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Research Article

Preparation and Characterization of a Novel Hybrid Hydrogel Composed of *Bombyx mori* Fibroin and Poly(*N*-isopropylacrylamide)

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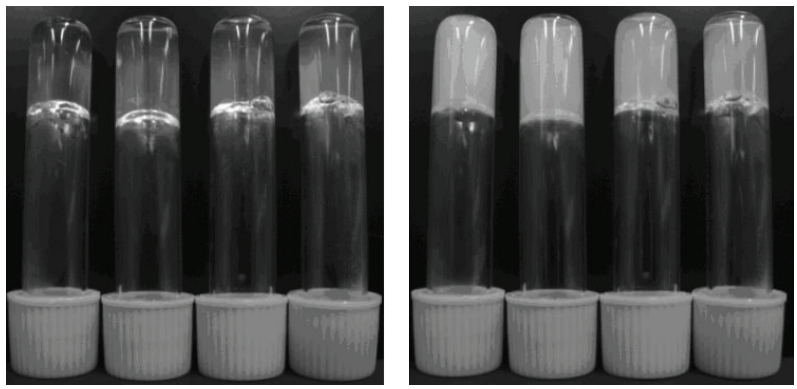
A novel hybrid hydrogel was prepared and investigated based on silkworm silk fibroin and poly(*N*-isopropylacrylamide) (PNIPAAm). PNIPAAm was introduced to silk fibroin, the resultant composite hydrogel was examined, and freeze-dried SF/PNIPAAm scaffold was analyzed using LB-550 dynamic light scattering particle-size analyzer, circular dichroism (CD), and scanning electron microscopy (SEM). Our results suggested that the hybrid hydrogels owned the porous sponge-like structures, and the gelation time of SF/PNIPAAm hybrids decreased with an increase in temperature and concentration of each polymer. Results of rheological analysis suggested that the rheological property of resultant SF/PNIPAAm gel depended on the concentration combinations as well as the aging time, which elapsed after mixing the two polymers. Results of CD spectra demonstrated that pH showed little influence on the secondary structure of silk fibroin, and significant changes of G', G'', and G^* as surrounding increase temperature above the lower critical solution temperature (LCST).

1. Introduction

Silks can be processed into many forms that suitable for a variety of biomedical and tissue engineering applications. They can be modified by chemical treatment or used in combination with other materials in order to vary the mechanical and surface chemistry properties. By these means, biomaterials with specific applications can be produced [1]. Silkworm (*Bombyx mori*) silk has been used to produce fabric by men for thousands of years and silk suture has also been utilized clinically for centuries. Natural silk consists primarily of two kinds of proteins: silk fibroin (SF) and sericin (gum), which form two cores and protective outlayer of silk fiber. Silk fibroin contains a heavy (390 kD) and a light (26 kD) chain polypeptides bridged by a disulfide bond and is dominated by glycine (G, 43%), alanine (A, 30%), and serine (S, 12%) [2]. The heavy SF chain consists of highly repetitive sequences which allow for tight packing into antiparallel-sheet crystalline domains in spun silks [3]. Degummed cocoon silk may be dissolved using some lithium or calcium salts solution at a high concentration and the regenerated SF has been prepared into 3D scaffolds [4, 5], fibers [6, 7], and hydrogels [8–10] for biomedical applications, due to its impressive biocompatibility and biodegradability.

Hydrogels prepared by proteins, such as collagen and gelatin, or designed peptides have gained particular interest due to their in situ gelation ability as well as their inherent biocompatibility [11, 12]. Tremendous attempts have been made to exploit SF as a gelling agent. Regenerated SF chains aggregate in aqueous solution and undergo random coil to cross-sheet transition which takes weeks to months to happen. Gelation dynamics of SF solution depends mainly on the concentration, temperature, pH, and ionic conditions [8, 10, 13]. The gelation rate can be facilitated by mixing with some super hydrophilic compounds, such as ethanol, PEG, and poloxamer, which would rival for water and decrease the hydration level on fibroin molecule surface [14–16]. Recently, physical stimulus like sonication has also been proven to be capable of inducing SF gelation [17].

Silk fibroin-based semi-interpenetrating polymer network (SIPN) hydrogel has been prepared by mixing SF with crosslinked polymers, such as collagen [18], chitosan



(a) (b)

Figure 1: The effects of temperature on SF/PNIPAAm hydrogels below (a) and above (b) the lower critical solution temperature. From left to right, these vials are F5P3, F5P2, F5P1, and F3P3 composite hydrogels, respectively.

[19], poly(ethylene glycol) diacrylate [20], and poloxamer macromer [21]. Poly(*N*-isopropylacrylamide) (PNIPAAm) was incorporated with SF to introduce SIPN, which displayed increased deswelling kinetics when crystalization of fibroin was postinduced using methanol. The fibroin molecules tend to form cross-beta-sheet structures spontaneous within these SIPNs and this process may be considered as some kind of "polymerization." Though crystallined SF would stabilize the SIPN networks in some degree, SF chains usually only contribute amorphous structure within SIPNs whose properties were largely determined by the cross-linked polymer, especially the physical one. On the other hand, such chemical cross-linking would significantly enhance the mechanical and functional properties of silk fibroin [22].

