Supporting Information

Enhancing the Carboxylation Efficiency of Silk Fibroin through the Disruption of Noncovalent Interactions

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This document includes experimental details and supplementary figures.

Experimental Methods

Reagents.

Bombyx mori (*B. mori*) silkworm cocoons were obtained from Tajima Shoji Co., Ltd. (Tokyo, Japan). Sodium carbonate (Na_2CO_3, ≥99%), succinic anhydride (≥99%), lithium bromide (LiBr, ≥99%), sodium dodecyl sulfate (SDS, ≥98.5%), *N*-Hydroxysuccinimide (NHS, 98%), dopamine hydrochloride (≥98%), and orange II sodium salt (≥85%) were purchased from Sigma Aldrich (St. Louis, MO). 1-butyl-3-methylimidazolium chloride (BMIM-Cl, TCI America, >98%), dimethylformamide (Alfa Aesar, 99%), urea (Alfa Aesar, 99.0-100.5%), Triton X-100 (electrophoresis), sodium hydroxide (NaOH, ACS Certified), hydrochloric acid (HCl, ACS Certified), phosphate buffered saline (PBS), methanol (HPLC), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), and methylene blue (Certified Biological Stain) were purchased from ThermoFisher (Waltham, MA). Deionized water (dH_2O) was obtained from an in-house purification unit.

Instruments.

The conductivity of solutions were obtained by using an Accumet AB200 pH meter (Fisher Scientific, Hampton, NH). ^{1}H NMR spectra were collected by an Avance 300 MHz spectrometer using Top Spin software (v. 3.5) (Bruker, Billerica, MA), and the spectra were analyzed with MestReNova (v. 9.1.0, Escondido, CA). The secondary structure of the silk and silk conjugates were analyzed by using a Magna 560 Fourier transform infrared (FTIR) spectrometer and a diamond attenuated total reflectance (ATR) accessory. The beta sheet content of the protein films was quantified using Origin software (v. 8.1, Northampton, MA) by deconvoluting the Amide I region of the ATR-FTIR spectra. The streaming potential of the silk films were measured using a SurPASS Electrokinetic Analyzer and Attract software (v 2.1, Anton Parr, Graz, Austria). The amount of dye released from the films was measured using a Synergy HT plate reader and Gen5 software (BioTek, Winooski, VT).

Extraction of Silk Fibroin.

Silk fibroin was extracted by following a previously reported protocol[1] with slight modification. The cocoons were boiled in a 0.02 M Na_2CO_3 solution for 5 min and allowed to dry overnight at room temperature to remove sericin that coats fibroin fibers. Rather than dissolving in aqueous lithium bromide solution, the degummed fibers (1 g) were dissolved in 6.25 g of molten 1-butyl-3-methylimdiazolium chloride (BMIM-Cl) for 4 h at 100°C.[2] The resulting solution was diluted with 15 mL of anhydrous dimethylformamide (DMF) and mixed until homogenous. This product is denoted as **$SF_{(IL)}$** and was used as a precursor for the following reactions.

Carboxylation of Silk Fibroin.

Three different sets of reaction conditions (Reactions A-C) were investigated for their effect on degree of carboxylation. All of the reactions employed the same primary molecule (succinic anhydride) to modify the hydroxyl groups of the serine and threonine residues and amine groups of the lysine, arginine, and histidine residues, resulting in the enrichment of carboxylic acid functional groups. For Reaction A, 2 g of succinic anhydride was added to the SF mixture described above and reacted for 1 h at 100°C. The solution was allowed to cool to room temperature before diluting 1:1 (v:v) with ultrapure water, dialyzing in regenerated cellulose tubing (3500 MWCO) against 8 M urea for 3 h, exchanging the urea for ultrapure water by stepping down the urea concentration in 2 M increments for 3 h each, and then finally dialyzing against ultrapure water for 48 h. During dialysis, SF precipitated due to the switch from DMF to water so the precipitate was collected by centrifugation, dissolved in 9.3 M lithium bromide (LiBr), and subsequently dialyzed against water to obtain carboxylated silk fibroin (**cSF**). This method is

