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Chemical Modification of Silk Proteins via Palladium-Mediated Suzuki–Miyaura Reactions

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Suzuki–Miyaura cross-coupling reactions are used to modify the tyrosine residues on *Bombyx mori* silkworm silk proteins using a water-soluble palladium catalyst. First, model reactions using tyrosine derivatives are screened to determine optimal reaction conditions. For these reactions, a variety of aryl boronic acids, solvents, buffers, and temperature ranges are explored. Qualitative information on the reaction progress is collected via high-performance liquid chromatography (HPLC), mass spectrometry (MS), and nuclear magnetic resonance (NMR). Optimized reactions are then applied to silk proteins. It is demonstrated the ability to modify silk fibroin in solution by first iodinating the tyrosine residues on the protein, and then carrying out Suzuki-Miyaura reactions with a variety of boronic acid derivatives. Modification of silk is confirmed with NMR, ion-exchange chromatography (IEC), UV-vis, and infrared spectroscopy (IR).

1. Introduction

Silk fibroin from *Bombyx mori* silkworm cocoons has been regarded for centuries as an exceptional material for wound healing and surgical sutures, and today its applications in the biomedical world are growing.[1,2] The low cytotoxicity, versatile mechanical properties and biodegradability of silk fibroin make it an ideal material for biomedical applications.[3] Silk fibroin can be processed from water into a variety of solid structures including films, hydrogels, scaffolds, and nanoparticles.[4] While the majority of silk is composed of unreactive amino acids, there are a few reactive sites in silk that can be chemically modified. The most abundant reactive amino acids include aspartic and glutamic acid (1.1 mol%), serine (12 mol%) and tyrosine (5.3 mol%).[5]

Emerging applications of chemically modified silk protein include materials for sustained drug delivery, site-specific protein labeling, antibiotic release, and gene delivery.[6–8] Several existing methods to chemically modify silk fibroin are available, such as carbodiimide coupling,[9–12] diazonium coupling to the tyrosine residues,[13–18] tyrosinase-catalyzed grafting[19] and cyanuric chloride coupling.[20] However, many of these strategies are limited by a low degree of functionalization or harsh reaction conditions.

In the past decade, advances have been established in the literature for protein cross-coupling reactions such as Mizoroki-Heck, Sonogashira, and Suzuki-Miyaura.[21,22] These reactions offer the ability to carry out selective, bio-orthogonal reactions on proteins, which presents enormous opportunities to expand the options for chemical modification of silk. Conventional cross-coupling reactions involve organic solvents, but the recent development of completely water-soluble, phosphine-free palladium-catalysts has expanded this already versatile class of reactions.[23] The most promising biocompatible palladium (Pd) catalysts reported have used Pd-pyrimidine ligands, which have shown good results in Sonogashira and Suzuki-Miyaura cross-coupling reactions on proteins under mild conditions.[24–27] Interestingly, silk fibroin has been used in Suzuki-Miyaura, Heck, and Ullmann cross-coupling reactions, but in these instances, the protein served only as a support for the Pd catalyst and was not the target for cross-coupling.[28–30] To the best of our knowledge, no work has been published for the functionalization of silk fibroin via Pd-catalyzed cross-coupling reactions.

Compared to the other methods mentioned above to modify the tyrosine residues in silk, Suzuki–Miyaura reactions have several advantages as they are fast, selective, and have the potential to modify each tyrosine residue multiple times. These reactions can occur under mild conditions making them acceptable for most biomolecules.[31] The ability to perform these reactions in mild conditions with a variety of boron derivatives makes them an advantageous choice for silk chemical modifications.[32] Aqueous Suzuki–Miyaura cross-coupling reactions have been employed to label proteins and DNA with fluorophores,[33] PEGylate halogenated amino acids through the coupling of PEG–boronic acid derivatives,[34] modify naturally iodinated human thyroglobulin,[35] and label proteins on mammalian cell surfaces.[27]

While the previous work demonstrates progress, it also exhibits some challenges to overcome. Most existing Suzuki–Miyaura reactions carried out in aqueous conditions involve peptides or proteins that have been genetically engineered to contain an iodinated phenylalanine residue, which would exclude the use of native silk proteins. In addition, most cross-coupling reactions reported in the literature require a considerable excess of

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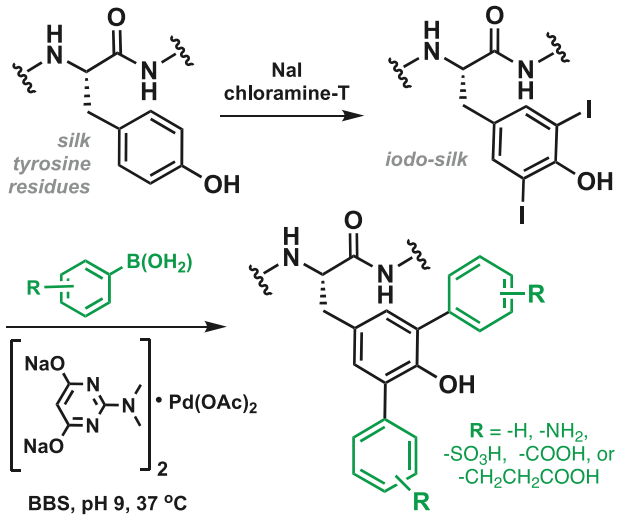


Scheme 1. General strategy to modify silk tyrosine residues through iodination followed by Pd-catalyzed cross-coupling with boronic acid derivatives.

Pd-catalyst and boronic acid relative to the number of tyrosine residues in order to achieve a high degree of modification.[31]

Here, new methods were developed to efficiently modify the native tyrosine residues in silk via aqueous Suzuki–Miyaura reactions (**Scheme 1**). Model reactions using small molecule tyrosine derivatives were first screened to optimize reaction conditions before applying the reactions to silk proteins. For several boronic acid derivatives, >95% conversion to the di-substituted tyrosine model products were observed in less than 30 min using only 5 mol% Pd. Next, a rapid, quantitative method to iodinate the tyrosine residues using mild, aqueous conditions was developed to produce silk derivatives containing two iodines per tyrosine ring. Suzuki-Miyaura reactions were then carried out between iodinated silk and a variety of functionalized aryl boronic acids using a water-soluble palladium catalyst. The resulting silk derivatives were characterized with NMR, UV-vis, and IR spectroscopy, and the ionic charge of the modified proteins were evaluated by binding to an ion exchange column.

2. Results and Discussion

2.1. Optimizing Suzuki–Miyaura Reaction Conditions with Tyrosine Model Compounds

Model reactions using commercially available iodinated tyrosine derivatives were first screened to optimize reaction conditions for silk (**Scheme 2**). For all reactions, a palladium complex with 2-(dimethylamino)−4,6-pyrimidinediol ligands was used as the catalyst as it has been successfully employed in aqueous Suzuki[24] and Sonogashira[25] modification reactions on proteins. HPLC chromatograms and MS data were used to identify the di-substituted product, monosubstituted product, and the starting materials present in reaction mixtures (Figure S1, Supporting Information). Relative integrations of these peaks were used to determine the reaction conversions under various conditions. For select derivatives, products were isolated and further characterized with NMR and MS (Figures S2–S4, Supporting Information).

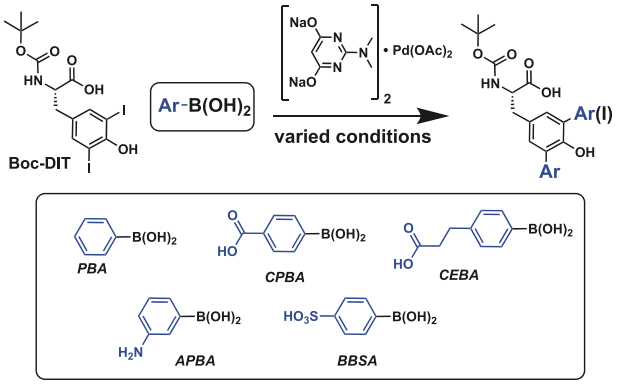
2.1.1. Temperature, Buffer and pH Screening

For the first round of screening, reactions employed *tert*-butyloxycarbonyl-3,5-diiodo-L-tyrosine (Boc-DIT), 4-carboxyphenyl boronic acid (CPBA; 4 mol. equiv. relative to tyrosine; 2 equiv. relative to each iodine) and 5 mol% Pd-catalyst relative to tyrosine (2.5 mol% relative to each iodine). Variables tested included temperature, co-solvent identity, and buffer. The compiled results are shown in **Figure 1**A.