Besides SIPN, simply mixing of SF with one or more polymers is also considered as an effective mean that was sought for versatile SF-based gels. Collagen and poly(vinyl alcohol) have been introduced to SF to form composite hydrogel [23, 24]. Hybrid gel composed of SF and gelatin was prepared simply through blending two polymers and the resultant hydrogel exhibited response to temperature due to the thermo-responsibility of gelatin [25]. These stimulate the exploration of new mechanisms, such as "physical cross-linking," which underlie gelation of SF mixtures and alternatives which can gel with SF, especially stimuli-responsive "smart" materials.

Thermoresponsive materials have earned considerable interest due to their value for both scientific researches and practical pharmaceutical applications [26–29]. Stimuli-sensitive hydrogels represent another advanced hydrogel system that, under intelligent design, can sense changes in complex *in vivo* environments and utilize these triggers to modify drug release rates [30]. Poly(*N*-isopropylacrylamide), with a lower critical solution temperature (LCST) around 32°C, is a typically thermoresponsive polymer which is hydrated below the LCST and undergo rapid volume decrease because of the hydrophilicity to hydrophobicity alteration. It undergoes a transition from hydrophilic to hydrophobic state in aqueous solution when the temperature is increased from below to above its LCST [31, 32]. Preparation of PNIPAAm homopolymer and copolymers, such as hydrogels and micelles, have been utilized in tissue engineering [33] and drug delivery systems [34–36]. Previously, Gil has investigated the effects of SF chain on the swelling/deswelling kinetics of PNIPAAm hydrogel [22]. However, the association between SF with different conformation and PNIPAAm was poorly understood.

In the present study, with an aim of screening new companions for SF-based blend gel and improving silk fibroin gelation, PNIPAAm was introduced to silk fibroin and resultant composite hydrogel was examined. Freeze-dried SF/PNIPAAm scaffolds were visualized using scanning electron microscopy (SEM). The effects of concentration, temperature, and pH on the gelation of SF/PNIPAAm solutions were rheologically investigated. The possible association between SF and PNIPAAm chains was also elucidated by comparing the change in CD spectra.

2. Experimental

Raw cocoon of *Bombyx mori* was used for preparation of silk fibroin (SF) solution. *N*-isopropylacrylamide (NIPAAm), calcium chloride, and ammonium persulfate (APS) were purchased from Sigma-Aldrich. N, N, N', N'-tetramethylethylenediamine (TEMED) and sodium dodecyl sulfate (SDS) were purchased from Bio-Rad laboratories and used as received. Millipore ultrapure water was used in all experiments.

The PNIPAAm homopolymer was synthesized by free radical polymerization of NIPAAm, modified by the method of Liu et al. [37]. Briefly, 1.2 g NIPAAm was completely dissolved in 20 mL of 1.2 mg/mL SDS aqueous solution, followed by magnetical stirring with N_2 bubbling for at least 20 min to remove residual oxygen. After placed still at room temperature for 30 min, 60 μL of APS (10%, w/v) aqueous solution and 10 μL of TEMED were added in turn while stirring vigorously. The polymerization was allowed at room temperature for 12 h with continuous stirring under nitrogen atmosphere. Subsequently, the polymerization mixture was dialyzed in a cellulous membrane bag (MWCO = 8,000) against deionized water for 3 days with 12 changes of water to remove unpolymerized monomers. The resultant mixture

