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Biomaterials - Engineering Cell Behavior

Enzyme-Mediated Conjugation of Peptides to Silk Fibroin for Facile Hydrogel Functionalization

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Abstract—Enzymatic crosslinking of tyrosine is a simple and modular method for adding functional peptides to silk fibroin (SF) hydrogels. Silk fibroin is a naturally derived polymer notable for its robust mechanical properties, biological compatibility, and versatility. Hydrogels fabricated from SF are elastic, optically clear, and have tunable moduli, however, they do not contain native biological epitopes for cell interactions. In this work we demonstrate the attachment of peptides to SF hydrogels through crosslinking of tyrosine with horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2). The goal was to understand the utility of this approach and to study how the addition of peptides affects the SF material properties. SF hydrogels conjugated to model peptides with different molecular weights and hydrophobic properties were studied by liquid chromatography/tandem mass spectroscopy (LC–MS/MS) (bond formation), fluorescent imaging (spatial distribution), Fourier transform infrared spectroscopy (FTIR) (protein secondary structure), and rheology (gelation time, modulus). As a proof of concept using a biologically relevant peptide, a peptide containing the cell binding domain Arg-Gly-Asp (RGD) was conjugated to SF, and the density and morphology of primary human fibroblasts were assessed. This work demonstrates a facile method for adding peptides to silk fibroin that can be adopted for a variety of biomaterials applications.

Keywords—Silk fibroin, Hydrogel, Peptide, Enzymatic reaction, Functionalization, Biopolymers, Horseradish peroxidase (HRP).

INTRODUCTION

Silk fibroin (SF) is a natural polymer derived from *Bombyx mori* silkworm cocoons that has found wide use as a biomaterial due to its remarkable material properties and versatility. The heavy chain of SF is ~ 390 kDa (5263 amino acids) and contains repetitive hydrophobic domains interspaced with hydrophilic domains, resembling a block copolymer. The most prevalent amino acids in the heavy chain are glycine (G, ~ 46%), alanine (A, ~ 30%), serine (S, ~ 12%), and tyrosine (Y, ~ 5%), which occur primarily in the motifs GAGAGS, GAGAGY, and GAAS. The repetitive domains form a repeated type II β-turn structure in solution[2,29] and can self-assemble into crystalline β-sheet structures, imparting strength and water insolubility.[33] Tyrosine is thought to play a role in self-assembly and chain registry, forming hairpin turns in the SF in solution and stabilizing the ends of β-sheets crystals during self-assembly.[23] In addition to influencing the secondary structure, tyrosine is notable in that it is amenable to chemical modification through its phenol group. Other residues that have been used for chemical modification include aspartic and glutamic acid, but are present in relatively low amounts (~ 0.5 and ~ 0.6%, respectively).[33]

Aqueous SF solution can be processed into a variety of material formats, including films,[12] porous sponges,[8] nano- and micro-particles,[16,32] fibers,[15] and coatings.[20] Silk hydrogels have also been generated through both physical[14,31] and chemical[1,24] approaches. Physically crosslinked SF hydrogels are formed by hydrogen bonds and physical entanglements. The sol–gel transition can been induced by sonication,[31] exposure to an electric field,[17] addition of ions, decrease in pH, or increase in temperature.[14] The resulting hydrogels contain β-sheet structures and are opaque. Alternatively, SF hydrogels can be synthesized by crosslinking tyrosines in the SF backbone through an enzymatic reaction.[24] Horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) generate phe-

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nolic radicals on the tyrosine groups, which can then form inter- or intrachain dityrosine bonds. The resulting crosslinked hydrogels are elastomeric and optically clear, reflecting the limited β-sheet content, with a modulus ranging from 0.2 to 10 kPa.[24] Previous work has shown that the crosslinking density, modulus, and diffusive properties can be varied by adjusting the SF and H_2O_2 concentrations.[21]

While SF hydrogels have the advantage of tunable mechanical properties, they can be limited in certain applications by their lack of biological activity. Silk fibroin is essentially inert to cells, lacking biological domains or epitopes for selective cell receptor interactions.[30] It is therefore desirable to enhance the functionality of SF while maintaining its advantageous mechanical properties. For guidance, we looked to other relatively inert hydrogels and the strategies that have been used to functionalize these scaffolds. The archetypical hydrogel polymer in biomaterial research is polyethylene glycol (PEG), widely used due to its versatile mechanical properties and biological inertness.[10] Adding cell binding domains to PEG hydrogels, namely RGD, Ile-Lys-Val-Ala-Val (IKVAV), and Tyr-Ile-Gly-Ser-Arg (YIGSR), which are derived from extracellular matrix (ECM) proteins, has been studied extensively.[6,9,19] The addition of these domains has been shown to influence cell attachment and differentiation.[10,13]

Existing strategies for adding functionality to SF-based constructs include blending silk with a biologically active component, such as collagen or ECM,[27,28] co-polymerizing with another biopolymer, such as hyaluronic acid,[25] synthesizing SF-like proteins through genetic engineering, such as silk–elastin proteins,[7] or chemically modifying reactive amino acids in SF using diazonium or carbodiimide coupling.[22] These strategies have advantages for certain applications, as well as associated limitations. Blending and co-polymerization are prone to phase separation and are non-specific, while genetic expression of silk-like proteins is highly specific but limited by scale. Existing chemical modification methods require multistep processes, harsh chemicals, pH conditions outside of physiological range, and in the case of diazonium coupling, induces a color change in silk.