First, product formation was significantly slower at room temperature (only 69% di-substituted after 1 h) compared to 37 °C (100% di-substituted after 30 min). Lower pH also hindered product formation. Literature reports using this or similar catalyst systems typically employ phosphate-buffered saline (PBS) at pH 8.[24–26] However, we found that PBS resulted in lower product yields and slower conversions than reactions carried out in borate-buffered saline (BBS). Therefore, BBS (100 mM, 136 mM NaCl) was used in all subsequent reactions. Reactions in BBS at pH 9 were also found to give superior results to reactions in BBS at pH 8. HPLC data suggested that product formation was significant (>90% di-substituted product) within approximately 10 min of catalyst addition for reactions in BBS pH 9 at 37 °C. Within 30 min, the reaction formed a 100% di-substituted product. Addition of an organic co-solvent also had a significant impact on the reaction. Adding 10% DMSO slowed the reaction, however, DMF was well tolerated. DMF only resulted in a minor decrease in di-substituted product conversion after 10 min (82% instead of 92%); after 30 min this decrease was negligible (97% instead of 100%). Adding 10% acetonitrile (MeCN) significantly inhibited reaction progress, and as a result, it was no longer used as a co-solvent in subsequent reactions.

2.1.2. Survey of Tyrosine Derivatives

The identity of the iodinated L-tyrosine derivative significantly impacted the reaction progress. Initial screening utilized Boc-3,5-diiodo-L-tyrosine (Boc-DIT). N-Acetyl-3,5-diiodo-L-tyrosine could



Scheme 2. Model Suzuki-Miyaura reactions with Boc-DIT and a variety of aryl boronic acids.

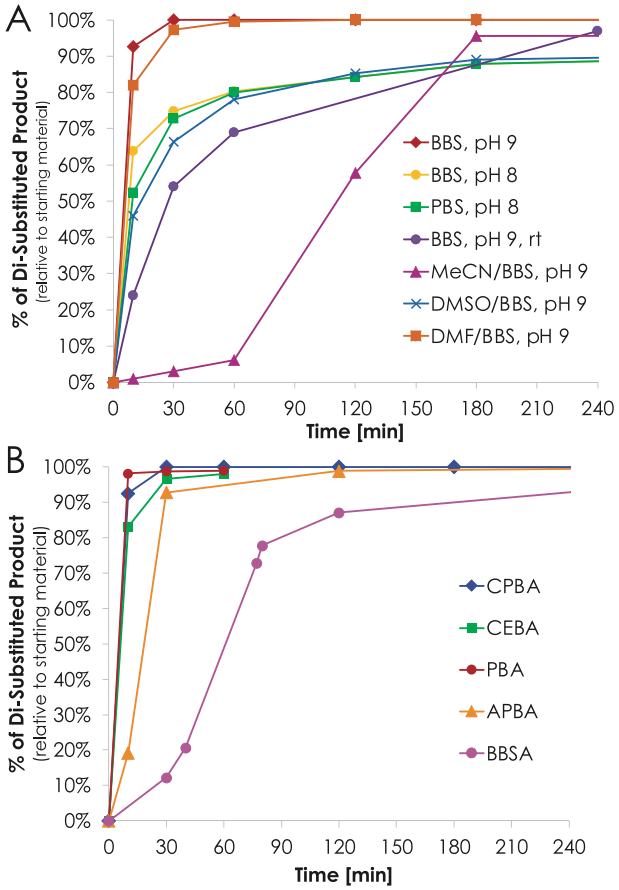


Figure 1. A) Survey of reaction conditions for Boc-DIT. Comparison of pH (8 vs. 9), buffer (PBS versus BBS), temperature (room temp versus 37 °C), and organic co-solvent. Total di-substituted product formation over time is given as a percent relative to the sum of Boc-DIT, mono-substituted product, and di-substituted product as determined by integrating 220 nm peak for each in HPLC. B) Product formation from boronic acid survey. Di-substituted product formation from Boc-DIT in BBS pH 9 at 37 °C, using 4 molar equiv. boronic acid and 5 mol% Pd catalyst relative to tyrosine.

also be used, but the starting material and products had similar retention times in the HPLC making analysis difficult (data not shown). No product formation was observed when tyrosine derivatives with a free amine terminus were used. Unprotected amino acids have been previously shown to coordinate to Pd, so we hypothesize that unprotected tyrosine bound to the catalyst and shuts down the reaction.[36,37] Given these results, the remaining model studies were completed with Boc-DIT only.

2.1.3. Model Compound Boronic Acid Survey

The reactivity of the boronic acids shown in Scheme 1 were compared side-by-side using Boc-DIT in BBS pH 9 at 37 °C, using 4 equiv. boronic acid and either 5 mol% of the Pd catalyst (Figure 1B) or 2.5 mol% of Pd relative to tyrosine (Figure S5, Supporting Information).

Overall, the carboxylic acid derivatives, 4-carboxyphenyl boronic acid (CPBA) and 4-(2-carboxyethyl)phenyl boronic acid (CEBA) performed the best in the Suzuki–Miyaura reactions. With these derivatives, >80% of the starting material was converted to a di-substituted product within 5 min and >95% conversion occurred within 30 min (Figure 1B). Rapid reactions were also observed with phenyl boronic acid (PBA). However, PBA was less soluble in water than the other boronic acids and required addition of 10% DMSO into the BBS solution. Reaction with 4-boronobenzene sulfonic acid (BBSA) was also successful, but was the slowest reaction of those evaluated. Only 87% di-substituted product was formed after 2 h, but eventually reached >90% product within ≈6.5 h.

Boronic acid derivatives containing amine groups were also explored. Rapid product formation was observed when 3-aminophenyl boronic acid (APBA) was employed, where >90% di-substituted product was formed within 30 min. However, when 4-aminomethylphenyl boronic acid was used, no product conversion was observed and only the starting materials remained (data not shown). The same issue was observed when using tyrosine derivatives with a free amine terminus, as discussed in the section above. The alkyl amine found in 4-aminomethylphenyl boronic acid likely binds to the Pd, however, the aryl amine in APBA did not seem to interfere with the catalyst. We attribute the difference to the higher pKa and lower nucleophilicity of the aryl amine as compared to the alkyl amine.

For further confirmation of product structure, products were isolated from reactions with Boc-DIT and CPBA, CEBA, and APBA (isolation procedures are given in Supporting Information). Structures of the major di-substituted products were confirmed using ^{1}H NMR and mass spectrometry (Figures S2–S4, Supporting Information).

Lastly, the model reactions described above were carried out again using a lower amount of palladium to mitigate potential concerns with metal contamination. The overall trends were the same, but the reactions were slightly slower when the Pd was reduced from 5 mol% to 2.5 mol% relative to tyrosine (2.5 and 1.25 mol% relative to each iodine) (Figure S5, Supporting Information. With 5 mol%, CPBA, CEBA, APBA, and PBA reached >90% conversion in 30 min, whereas 60 min were needed to achieve the same conversion when the catalyst was reduced to 2.5 mol%.

2.2. Silk Iodination Reactions

As mentioned in the introduction, most of the literature utilizes genetically engineered proteins containing iodinated phenylalanine residues to carry out Suzuki-Miyaura reactions.[24,27,31] Here, the goal was to modify native silk proteins, so it was necessary to first determine methods to iodinate the tyrosine residues. Initial attempts to iodinate silk solutions utilized N-iodosuccinimide[38] and IPy_2BF_4,[39] both reported to be mild iodinating reagents. However, both of these reagents are insoluble in water, so homogeneous reactions employing co-solvents and heterogeneous reactions where the protein is exposed to solid iodogen were explored. In all cases, either protein precipitation or very low conversion to the iodinated silk was observed.

Next, we turned to a water-soluble oxidant, chloramine-T.[40] When used in conjunction with NaI, very rapid iodination in under 5 min could be achieved. When left to react more than 5 min, the solution turned cloudy suggesting the silk was precipitating. For this reason, reactions were limited to less than 3 min.

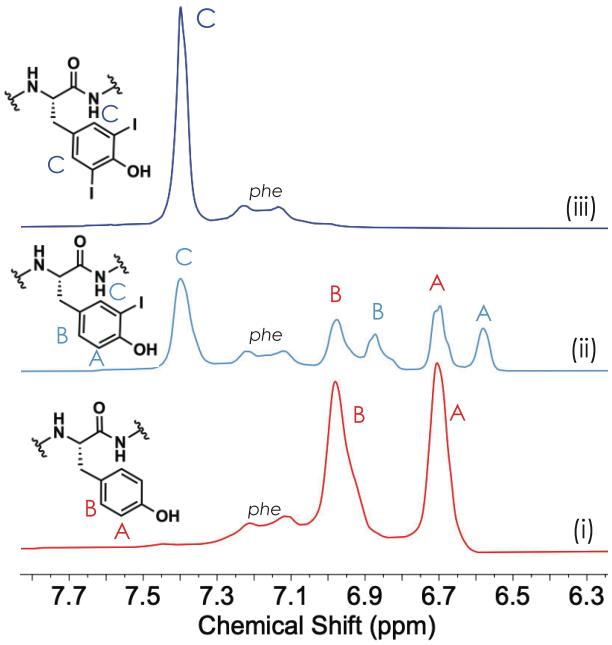


Figure 2. ^{1}H NMR spectra of iodinated silk. Stacked NMR spectra zoomed in on the aromatic region of i) unmodified silk; ii) partially iodinated silk produced with 2 equiv. NaI and chloramine-T; iii) fully iodinated silk after reaction with 3 equiv. NaI and chloramine-T. All samples are ≈1–2 wt.% silk in D_2O. Peaks are normalized to the valine methyl peak at 0.9 ppm.