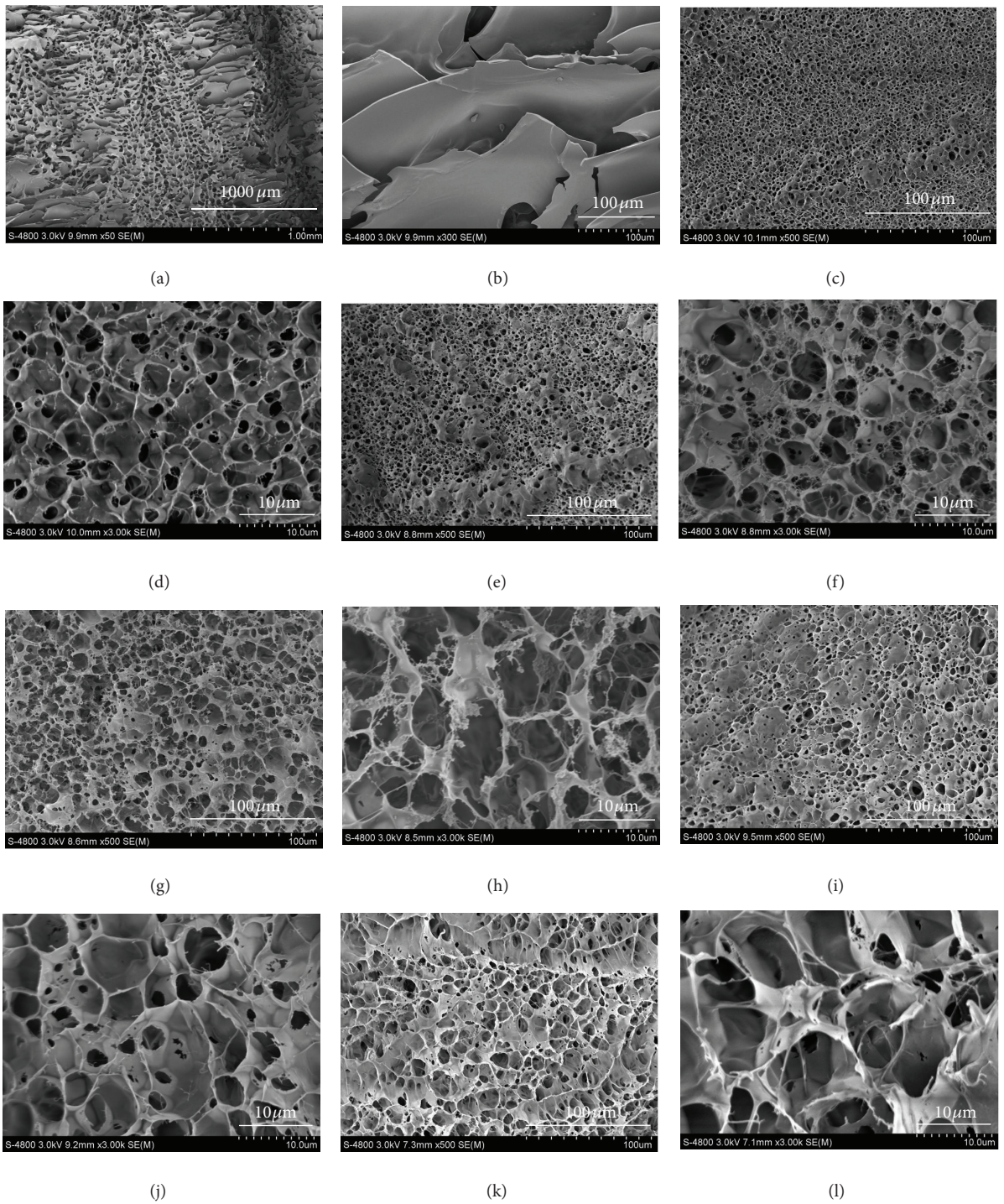


Figure 2: SEM images for the freeze-dried 5.0 wt% silk fibroin solution ((a)-(b)) and SF/PNIPAAm composite gel prepared with various concentration of each polymer. ((c)-(d)) 5.0 wt% SF and 3.0 wt% PNIPAAm; (e)-(f) 5.0 wt% SF and 2.0 wt% PNIPAAm; (g)-(h) 5.0 wt% SF and 1.0 wt% PNIPAAm; (i)-(j) 3.0 wt% SF and 3.0 wt% PNIPAAm; (k)-(l) 1.0 wt% SF and 3.0 wt% PNIPAAm.

was concentrated using PEG (MW = 8,000) powder to get an ultimate concentration of 6.0 wt%.

Raw cocoons of *Bombyx mori* were degummed by boiling for 60 min in 20 mM Na_2CO_3 aqueous solution. The silk fibroin was then extensively washed in distilled water for 5 times to remove other impurities. Blow-dried silk fibroin was completely dissolved in 100 mL $CaCl_2$ aqueous solution at 100°C for 60 min, yielding a 5.0 wt% fibroin solution which was then dialyzed against distilled water using a cellulous membrane bag (MWCO = 3,500) for 3 days. After filtration,

the pure fibroin solution was concentrated to 10.0 wt % by osmotic stress. The resultant fibroin solution was stored at 4°C before use.

For preparing SF/PNIPAAm hybrid hydrogel, silk fibroin (10.0 wt.%) and PNIPAAm (6.0 wt.%) stock solutions were mixed homogenously with varied concentration of each polymer and resultant mixtures were adjusted to demanded pH with 10 mM hydrochloric acid. The composition of the SF/PNIPAAm mixture or gel was expressed as FxPy, where x and y indicated the ultimate concentration of SF and PNIPAAm in the blended system. A total of 1.0 mL of SF/PNIPAAm mixture was pipetted into a flat-bottomed vial with a diameter of 1.0 cm and placed at 25°C or 30°C for gelation. The relative gelation time was determined as the spectrum did not fall from the inverted vial.

The particle-size distribution of F5P3 mixture at different gelation stages was recorded using an LB-550 dynamic light scattering particle-size analyzer (HORIBA, Kyoto, Japan). Gelation temperature was specifically controlled according to an inserted needle temperature sensor.