The objective of the present study was to utilize enzymatic tyrosine crosslinking reactions to covalently bind tyrosine-terminal peptides onto the SF backbone, and to study how the addition of peptides affects the SF material properties. Model peptides with different hydrophobic properties and molecular weights (MW) were used to study bond formation, gelation time, and spatial distribution of the peptides, as well as the effect on the modulus and secondary structure of SF. As a proof of concept, peptides with the cell binding domain RGD were conjugated to SF and the hydrogels were seeded with primary human fibroblasts to study functional changes *in vitro*. Ultimately, this study established a selective approach for synthesizing peptide-functionalized SF, expanding the types and utility of these hydrogels for a range of potential biomaterial and tissue applications.

MATERIALS AND METHODS

Silk Fibroin Purification and Dissolution

Silk fibroin was purified from *B. mori* cocoons as previously described.[26] Cocoons were boiled in 0.02 M sodium carbonate (Na_2CO_3, Sigma Aldrich, St. Louis, MO) solution for 60 min to separate fibroin from sericin. SF fibers were then washed under deionized (DI) water to remove residual Na_2CO_3, dried, and dissolved in 9.3 M lithium bromide (LiBr, Sigma Aldrich, St. Louis, MO) solution at 60 °C. The SF solution was then dialyzed in regenerated cellulose tubing (molecular weight cut off 3.5 kDa, Fisher Scientific, Waltham, MA) against DI water to remove LiBr. The dialysis water was changed six times, or until no LiBr remained (as measured by the conductivity of the water). The concentration of the resulting SF solution was calculated from the dried mass of a 1 mL sample; typical concentration was 50–60 mg/mL. The SF solution was concentrated in regenerated cellulose tubing in a fume hood until it reached approximately 120 mg/mL, and the concentration was then adjusted as needed by diluting the solution with DI water.

Silk and Silk/Peptide Hydrogel Fabrication

Peptides with one or two terminal tyrosine groups (Table 1) were designed with different hydrophobic properties, MWs, and labels (> 90% purity, LifeTein, Somerset, NJ). Isotopically labeled peptides were used for liquid chromatography tandem mass spectroscopy (LC–MS/MS), fluorescein isothiocyanate (FITC) labeled peptides were used for fluorescent imaging, unlabeled peptides were used for Fourier transform infrared spectroscopy (FTIR) and rheology experiments, and a peptide with the cell binding domain Y-RGDS (Bachem, Torrance, CA > 98%) was used for *in vitro* studies.

Peptides were conjugated to SF via enzymatic crosslinking reaction with HRP and H_2O_2. SF solution (concentration 50–120 mg/mL) and peptides dissolved in DI water (5 mg/mL) were mixed at various ratios on a molar basis of peptide tyrosine to SF tyrosine. Ratios ranged from 0.1000 to 0.0010 moles of peptide tyrosine to SF tyrosine, where there were fewer peptide tyrosi-

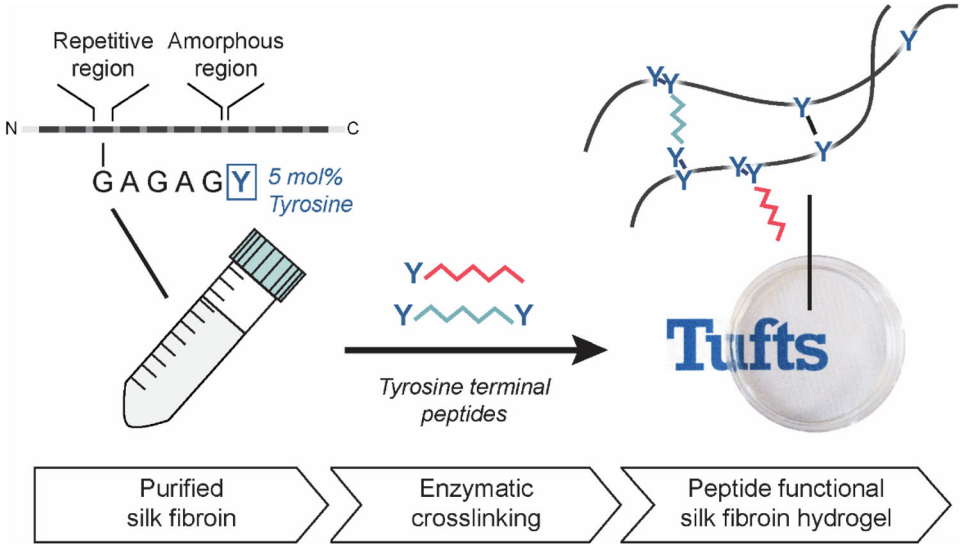

FIGURE 1. Schematic of the enzymatic conjugation of tyrosine-terminal peptides to SF. Aqueous SF solution (5 mol% tyrosine) was blended with peptides containing one or more tyrosine groups and crosslinked with horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2). Dityrosine bonds were formed between the peptides and the silk as well as the silk itself, resulting in a peptide-decorated SF hydrogel.