When 2 equiv. (relative to each tyrosine) of NaI and chloramine-T were used, residual unmodified silk and mono substituted products were seen in ^{1}H NMR (**Figure 2**). However, tyrosine residues could be quantitatively modified when 3 equiv. of NaI and chloramine-T were added. When 6 equivalents of NaI and chloramine-T were used, no further modification occurred and residual p-toluenesulfonamide by-product from the chloramine-T was observed in the ^{1}H NMR. Therefore, 3 equiv. were used for all subsequent experiments. Figure 2 shows the aromatic region of the NMR spectra of i) unmodified silk, ii) partially iodinated silk containing mono- and di-iodotyrosine, and iii) completely di-iodinated silk where the tyrosine residues converge into one signal from the symmetric di-iodinated species. The full ^{1}H NMR spectra with peak assignments can be found in the Supporting Information (Figure S6, Supporting Information). For the remaining text, silk samples where 100% of the tyrosine residues were di-iodinated will be referred to as iodo-silk. Further characterization of iodo-silk will be discussed below alongside the silk derivatives modified by Suzuki-Miyaura reactions.

2.3. Suzuki-Miyaura Reactions on Iodinated Silk Solutions

Cross-coupling reaction conditions identified in the model studies were then applied to solutions of iodo-silk and refined as needed. All boronic acids shown in Scheme 2 were tested under the same conditions to compare their reactivity and extent of modification. The general procedure combined iodo-silk (≈2% w/v in BBS, pH 9), 3–6 equiv. of boronic acid, and 2.5–16 mol% Pd catalyst (equivalents all calculated relative to tyrosine; 277 tyrosine residues per protein). The boronic acid derivatives were pre-dissolved in 1 M NaOH prior to mixing with the silk solution to ensure complete dissolution and to maintain the final reaction pH at 9. The reaction solutions were stirred in an aluminum bead bath pre-equilibrated to 37 °C for times ranging from 30 min to 24 h. At selected time points, the solutions were purified by size exclusion as described in the Experimental Section.

2.3.1. General Reaction Trends Analyzed by NMR

When silk was reacted with CPBA, CEBA, BBSA, and APBA, the protein remained soluble and the success of the reactions was evaluated with ^{1}H NMR. The full spectra and an expansion of the aromatic region for each derivative following reaction with i) 3 equiv. boronic acid and 12 mol% Pd or ii) 6 equiv. boronic acid and 16 mol% Pd (calculated relative to tyrosine) are provided in **Figure 3**. For clarity, the large water solvent peaks between 4.5 and 5 ppm have been excluded from the spectra.

In all cases, new peaks were observed in regions expected for each aryl boronic acid derivative. Peak assignments were made based on comparison with model compound spectra (Figures S2–S4, Supporting Information), 2D COSY NMR spectra (Figure S7, Supporting Information), and peak predictions obtained from MestrReNova software. In general, product peaks continued to increase up to 3 h of reaction time. Longer times did not result in further conversion, so all data provided is from products isolated after 3 h. For all derivatives, low conversion was seen using the 2.5 mol% catalyst loading employed in the model studies. However, increasing to 12 mol% Pd showed evidence of product formation in all cases (Figure 3-i). A further increase to 16 mol% Pd gave only modest increases over reactions utilizing 12 mol% Pd. However, when 6 equiv. boronic acid and 16 mol% Pd was employed, peaks attributed to the products were much more significant for APBA, CEBA, and BBSA (Figure 3-ii). Unfortunately, in most cases, we were unable to establish a reliable calculation of percent conversion due to poorly resolved or overlapping peaks for the newly installed aromatic rings.

For CPBA and BBSA, the deshielding effect of the electron withdrawing groups result in resonances for the newly-installed aromatic groups between 7.5 and 8.0 ppm. Both derivatives showed evidence for the presence of two different aromatic rings. The 2D COSY NMR spectra showed clear interactions between resonances labeled D and E and resonances labeled D' and E', indicating that these peaks were from adjacent protons on different rings (Figure S7, Supporting Information). We hypothesize that the distinct aromatic rings correspond to the mono-substituted (D'E') and di-substituted (DE) products. The spectrum for CPBA-silk was nearly identical even after adding more boronic acid and catalyst, indicating that the addition of more reagents is not useful for this derivative. Peaks for the new aromatic rings (D) in CPBA-silk overlap with the iodo-tyrosine resonance (C), making it impossible to determine if all the iodo-tyrosines have been modified. However, for the BBSA derivative, the addition of more boronic acid and catalyst did have a marked effect. Peaks corresponding to the products (D/E, D'/E') grew significantly and the peak from the initial iodo-silk (C) was no longer visible after reaction with 6 equiv. BBSA and 16 mol% Pd.

The alkyl spacer between the COOH group and the benzene ring in the CEBA derivative shifted the new aromatic resonances

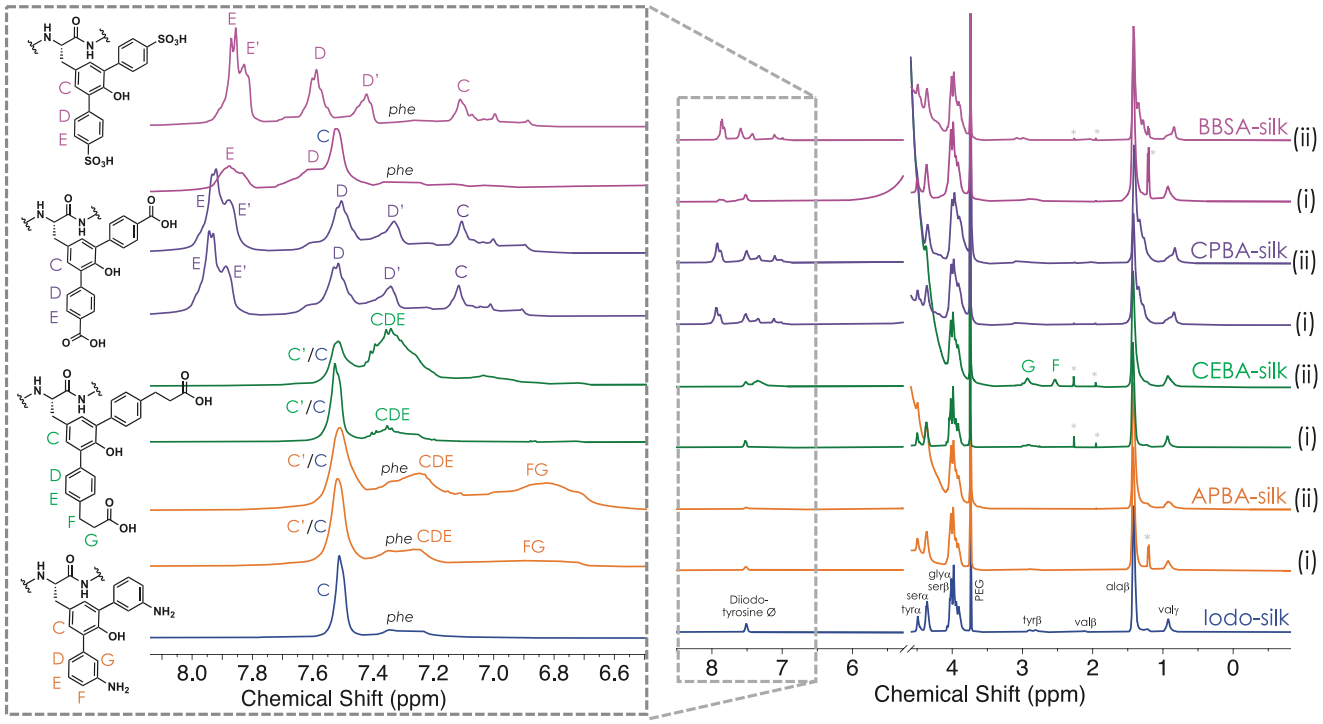


Figure 3. ^{1}H NMR of Suzuki-Miyaura modified silks.Spectra of the products following reaction of iodo-silk for 3 h with the boronic acids noted using i) 3 eq. boronic acid and 12 mol% Pd catalyst or ii) 6 eq. boronic acid and 16 mol% Pd catalyst. 2D NMR supporting peak assignments are given in the Supporting Information (Figure S7, Supporting Information). All samples are ≈0.5–1 wt.% silk in D_2O. The large water peak at ≈4.5 ppm has been removed from the spectra for clarity. Small impurity peaks are labeled with an asterisk (*), although these can typically be removed by passing the product through a second-size exclusion column.

