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In situ formation of silk-gelatin hybrid hydrogels for affinity-based growth factor sequestration and release†

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Silk fibroin (SF) and gelatin are natural polymers suitable for biomedical applications, including controlled protein release. SF offers high mechanical strength and slow enzymatic degradability, whereas gelatin contains bioactive motifs that can provide biomimicry to the resulting scaffolds. Owing to their complementary material properties, SF and gelatin are increasingly being used together to afford hybrid scaffolds with adjustable properties. Here, we report the use of *in situ* crosslinked SF/gelatin hydrogels as a platform for tunable growth factor sequestration and delivery. We demonstrate that the physical assembly of SF into insoluble networks could be accelerated by sonication even in the presence of gelatin. However, the processing conditions from which to prepare SF aqueous solution (*e.g.*, heating duration and number of processing steps) drastically altered the resulting hydrogel physical properties. Furthermore, the stiffness of SF/gelatin hybrid gels displayed temperature dependency. Specifically, incorporation of gelatin increased gel stiffness at 25 °C but decreases hydrogel mechanical stability at 37 °C. The thermostability of SF/gelatin gels can be restored by using a low concentration of genipin, a naturally derived chemical crosslinker. We also incorporate heparin-conjugated gelatin (GH) into the hydrogels to create a hybrid matrix capable of sequestering growth factors, such as basic fibroblast growth factor (bFGF). Both sonicated SF (SSF) and hybrid SSF-GH gels exhibit moderate bFGF sequestration, but only SSF-GH gels afford slow release of bFGF. On the other hand, genipin-stabilized network exhibited the highest retention and sustained release of bFGF, suggesting the suitability of this particular formulation as a scaffold for tissue engineering applications.

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Introduction

Hydrogels are ideal for *in vitro* cell culture owing to their high water content and tunable material properties that can mimic the extracellular matrix (ECM). Naturally-derived biomacromolecules, such as silk fibroin (SF) and gelatin, are increasingly used for hydrogel fabrication as these molecules possess biologically relevant structure and function that emulate aspects of ECM properties.[1] SF derived from *Bombyx mori* silkworm cocoons is an appealing material for biomedical applications because of its biocompatibility, high tensile strength, slow enzymatic degradation, and versatile processing methods to produce scaffolds with a variety of forms and shapes.[2,3] SF has been formulated into hydrogels for 3D cell culture,[4–6] particles for drug loading and delivery,[7] and spun fibers for scaffolding materials.[8]

Among the various gelation mechanisms, chemical crosslinking is ideal for biomedical applications requiring long-term network stability. However, modifying SF chemically is challenging due to its hydrophobicity and poor solvent solubility. SF is composed of paired heavy and light chains linked by a disulfide bond.[9,10] The primary structure of SF heavy chain is mostly hydrophobic owing to the Gly-X repeats (X being primarily Ala and Ser).[2,7,10] Despite its hydrophobicity, SF can be prepared in aqueous solution through high salt and ethanol-based dissolution and dialysis processes. Solubilized SF exhibits strong intra- and intermolecular interactions, which facilitate their self-assembly into anti-parallel β-sheets and ultimately lead to physical gelation.[2,4,6,11,12] These processes, however, can take several hours to days, depending on SF concentration, solution compositions, and storage conditions.[12] Physical gelation could be accelerated by exposing SF to methanol, which dehydrates the protein chains and accelerates the formation of β-sheet crystalline domains.[13,14] Physical gelation of SF can also be accelerated by sonication,[6] which forgoes

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the use of methanol whose residual presence in the SF network could pose cytotoxic effect on cells.[4,6] Sonication causes cavitation and elevated temperature, pressure, and strain rate, all of which disrupt water molecules surrounding the SF protein chains and accelerate SF self-assembly.[4,6,16] Sonication decreases the time of SF physical gelation from days to hours or even minutes.[6,15] SF gelation kinetics can be readily tuned by adjusting the sonication duration and intensity.[6,15]

Another natural macromolecule commonly used to fabricate hydrogels is gelatin, a collagen-derived water-soluble protein. Gelatin is a popular choice in hydrogel fabrication because it contains peptide sequences for cell adhesion (*e.g.*, integrin binding site RGD) and protease-mediated cleavage (*e.g.*, substrates for matrix metalloproteinase (MMPs)).[4,17] Gelatin experiences thermo-reversible physical gelation, where it is a gel at room temperature and undergoes gel–sol transition at an elevated temperature (*e.g.*, 37 °C).[18] Gelatin can be chemically modified to provide additional features (*e.g.*, heparinization[17] or PEGylation[19]) or to provide covalently crosslinkable motifs (*e.g.*, methacrylates,[20] norbornene,[17,20] *etc.*). For example, *via* carbodiimide chemistry,[17,21,22] the amino groups on gelatin can be conjugated with carboxyl groups on heparin, a sulfated glycosaminoglycan that binds to various growth factors for controlling their bioavailability and for protecting them from proteolysis.[23–25] Sequestering growth factors by heparin near the cell surface provides a mechanism for controllable amplification of specific growth factor signaling to direct cell fate. For example, our laboratory has reported an orthogonal thiol–ene crosslinked gelatin-heparin hybrid hydrogel for studying the effect of matrix properties on hepatocellular carcinoma cell fate *in vitro.*[17] In that application, gelatin was dually functionalized with norbornene and heparin. While the norbornene motif affords facile thiol–ene crosslinking, the conjugated heparin permits binding and slow release of hepatocyte growth factor (HGF).