referred to as **cSF$_{(IL)}$(no additives)** and was recovered at a typical concentration of 10 mg/mL and a yield of about 60%. Reaction B introduced surfactants, where the rationale was that surfactants may associate with the hydrophobic regions of silk to permit access to more serine residues, some of which are located in the hydrophobic blocks. The surfactants selected were sodium dodecyl sulfate (SDS) (0.1% w/v), Triton X-100 (0.1% w/v), and sodium deoxycholate (1% w/v) for their ability to dissolve hydrophobic membrane proteins.[3] The surfactants were added to the **SF$_{(IL)}$** mixture at 100°C. After stirring for 30 min, 2 g of succinic anhydride was added and permitted to react under magnetic stirring for 1 h. The solution was then diluted 1:1 with ultrapure water and worked up as described above. This product is denoted as **cSF$_{(IL)}$(surfactant)**, and was collected at a concentration of 5 mg/mL and at a yield of 57%. Reaction C used urea, where the rationale was that urea would disrupt the hydrogen bonding in the hydrophobic sections of the protein and again increase the number of serine residues available to react. Urea was added to the **SF$_{(IL)}$** mixture at a concentration of 8 M and the solution was magnetically stirred at 100°C for 30 min to allow the urea to dissolve. Succinic anhydride (2 g) was added to the solution, and the proceeded for 1 h at 100°C. The reaction mixture was diluted with ultrapure water at 1:1 (v:v) and dialyzed as described above. This product is denoted as **cSF$_{(IL)}$(urea)** and was recovered at a concentration of 1 mg/mL and a yield of 51%. For each reaction, the main losses of the silk product are attributed to the workup of the reaction. The switch from IL/organic solvent to water caused all of the SF to precipitate during dialysis. The dialysate (liquid and solids) was removed from the dialysis tubing and centrifuged. The supernatant after centrifuging was dried, but there was no silk product observed in this phase. The main losses are thus thought to arise from solids that were not fully collected from the tubing.

Characterization

The degrees of carboxyl substitution were characterized using ^{1}H NMR and conductometric titrations. All silk and conjugate solutions were concentrated using centrifugal filter units (3000 MWCO) to 70 mg/mL and diluted 1:10 (v:v) with D_2O to prepare ^{1}H NMR samples. The NMR spectra were then used quantify the number of moles of carboxylic acid groups added. The degree of carboxylation was determined by first normalizing the spectra to one mole of valine. This was accomplished by integrating the valine γ-CH_3 protons (~0.95 ppm) and setting the integration to 6 protons. The new peaks present in the carboxylated samples (2.44-2.40 ppm) were attributed to the methylene protons of succinic anhydride and integrated. The number of protons present in the unmodified silk was subtracted from the number of protons in the carboxylated silk. This difference, which represents the number of new protons in the spectra of the carboxylated

conjugate, was then divided by the number of protons expected from the modification with succinic anhydride (4 protons). The result is the number of moles of COOH substitution per mole of valine in the silk. This number was then divided by the number of serine, threonine, lysine, arginine, and histidine residues (these are the residues that are subject to modification) to calculate the degree of substitution.

To prepare the conductometric titration samples, the conjugates were diluted to a concentration of 1 mg/mL and incubated with 0.1 M sodium hydroxide (NaOH) for 30 min at room temperature to completely deprotonate the carboxylic acid groups. Hydrochloric acid (HCl, 0.1 M) was then added to the solution in 0.2 mL aliquots, and the samples were stirred at room temperature for 5 min before measuring pH and conductivity. The HCl aliquots were added until the SF solutions began to precipitate and the conductivity value began to increase, both of which occurred at the same time. The number of carboxylic acid groups was calculated by determining the amount of acid required to protonate the carboxylic acid groups, which was established by the plateau region of the conductometric titration.