upfield to ≈7.3 ppm, which matches predicted values. CEBA was the only derivative containing aliphatic methylene protons, and these peaks could be readily identified in the products at 2.5 and 2.9 ppm, further confirming the modification. The electron-donating nature of the amine groups in the APBA derivative also result in the resonances for the newly installed aromatic rings to appear upfield in the 6.8–7.3 ppm range. For both the CEBA and the APBA derivatives, marked increases in the product peaks were observed following the reaction with 6 equiv. of boronic acid and 16 mol% Pd. No clear evidence of mono-substituted products was found for either derivative, but the ill-defined, overlapping aromatic peaks could be masking peaks for the mono-substituted products. For the CEBA-silk and APBA-silk, a peak at 7.4 ppm was present after reaction suggesting that di-iodotyrosine and/or mono-substituted residues remained in the products.

Reactions with PBA and iodo-silk gelled within 10 min, and as a result no product could be purified to analyze. The gelation occurred as a result of adding hydrophobic benzene groups to the tyrosine, rendering the substituted protein insoluble in water. The APBA-silk discussed above also had a tendency to gel within a few days of reaction. Therefore, if modification of silk in a soluble form is desired, this reaction sequence should be carried out using boronic acids with polar or preferably charged functional groups. This is a limitation common to all of the silk modification methods. However, the use of this reaction to modify the surface of solid silk structures can accommodate a broader range of boronic acids since the potential for protein precipitation is not an issue.

Control reactions were also carried out to verify that the iodinated tyrosine, boronic acid, and Pd catalyst are all necessary for the reaction to occur, and the new peaks found in the NMR spectra were not simply due to excess reagents. Three samples were prepared: 1) iodo-silk + 14 mol% Pd catalyst (relative to tyrosine), 2) iodo-silk + 8 equiv. CPBA, and 3) Unmodified silk + 14 mol% Pd catalyst + 8 equiv. CPBA. Components were mixed, allowed to react for 1 h at 37 °C, and then purified by size exclusion. ^{1}H NMR spectra from the resulting products are given in the Supporting Information (Figure S8, Supporting Information). Samples of iodo-silk exposed to Pd or boronic acid individually are virtually identical to the starting material. Likewise, when unmodified silk was exposed to Pd and CPBA, no significant changes were observed in the silk peaks consistent with the fact that the iodination step is key for reactivity. Finally, only small peaks corresponding to impurities such as unreacted CPBA, can be seen in the samples, indicating that size exclusion is an effective method for small molecule purification after reaction.

2.3.2. Ion Exchange Chromatography (IEC)

IEC was used to evaluate changes in the overall ionic charge of the proteins after iodination and subsequent Suzuki-Miyaura reactions. The silk samples were dissolved in Tris buffer at pH 8.5 and loaded onto a cationic column. The modified proteins were then eluted by continuously increasing the salt concentration in the buffer and monitoring the UV signal of the eluent at 280 nm.

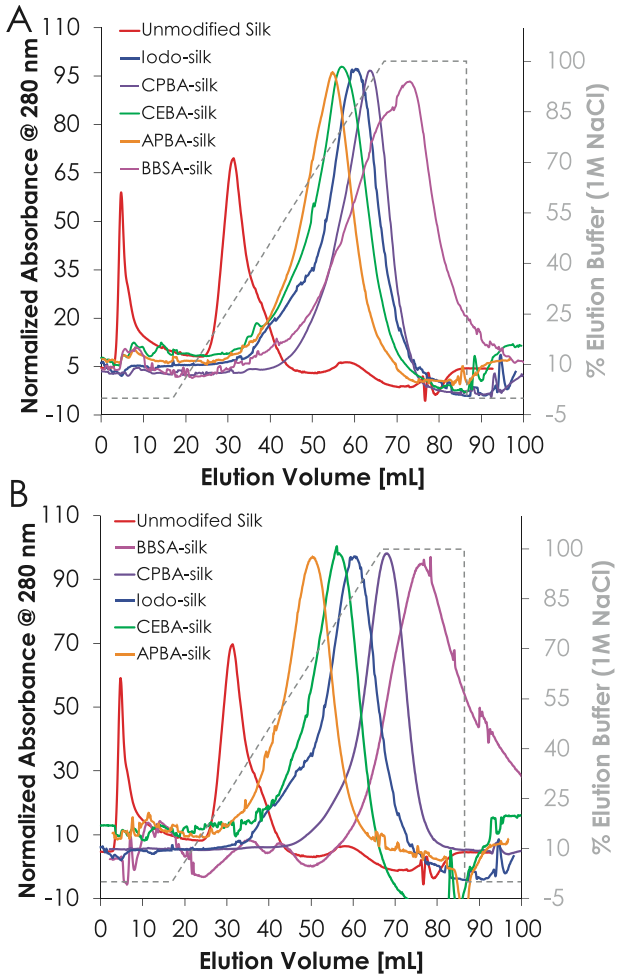


Figure 4. Ion exchange chromatograms. Comparison of the elution profile of A) silk samples reacted with 3 equiv. boronic acid and 12 mol% Pd for 3 h and B) silk samples reacted with 6 equiv. boronic acid and 16 mol% Pd for 3 h. The unmodified silk solution (red) bound weakly to the column while iodo-silk solution (blue, 100% di-iodinated) bound strongly and required high salt concentrations to flush it from the column. The addition of CPBA and BBSA further increased binding, while silk reacted with CEBA and APBA had weaker binding than iodo-silk. The changes in binding became more apparent with samples reacted with 6 equiv. boronic acid and 16 mol% Pd indicating a higher level of modification.

A significant portion of the unmodified silk protein failed to bind to the column and was detected before the salt gradient was introduced (**Figure 4**). Some unmodified silk protein did bind weakly to the column but began to elute when the gradient reached ≈30% 1 M NaCl (≈31 mL). In sharp contrast, the elution volume for iodo-silk was ≈60 mL (≈85% 1 M NaCl) suggesting a significant increase in negative charge over unmodified silk. Samples that were ≈50% iodinated eluted earlier than samples that were 100% diiodinated (data not shown). These observations can be explained by evaluating pK_a of tyrosine before and after iodination. The pI of unmodified silk is ≈4.2.[41] In buffer at pH 8.5, silk should have a slight net negative charge from its natural COOH groups (≈55 aspartic/glutamic acids per protein), while its tyrosine side chains should be protonated (pK_a ~10.4).[42] However, iodination of the aromatic ring in tyrosine strongly effects the acidity of the phenol, and the pK_a of the –OH in diiodotyrosine is predicted to be ≈7.3 (Figure S9, Supporting Information). Thus, the diiodotyrosine residues in silk should be mostly deprotonated in the pH 8.5 buffer used in the IEC, and the net-negative charge of iodo-silk would be expected to increase as compared to unmodified silk. For further confirmation, the same samples were re-run using phosphate buffer at pH 6 as the eluent in the IEC, where the diiodotyrosine groups would be largely protonated. In this case, the iodo-silk began to elute when the gradient reached ≈50% NaCl as opposed to the ≈80% NaCl required to elute the iodo-silk at pH 8.5 (data not shown).

The silks reacted under Suzuki–Miyaura conditions also eluted at volumes clearly distinct from the unmodified silk and the iodo-silk starting material, which indicated further changes to the net ionic charge of the protein. Chromatograms of the silk derivatives reacted with 3 equiv. boronic acid and 12 mol% Pd are provided in Figure 4A. In all cases, the changes in binding showed the same trends but were more pronounced when iodo-silk was reacted with 6 equiv. boronic acid and 16 mol% Pd, as shown in Figure 4B. The CPBA and BBSA-modified silks required significantly higher salt concentrations to elute them from the cationic column, indicating that the derivatives had more negative charge due to the introduction of carboxylic and sulfonic acid functional groups, respectively (Figure S9, Supporting Information). As expected, the APBA-modified silk eluted at a lower volume than the iodo-silk (51 mL versus 60 mL, respectively). The aromatic amine groups are expected to be neutral above pH ≈6 and the pKa of the tyrosine is predicted to rebound to ≈9.7 once the iodine groups are replaced with aryl groups (Figure S9, Supporting Information). Therefore, fully modified tyrosine residues in APBA-silk should not be charged in the pH 8.5 buffer. The result is weaker binding to the cationic column, which was observed in the form of smaller elution volumes. However, the NMR suggests that the reaction does not fully go to completion, so it is expected that some of the tyrosine residues are still iodinated. Therefore, the APBA-silk is expected to have a larger net negative charge than unmodified silk and elute at higher salt concentrations.

