported by Partlow et al., the presence of H_2O_2 and HRP leads to the formation of dityrosine cross-linked bridged networks in fibroin [34]. So, polymerized NVP entangles with these to the formation of optically clear, highly elastic protein hydrogels.

The secondary structure of SF in the SF/PVP hydrogel was dominated by random coils, and the pure silk fibroin was a β -sheet structure. Of course, the photocurable hydrogel still has a β -sheet absorption peak at 1635 cm^{-1} , but no corresponding crystallization peak appears on the XRD curve. This is because a small amount of β -sheet structure does exist in the photocurable hydrogel, but a long-range ordered crystal structure cannot be formed due to presence of PVP chains. This can be attributed due to the formation of IPN due to the entanglements that takes place physically between the fragments of PVP and that of SF macromolecules. The gel crosslinking point is interpenetrating network structure between SF and PVP. This crosslinking limits the self-assembly of SF chains, as a result, it becomes arduous for the SF chains to rotate freely to achieve a stable β -sheet structure (Fig. 8). Consequently, the SF hydrogels are mainly composed of predominantly β -sheet while the SF/PVP IPN consists of predominantly random coil aggregate structure [36]. It is also a reasonable explanation for the highly elastomeric and highly transmittance of photocurable hydrogels. The randomly coiled silk fibroin molecular chain has a good energy storage capacity. When subjected to pressure, the energy can be stored and released when the external force is removed to restore deformation. However, the aggregate structure of pure silk fibroin is a rigid silk II crystal structure, which is easily broken when compressed by external force. The compressive strength of the SF increased when photocured with PVP compared to the native SF only. This is in accordance with the findings by Yoo et al. where they prepared a semi-interpenetrating network hydrogel by combining SF with poloxamer 407 macromer [35]. The crystalline SF hydrogel consists of a number of long-range ordered grains that are up to several tens of microns in size. The size of these grains can be as much as several tens of microns far greater than the wavelength of visible light, so the milky white silk fibroin will reflect visible light. Since the β -sheet structure of the photocured hydrogel observed in the FTIR is nanoscale, diffraction occurs when light passes through these β -sheet structures.

In our earlier study [36] we used protease XIV. where only SF gel showed faster degradation, compared to the IPN gel. Upon immersion of the SF/PVP hydrogels in collagenase, the enzyme would hydrolyze the X-Gly covalent bond of the X-Gly-Pro regions of the SF chain, while keeping the PVP chain unaffected. In the heavy chain of SF, Gly content is 46%, and X-Gly-Pro appears 12 times. In the light chain, X-Gly-Pro appears 2 times. However, the disintegration of SF molecule from longer chain to short fragments would damage the physical entanglement between the SF and PVP at the crosslinking point resulting in breaking up of the IPN structure. The rate of degradation of SF/PVP IPN is faster in comparison to the native SF hydrogels due to the higher availability of random coils instead of β -sheet, resulting in higher amorphous content.

Taken together our developed photocured SF/PVP IPN hydrogels have superior characteristics in comparison to the already available pure SF hydrogels with tunable features optimum for biomedical applications. This fabricated photocurable SF/PVP hydrogel has the best light transmission properties and resilience when the mass concentration is 30 mg/mL and the feed ratio is 8:2, which might be highly promising for ophthalmology related applications, as well as regenerative medicine needs.

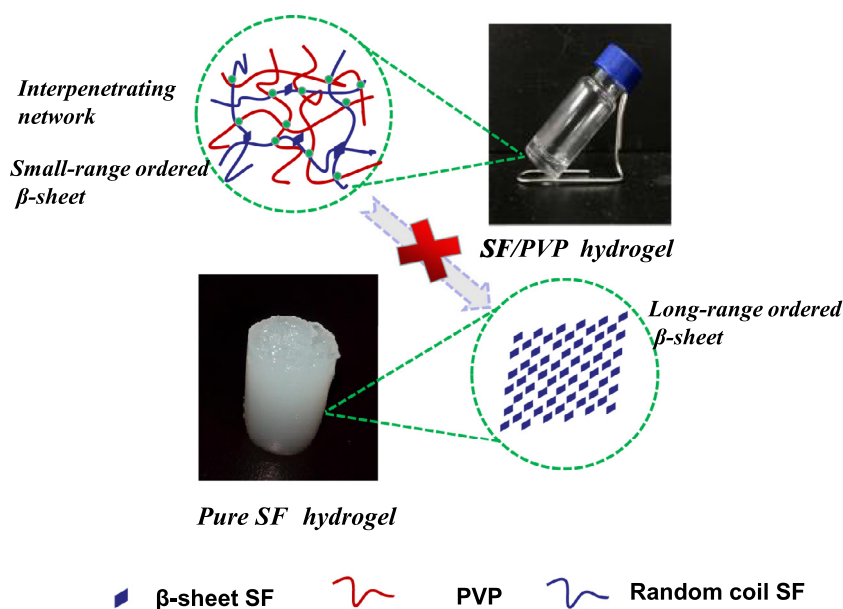


Fig. 8. The internal structure of different hydrogels.

5. Conclusion

By studying morphology, structure, optical properties and mechanical properties of hydrogels at different solid rates and different ratios, we found that photocurable SF/PVP hydrogels have a regular three-dimensional porous structure. Light-cured SF/PVP hydrogels have high light transmission and high resilience due to the presence of a large number of random coil structures. When the solid content is 30 mg/mL and the mass ratio is 8:2, the light transmittance is nearly 100%, which provides these photocurable crosslinked silk fibroin hydrogels for many biomedical applications.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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