Recently, silk fibroin and gelatin were combined to form hybrid hydrogels through different crosslinking mechanisms. For example, Das *et al.* used an SF-gelatin blend as bioink for bioprinting three-dimensional SF-gelatin hydrogel constructs to study multi-lineage differentiation of stem cells.[4] The bioprinted SF-gelatin hydrogels were crosslinked *via* either sonication or tyrosinase-mediated enzyme crosslinking. In another example, genipin was used to crosslink SF-gelatin hybrid hydrogels for studying stem cell behavior.[26] One common feature of these prior studies was that SF provides mechanical strength and stability, whereas gelatin lends its bioactive motifs for promoting cell–materials interactions. Additionally, gelatin could be modified with heparin conjugates to affect cell fate *in vitro via* growth factor sequestration and signaling.[23]

In this contribution, we report the development of an *in situ* crosslinked hydrogel platform using sonicated SF (*i.e.*, SSF) and gelatin (*i.e.*, G) or heparin-conjugated gelatin (*i.e.*, GH). This hybrid hydrogel system differs from previously presented SF/gelatin hydrogels by employing sonicated SF to entrap bioactive gelatin or heparin-conjugated gelatin, thus providing additional control of growth factor sequestering and release over time. This system also differs from previous SF/gelatin gels by using genipin as the secondary crosslinking mechanism to reinforce the hybrid hydrogels. We systematically evaluated the gelation kinetics of sonicating SF-reinforced hybrid hydrogels. The effect of macromer concentration and SF processing conditions on gel modulus, as well as the effect of gelatin on physical gelation kinetics, were evaluated *via* rheometry. We further examined the thermostability of the hybrid hydrogels in the absence or presence of additional genipin crosslinking. Additionally, retention of GH and fibroblast growth factor basic (bFGF) in the hydrogels were quantified to assess the suitability of this class of hybrid matrix as a growth factor delivery vehicle.

Materials and methods

Materials

Type A Gelatin (Bloom 238–282) was obtained from Amresco. Heparin sodium salt was obtained from Celsius Laboratories. 1-(3-(Dimethyl amino) propyl)-3-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were obtained from Fisher and TCI chemicals, respectively. Genipin was obtained from Enzo Life Sciences. Recombinant human bFGF and Mini ABTS ELISA Development Kit were obtained from Peprotech. 1,9-Dimethyl-methylene blue (DMMB) was obtained from Sigma-Aldrich. All other chemicals were obtained from Fisher Scientific unless otherwise noted.

Preparation of SF aqueous solution

Silk fibroin was purified from *Bombyx mori* silkworm as previously described.[27,28] Two types of SF were used in this work: degummed SF (D-SF) and regenerated SF (R-SF). D-SF describes the silk fibroin protein after the removal of sericin from silk worm cocoons. On the other hand, R-SF designates SF produced from degummed SF solution that was lyophilized and stored for later use. R-SF was prepared as previously described.[29] To solubilize SF, D-SF was dissolved at 6 wt% in aqueous solution composed of 9.3 M $CaCl_2$, and 20% (v/v) of absolute ethanol (molar ratio of $CaCl_2 : H_2O$: ethanol = 1 : 8 : 2). The solution was refluxed at 95 °C for 1 h, cooled to room temperature, filtered, and dialyzed against ddH_2O (MWCO 6–8 kDa, Fisher) for 3 days to remove the salts. Following dialysis, the SF solution (still within the dialysis membrane) was concentrated in a bath of dry poly(ethylene glycol) (PEG, 10 kDa). The final concentration of SF solution (5–6 w/v%) was determined gravimetrically by drying a small sample of dialyzed and concentrated SF solution. Sonication of SF solution was performed using a Branson S450 Sonifier. Regenerated SF solution was sonicated at 25% amplitude for 25 s pulse (5 s on, 2 s off), and degummed SF solution at 20% amplitude for 5 s.

Fabrication of SSF/gelatin physical hydrogels

Prepolymer solution was prepared in pH 7.4 PBS containing SSF and gelatin or GH. In select experiments, genipin (GN, final concentration at 0.1 wt%) was added to provide partial chemical crosslinking for improving the stability of the hybrid network. Hydrogels were formed between two glass slides separated by 1 mm thick Teflon spacers.[30] The glass slides containing

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prepolymer aliquots were incubated at room temperature for 24 hours within a humidified chamber.

Synthesis, characterization, and retention of GH by SSF-GH hydrogels

Heparin was conjugated onto type A gelatin through standard carbodiimide chemistry following an established protocol.[17] The degree of heparin substitution (DS) was quantified by DMMB assay using unmodified heparin sodium salt solutions as standards. The results were quantified using a microplate reader (abs 525 nm) and determined to be about 0.5% (~5 μg heparin per 1 mg of gelatin). GH retention by SSF-GH gels was evaluated by DMMB assay. Briefly, SSF-GH and SSF-GH-GN gels were prepared as described above, except one group of SSF-GH-GN gels was allowed to crosslink at 37 °C for 24 hours. Gels were incubated in 3 mL pH 7.4 PBS at 37 °C for 72 hours. At 24 hour intervals, 500 μL of solution was collected and replaced with fresh PBS to maintain total solution volume. Samples were stored at −20 °C until analysis by DMMB assay as described above.