Contrary to expectations, CEBA-modified silk eluted slightly before the iodo-silk ($V < 60$ mL) suggesting it binds less strongly to the column than iodo-silk. There is no clear explanation for why CEBA-silk did not bind strongly to the column similar to CPBA-silk.

2.3.3. UV-Vis Spectroscopy

The UV-Vis spectra of unmodified and the modified silks were also compared, as shown in **Figure 5**A. After iodination, a clear shift of the tyrosine maximum absorbance to a higher wavelength was observed, shifting from 276 nm to 312 nm after iodination. The absorbances only shifted slightly for the Suzuki-modified silk derivatives, resulting in max absorbances in the range of 306–310 nm.

2.3.4. ATR-FTIR Spectroscopy

A comparison of FTIR spectra from silk films before and after treatment with ethanol is a straightforward way to observe

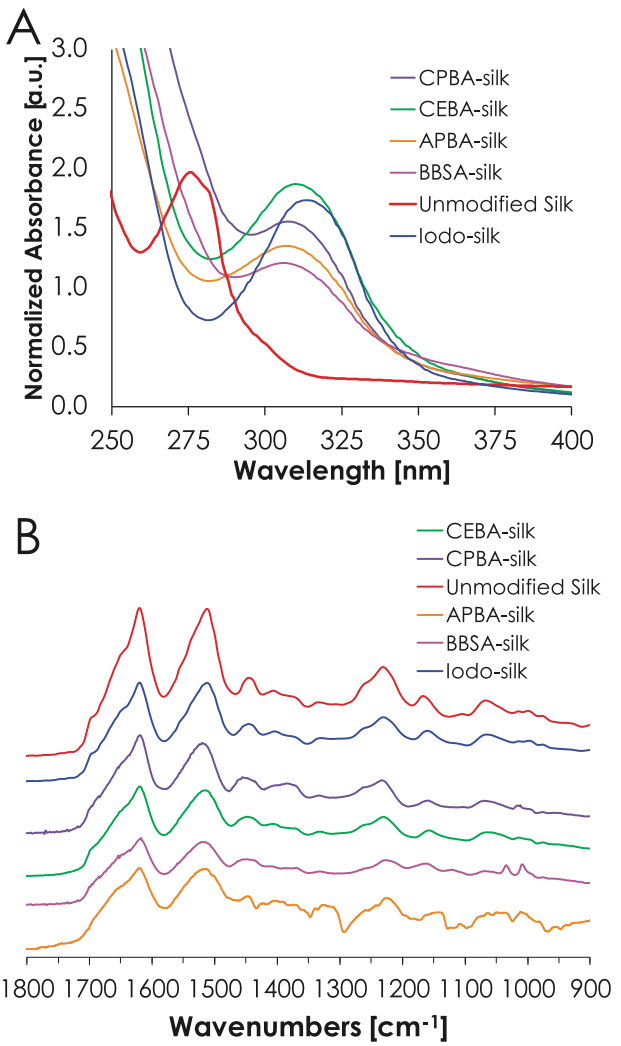


Figure 5. Additional characterization of iodo-silk and Suzuki-Miyaura modified silks reacted with 3 equiv. boronic acid and 12 mol% Pd for 3 h. A) UV-Vis spectra. Unmodified silk solution (red) had a λ_{max} of 276 nm, while iodo-silk solution (blue) exhibited a shifted λ_{max} of 313 nm. The Suzuki-Miyaura modified silks all had similar spectra with λ_{max} ranging from 306 to 310 nm. B) FTIR-ATR spectra. The amide bond region is shown to highlight characteristic absorbances after treatment with ethanol.

the protein assembly in silk fibroin. Treatment with ethanol has been shown to prompt beta-sheet structure assembly and render the films insoluble.[43] Unstructured silk typically has broad bands ≈1640–1650 nm and ≈1540 nm, corresponding to the C≐O stretching and N–H bending modes, respectively. After silk assembles into its beta-sheet structure the vibrational modes shift to lower wavenumbers and appear sharper.[44] FTIR spectroscopy of all chemically modified silk films showed vibrations characteristic of silk assembled into a beta-sheet structure at 1620 cm^{-1} and 1512 cm^{-1} corresponding to the C≐O stretching and N-H bending modes, respectively (Figure 5B). Therefore, the iodo-silk and Suzuki-modified proteins retained their ability to form a beta-sheet structure. This is important as the mechanical strength of the protein is dependent on the extent of beta-sheet formation. While most of the spectra were indistinguishable from plain silk, new peaks corresponding to the S≐O bonds in the BBSA derivative were observed ≈1000–1050 cm^{-1} (Figure 5B).

2.3.5. Elemental Analysis of the Products

One drawback for the use of transition metal catalysts in bioconjugation reactions is the potential for the metal to bind to the protein. Here, no specific efforts were made to scavenge residual palladium retained in the silk after the reaction. To determine the amount of Pd remaining, the elemental composition of the samples were analyzed using energy-dispersive X-ray spectroscopy (EDS). Suzuki–Miyaura modified silks were reacted with 3 equiv. boronic acid and 12 mol% Pd for 3 h, purified, and then dried into a film prior to analysis. Films cast from plain silk and iodo-silk solutions were also analyzed. Representative EDS spectra (Figure S10, Supporting Information) and a compiled table containing the average elemental composition of modified silk samples (Table S1, Supporting Information) are given in the Supporting Information. All Suzuki-modified samples were found to contain ≈0.1%–0.15% palladium (atomic%). No clear trends were observed among the different derivatives, but the amount of Pd retained did scale with the original mol% of Pd catalyst added. However, the amount retained was only a fraction of what was added initially, suggesting that the majority of the Pd was removed during purification. However, if lower levels of Pd are required for future applications, there are several soluble and resin-bound Pd scavengers that have been developed and utilized in the pharmaceutical industry.[45] Of particular interest, 3-mercaptopropanoic acid could be employed as it has been shown to effectively scavenge Pd from other proteins modified using Suzuki-Miyaura reactions.[31]

The elemental analysis also revealed other trends. As expected, the iodine content was highest in the iodo-silk and then dropped after the Suzuki-Miyuara reactions (Table S1, Supporting Information). The level of iodine was lowest in the CPBA-silk, consistent with the NMR data (Figure 3) that showed it reacted to a greater extent than the BBSA and APBA derivatives under these reaction conditions. Likewise, the sulfur content increased significantly in the BBSA-silk due to the newly installed sulfonic acid-functional groups but no increase was observed in the CPBA or APBA-silk samples, as expected.

3. Conclusion

First, in order to optimize the reaction conditions for Suzuki-Miyaura cross-coupling, a screen was carried out using the model compound Boc-3,5-diiodo-L-tyrosine (Boc-DIT) and characterized with HPLC. The screening revealed that these reactions were most efficient with heat (37 °C) in borate buffer pH 9 without co-solvent. Under those conditions, a reaction with 4 eq. CPBA and 5 mol% Pd catalyst (rel. to Boc-DIT) formed >90% di-substituted product within 10 min. The model compound studies also included a survey of various boronic acids. Of the boronic acids, CPBA, CEBA, PBA, and APBA performed the best, achieving more than 90% di-substituted product within 30 min in a reaction with 4 eq. boronic acid 5 mol% Pd. In contrast, BBSA formed less than 40% di-substituted product in the same amount of time.

From the silk iodination reactions, we concluded that 3 equivalents of NaI (per tyrosine) was necessary to fully iodinate 100% of the silk tyrosine residues. The di-iodinated silk product was then successfully modified using Suzuki-Miyaura reactions. Some common trends were observed when variables including reaction time, equivalents of boronic acid and mol% Pd were changed. In general, reactions formed more product after 3 h compared to 1 h. Typically more product was formed by increasing the amount of catalyst from 12 mol% to 16 mol% Pd. IEC demonstrated a change in overall charge of the protein after modification which supported initial ^{1}H NMR findings. For all the boronic acids, silk retained the ability to form its beta-sheet structure following modification. This was confirmed by FTIR and the results were promising for future applications of modified silk material. Altogether this research demonstrated the successful chemical modification of silk tyrosine through aqueous Suzuki-Miyaura cross-coupling, giving access to previously unexplored silk derivatives.