One of the most desirable features of silk fibroin is its ability to be cast as a liquid into different forms[1] and subsequently be triggered to form beta sheet structures,[4–6] the extent of which are often exploited to control the silk biomaterial's mechanical properties and degradation profile.[4,7,8] To examine the effect of carboxylation on beta sheet formation, films were cast by applying 0.2 mL of 2% (w/v) silk or silk conjugate to aluminum foil pan with an area of 1.12 cm^2. "As Cast" films were dried at room temperature overnight, and then transferred to a vacuum oven, where they were further dried for 72 h. "Methanol Treated" films were dried at room temperature overnight, treated with 95% (v/v) methanol in water for 6 h to induce beta sheet formation, and then dried in a vacuum oven for 72 h. Both sets of films were stored in a desiccator until use to reduce water adsorption. ATR-FTIR spectra were obtained for the films, and the Amide I region was assessed and deconvoluted using established methods[9] to determine the beta sheet content of the films.

The hydrophilicity of the films of the carboxylated SF conjugates were also measured using water contact angle. The films were cast using the same method described above. 5 µL of ultrapure water was deposited onto each film and the contact angle was measured using a Ramé-Hart contact angle goniometer.

To assess the surface charge of the carboxylated SF conjugates, streaming potential was used. Surface potential entails flowing an electrolyte across a sample at a known pressure to obtain a potential difference, which relates to the charge.[10] Films of the precursor, **$SF_{(IL)}$**, and the **$cSF_{(IL)}$** conjugates were cast in 85 mm petri dishes from their respective solutions at concentrations of 2% (w/v) and were dried overnight at 25°C. The testing setup requires flat and

insoluble films, but it was found that methanol treatment resulted in some film wrinkling. Therefore, the films were then exposed to water vapor to induce beta sheets,[9] rendering the films insoluble while maintaining their flatness. Prior to loading the films into the sample clamping cell, they were cut in 55 mm x 25 mm rectangles. Zeta potential was measured using 10 mM potassium chloride as the electrolyte buffer.

The binding and release of charged dyes from the **cSF$_{(IL)}$** conjugates were also analyzed. Free standing films of **SF$_{(IL)}$**, and the **cSF$_{(IL)}$** conjugates were prepared by casting a 2% (w/v) solution into aluminum pans with an area of 1.9 cm^2. The films were dried overnight and were treated with 95% (v/v) methanol to induce beta sheet formation and render the films insoluble. After inducing insolubility, films of each composition were transferred to solutions of 10 mg/mL methylene blue (MB) (positively charged at neutral pH) or orange II sodium salt (negatively charged at neutral pH), where the films were incubated at 25°C for 12 h. After binding, the films were removed from the respective dye solutions, blotted with a Kimwipe to remove excess dye, and then the films were loaded into a UV-Visible spectrophotometer to determine the amount of initial dye bound to the films using the absorbance measured and the molar extinction coefficient of the dyes. For release studies, the films were and placed into an empty well of a 12 well plate. Each well was filled with 5 mL of 1X PBS, and 2.5 mL of solution (for MB) and 0.1 mL of solution (for orange II) were extracted at 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 10 h, 24 h, and 48 h. After the sampling, 2.5 mL or 0.1 mL of 1X PBS was added to replenish the well. Preliminary experiments showed that more MB releases than orange II, thus a larger volume of fresh buffer was used in the MB experiments compared to the orange II experiments. The absorbances of each sample were read at 665 nm for methylene blue or 484 nm for orange II, and the concentration of dye released was calculated using a standard curve constructed for each dye.

Carbodiimide Coupling to Silk and Carboxylated Silks

To demonstrate carbodiimide coupling efficiency using EDC, dopamine was selected as the model molecule because it has distinct protons that can be readily quantified using ^{1}H NMR. For the EDC coupling of dopamine, two synthetic routes were investigated – one without any additives in the reaction (Reaction i), and one with adding sodium dodecyl sulfate and Triton X-100 to the reaction mixture (Reaction ii). Urea could not be used as an additive to disrupt the hydrogen bond formation because the primary amine competes with the primary amine of the molecule that is being conjugated. For Reaction i, **SF$_{(IL)}$**, **cSF$_{(IL)}$(no additives)**, **cSF$_{(IL)}$(surfactant)** or **cSF$_{(IL)}$(urea)** were prepared at a concentration of 4 mg/mL in 1X PBS. The pH was adjusted to pH 7.4, and EDC (2 mg/mL) and NHS (0.5 mg/mL) were added to the solution. Next, dopamine was added to