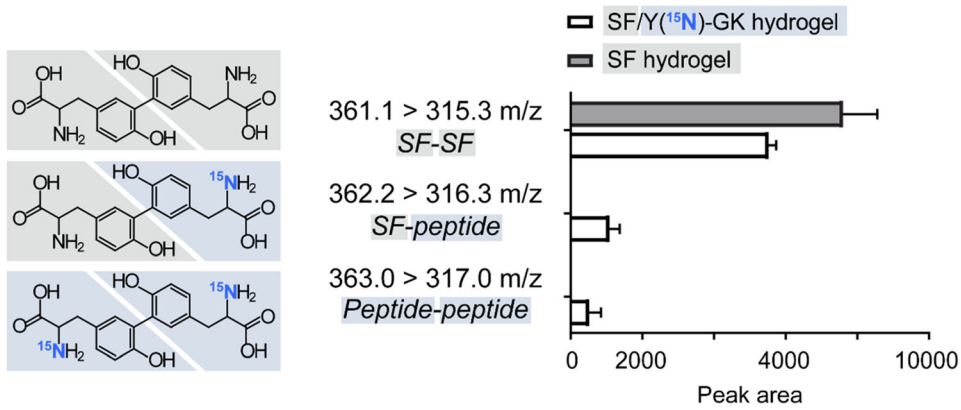

FIGURE 2. Three different dityrosine bonds formed in SF/peptide hydrogels, as detected by LC–MS/MS. Peptide tyrosine groups were isotopically labeled (^{15}N) to differentiate between different bonds. SF–SF dityrosine was seen in both the control SF hydrogels (grey bar) as well as the SF/Y(^{15}N)–GK hydrogels (white bars); SF–peptide bonds and peptide–peptide bonds were also detected in SF/Y(^{15}N)–GK hydrogels (white bars).

nes than SF tyrosines. The SF concentration was adjusted as needed to maintain the final SF concentration at 50 mg/mL after the peptide was added. HRP (Type VI, Sigma Aldrich, St. Louis, MO) solution was prepared at 1000 U/mL in DI water, and H_2O_2 (Sigma Aldrich, St. Louis, MO) solution was prepared at 245 mM in DI water. For all experiments, the hydrogels were prepared by adding the reactants at 20 U/mL of HRP and 4.9 mM H_2O_2 and allowed to react for 2 h at 37 °C.

Liquid Chromatography Tandem Mass Spectroscopy (LC–MS/MS)

The covalent crosslinking of peptides to SF was confirmed by LC–MS/MS, based on a modified version of a previously established method.[21] SF hydrogels were conjugated to peptides containing isotopically labeled tyrosine, Y(^{15}N)-GK, at a ratio of 0.1000 to 1 (moles peptide tyrosine to SF tyrosine) as described above. To prepare samples for analysis, the SF/peptide hydrogels were hydrolyzed in 6 M hydrochloric acid (HCl, Sigma Aldrich, St. Louis, MO) for 4 h at 60 °C. Next, the water and HCl were evaporated, and the samples were reconstituted in 75%