The CD spectra of extracted silk fibroin with various pH conditions and F5P3 hydrogel were measured on a Model 400 spectrophotometer (Aviv Biomedical, Lakewood, NJ, USA) at room temperature. Samples with appropriate concentrations around 0.1 mg/mL and 2 mm pathlength cells were used in all CD experiments.

SF/PNIPAAm hydrogels were frozen at −80°C and then freeze-dried at −50°C. The sponge-like mats were then visualized using scanning electron microscope (SEM, Hitachi S-4800, Hitachi High-Technologies Co., Tokyo, Japan) at room temperature when they were mounted on a stub and splutter coated using gold. To examine the morphological changes due to the addition of PNIPAAm, pure silk fibroin scaffold was prepared from 5.0 wt% SF aqueous solution by freeze-drying, too.

The gelation dynamics of SF/PNIPAAm mixtures was studied on an AR2000 rheometer (TA Instruments, New Castle, DE, USA) at 30°C in plate-plate geometry with a diameter of 40 mm. A 20 mm diameter, 1° stainless-steel cone with a truncation at 25 μm was used and covered with a split solvent trap cover. The elastic modulus (G) was averaged with a frequency of 1 rad/s and constant strain of 0.5% for 1 min at 25°C.

All the experimental data were expressed as the mean ± SD, and statistical analysis was performed by using SPSS 11.5 at two-tailed Student's t-test.

3. Results and Discussion

Sol-gel transition of pure silk fibroin solution with a concentration up to 10.0 wt% was not observed within 60 days of incubation at 30°C. This might imply that the random coil to cross-sheet transition of SF solubilized using 5.5 M $CaCl_2$ aqueous solution was not as robust as compared with that regenerated using saturated LiBr [8, 10]. Introducing of PNIPAAm polymers significantly facilitated the gelation process of silk fibroin aqueous solution. SF/PNIPAAm composite hydrogel yielded transparence and achromaticity

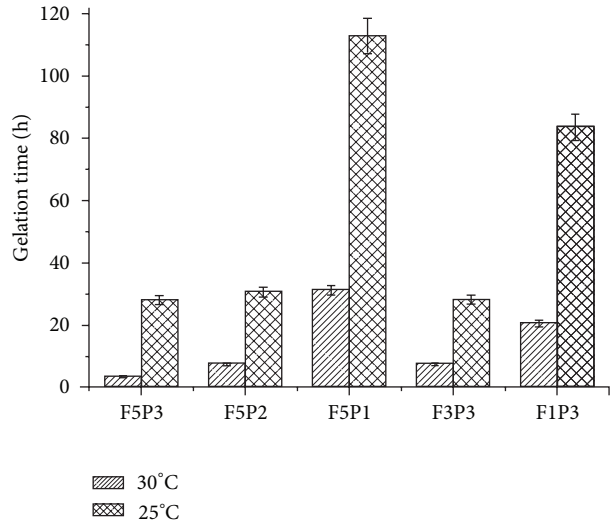


Figure 3: Relative gelation time of SF/PNIPAAm mixtures incubated at 25°C and 30°C.

(Figure 1(a)) below the LCST of PNIPAAm due to the additional PNIPAAm which interacts with SF and restrain SF aggregation and transition into cross-sheet dominated heterogeneous complex. As coil-to-globule transition occurred to PNIPAAm chains above the LCST, the composite hydrogel exhibited an opaque white color (Figure 1(b)), and probably internal changed structure.

For eventual 3D cell culture and *in vivo* tissue repairing, it is important for hydrogel matrices to have microporous structures so that mass transfer through the matrices is efficient [38]. The microstructures of the hydrogels with different concentrations were observed by SEM. As shown in Figure 2, the hydrogels owned the porous sponge-like structures. The formation of this structure may be attributed to the addition of PNIPAAm. The pure SF solution, observed by SEM, showed irregular lamellar structure and also some porous sponge-like structures, but the scales were large (Figures 2(a) and 2(b)). When the two polymers were mixed to form hydrogel, the SEM images showed mesh-like structures with good permeability, which was conducive to cell crawling and nutrient delivery. Better mesh-like structures were present in the case of higher concentrations of SF, and higher concentrations of PNIPAAm made more integrity of the pore wall, that is, more intensity of the material. Both had a high proportion of F5P3, showing the best microscopic appearance (Figures 2(c) and 2(d)). In the *in vivo* condition, cells are located in three-dimensional (3D) microenvironments, where they are complex mixture of pores, ridges, and fibers of ECM at nanometer scales. Such structures act as a scaffold to support cells, allow transport of nutrients and metabolic wastes, and promote tissue development [38]. The present results suggested that the structure of this hydrogel has similar interior porous morphology consisting of honeycomb-like pores. Our findings indicated that the porous structure of the hydrogel may provide the proper spatial scale for cell infiltration and growth *in vitro*.