4. Experimental Section

Instrumentation: Ultraviolet–Visible spectroscopy (UV–Vis) of silk solutions were measured on a BioTek Synergy/H1 microplate reader, in BBS pH 9.4. Fourier transform Infrared (FTIR) spectra of solid silk films were recorded on a Thermo Nicolet 6700 FTIR in the range of 4000 to 400 cm^{-1}. The concentration of Pd in the catalyst was determined by flame atomic absorption spectrometry (FAAS) and recorded on a Varian SpectrAA 220FS. Proton nuclear magnetic resonance (^{1}H NMR) spectra of reagents, silk solution, and isolated model compounds were recorded on a Bruker Avance III 500 MHz spectrometer. HPLC data collection for model compound studies was performed using a reverse-phase C_{18} column with a Varian Prostar HPLC with PDA UV/Vis detector. A gradient of MeCN/H_2O (with 0.1% TFA or formic acid) was used at a flow rate of 1.0 mL min^{-1}, and peaks detected at $\lambda = 220$ nm were used for analysis. Mass spectrometry data was collected using an Advion Expression LCMS with a Thermo Scientific Dionex UltiMate 3000 HPLC. Ion exchange chromatography (IEC) was performed on silk samples using a General Electric ÄKTA prime plus with a HiTrap Q HP cartridge. Elemental analysis was carried out on dried silk films using an Oxford X-Max EDS detector within a JEOL JSM-7200F Field Emission SEM.

Catalyst Preparation: Palladium(II) (10 mM) catalyst was prepared as follows. In a 10.00 mL volumetric flask, 2-(dimethylamino)−4,6-pyrimidinediol (31 mg, 0.2 mmol) and 4.0 mL of a 0.10 M NaOH stock solution were combined. The pyrimidine ligand was dissolved completely by stirring for 5 min in a bead bath preheated to 65 °C at which point the solution turned light pink. $Pd(OAc)_2$ (22 mg, 0.1 mmol) was then added to the flask. The mixture was stirred vigorously at 65 °C for 2 h (open air) to give a homogenous clear brown-orange solution. After cooling to room temperature, the stir bar was removed and the solution was diluted to 10.00 mL with nanopure water to give a 10 mM Pd(II) catalyst solution. This solution was stored in a capped glass vial in a dark refrigerator at 4 °C.

General Procedure for Suzuki-Miyaura Reactions with Model Compounds: Tyrosine derivatives tested include 3,5-diiodo-L-tyrosine (DIT), Boc-DIT, and N-acetyl-3,5-diiodo-L-tyrosine. Boronic acids screened are shown in Scheme 2. The buffer solution used in the reaction was either PBS (pH 8, 100 mM phosphate, 136 mM NaCl) or BBS (pH 8, 100 mM phosphate, 136 mM NaCl). Co-solvents tested with Boc-DIT and CPBA include acetonitrile (MeCN), dimethylformamide (DMF), and dimethyl sulfoxide (DMSO). The co-solvents were added to BBS pH 9 to obtain a 10% co-solvent mixture (total volume 2.5 mL).

The following procedure is for a 2.5 mL reaction with Boc-DIT, 4 molar equivalents of boronic acid, and 5 mol% catalyst (both relative to tyrosine). However, reactions were also carried out on scales ranging from 1 to 5 mL with catalyst concentrations ranging from 2 to 5 mol% Pd and boronic acid ranging from 2 to 8 molar equivalents relative to each tyrosine.

Boc-DIT (15 mg, 0.028 mmol) and boronic acid (variable mass, 0.112 mmol) were added to a 20 mL glass vial and dissolved in 2.5 mL buffer using mild heat and sonication when needed. An aliquot of the solution was collected as a $T = 0$ time point before the addition of the Pd-catalyst and used as a reference for HPLC. After the addition of Pd-catalyst solution (140 µL, 0.0014 mmol), the mixture was capped and stirred in an aluminum bead bath pre-equilibrated to 37 °C. Aliquots of the reaction mixture were collected and diluted for HPLC analysis at designated time points. MeCN was found to severely inhibit the reaction, so aliquots were diluted 10-fold in 1:1 MeCN/H_2O to prevent further reaction prior to analysis. HPLC: Boc-DIT $R_t = 7.9$ min, CPBA $R_t = 5.0$ min, di-substituted product $R_t = 7.4$ min, mono-substituted product $R_t = 7.6$ min

Procedures used to isolate model compounds for further characterization are given in the Supporting Information.

Preparing Silk Solution: Silk fibers were isolated and purified to remove the sericin protein from the silk. First, 20 cocoons were cut into several pieces and the worm was discarded, and then boiled in 3 L of 0.02 M Na_2CO_3 in DI water for 1 h. Afterward, fibers were rinsed for 10 min in boiling DI water, then rinsed three times for 10 min each with room temperature DI water and spread out to dry overnight. To make the silk solution, the dry fibers were weighed and added to a LiBr (9 M) solution (5 mL of LiBr per gram of silk fibers). The fibers were placed in a 60 °C oven and dissolved into the solution after ≈45 min. Next, the silk solution was transferred to hydrated dialysis tubing (Fisherbrand, 3500 MWCO) and dialyzed against 3 L of DI water. The dialysis water was changed after 1 h, and again after 3 h, then was left to dialyze overnight. The dialysis water was changed 3 more times over the course of 12 h then the silk solution in dialysis tubing was removed. A small amount of the solution (0.5 mL) was used to check that the concentration was within the target concentration (either 2% or 7% w/v). In cases where the silk concentration was not within the desired range, the silk solution was either diluted with nanopure water (or buffer) or alternatively concentrated by laying the dialysis bag containing the silk solution on dry polyethylene glycol (PEG 12k MW) pellets for 2–5 h. The solution was pipetted out of the tubing and stored in the refrigerator. For buffered silk, the dialysis tubing was placed in either borate-buffered saline (BBS, 100 mM borate, 136 mM NaCl, pH 9) or phosphate buffer (50 mM, pH 7.5) on dialysis day two and the same procedure was followed except fresh buffer was used in place of water changes. ^{1}H NMR (500 MHz, D_2O, ppm) δ 7.2 (m, 1H), 7.0 (m, 2H), 6.7 (m, 2H), 4.5-4.3 (m, 3H), 4.2 (m, 5H), 3.9 (m, 19H), 3.0-2.7 (m, 2H), 2.0 (m, 1H), 1.3 (m, 15H), 0.8 (m, 3H). UV-Vis (BBS pH 9): λ_{max} 276 nm.

Silk Molarity Calculations: Silk is known to degrade during the purification and solubilization process leading to a dispersity in molecular weight that makes the reagent calculations imprecise. To estimate the amounts of reagents needed for each reaction, the molar ratios reported below were calculated assuming the full molecular weight of silk (391 kDa) and the fact that silk contains 5.3 mol% tyrosine (277 tyr residues per protein). The concentration (mg/mL) of silk solutions were determined by drying aliquots of the solution in an oven at 60 °C for at least 1 h and weighing the residual protein.

Iodination of Silk Solution with Chloramine-T: Representative conditions are given below to convert 100% of the tyrosine residues to diiodotyrosine on a 3 mL scale using silk at ≈5% w/v. However, reactions were also carried out on scales ranging from 0.5 to 8 mL, or with less of the iodinating reagents to produce partially modified silk. In a 20 mL scintillation vial, chloramine-T trihydrate (92 mg, 0.33 mmol; 3 equiv.) was dissolved in 0.80 mL phosphate buffer (50 mM, pH 7.5) and then solid NaI (50 mg, 0.33 mmol; 3 equiv.) was added to the vial. Then, silk solution (2.2 mL, 7% w/v silk; est. 0.4 µmol silk and 110 µmol tyrosine) in 50 mM phosphate buffer (pH 7.5) was immediately added to the mixture and stirred gently to combine. After 2 min, the reaction was quenched with 0.3 mL of a sodium thiosulfate solution (2.4 M in phosphate buffer pH 7.5). Upon addition of the quenching solution, the reaction mixture was stirred gently for 3 min, and then was purified by size exclusion (illustra NAP columns). BBS (pH 9) was used both to equilibrate the column and elute the product. In some cases, the product was purified with a second column using

either BBS, D_2O, or nanopure water. ^{1}H NMR (500 MHz, D_2O, ppm) δ 7.40 (s, 2H), 7.2 (m, 1H), 4.4–4.1 (m, 11H), 3.9–3.7 (m, 20H), 2.9–1.8 (m, 3H), 1.3 (m, 20H), 0.8 (m, 3H). UV-Vis (BBS pH 9): λ_{max} = 312 nm.

General Procedure for Suzuki-Miyaura Reactions with Silk: Iodotyrosine silk used in Suzuki-Miyaura reactions was prepared as described above. Representative conditions are given below to modify silk samples where 100% of the tyrosine residues were diiodinated as described in section 5.6. The general reaction described used CPBA, however, reactions were also carried out with the remaining boronic acids shown in Scheme 2. Reaction volumes ranged from 0.25 to 6 mL, equivalents of boronic acid ranged from 3 to 6 (relative to tyrosine), and the Pd catalyst loading ranged from 2.5 to 16 mol% (relative to tyrosine).