a concentration of 0.5 M, and the reaction was magnetically stirred for 16 h at 25°C. The products, **dopaSF**, were dialyzed (3500 MWCO tubing) against ultrapure water for 48 h to achieve a purified solution, with yields of about 67% at a concentration of 2 mg/mL. For Reaction ii, **$SF_{(IL)}$**, **$cSF_{(IL)}$(no additives)**, **$cSF_{(IL)}$(surfactant)** or **$cSF_{(IL)}$(urea)** were mixed with 1X PBS to obtain a concentration of 4 mg/mL and the pH was adjusted to 7.4. Then, SDS (0.1 w/v%) and Triton X-100 (0.1 v/v%) were added to the solution. The mixture was allowed to stir for 15 min to allow the surfactants to dissolve before adding EDC (2 mg/mL) and NHS (0.5 mg/mL). Dopamine (0.5 M) was added to the solution, which was stirred at 25°C for 16 h. The products, **dopaSF(surfactant)**, were purified by dialyzing against ultrapure water for 48 h, and resulted in yields of approximately 63% at a concentration of 0.75 mg/mL.

Statistical Methods

Statistics were measured by using one-way analysis of variance (ANOVA) using the Holm-Sidak means comparison. A confidence level of 95% was used to determine significant differences.

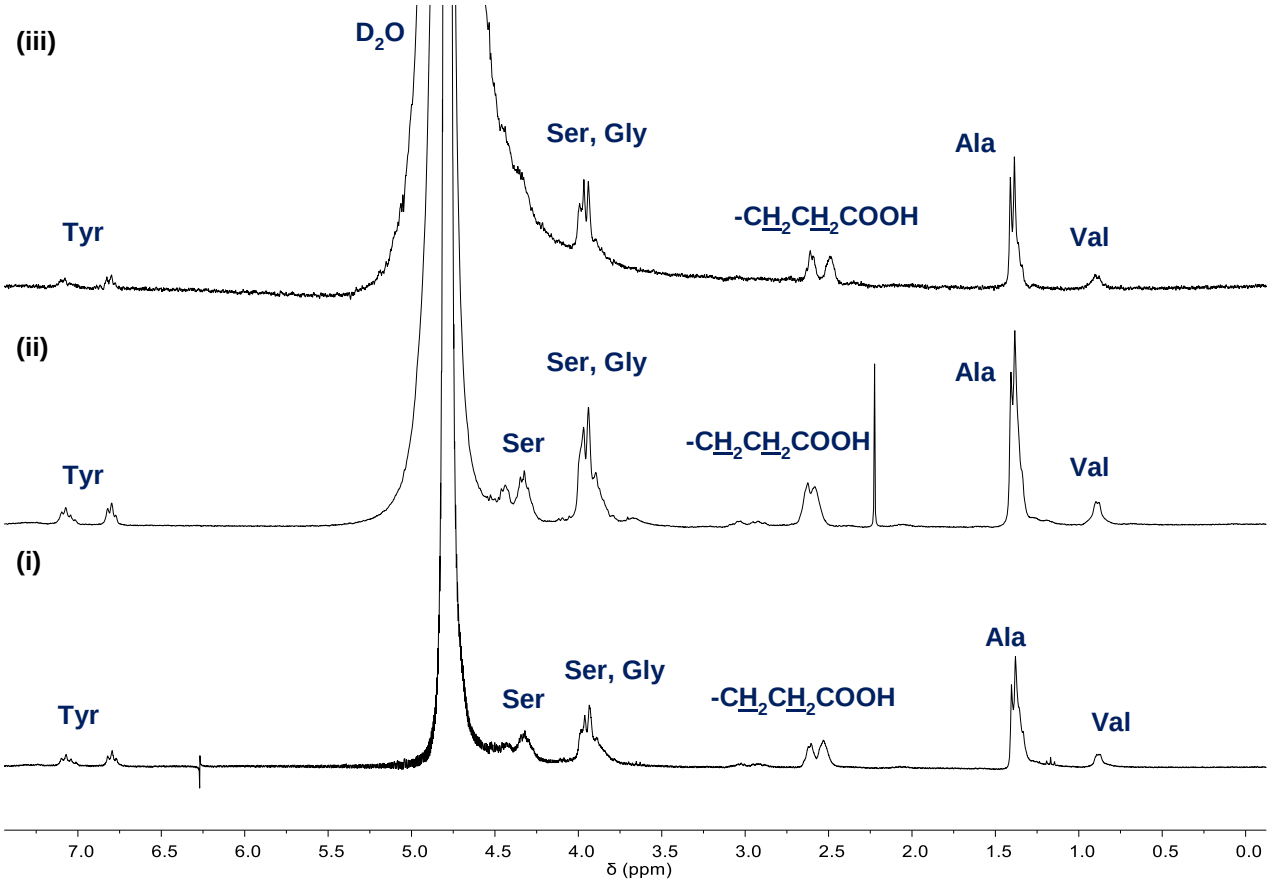


Figure S1. ^{1}H NMR spectra of (i) **cSF$_{(IL)}$(no additives)**, (ii) **cSF$_{(IL)}$(surfactants)**, and (iii) **cSF$_{(IL)}$(urea)** in D_2O.

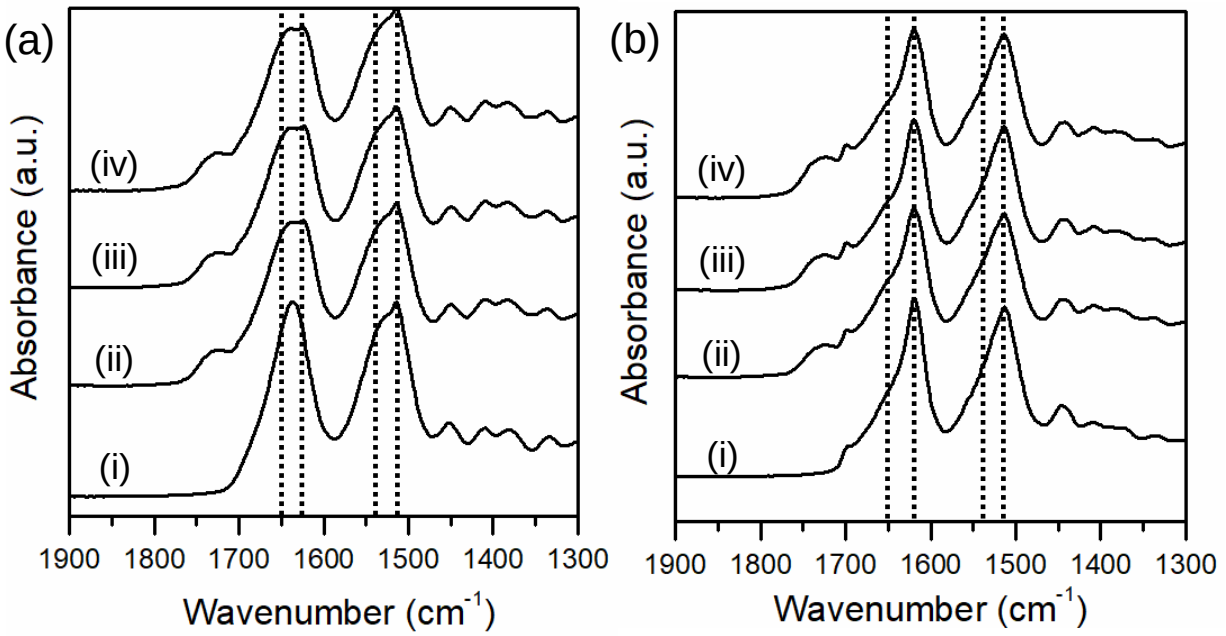
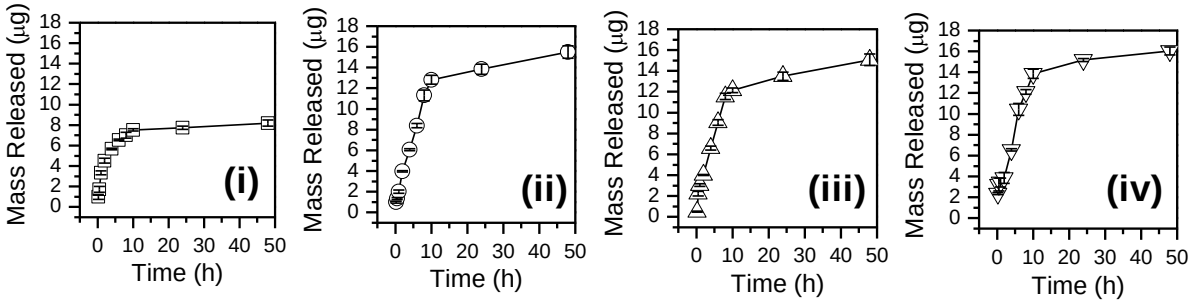