In situ physical gelation

To evaluate gelation kinetics, *in situ* rheometry was performed in time-sweep mode on a Bohlin CVO 100 digital rheometer. Gel point was defined as the time at which storage modulus (G') surpasses loss modulus (G''). Immediately post-sonication, prepolymer solution was aliquoted between the rheometer platform and 8 mm parallel plate geometry. The rim of the geometry was lightly sealed with mineral oil to prevent drying. Time-sweep rheometry was operated at 0.5% strain, 1 Hz frequency, 90 μm gap size, and at 37 °C (temperature controlled by a Peltier controller).

Characterization of gel properties

G' and G'' of SSF/gelatin hybrid hydrogels were measured using a Bohlin CVO 100 digital rheometer in oscillatory strain-sweep (0.1–2%) mode with 8 mm parallel plate geometry at 1 Hz frequency and 680 μm gap size. With a Peltier controller, the effect of temperature on gel modulus was evaluated (0.5% strain, 1 Hz frequency) at a temperature range of 25 to 37 °C.

In vitro bFGF sequestering and release from hybrid hydrogels

The SSF, SSF-G, SSF-GH, SSF-GH-GN hydrogels (compositions listed in Table 1) were prepared in 96-well plates for the bFGF sequestering and release experiments. 50 μL of prepolymer solution was added per well. The plates were sealed and incubated for 24 hours at room temperature. For sequestration study, gels were cast in 96-well plate, followed by adding 250 μL of bFGF solution (3 ng mL^{-1} in pH 7.4 PBS containing 0.1% BSA) in each well. The plate was sealed and incubated at 37 °C for 24 hours. After incubation, the release buffer with bFGF was collected and replaced with 250 μL fresh release buffer (0.1% BSA in pH 7.4 PBS) per well. This process was repeated at 48 and 72 hours of incubation. Immediately following collection, the samples were stored at −80 °C until analysis with Human FGF-basic Mini ABTS ELISA Development Kit following manufacturer's protocol. Results were displayed as percentages of the total bFGF originally introduced either in solution or within the gels. Release study was performed as described above, except recombinant human bFGF (3 ng mL^{-1}) were added to prepolymer solutions and not included in the release buffer.

Table 1 Hydrogel Formulations used in Fig. 5–7. All numbers indicate the final wt% of each component in the hydrogels

Group	SSF	SSF-G	SSF-GH	SSF-GH-GN
Sonicated silk fibroin (SSF)	3	3	3	3
Gelatin (G)	0	3	0	0
Gelatin-heparin (GH)	0	0	3	3
Genipin (GN)	0	0	0	0.1

Statistics

All statistical analyses and curve fittings were conducted using GraphPad Prism 5 software. Gel modulus, and gel point were analyzed by One-Way ANOVA followed by Tukey's post-hoc test. Heparin and bFGF retention were analyzed by Two-Way ANOVA followed by Bonferroni post-hoc test. All data was presented as mean ± SEM. Single, double, and triple asterisks represent $p <$ 0.05, 0.001, and 0.0001, respectively. $p < 0.05$ was considered statistically significant.

Results and discussion

Effect of sonication on physical gelation of SF/gelatin hydrogels

The primary goal of the current study was to fabricate *in situ* forming silk-gelatin hybrid hydrogels suitable for growth factor sequestration and delivery. Unlike a previous work by our laboratory that used sonicated SF (SSF) physical gelation as a secondary mechanism to stiffen chemically crosslinked hydrogels,[29] physical gelation was the main focus of this work. While sonication has been shown to accelerate physical gelation of SF, it is not clear whether the presence of gelatin in the mixture would adversely affect the physical gelation of the hybrid hydrogels. Here, we performed conventional tilt tests to demonstrate the influence of sonication and/or gelatin incorporation on physical gelation of SF (Fig. 1). Solutions of pure regenerated SF, and SF-gelatin (SF-G) were incubated at 37 °C. As shown in Fig. 1A, both SF and SF-G were still in solution after 2 hours of incubation. Conversely, SSF and SSF-G appeared to gel by 20 minutes of incubation (Fig. 1B). Sonication causes localized increases in temperature, pressure, and strain rate that accelerate SF self-assembly.[4,6,16] This simple tilt test showed that the presence of gelatin did not adversely impact physical gelation of sonicated SF. To understand the influence of gelatin incorporation on SSF physical gelation, additional rheometry studies were performed (see section below).

Effect of SSF and gelatin content on gel modulus

To quantitatively assess the effect of SSF and gelatin contents on physical gelation of the hybrid hydrogels, we prepared gels with 1–3 wt% of SSF at constant gelatin content (*i.e.*, 3 wt%) or 0–4

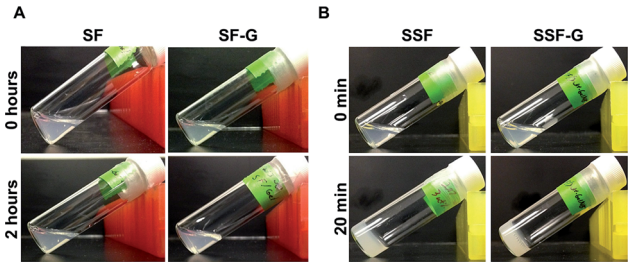

Fig. 1 Tilt tests of SF physical gelation using (A) pure SF solution without or with gelatin (SF and SF-G, respectively), and (B) sonicated (25% amplitude, 25 s pulse) SF solution without or with gelatin (SSF and SSF-G, respectively). Components were added at 3 wt% for SF and SSF and 6 wt% for SF-G and SSF-G (equal weight ratio of SF and gelatin).