In a 20 mL glass vial, CPBA (14 mg, 85 µmol, 6 eq. rel. to tyrosine) was dissolved in 96 µL NaOH (1 M) and then combined with 1.0 mL of 2% w/v diiodotyrosine silk in BBS (20 mg silk; est. 5E-5 mmol silk and 14 µmol tyrosine). This solution was stirred in an aluminum bead bath pre-equilibrated to 37 °C. The Pd-catalyst solution (227 µL, 2.2 µmol, 16 mol% rel. to tyrosine) was added to the solution and left to stir in an aluminum bead bath pre-equilibrated to 37 °C. At select time points (30 min,1 h, 2 h, 3 h, or 24 h) the solution was purified by size exclusion (illustra NAP columns). BBS was used both to equilibrate the column and elute the product. In some cases, the product was purified with a second column using either BBS, D_2O or nanopure water to equilibrate and elute the product. In some cases, the BBS purified solution was concentrated by centrifugal concentration (Vivaspin 6; 10 kDa MWCO) until the volume was reduced by half. The product was then analyzed with ^{1}H NMR and UV-Vis.

[CPBA-modified silk]: ^{1}H NMR (500 MHz, D_2O) δ 7.8 (m, 3H), 7.5-6.7 (m, 5H), 4.4–4.1 (m, 19H), 3.9–3.7 (m, 25H), 3.1–1.7 (m, 2H), 1.3 (m, 24H), 0.8 (m, 3H). UV-Vis (BBS pH 9): λ_{max} = 306 nm.

[CEBA-modified silk]: ^{1}H NMR (500 MHz, D_2O) δ 7.7–6.5 (4H), 3.9 (m, 27H), 3.1–1.9 (m, 3H), 1.3 (m, 20H), 0.8 (m, 3H). UV-Vis (BBS pH 9): λ_{max} = 308 nm.

[APBA-modified silk]: ^{1}H NMR (500 MHz, D_2O) δ 7.6–6.4 (3H), 3.9-3.7 (m, 28H), 3.1–1.7 (m, 2H), 1.3 (m, 22H), 0.8 (m, 3H). UV-Vis (BBS pH 9): λ_{max} = 308 nm.

[BBSA-modified silk]: ^{1}H NMR (500 MHz, D_2O) δ 7.8 (2H), 7.6–6.8 (m, 4H), 4.4 (m, 8H), 4.2 (m, 6H), 4.0–3.7 (m, 19H), 3.1–1.9 (m, 3H), 2.0 (m, 1H), 1.3 (m, 17H), 0.8 (m, 3H). UV-Vis (BBS pH 9): λ_{max} = 302 nm.

Procedure for Ion-Exchange Chromatography: The binding affinity of the modified silk derivatives to a cation-exchange column (5 mL HiTrap Q HP cartridge) was evaluated. A 50 mM Tris buffer (25 mM NaCl, pH 8.5) solution was used for instrument equilibration and a 50 mM Tris buffer (1 M NaCl, pH 8.5) was used as the elution buffer. Prior to data collection the silk derivatives were purified and concentrated according to the procedure described in section 4.7, resulting in ≈20 mg mL^{-1} silk solutions in BBS. To prepare samples for IEC injection, 200 mL of silk was mixed with 400 mL of equilibration buffer and then 500 mL of this solution was loaded onto the column and washed with three column volumes (15 mL) before eluting over a 50 mL gradient. A constant flow rate of 5 mL mi^{-1}n was maintained for the entire experiment, and protein elution was monitored using a UV detector at 280 nm. Data was recorded for unmodified, iodinated, and Suzuki-Miyaura-modified silk. Suzuki products from reactions with CPBA, CEBA, APBA, and BBSA were analyzed. The pK_a predictions for modified silk proteins were estimated using Chemicalize – Instant Cheminformatics Solutions software.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

iodination, palladium catalyst, protein modification, Suzuki-Miyaura reaction, silk, tyrosine

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Supporting Information

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Chemical Modification of Silk Proteins via Palladium-Mediated Suzuki–Miyaura Reactions

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Supporting Information

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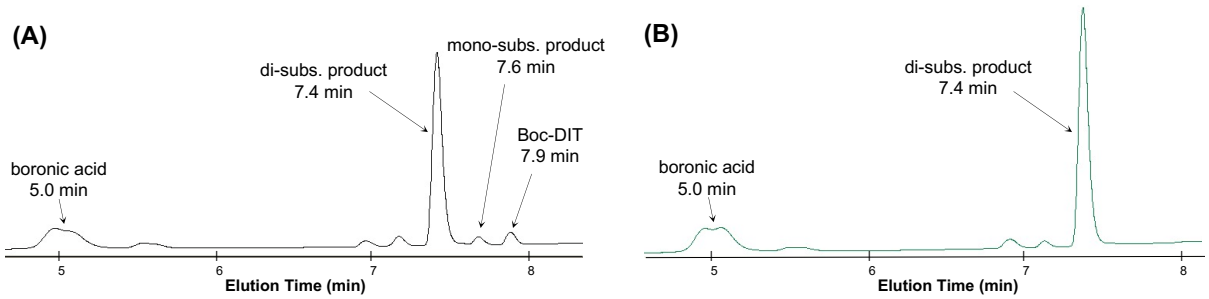


Figure S1. Representative HPLC chromatograms of Suzuki-Miyaura product formation using the model compounds over time. Reaction progress in BBS pH 9 at 37 ºC using Boc-DIT, 4 eq. CPBA and 5 mol% Pd-catalyst relative to tyrosine after (A) 10 min and (B) 30 min (220 nm).

General Procedures for Isolation of Products from Model Compound Reactions

A Suzuki-Miyaura reaction was carried out according to the general procedure with 2.5 mol% Pd-catalyst (70 µL, 0.7 µmol) and 4 molar equivalents of boronic acid (varying mass, 0.112 mmol) relative to Boc-DIT (15 mg, 0.028 mmol) for 24 h. Afterwards, any black solid palladium that had accumulated in the vial was gravity filtered from the solution. Approximately 4 mL of 1 M hydrochloric acid (HCl) was added, turning the solution pink and producing a white precipitate. The precipitate was immediately filtered with a Buchner funnel and rinsed with water. The solid was dissolved in 20-30 mL ethyl acetate and placed in a separatory funnel. The solution was washed 3 times with ~10 mL HCl (1 M) and 3 times with ~10 mL of nanopure water. The ethyl acetate layer was drained and dried over sodium sulfate. The solution was decanted into a flask, the solvent was removed by rotary evaporation, and the resulting solid was dried *in vacuo*. The isolated white powder was dissolved in deuterated dimethyl sulfoxide (DMSO) and analyzed with ^{1}H NMR; 20 µL of the NMR sample was diluted with 200 µL of a 1:1 MeCN/H_2O mixture and analyzed with LC-MS. LCMS was carried out on an Advion Express LCMS coupled to a ThermoScientific Dionex UltiMate 3000 HPLC equipped with a reverse-phase C_{18} column and a diode array UV-Vis spectrophotometer. A MeCN/H_2O gradient containing 0.1% formic acid was used to elute the products.

Product from CPBA [(*S*)-5'-(2-((*tert*-butoxycarbonyl)amino)-2-carboxyethyl)-2'-hydroxy-[1,1':3',1"-terphenyl]-4,4"-dicarboxylic acid]: ^{1}H NMR (500 MHz, DMSO) δ 8.19 (d, J = 10 Hz, 4H), 7.84 (d, J = 10 Hz, 4H), 7.37 (s, 2H), 6.24 (d, J = 8.4 Hz, 1H), 4.61 (m, 1H), 3.37 (m, 1H), 3.15 (m, 1H), 1.40 (s, 9H).

Product from CEBA [(R)-3,3'-(5'-(2-((tert-butoxycarbonyl)amino)-2-carboxyethyl)-2'-hydroxy-[1,1':3',1"-terphenyl]-4,4"-diyl)dipropionic acid]: ^{1}H NMR (500 MHz, DMSO) δ 8.03 (s, 1H), 7.53 (d, *J* = 8 Hz, 4H), 7.27 (d, *J* = 8 Hz, 4H), 7.05 (s, 2H), 6.61 (s, 1H), 4.14 (m, 1H), 3.00 (m, 2H), 2.87 (m, 4H), 2.57 (m, 4H), 1.32 (s, 9H).