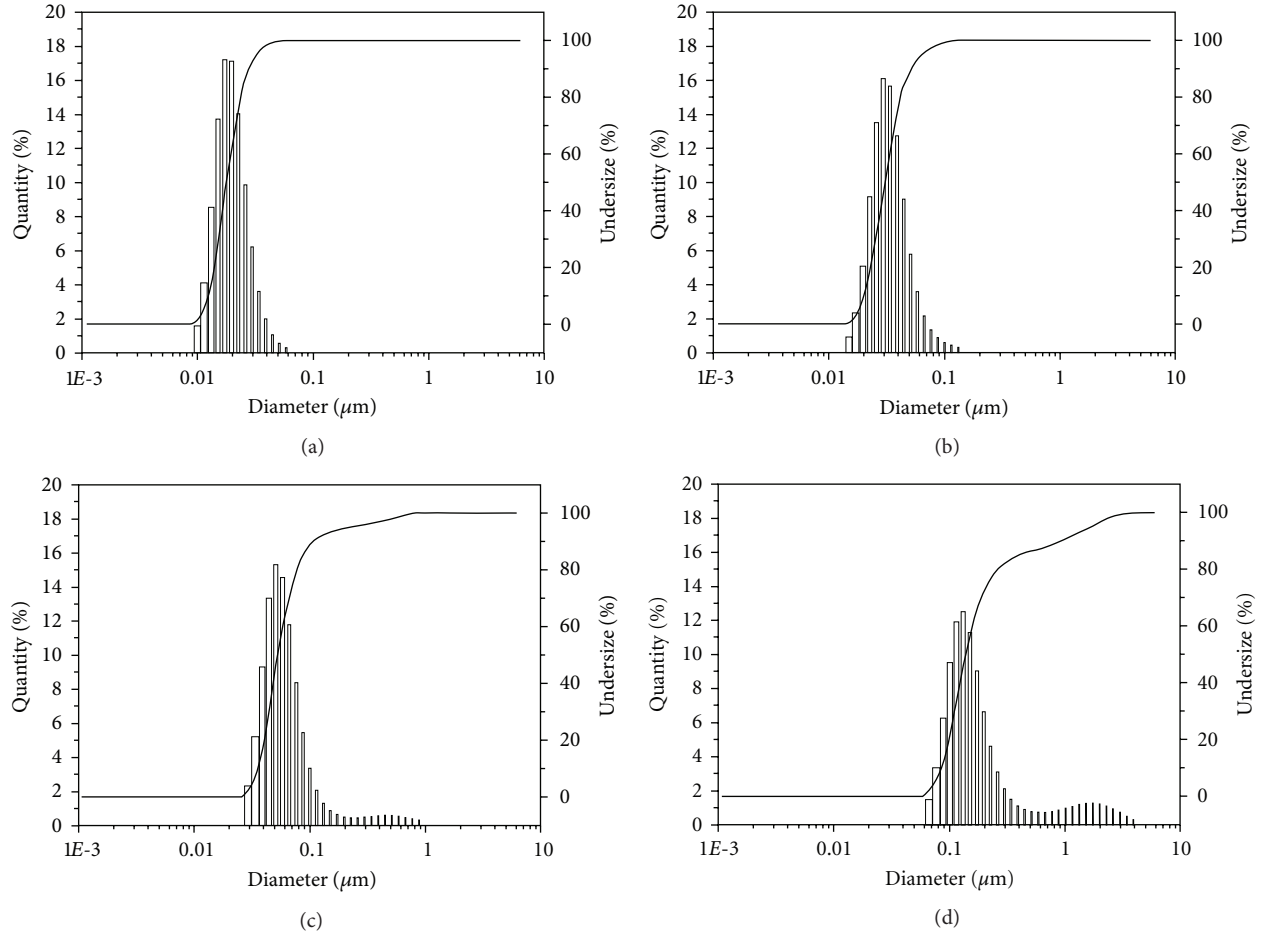


Figure 4: (a) Typical size distribution of SF/PNIPAAm aggregates which remained at 19.2 ± 6.8 nm during the first 12 h after mixing of two polymers. (b) Upon gelation, the size of SF/PNIPAAm aggregates increased slightly to 33.7 ± 15.0 nm, and the size distribution profile does not change with varied temperature. ((c)-(d)) During early stage of gelation process, SF/PNIPAAm aggregates increased in size with elevated temperature and wider size distribution profile can be observed. 78.7 ± 99.6 for 28°C; 341.6 ± 566.3 for 30°C.