wt% gelatin at constant SSF content (*i.e.*, 3 wt%) and conducted shear modulus measurements at room temperature (25 °C). For the oscillatory rheometry studies, frequency of 1 Hz was selected because it lies within the linear region of SSF gel frequency response curve (Fig. S1†). Note that without SSF, pure gelatin at below 4 wt% could not gel at ambient temperature. Prior to mixing SF with gelatin, regenerated SF solution (R-SF) was sonicated at 25% amplitude for 25 s pulse mode (5 s on, 2 s off). After mixing in gelatin at desired concentration, the mixture solution was pipetted into the glass slides assembly and allowed to form physical gels in a humidified chamber overnight. While 20 min at 37 °C was sufficient for sol–gel transition when SSF was incorporated (Fig. 1B), we used longer incubation time at 25 °C for the modulus testing to ensure complete gelation. To assess the effect of SSF concentration on gelation, we measured moduli of the physical gels with varying SSF concentration (1–3 wt%) while holding gelatin concentration constant (3 wt%). Not surprisingly, gel moduli increased significantly with increasing SSF concentration. Specifically, there was an approximately 6-fold increase in gel shear modulus when SSF content was increased from 1 wt% to 3 wt% ($G' = 1181 \pm 30$ Pa, 3461 ± 141 Pa, and 7573 ± 398 Pa for 1, 2, and 3 wt% SSF, respectively Fig. 2A). Next, we evaluated the effect of gelatin incorporation on the mechanical properties of the hybrid hydrogels using constant SSF content (*i.e.*, 3 wt%). Increasing gelatin content from 0 to 4 wt% caused an approximately 4-fold increase in G' ($G' = 2320 \pm 134$ Pa, 6652 ± 178 Pa, 7573 ± 398 Pa, and $10\,057 \pm 128$ Pa for 1, 2, 3, and 4 wt% gelatin, respectively Fig. 2B). Increase in SSF content caused a greater increase in G' as compared with an equivalent increase in gelatin content since semi-crystalline SF β-sheets provided greater mechanical stability than amorphous gelatin.[11] It is worth noting that gels with lower SSF concentration, particularly at 1 wt%, were too brittle and difficult to handle without breaking. Based on these results, a single formulation (3 wt% SSF + 3 wt% G) was chosen for all subsequent experiments unless otherwise stated. These formulations also yield gels with physiologically relevant moduli (*i.e.*, ~1–10 kPa).

Effect of SF solution processing on hydrogel physical properties

Fig. 1 and 2 show that sonication accelerates silk physical gelation and that increasing SSF or gelatin concentration increases gel stiffness. Gel physical properties can also be affected by altering SF processing conditions. Several processing steps are necessary to obtain aqueous SF solution suitable for biomedical applications. The solvent system, temperature and duration of dissolution, storage conditions, as well as intensity and duration of sonication all affect the properties and behavior of aqueous SF. To illustrate the effect of SF solution processing conditions on SF hydrogel physical properties, we compared the moduli of pure SF hydrogels (3 wt% SSF) fabricated from sonicated regenerated-SF (R-SSF) and sonicated degummed-SF (D-SSF). In particular, D-SSF hydrogels presented about 3-fold higher G' than R-SSF gels ($G' = 2320 \pm 134$ Pa and 8572 ± 306 Pa for R-SSF and D-SSF, respectively Fig. 3). In this study, D-SF was refluxed for 1 hour at 95 °C while the R-SF was derived from SF that was refluxed for longer than 2 hours. The increased dissolution duration causes a higher degree of SF protein breakdown leading to lower molecular weight SF.[31,32] While R-SF solution was sonicated at 25% amplitude for 25 s pulse (5 s on, 2 s off), D-SF was only sonicated at 20% amplitude for 5 s. Attempting to sonicate D-SF using the same conditions as for R-SF resulted in rapid gelation of D-SSF during sonication. The objective of sonication was to accelerate gelation of SSF, while still allowing SSF to be soluble long enough for preparing the prepolymer solution. Sonication parameters for R-SSF were selected based upon a previous work[29] but slightly increased to accelerate gelation even more. Sonication at 20% amplitude for 5 s was selected for D-SSF because sonication with higher intensity or duration caused the silk to gel during, or seconds after sonication. Since

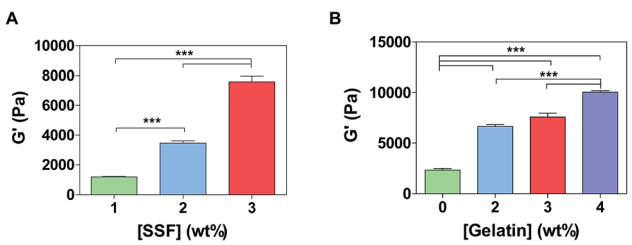

Fig. 2 (A) Effect of SSF content on shear modulus (G') of SSF-G hydrogels with constant gelatin concentration (3 wt%) at 25 °C. (B) Effect of gelatin concentration on shear modulus of SSF-G hydrogels with constant SSF concentration (3 wt%). Data represent mean ± SEM ($n = 5$); ***$p < 0.0001$.