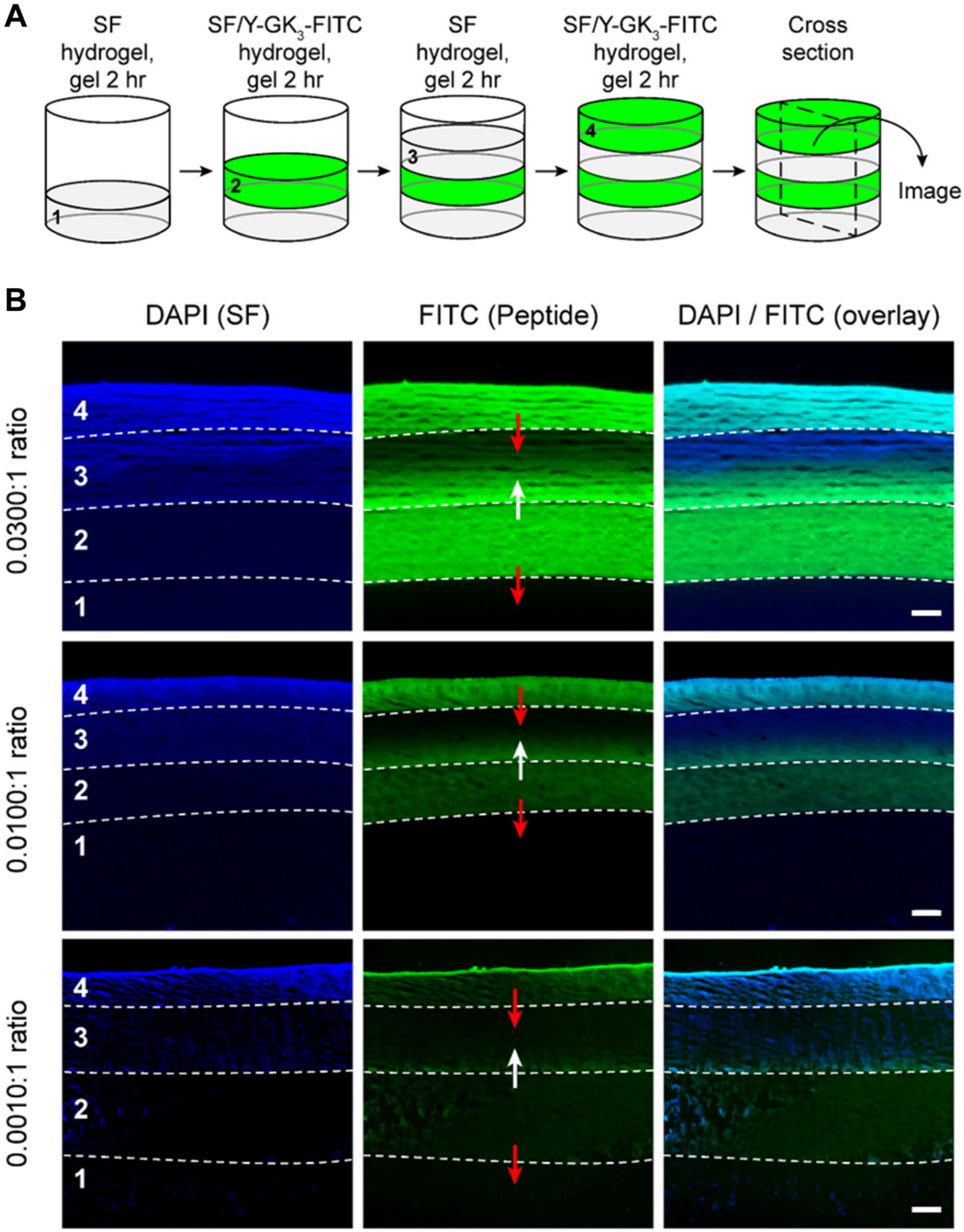

FIGURE 3. Alternating layers of control SF hydrogels (layers 1 and 3) and SF/peptide hydrogels (layers 2 and 4) with a FITC-labeled peptide to visualize spatial distribution and leaching. Layers were 100-400 μm in height and hydrogel sections were 15 μm in thickness. (a) Schematic of layered gel fabrication method. (b) Cross sections of layered gels made with Y-GK_3-FITC peptide at 0.0300:1 (top), 0.0100:1 (middle), and 0.0010:1 (bottom) ratios (moles peptide tyrosine to SF tyrosine). The first column shows SF, which auto-fluoresces under the DAPI filter, the second column shows the FITC-labeled peptide, and the third column is the overlay. FITC-peptide leached into the newly formed hydrogel layer, forming a gradient (white arrows), but did not penetrate the previously gelled layer below (red arrows). Scale bar 100 μm.

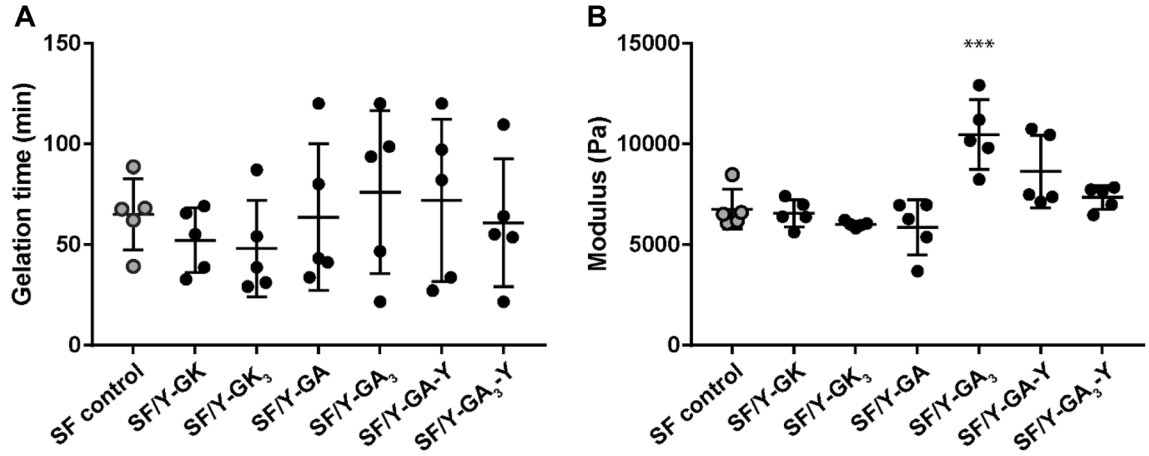

FIGURE 4. Gelation time and modulus of SF/peptide hydrogels, as measured by rheology. All groups had a 0.0100:1 molar ratio of peptide tyrosine to SF tyrosine. (a) Gelation time was measured from the time that reactant was added to the hydrogel precursor solution to the time that the hydrogel modulus stabilized (defined as the time when slope of time vs. *G*' was zero). (b) Storage modulus was extracted from the zero-slope region of time vs. *G*'. Statistical significance indicated ***$p < 0.001$.

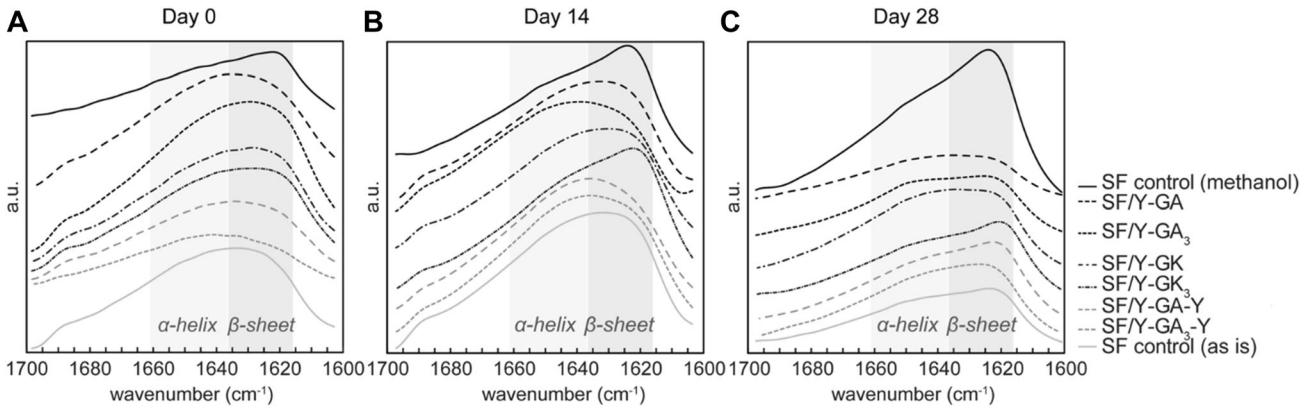

FIGURE 5. FTIR spectra of each peptide at 0.0100:1 ratio (moles peptide tyrosine to SF tyrosine) 0, 14, and 28 days post-synthesis. Two control samples were included, SF control as is (solid light grey), and SF control methanol (solid black). The α-helix region is shaded in light grey and the β-sheet region is shaded in dark grey. Different peptides may increase or decrease the amount of β-sheet in the hydrogels as they self-assemble over time.