To study the effect of temperature and concentration of each polymer on the gelation kinetics, the flat-bottomed vials were fed with 1.0 mL of SF/PNIPAAm solution, were placed at 25°C or 30°C, and were monitored every hour. As illustrated in Figure 3, gelation time of SF/PNIPAAm hybrids decreased with increase in temperature and concentration of each polymer. Particle-size distribution profile for F5P3 remained almost constant at 25°C in the first 12 h after mixing of two polymers (Figure 4(a)). During early stage of gelation, the size of SF/PNIPAAm nanoaggregates increased abruptly with rising of temperature and, more interestingly, this process can be reversed by lowering the temperature (Figures 4(c) and 4(d)). In late stage of gelation, particle size showed no response to changed temperature below the LCST (Figure 4(b)) and stabilized around 30 nm, which is just slightly higher than the size distribution during the early stage at 25°C. It has been well established that in the temperature range below LCST, a higher fraction of intramolecular hydrogen bonding forms with increase of temperature. PNIPAAm chains undergo intrachain contraction due to the dehydration, but the coil conformation remains [39]. Accordingly, the hydrophobicity of the PNIPAAm chain surface increased, and a higher degree of hydrophobic interaction occurs between PNIPAAm and silk fibroin molecules. This change can explain the faster solidation under 30°C as well as the increase in particle size with rising temperatures.

It is well known that the storage modulus (G') is an important parameter of the elastic behavior hydrogel. The changes of the values may respond to the changing strength in the hydrogels [40]. The liquid-solid transition dynamics of a series of SF-PNIPAAm mixtures were rheologically analyzed on a shear rate controlled rheometer (TA, AR2000) with a steel plate geometry at 30°C. The viscoelastic response of each sample was recorded after exposing to a sinusoidal strain, with 0.02 and 6.28 rad/s. At the beginning of the experiments, the storage modulus (G') and the loss modulus (G'') are of similar order of magnitude with $G'' > G'$. During early stage, G' rises more rapidly than the G'' and G' crossed over G'' in minutes after mixing due to the formation of SF-PNIPAAm nonequilibrium complexes. However, the cross-over point varied significantly as sweep frequency changed from $f = 0.5$ to $f = 1.5$. The ratio of G'' to G' (tan) is characteristic for the changes of the material's structure during gelation and tan curves cross at the same time for