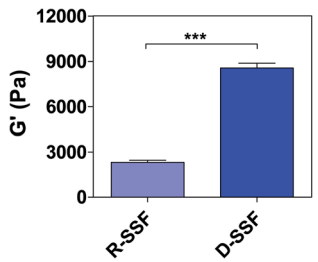

Fig. 3 Effect of SF solution processing conditions on shear modulus (G') of pure SSF physical hydrogels (R-SSF: regenerated SSF, D-SSF: degummed SSF). Data represent mean ± SEM ($n = 5$); ***$p < 0.0001$.

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D-SSF exhibited higher gelation efficiency compared with R-SSF, it was used in the subsequent experiments.

Physical gelation kinetics of SSF/gelatin hydrogels

We investigated whether the presence of gelatin or gelatin-heparin (*i.e.*, GH) would accelerate physical gelation kinetics of SSF hydrogels (Fig. 4). Real time *in situ* rheometry was conducted at 37 °C immediately after mixing the solution components with pure gelatin (Fig. S2A†) or pure SSF (Fig. 4A) as controls. The gel point (the time at which G' exceeds G'') is a metric of when the sol–gel transition occurs and was identified from the *in situ* rheometry results. *In situ* rheometry results show that all formulations tested, except for 3 wt% of pure gelatin (Fig. S2A†), formed physical hydrogel at 37 °C. Specifically, gel point for SSF (Fig. 4A), SSF-G (Fig. 4B), and SSF-GH (Fig. 4C) was 764 ± 47, 270 ± 25, and 374 ± 34 s, respectively (Fig. S2B†). Notably, the inclusion of gelatin or GH accelerated the gelation kinetics. Although gelatin by itself did not gel under the testing conditions (Fig. S2A†),[18] it did act as a crowding agent for the SSF chains and increase their local concentration that led to accelerated gelation.[15,29] GH also accelerated SSF gelation, but to a lesser extent than unmodified gelatin. This is potentially due to charge repulsion between the negatively charged heparin and SSF that disrupted the folding of SF β-sheets and slowed the self-assembly process.[33]

Effect of temperature on SSF/gelatin hydrogel modulus

Previous measurements of G' to determine average gel stiffness (Fig. 2 and 3) were carried out at room temperature (*i.e.*, 25 °C). However, these silk fibroin-gelatin gels are intended for applications under physiological temperatures where gelatin is likely unstable due to its thermo-reversibility. Hence, the effect of temperature on gel modulus was evaluated in real time (Fig. 5A) and as average values at 25 °C and 37 °C (Fig. S3†). While SSF gels were thermostable with little change in modulus within the testing temperatures, the incorporation of gelatin or GH decreased gel modulus noticeably when the temperature were raised above ~32 °C. Due to the thermo-reversible nature of gelatin physical crosslinking, moduli of SF-G hydrogels were also temperature dependent. To improve thermostability of the physical silk fibroin-gelatin hydrogels, genipin (0.1 wt% final conc.) was incorporated in the prepolymer mixture. Genipin is a natural crosslinker derived from geniposide, a compound found in gardenia fruit[26] and has been used to covalently crosslink proteins with abundant primary amine groups (*e.g.*, gelatin and silk fibroin).[5,26,34] Typically, the standard reaction time allowed for genipin crosslinking has been approximately 24 hours.[5,26,34] For example, Bigi *et al.* showed that a high degree of gelatin crosslinking occurs after 24 hours with 0.15 wt% genipin.[34] As shown in Fig. 5B, the addition of genipin improved SSF-GH-GN hydrogel thermostability, most likely a result due to genipin-induced crosslinking.[35–37] Since silk fibroin has relatively few primary amines for genipin to react with, the majority of crosslinking would occur within gelatin chains.[5]

GH retention in SSF-GH hydrogels

Fig. 5 shows that physically entrapped gelatin or GH caused decreased stability of the hybrid hydrogels and this might lead to leaching of G or GH from the physical hydrogels. The retention of GH within the SSF-GH hydrogels was particularly

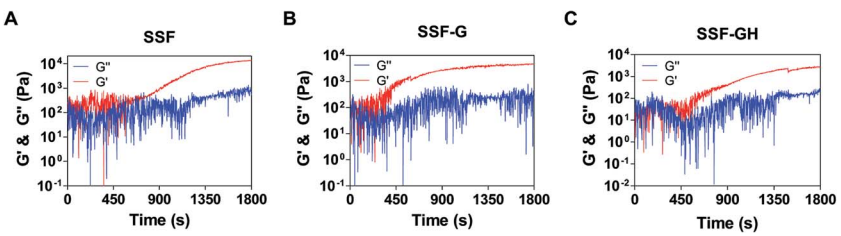

Fig. 4 *In situ* rheometry of SSF (A), SSF-G (B), and SSF-GH (C). All rheometry experiments were conducted at 37 °C. Compositions of the macromer solutions were 3 wt% SSF and/or 3 wt% G/GH. Data shown were representative of at least three independent experiments for each condition.