v/v acetonitrile in water to a concentration of 400 μg/mL, and then transferred to a 96-well plate. 20 μL samples were injected into a hydrophobic interaction liquid chromatography column (HILIC, Agilent Technologies, Santa Clara, CA). An elution gradient from 95% acetonitrile (0.1% formic acid) to 95% water (0.1% formic acid) was run over 10 min at a rate of 1.0 mL/min (all reagents MS grade, Fisher Scientific, Waltham, MA). The product ions and optimal collision energy for each analyte of interest were determined using a product ion scan, and then a multiple reaction monitoring method (MRM) was developed (Table 2). The abundance of each analyte was determined by calculating the area under the curve of the LC chromatogram in Mass Hunter Qualitative Data Analysis software (Agilent Technologies, Santa Clara, CA). To account for naturally occurring isotopes in the native SF, control samples were run and used to set a baseline level of isotopic tyrosine.

Fluorescent Imaging

Hydrogels consisting of alternating layers of SF hydrogel and SF/peptide hydrogel (FITC-labeled peptide, Y-GK_3-FITC) were synthesized, cross-sectioned, and imaged to visualize the leaching and spatial distribution of the peptides. First, a 300 μL SF hydrogel was prepared as described above and allowed to react for 2 h at 37 °C (layer 1). Next, 150 μL of SF/Y-GK_3-FITC at a ratio of 0.0300, 0.0100, or 0.0010 to 1 (moles peptide tyrosine to SF tyrosine) was mixed with HRP and H_2O_2, immediately deposited on top of layer 1, and allowed to react for 2 h at 37 °C (layer 2). This was followed by another SF hydrogel (layer 3)

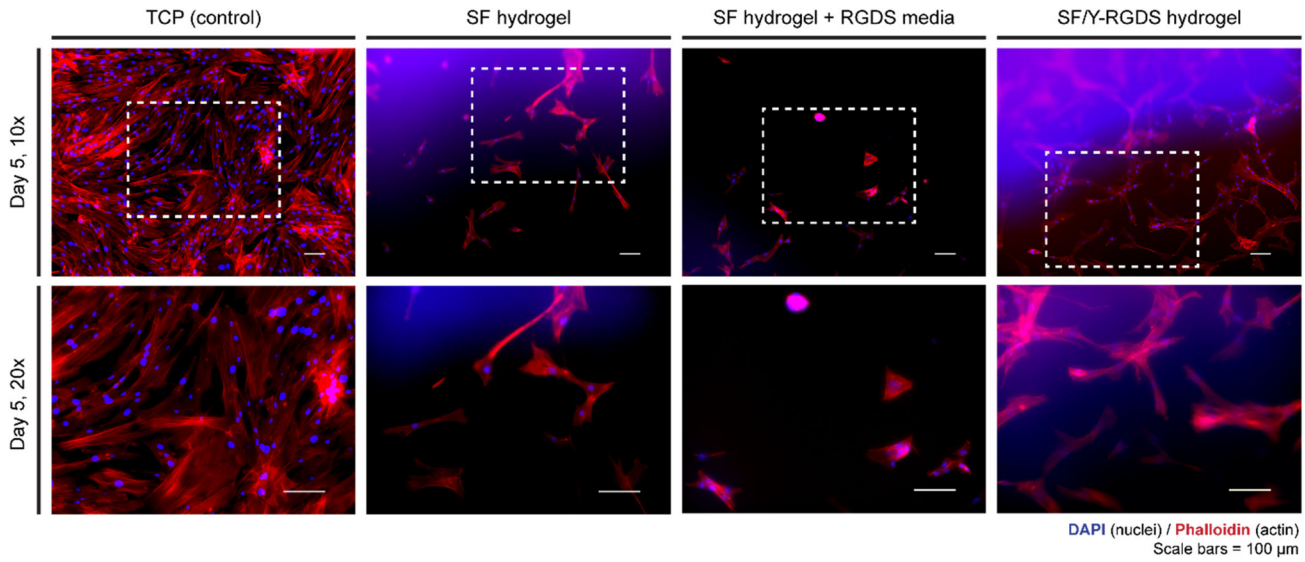

FIGURE 6. Fluorescent imaging of HFF cells stained with DAPI (nuclei) and phalloidin (actin) after 5 days of culture. These results suggest that the covalent binding of the Y-RGDS had a positive effect on the cell density of HFFs cells. Scale bars 100 μm. The dashed rectangle in the top row of images indicate the location of the area shown in the image directly below.