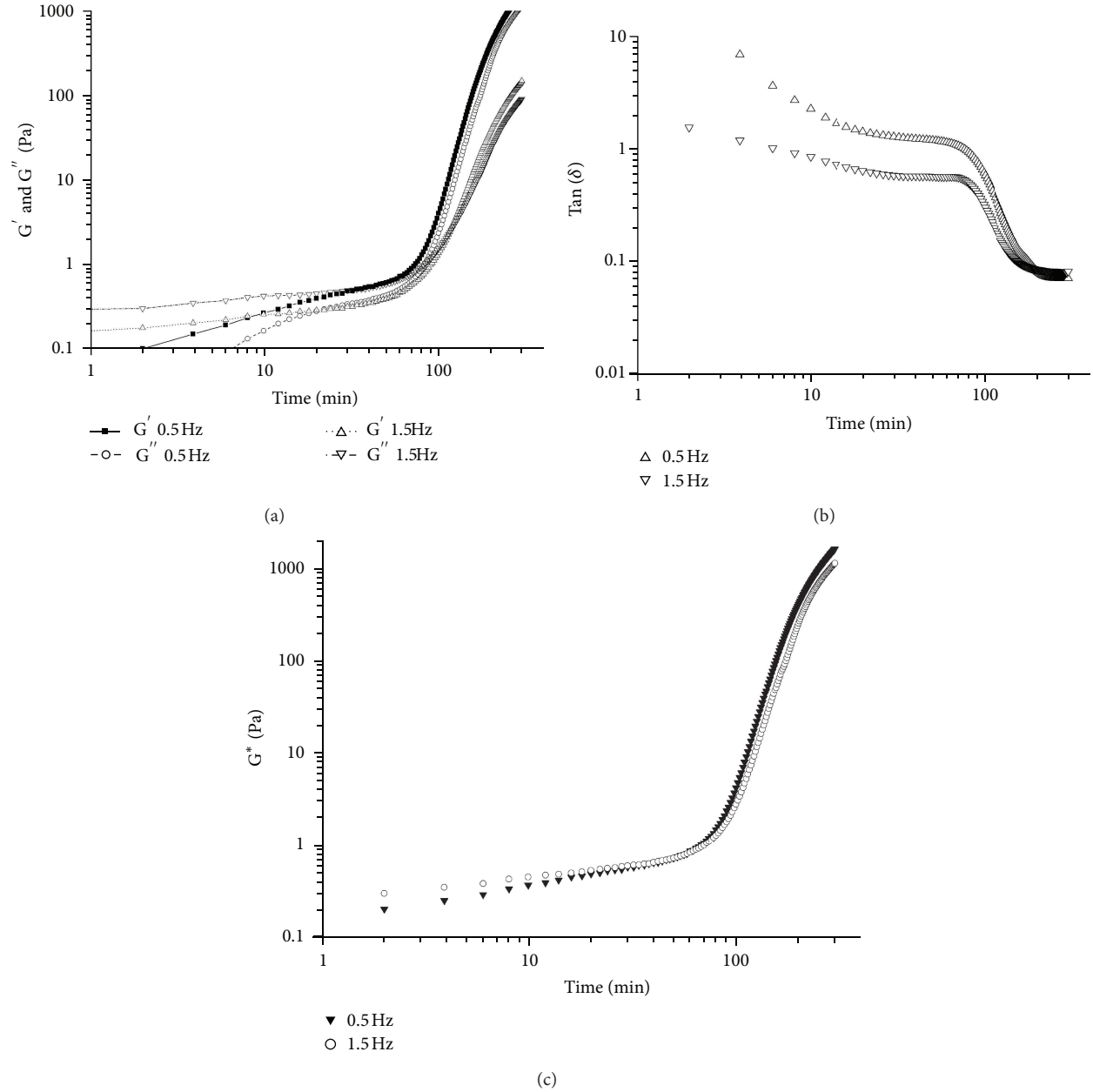


Figure 5: For F5P3, the G″ is about one order of magnitude larger than the G′ initially after mixing of silk fibroin and PNIPAAm aqueous solutions. As the system ages, the G′ increases rapidly and crosses over G″ about 8 min after mixing with a sweep frequency of 0.5 Hz, while the crossing of G′ over G″ occurred about 80 min later when the sweep frequency was set to 1.5 Hz. The complex modulus G* shows slight difference at varied sweep frequency.

different sweep frequencies [41–43]. But in our experiments, no intersection point of tan was identified around tan = 1, indicating that the aging of SF-PNIPAAm mixture did not obey typical sol-gel transition dynamics. Therefore, it is reasonable to consider that the liquid-solid transition should take place at some point between plateau and followed significant increase of complex modulus (G*) spectrum. This phase transition of SF/PNIPAAm exhibited such as setting of structural concentrate and the set time can be conveniently defined as the intersection of the straight lines fitted to the G* slope of plateau and followed increase [42]. As shown in Figure 5, much more time was needed for setting of mixtures with lower concentrations of silk fibroin and PNIPAAm. The slope increase of G* for F1P3 was not as steep as the other four recipients (Figure 6), indicating a smoother set process. This difference may be explained by the highest ratio of PNIPAAm to silk fibroin, implying the important role of silk fibroin in the solidation process. With higher concentration of each polymer, one would expect fast and abrupt setting dynamics. As shown in Figure 6, the rheological property of resultant SF/PNIPAAm gel depended on the concentration combinations as well as the aging time, which elapsed after

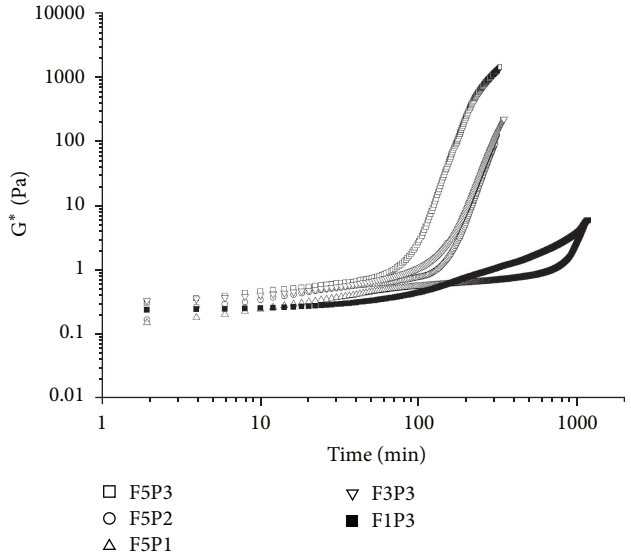


Figure 6: Typical complex modulus spectrum for SF/PNIPAAm mixtures at different concentration of each polymer.

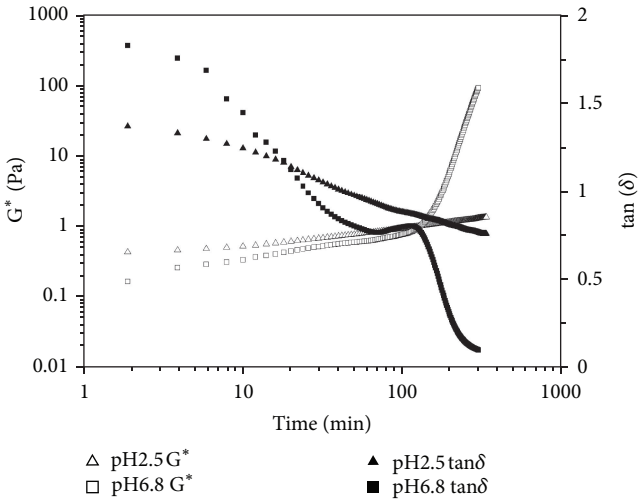


Figure 7: Typical complex modulus spectrum for SF/PNIPAAm mixtures at different pH values.

mixing of the two polymers. Within a typical experimental time scale, tan is reduced continually (Figure 5), even after the setting. This implies that the networking within the system evolved continually beyond the set point and leads the whole system to a high modulus plateau. The higher strength of peptide hydrogel may be associated with the stronger 3D network form and physical interactions between fibroin chains of silk fibroin [25, 38]. Thus, the hydrogel have a higher strength that might also agree with the excellent strength properties of silk fibroin.