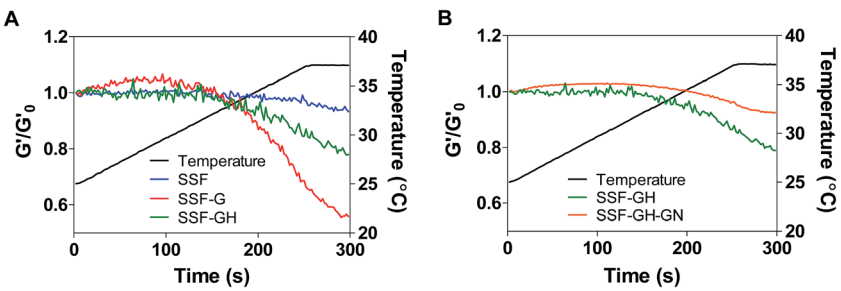

Fig. 5 Thermostability of silk fibroin-gelatin physical gels (A) and the effect of genipin crosslinking on thermostability (B). Shear moduli (G') of gels were measured over 300 s with temperature increasing from 25 °C to 37 °C. Data represent mean of three independent experiments for each formulation. Error bars were omitted for clarity of results.

important as it would affect the sequestration of growth factors. To this end, GH retention was qualitatively evaluated by a modified DMMB assay (Fig. S4†).[17] After 24 hours incubation in DMMB solution, dark pink precipitates formed on top of the SSF-GH gels, indicating the formation of DMMB/heparin complex. This formation of DMMB/heparin complex over the SSF-GH gels also signified the low retention of GH within the physical hydrogels. Low GH retention was of particular concern given the assay was carried out at 25 °C and the loss of GH would be even more pronounced at physiological temperatures due to gel–sol transition of gelatin. We hypothesized that additional genipin crosslinking would limit GH leaching from the hydrogels. To test this hypothesis, small quantity of genipin (0.1 wt%) was added during physical gelation of SSF-GH and the resulting SSF-GH-GN gels were formed at 25 °C or 37 °C. The SSF-GH-GN hydrogels showed statistically significant improvement in heparin (in the form of GH) retention over SSF-GH (Fig. 6). Also, SSF-GH-GN crosslinked at 37 °C exhibited improved heparin retention over gels crosslinked at 25 °C at 72 hours incubation. However, heparin retention was still less than 50%. This was likely due to the low genipin content available for crosslinking and some genipin crosslinking occurred between SSF chains.

Sequestering of basic fibroblast growth factor

To evaluate the ability of the SSF/gelatin hybrid hydrogels to sequester and release growth factors, we designed two set of experiments: bFGF sequestration (Fig. 7) and sustained release (Fig. 8). Growth factor sequestering can be broadly applied during *in vitro* cell culture to help direct cell fate.[23] The goal of this study was to obtain high sequestration of growth factor (*i.e.*, bFGF) from solution to the surface of the hybrid hydrogel. As shown in Fig. 7B, pure SSF gels sequestered about 50% of bFGF from the solution after 24 hours of incubation. The sequestration reduced slightly to 42% after 72 hours of incubation. SSF gels were able to sequester bFGF because at physiological pH, SF was negatively charged and is attracted to positively charged bFGF.[33] Conversely, type A gelatin is positively charged at physiological pH,[18] which repels bFGF and so little to no bFGF is sequestered by the SSF-G gels. When gelatin was replaced with gelatin–heparin conjugate (*i.e.*, GH), the resulting SSF-GH hydrogels displayed comparable bFGF sequestration to SSF

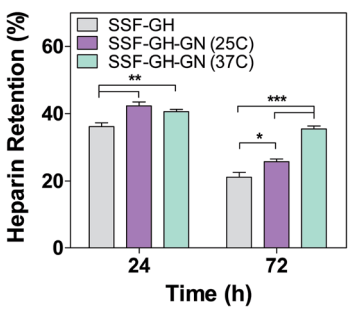

Fig. 6 Retention of heparin by SSF-GH hydrogels with (SSF-GH-GN) or without (SSF-GH) genipin crosslinking. Data represent mean ± SEM of three independent experiments for each formulation; *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$.

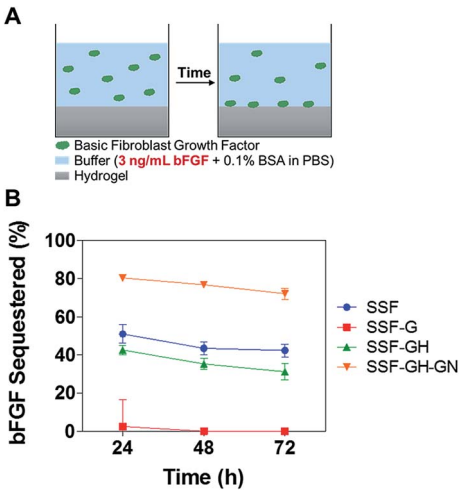

Fig. 7 (A) Schematic of human fibroblast growth factor basic (bFGF) sequestering from the buffer to the surface of different SF-gelatin hybrid hydrogels. (B) Sequestration of bFGF from buffer solution to the gel surface at 24, 48, and 72 h of incubation. Data represent mean ± SEM ($n = 4$).

gels (Fig. 7B). The slight decrease in bFGF sequestration on SSF-GH gel surface when compared with SSF gels was likely caused by leaching of GH to which bFGF was bound. SSF-GH-GN gels showed the highest sequestration of bFGF (~70% after 72 hours, Fig. 7B) due to additional genipin crosslinking. The improved bFGF sequestration is at least partially due to the improved GH retention from genipin crosslinking, presenting more available heparin on the gel surface to sequester bFGF. For all groups, the slight decrease in sequestered bFGF after each 24 hour interval was due to the release of some surface-sequestered bFGF back to the solution.