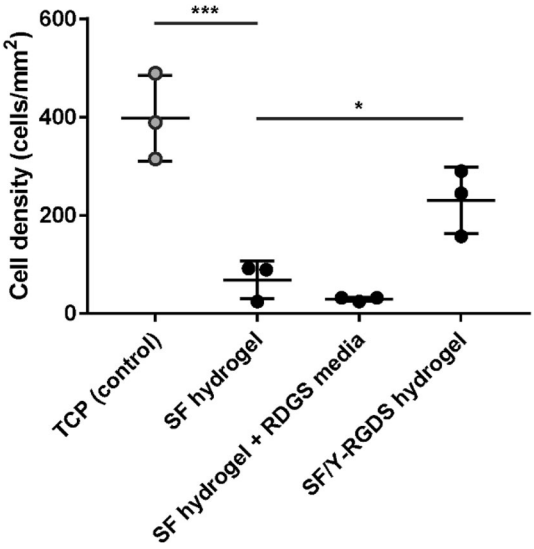

FIGURE 7. Cell density of HFF cells cultured under different conditions. The TCP (positive control) had a significantly higher cell density than the SF hydrogel, as did the SF/Y-RGDS hydrogel, while the addition of RGDS to the media of the SF hydrogel did not increase cell density. Cell count was done on images from *N* = 3 sample replicates with DAPI stain at day 5.

and a SF/Y-GK_3-FITC hydrogel (layer 4). A sample was removed from the layered construct using a 10 mm biopsy punch, and it was embedded in Optimal Cutting Temperature (OCT) compound (Fisher Scientific, Waltham, MA) and frozen for 24 h at – 80 °C. The frozen sample was cut cross-sectionally with a cryostat (CM 1950, Leica Biosystems, Buffalo Grove, IL) to a thickness of 15 μm at – 20 °C, and then sealed under a glass coverslip with aqueous mounting media (VectaMount AQ, Vector Laboratories, Burlingame, CA). The layered hydrogel cross sections were imaged using a fluorescent microscope (BZ-X700, Keyence, Itasca, IL).

TABLE 1. Model peptides, cell binding peptide, and abbreviations used in text and figures.

Peptide	Abbreviation
Y-GAGAGA	Y-GA
Y-$(GAGAGA)_3$	Y-GA_3
Y-GGKGGK	Y-GK
Y-$(GGKGGK)_3$	Y-GK_3
Y-GAGAGA-Y	Y-GA-Y
Y-$(GAGAGA)_3$-Y	Y-GA_3-Y
Y-RGDS	Y-RGDS

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR was used to identify changes in the secondary structure of the SF/peptide hydrogels on a Jasco FTIR 6200 spectrometer with a MIRacle attenuated total reflection (ATR) crystal (JASCO, Easton, MD). SF was conjugated to model peptides (Y-GA, Y-GA_3, Y-GK, Y-GK_3, Y-GA-Y, Y-GA_3-Y) at ratios of 0.0100, 0.0050, or 0.0010 to 1 (moles peptide tyrosine to SF tyrosine) as described above. Samples were stored in DI water and measured at 0, 14, and 28 days post-synthesis. Prior to analysis, samples were soaked in deuterium oxide (D_2O, Sigma Aldrich, St. Louis, MO)

TABLE 2. Analytes and conditions for LC–MS/MS MRM method.

Analyte	Precursor ion (m/z)	Product ion (m/z)	Collision energy
L-Tyrosine (SF)	182.1	136.1	7
L-Tyrosine, Y(^{15}N) (peptide)	183.1	137.1	7
Dityrosine (SF–SF)	361.1	315.3	15
Dityrosine (SF–peptide)	362.2	316.3	15
Dityrosine (peptide–peptide)	363.0	317.0	15

The tyrosines on the peptides contained an isotopic label in order to differentiate between silk (SF–SF), silk and peptide (SF–peptide), and peptide (peptide–peptide) dityrosine bonds.

to remove water, which has a peak in the amide I region (1600–1700 cm^{-1}). Hydrogels were soaked for 30 min, after which the D_2O was replaced. After three D_2O changes, the water peak was no longer present. The samples were analyzed over 32 scans at a resolution of 4 cm^{-1} from 400 to 4000 cm^{-1}. A background scan was run between each sample.

Rheology

Gelation time and storage modulus were assessed using an ARES-LS2 rheometer (TA Instruments, New Castle, DE) with a 25 mm stainless steel conical upper plate (angle: 0.0994 rad) and a temperature-controlled Peltier lower plate at 37 °C. SF was conjugated to model peptides (Y-GA, Y-GA_3, Y-GK, Y-GK_3, Y-GA-Y, Y-GA_3-Y) at a ratio of 0.0100 to 1 (moles peptide tyrosine to SF tyrosine) as described above, $N = 5$ sample replicates. 420 μL of SF was homogenized with 8.4 μL of HRP (1000 U/mL) (final concentration 20 U per 1 mL of silk) and dispensed onto the center of the lower plate. The top plate was then lowered to a 0.047-in. gap. The program was initiated and immediately 8.4 μL of 245 mM H_2O_2 solution was added to the sample (final concentration 4.9 mM). A 10 s pre-shear step was performed at the beginning of the program to homogenize the H_2O_2 with the SF/peptide/HRP solution. The storage (G') and elastic (G'') moduli were measured for 120 min with measurements at 30 s intervals at 1 Hz frequency and 1% strain. The slope of the storage modulus vs. time was calculated, and gelation time was determined to be the point at which the slope was zero. The final modulus was determined from the average storage modulus in the zero-slope region.