Figure S2. ATR-FTIR spectra of $SF_{(IL)}$ and $cSF_{(IL)}$ films, where the spectra shown in (a) are from "as cast" films and the spectra shown in (b) are after treatment with methanol to induce beta sheet formation. For both graphs, the traces are labeled as follows: (i) **$SF_{(IL)}$**, (ii) **$cSF_{(IL)}$(no additives)**, (iii) **$cSF_{(IL)}$(surfactant)**, (iv) **$cSF_{(IL)}$(urea)**. Reference lines are shown at wavenumbers of 1650, 1625, 1540, and 1515 cm^{-1}.

Table S1. Water contact angle of **$SF_{(IL)}$** and **$cSF_{(IL)}$** conjugates.

Conjugate	Contact Angle (°)
$SF_{(IL)}$	52 ± 6
$cSF_{(IL)}$(no additives)	46 ± 4
cSF(IL)(surfactant)	45 ± 3
$cSF_{(IL)}$(urea)	42 ± 4

(A)



(B)

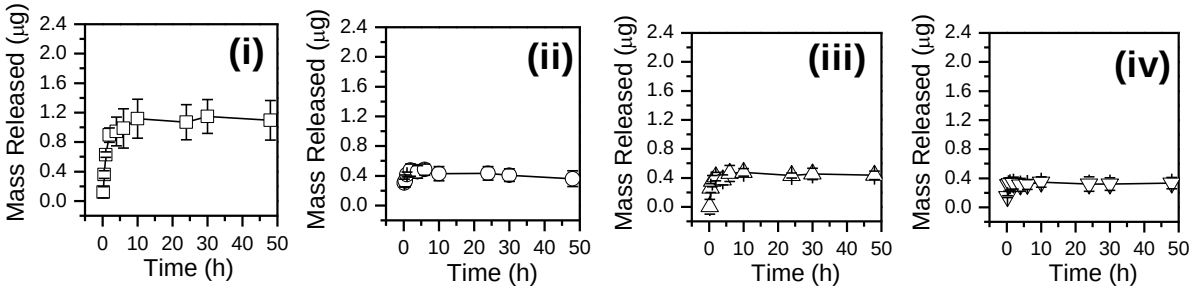
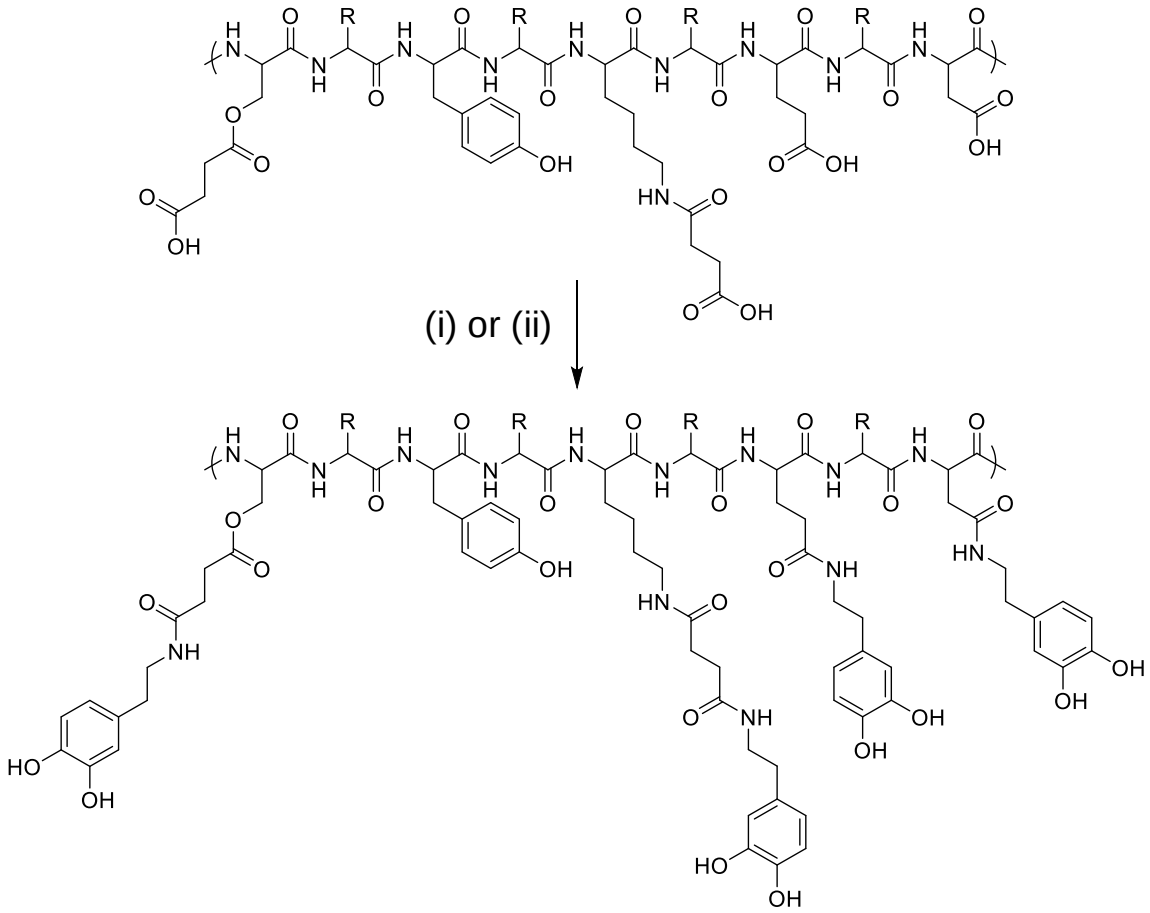


Figure S3. Dye release profiles for (A) methylene blue and (B) orange II. For both sets of plots: (i) **$SF_{(IL)}$**, (ii) **$cSF_{(IL)}$(no additives)**, (iii) **$cSF_{(IL)}$(surfactant)**, (iv) **$cSF_{(IL)}$(urea)**.



Scheme S1. Coupling of dopamine to **cSF$_{(IL)}$** conjugates using EDC. Reaction conditions: (i) 4 mg/mL cSF$_{(IL)}$ in 1X PBS, 2 mg/mL EDC, 0.5 mg/mL NHS, 0.5 M dopamine, 16 h, 25°C; (ii) 4 mg/mL cSF$_{(IL)}$ in 1X PBS, 0.1% (w/v) SDS, 0.1% (v/v) Triton X-100, 2 mg/mL EDC, 0.5 mg/mL NHS, 0.5M dopamine, 16 h, 25°C.