Release of basic fibroblast growth factor

Sequestration of growth factor onto hydrogel surface is useful in promoting cell attachment, proliferation, and/or differentiation

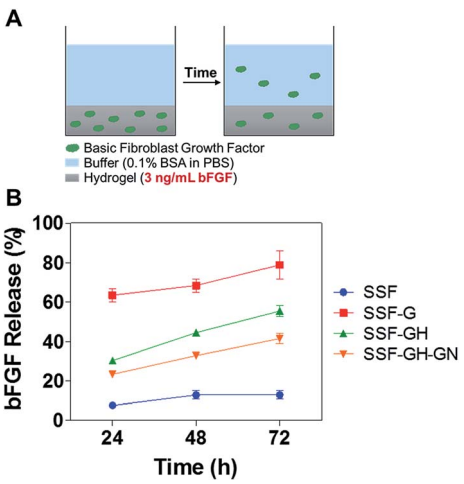

Fig. 8 (A) Schematic of bFGF release from the silk fibroin-gelatin gels into the solution. (B) Release of bFGF from silk fibroin-gelatin gels at 24, 48, and 72 hours of incubation. Data represent mean ± SEM ($n = 4$).

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when the gel matrix is used as a substrate for 2D cell culture. On the other hand, sustained release of growth factors from hydrogels is useful for promoting tissue regeneration. The objective of this study was to evaluate the release of bFGF from different SF-based hydrogels. Here, bFGF was initially encapsulated in the hydrogels during the gelation process, followed by gradual release into the buffer covering the gel (Fig. 8A). As shown in Fig. 8B, the release of bFGF from SSF gel was only about 13% of total bFGF loaded in the hydrogel after 72 hours. The limited release of bFGF from SSF gels was likely caused by the molecular attraction occurring between oppositely charged bFGF and SF, and/or entrapment of bFGF in the SSF β-sheet crystalline domains. When gelatin was added in the hybrid hydrogel (*i.e.*, SSF-G), bFGF release reached ~80% after 72 hours (Fig. 8B), which could be explained by the charge repulsion effect between gelatin and bFGF as described earlier. It was also possible that gelatin was soluble at 37 °C, which led to low retention (and hence high% release) of most bFGF within the gels. The incorporation of heparinized gelatin into the hybrid hydrogel (*i.e.*, SSF-GH) reduced the amount of bFGF release (~55% after 72 hours), confirming the growth factor binding capability of the immobilized heparin. Finally, SSF-GH-GN gels showed the lowest degree of bFGF release (*i.e.*, ~40% after 72 hours, Fig. 8B) among all gelatin-containing gels. This was likely due to genipin-mediated GH retention, and hence bFGF sequestration, in the hybrid hydrogel network.

Conclusion

In summary, we have developed a simple *in situ* forming silk fibroin-gelatin hybrid hydrogel system as a platform for growth factor delivery. At room temperature, increasing silk fibroin and gelatin concentration increased gel moduli. However, at physiological temperature the presence of gelatin and heparin-conjugated gelatin accelerated SSF gelation, but decreased gel moduli. Hydrogels containing gelatin or gelatin-heparin had decreased stability at physiological temperature and significant leaching of GH was detected in SSF-GH hydrogels after 72 hours of incubation. On the other hand, hydrogel thermostability and GH retention were improved upon introducing low concentrations of genipin, which formed chemical crosslinks to stabilize the physical gels. In terms of growth factor sequestration, providing additional genipin crosslinking improved the sequestration of bFGF from ~30% (SSF-GH) to ~75% (SSF-GH-GN) after 72 hours. Genipin crosslinking also slowed bFGF release from 55% (SSF-GH) to ~40% (SSF-GH-GN) after 72 hours. In addition to serving as a carrier for sustained growth factor delivery, this hybrid hydrogel system can be used for *in vitro* cell culture. Future work will focus on exploiting the growth factor sequestration feature of the system to improve stem cell proliferation and differentiation.