In Vitro Study: SF/Y-RGDS Peptide Hydrogels

An RGD domain was conjugated to silk hydrogels and the effect on cell proliferation was studied *in vitro*. Primary neonatal human foreskin fibroblasts (HFF, passage 12, Lonza, Morristown. NJ) were maintained in media (DMEM, 10% fetal bovine serum, 1% 100 × Antibiotic-Antimycotic, all Thermofisher, Waltham, MA) at 37 °C and 5% CO_2. Cells were seeded at 5000 cells/cm^2 in a 24-well tissue culture treated (TCP) plate, $N = 3$ sample replicates per group. The sample groups were as follows: tissue culture plastic (TCP) (positive control), SF hydrogel, SF/Y-RGDS hydrogel at a ratio of 0.0070 to 1 (moles peptide tyrosine to SF tyrosine), and SF hydrogel with soluble RGDS (Sigma Aldrich, St. Louis, MO) added to the media (0.015 mg/mL). On day 3 the media was changed, and on days 3 and 5 the cells were imaged under a brightfield microscope. On day 5 the cells were fixed with 10% buffered formalin (Fisher Scientific, Waltham, MA) for 20 min, permeabilized with 0.1% Triton X-100 (Sigma Aldrich, St. Louis, MO) in phosphate buffered saline (PBS) for 20 min, blocked with 3% bovine serum albumin (BSA, Sigma Aldrich, St. Louis, MO) in PBS overnight, and stained for nuclei (DAPI Nucblue, Life Technologies, Carlsbad, CA) and actin (Alexa Fluor 647 Phalloidin, Thermofisher, Waltham, MA). Three 20 min PBS washes were performed after the fixing, permeabilizing, and staining steps. The hydrogels were then imaged using a fluorescent microscope (BZ-X700, Keyence, Itasca, IL). To determine cell density, one representative image taken with 10X objective was selected from each $N = 3$ replicates for each sample group. The DAPI images were converted to greyscale, cell nuclei were counted using ImageJ software (NIH), and cell density was reported as cells per unit area.

TABLE 3. Mean gelation time and coefficient of variance (%CV) of $N = 5$ hydrogels.

Analyte	Mean gelation time (min)	%CV
SF control	65.2	27.2
Y-GK	52.2	30.9
Y-GK_3	48.0	49.9
Y-GA	63.6	57.1
Y-GA_3	76.1	53.3
Y-GA-Y	72.0	56.1
Y-GA_3-Y	60.9	52.1

Compared with SF control hydrogels, all SF/peptide hydrogels except for Y-GK had increased %CV.

Statistics

LC–MS/MS peak areas are presented as an average and standard deviation of $N = 3$ sample replicates and $N = 3$ technical replicates ($N = 9$ per sample group). Cell count was done in ImageJ (NIH) and is presented as an average and standard deviation of $N = 3$ sample replicates. Gelation time and modulus are presented as an average and standard deviation of $N = 5$ sample replicates, and coefficient of variation was calculated from the mean gelation time. Significance was determined via one-way analysis of variance (ANOVA) with a Tukey *post-hoc* test in Prism 7 Software (GraphPad, La Jolla, California). Statistical significance was defined as $p < 0.05$ and is indicated in figures as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

RESULTS

Confirmation of Covalent SF–Peptide Bonds with LC–MS/MS

SF hydrogels functionalized with various peptides (Table 1) were formed through enzymatic reaction with HRP and H_2O_2 (Fig. 1). LC–MS/MS was used to verify that a covalent bond was formed between the SF and the peptides. In order to differentiate between silk tyrosines and peptide tyrosines within the dityrosine bonds, peptides were synthesized with an isotopically-labeled tyrosine group (^{15}N). A previously established MRM method was modified[21] to detect the additional tyrosine and dityrosine analytes introduced by the isotopic tyrosine (Table 2).

Since isotopic tyrosine could be present in the native silk as well as in the peptides, the amount of isotopic tyrosine in control SF hydrogel samples was determined and used as a baseline (Supplemental Fig. S1). After normalizing to the control SF hydrogels to account for naturally present isotopes, SF–SF dityrosine bonds (361.1 m/z) were shown to be present in both the SF/peptide hydrogels and control SF hydrogels, while SF–peptide (362.2 m/z) and peptide–peptide (363.0 m/z) bonds were present in the SF/peptide hydrogels (Fig. 2).

Peptide Gradients Seen in Layered Hydrogels

In order to understand the spatial distribution of the peptides, FITC-labeled peptides were conjugated to SF hydrogels. SF hydrogels and SF/Y-GK_3-FITC hydrogels were fabricated on top of one another in alternating layers (i.e. SF–HRP–H_2O_2 or SF–peptide–HRP–H_2O_2 hydrogel precursor solution was deposited directly onto the previously formed layer). Results showed that the peptide leached into the newly formed hydrogel layers, creating a gradient (Fig. 3, white arrows), but did not leach into the previously formed hydrogel layer (Fig. 3, red arrows). This leaching gradient effect was most prominent at the highest peptide ratio tested, 0.0300:1 (moles peptide tyrosine to SF tyrosine). It was also seen at the next highest ratio, 0.0100:1, and to a lesser extent in the 0.0010:1 ratio. The hydrogel layers did not separate, suggesting that dityrosine bonds formed at the layer interfaces.

Gelation Time More Variable with Addition of Peptides

The effect of peptide length and hydrophobicity on the gelation time and final modulus of SF hydrogels was studied using a rheometer (Fig. 4). There was no significant change in gelation time with the addition of peptide in any group (Y-GA, Y-GA_3, Y-GK, Y-GK_3, Y-GA-Y, Y-GA_3-Y); however, there was an increase in the coefficient of variance (CV%) for all groups except for SF/Y-GK (Table 3). The final modulus of the SF/Y-GA_3 peptide was significantly higher than the control ($p = 0.0007$); no other groups showed a significant change in modulus.