The solidation dynamics for SF/PNIPAAm mixtures also depended on the pH condition. For F3P3, the preparation with modification to pH 2.5 using 0.1 M HCl exhibited significant setting delay as compared with the recipient without pH adjustment (Figure 7). To demonstrate if the

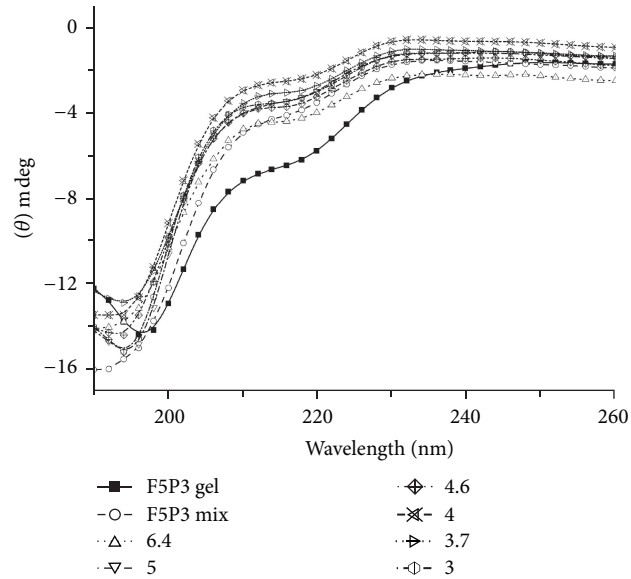


Figure 8: CD spectra of SF/PNIPAAm hydrogel (red line), mixture (black line) and SF solutions with different pH.

secondary structure of silk molecules would be affected by the pH condition, 0.1 mg/mL of fibroin was prepared in buffer with pH = 3.0 to pH = 6.5. Circular dichroic (CD) studies showed a strong negative peak near 194 nm, and two small shoulders near 209 and 231 nm. This spectrum indicated the absence of either α-helix or β-sheet in secondary structure of the oligopeptide, which has been concluded in some reports that this type of CD spectrum represents random-coil structure [44]. CD spectra demonstrated that pH showed little influence to the secondary structure of silk fibroin (Figure 8). On the other hand, the LCST of PNIPAAm increases with the decrease of pH within the range of 1.0–7.0 [45]. It might be considered that the hydration degree on PNIPAAm chain surface would increase under low pH just like under the low temperature. Thus higher fraction of both polymers established SF-PNIPAAm complexes as pH approaching to 7.0.

Results for F3P3 was plotted in Figure 9, in which we observe a significant increase of G', G'', and G^* as surrounding temperature increase above the LCST (around 32°C at pH 7.0). The modulus of the gel increased significantly due to the rapid hydrophilicity to hydrophobicity transition of PNIPAAm chains above the LCST.

4. Conclusion

In the present study, a novel hybrid hydrogel biomaterial has been prepared successfully silkworm silk fibroin and poly (*N*-isopropylacrylamide). Results of SEM images suggested that the hybrid hydrogel shows the porous sponge-like structures. Results of kinetics of this hydrogel showed that the gelation processes of silk fibroin aqueous solution were significantly shorted and the mechanical properties were significantly improved with the increasing concentration of PNIPAAm and silk fibroin. The gelation processes may be

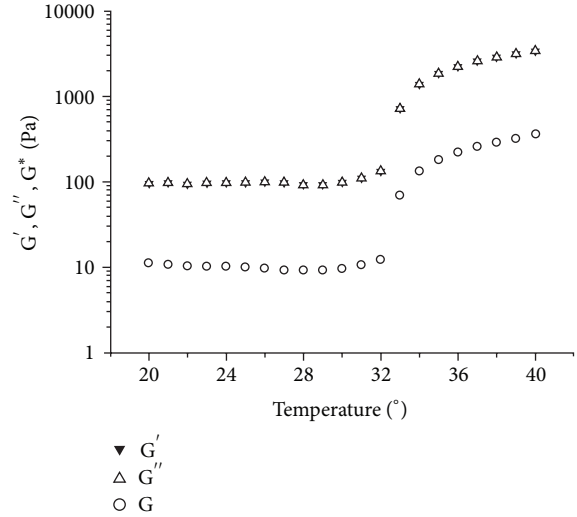


Figure 9: Temperature sweep of SF/PNIPAAm hydrogel with the concentration of F3P3.

also accelerated with the elevated temperature, and G′ will be increased immediately when the temperature is higher than the LCST of the PNIPAAm. The effects of pH on gelation processes were mainly achieved by PNIPAAm, and neutral environment will help to the formation of the hydrogel. The present results suggested that this hydrogel biomaterial will be potential to become widely used in the field of tissue engineering and medical biological materials.

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