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References

1 L. Gasperini, J. F. Mano and R. L. Reis, *J. R. Soc., Interface*, 2014, **11**, 20140817.
2 B. Kundu, R. Rajkhowa, S. C. Kundu and X. Wang, *Adv. Drug Delivery Rev.*, 2013, **65**, 457–470.
3 S. Talukdar, M. Mandal, D. W. Hutmacher, P. J. Russell, C. Soekmadji and S. C. Kundu, *Biomaterials*, 2011, **32**, 2149–2159.
4 S. Das, F. Pati, Y. J. Choi, G. Rijal, J. H. Shim, S. W. Kim, A. R. Ray, D. W. Cho and S. Ghosh, *Acta Biomater.*, 2015, **11**, 233–246.
5 W. H. Elliott, W. Bonani, D. Maniglio, A. Motta, W. Tan and C. Migliaresi, *ACS Appl. Mater. Interfaces*, 2015, **7**, 12099–12108.
6 X. Wang, J. A. Kluge, G. G. Leisk and D. L. Kaplan, *Biomaterials*, 2008, **29**, 1054–1064.
7 X. Wang, T. Yucel, Q. Lu, X. Hu and D. L. Kaplan, *Biomaterials*, 2010, **31**, 1025–1035.
8 D. N. Rockwood, R. C. Preda, T. Yucel, X. Wang, M. L. Lovett and D. L. Kaplan, *Nat. Protoc.*, 2011, **6**, 1612–1631.
9 T. Yucel, P. Cebe and D. L. Kaplan, *Biophys. J.*, 2009, **97**, 2044–2050.
10 A. Matsumoto, J. Chen, A. L. Collette, U. J. Kim, G. H. Altman, P. Cebe and D. L. Kaplan, *J. Phys. Chem. B*, 2006, **110**, 21630–21638.
11 X. Hu, D. Kaplan and P. Cebe, *Macromolecules*, 2006, **39**, 6161–6170.
12 W. Xiao, J. He, J. W. Nichol, L. Wang, C. B. Hutson, B. Wang, Y. Du, H. Fan and A. Khademhosseini, *Acta Biomater.*, 2011, **7**, 2384–2393.
13 X. Wang, X. Hu, A. Daley, O. Rabotyagova, P. Cebe and D. L. Kaplan, *J. Controlled Release*, 2007, **121**, 190–199.
14 L. Yang, M. Yaseen, X. Zhao, P. Coffey, F. Pan, Y. Wang, H. Xu, J. Webster and J. R. Lu, *Biomed. Mater.*, 2015, **10**, 025003.
15 X. Wang, B. Partlow, J. Liu, Z. Zheng, B. Su, Y. Wang and D. L. Kaplan, *Acta Biomater.*, 2015, **12**, 51–61.
16 X. Hu, Q. Lu, L. Sun, P. Cebe, X. Wang, X. Zhang and D. L. Kaplan, *Biomacromolecules*, 2010, **11**, 3178–3188.
17 T. Greene and C. C. Lin, *ACS Biomater. Sci. Eng.*, 2015, **1**, 1314–1323.
18 R. K. Singh Saranjit, K. Venugopal and R. Manikandan, *Pharm. Technol.*, 2002, 36–58.
19 D. Narayanan, M. G. Geena, H. Lakshmi, M. Koyakutty, S. Nair and D. Menon, *Nanomedicine*, 2013, **9**, 818–828.
20 Z. Munoz, H. Shih and C. C. Lin, *Biomater. Sci.*, 2014, **2**, 1063–1072.
21 Z. Li, T. Qu, C. Ding, C. Ma, H. Sun, S. Li and X. Liu, *Acta Biomater.*, 2015, **13**, 88–100.
22 G. Niu, J. S. Choi, Z. Wang, A. Skardal, M. Giegengack and S. Soker, *Biomaterials*, 2014, **35**, 4005–4014.
23 G. A. Hudalla, N. A. Kouris, J. T. Koepsel, B. M. Ogle and W. L. Murphy, *Integr. Biol.*, 2011, **3**, 832–842.
24 M. Kim, J. Y. Lee, C. N. Jones, A. Revzin and G. Tae, *Biomaterials*, 2010, **31**, 3596–3603.
25 S. Nakamura, T. Kubo and H. Ijima, *J. Biosci. Bioeng.*, 2013, **115**, 562–567.

26 W. Sun, T. Incitti, C. Migliaresi, A. Quattrone, S. Casarosa and A. Motta, *J. Tissue Eng. Regener. Med.*, 2016, **10**, 876–887.
27 H. Kweon, J. H. Yeo, K. G. Lee, H. C. Lee, H. S. Na, Y. H. Won and C. S. Cho, *Biomed. Mater.*, 2008, **3**, 034115.
28 S. K. Kim, Y. Y. Jo, K. G. Lee, J. H. Lee Heui-Sam, H. Yeo and H. Kweon, *Int. J. Indust. Entomol.*, 2014, **2**, 66–70.
29 J. C. Bragg, H. Kweon, Y. Jo, K. G. Lee and C. C. Lin, *J. Appl. Polym. Sci.*, 2016, **133**, 43075.
30 T. Y. Lin, J. C. Bragg and C. C. Lin, *Macromol. Biosci.*, 2016, **16**, 496–507.
31 H. J. Cho, C. S. Ki, H. Oh, K. H. Lee and I. C. Um, *Int. J. Biol. Macromol.*, 2012, **51**, 336–341.
32 H. J. Cho and I. C. Um, *Int. J. Indust. Entomol.*, 2011, **23**, 183–186.
33 S. Nagarkar, A. Patil, A. Lele, S. Bhat, J. Bellare and R. A. Mashelkar, *Ind. Eng. Chem. Res.*, 2009, **48**, 8014–8023.
34 A. Bigi, G. Cojazzi, S. Panzavolta, N. Roveri and K. Rubini, *Biomaterials*, 2002, **23**, 4827–4832.
35 C. Mu, K. Zhang, W. Lin and D. Li, *J. Biomed. Mater. Res., Part A*, 2013, **101**, 385–393.
36 J. B. Rose, S. Pacelli, A. J. El Haj, H. S. Dua, A. Hopkinson, L. J. White and F. R. A. J. Rose, *Materials*, 2014, **7**, 3106–3135.
37 H. G. Sundararaghavan, G. A. Monteiro, N. A. Lapin, Y. J. Chabal, J. R. Miksan and D. I. Shreiber, *J. Biomed. Mater. Res., Part A*, 2008, **87**, 308–320.

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