Temporal Self-assembly Affected by Peptide Addition

The secondary structure of SF hydrogels conjugated to peptides of different lengths and hydrophobic properties was studied over 28 days (Fig. 5). Two control SF hydrogels were included: one as is, and one treated with methanol. The methanol induced a conformational change, increasing the β-sheet content, and served as a positive control. This group showed little change in peak shape and peak location in the FTIR spectra over the duration of the experiment, with a strong peak at 1620 cm^{-1} characteristic of antiparallel β-sheets.[11] The other control, as is, showed minimal β-sheet content at Day 0 but shifted to a peak at 1620 cm^{-1} by Day 28. Different peptides were observed to enhance or retard the tendency to self-assemble over time, based on changes in peak shape as compared with the positive and negative controls. SF/Y-GK_3 hydrogels were similar to the as is control at Day 0 but had a β-sheet shift by Day 14, increasing the rate of self-assembly. Meanwhile, SF/Y-GA and SF/Y-GK delayed the progression of β-sheet formation at Day 28. FTIR spectra for all peptides at all three ratios tested are shown in Supplemental Fig. S2.

In Vitro Studies Demonstrate Increased Cell Density on RGD-Decorated SF Hydrogels

The effect of conjugating a tyrosine-terminal peptide containing the cell binding domain RGD to SF hydrogels was studied *in vitro* with primary neonatal

human foreskin fibroblasts (HFF) cells over 5 days (Fig. 6). HFF cells proliferated and spread on the TCP control surface, as expected. There was a significantly higher cell density at Day 5 on SF/Y-RGDS hydrogels compared with both control SF hydrogels and SF hydrogels with RGDS in the media (Fig. 7), based on cell count.

DISCUSSION

A previously established enzymatic crosslinking reaction was used to incorporate peptides into SF hydrogels, which have no cell-specific epitopes related to integrin signaling. The utility of this method was studied using model peptides with different molecular weights and hydrophobic properties through mass spectroscopy, fluorescent imaging, rheology, and FTIR. The goal was to understand the utility of the method in terms of peptide chemistry and concentration, and to study how the peptides affected the material properties of SF.

LC–MS/MS results revealed that there were covalent bonds between the SF and peptides, eliminating the possibility that the peptides were solely incorporated by entrapment in the matrix. However, it is possible that some portion of the peptides were entrapped in addition to being covalently bound. To get a better understanding of the reaction efficiency and the tendency of the peptides to diffuse out of the matrix, layered hydrogels were fabricated with FITC-peptides. Fluorescent imaging showed that unbound FITC-peptides could diffuse out of the hydrogel into a hydrogel precursor solution. Greater intensity and a longer diffusion front were seen with increasing FITC-peptide ratios, while relatively lower intensity was seen at the lowest ratio, although some diffusion was still visible. This revealed that while the LC–MS/MS results indicated that covalent bonds were formed, the reaction efficiency was not 100%, which was expected. The LC–MS/MS results showed that some of the peptides dimerized (363.0 m/z/), and therefore they could not bind to the SF. Additionally, some SF tyrosines were expected to be unavailable for binding due to steric hindrance,[3] and FITC-labeled peptides may adsorb to the silk. The layered hydrogel results revealed that this method can be used to generate hydrogels with not only uniform peptide distributions, but also with peptide concentration gradients. Gradients of peptides or other cellular cues on scaffolds are useful to instruct cell behavior and guide tissue formation.[5,9]

In rheology experiments, we saw that the addition of peptides to the SF hydrogel increased the variance of the gelation time. A possible explanation is that peptide binding to the SF chains induces local structural changes, which in turn affect gelation kinetics. The SF hydrogel system itself is complex; the rate of the reaction depends not only on the formation of phenolic radicals, but also on the collision of radicals to form bonds. This second event could be affected by polymer chain mobility and steric hindrance.[3] Only one peptide, Y-GA_3, significantly changed the modulus of the final hydrogel (6563 ± 686 Pa to $10{,}473 \pm 1742$ Pa, $p = 0.0007$). One hypothesis for this is that Y-GA_3 mimics the repetitive region of silk. Poly(AG) chains can achieve tight packing conformations in SF[4]; it's plausible that these peptides were incorporated into the repetitive regions by electrostatic interaction and contributed to the modulus.

Chemically crosslinked SF hydrogels form β-sheet structures over time, resulting in increased opacity and stiffness.[23,28] In this work, we saw that the addition of peptides to SF hydrogels affected their propensity to self-assemble. From our fundamental understanding of SF structure, it makes sense that modifying the tyrosine groups with peptides of varying charge would affect self-assembly. In solution, self-assembly of silk proteins is driven by concentration, molecular mobility, charge, and hydrophilic interactions.[18] Tyrosine groups are thought to play a role in the stabilization of silk in solution state[29] and tyrosine–tyrosine interactions provide a template for β-sheet formation.[23] Previous work has also shown that SF and hyaluronic acid (HA) composite hydrogels had reduced stiffening and β-sheet formation over time compared to SF controls, and this stability was directly proportional to the concentration of HA.[25]

In the next part of this work, we sought to study a peptide with biological function. Y-RGDS was bound to SF at a ratio of 0.0070:1, one of the lower ratios used in the first part of this work. The purpose of this was twofold: (1) to test the biological significance at a low ratio, with the expectation that a functional peptide may be limited by cost or quantity, and (2) to study a ratio where we would not expect secondary structure or modulus to be affected. There is a tendency for low cell count and spherical morphology when fibroblasts are seeded on SF hydrogels, and we saw that these outcomes were improved when Y-RGDS was bound to the SF hydrogel, but not when RGDS was doped into the media. This experiment using a well-studied ligand opens the door for other functional domains to be bound to silk using this method. This work established enzymatic crosslinking as a modular method for conjugating peptides to SF hydrogels, effectively expanding the utility of these hydrogels for a range of biomaterial and tissue applications.

ELECTRONIC SUPPLEMENTARY MATERIAL

The online version of this article (https://doi.org/10.1007/s10439-020-02503-2) contains supplementary material, which is available to authorized users.

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