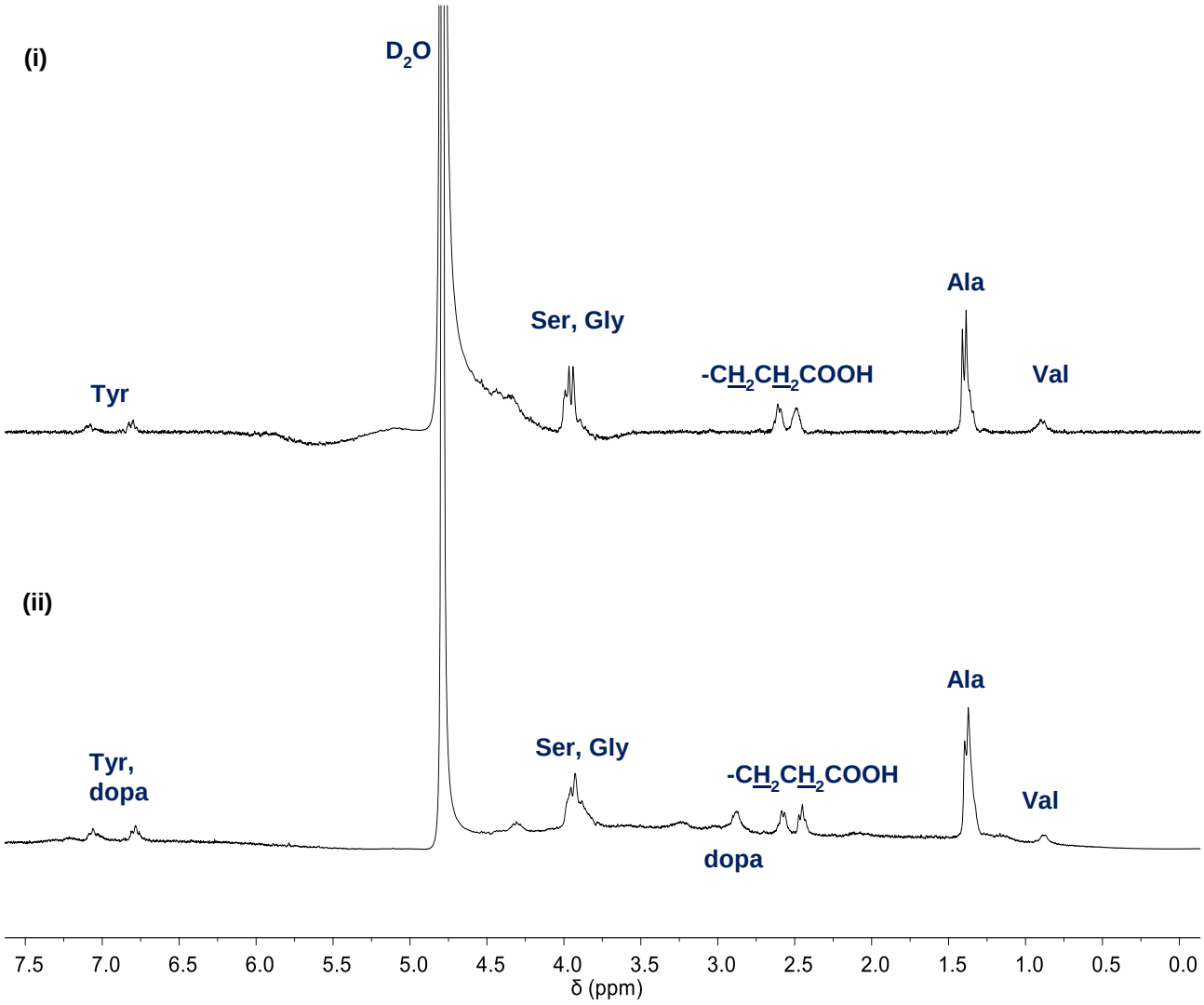


Figure S4. ^{1}H NMR in D_2O of (i) **cSF$_{(IL)}$(urea)** and (ii) dopamine conjugated to **cSF$_{(IL)}$(urea)** using a reaction run without the addition of surfactants.

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