Product from APBA [(S)-2-((tert-butoxycarbonyl)amino)-3-(3,3"-diamino-2'-hydroxy-[1,1':3',1"-terphenyl]-5'-yl)propanoic acid]: ^{1}H NMR (500 MHz, DMSO) δ 7.27 (m, 2H), 7.06 (m, 2H), 6.85 (s, 2H), 6.80 (m, 2H), 5.77 (s, 1H), 4.68 (s, 2H), 4.09 (s, 1H), 3.20 (m, 2H), 1.35 (s, 9H).

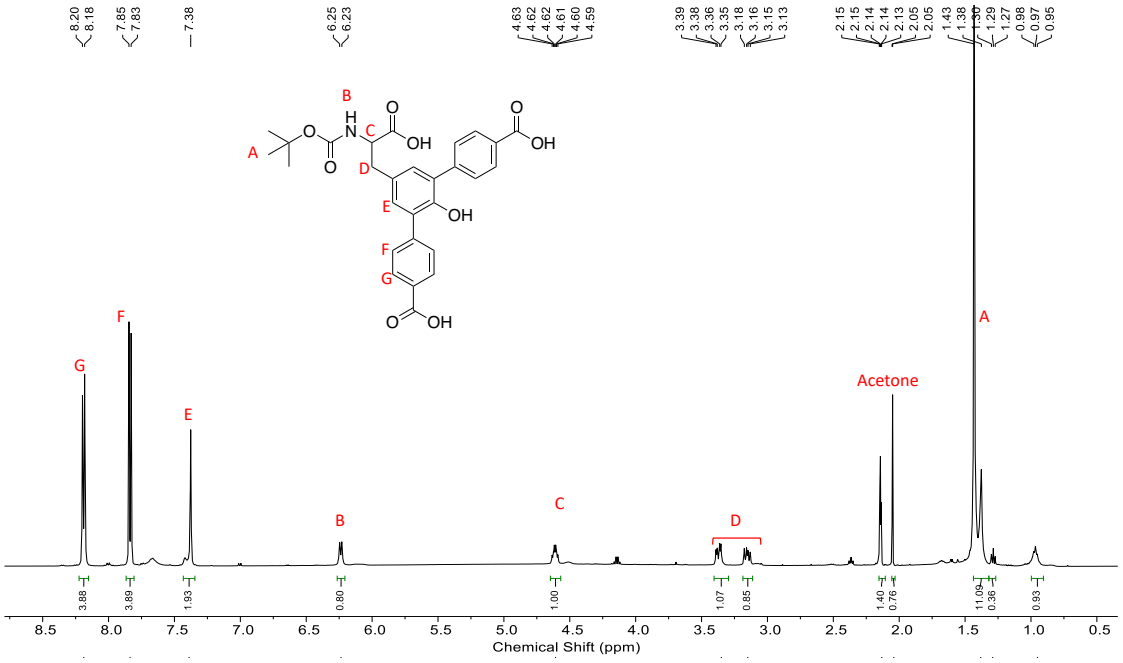
This isolation procedure was unsuccessful with BBSA reactions, and therefore no ^{1}H NMR could be performed.

Characterization of Products from Model Compound Reactions

Isolated products from reactions with Boc-DIT and CPBA, CEBA and APBA were examined with ^{1}H NMR and mass spectrometry as shown in **Figures S2-S4** below. In all cases, relative integrations of the aromatic peaks relative to the aliphatic peaks suggest that two aryl boronic acid derivatives were added to each tyrosine.

Mass spectrometry also confirmed the formation of the di-substituted products. However, all products were observed to lose the Boc protecting groups, presumably due to the formic acid in the mobile phase combined with the elevated temperature in the ESI source. In addition, while the [M+H] was present, the most abundant peak for all products had a mass consistent with an acetonitrile adduct. Both of these effects were also seen when collecting mass spectra of the starting material Boc-DIT.

A)



B)

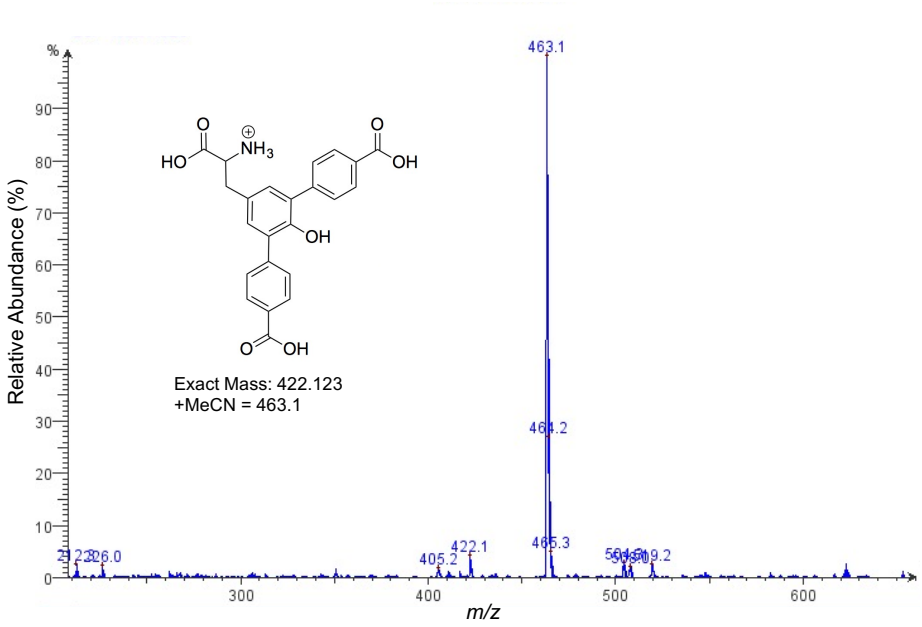
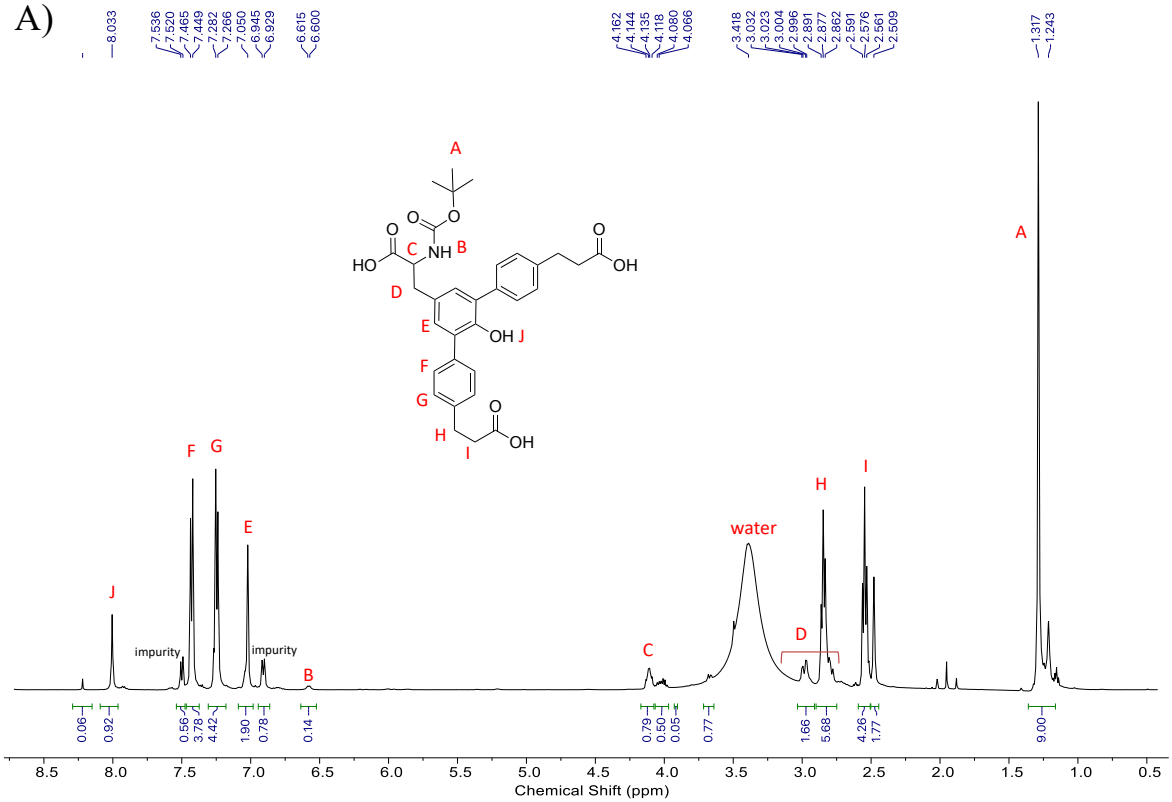


Figure S2. A) ^{1}H NMR spectrum and B) Mass spectrum of the isolated product synthesized from a Suzuki-Miyaura reaction with Boc-DIT and CPBA.

A)



B)

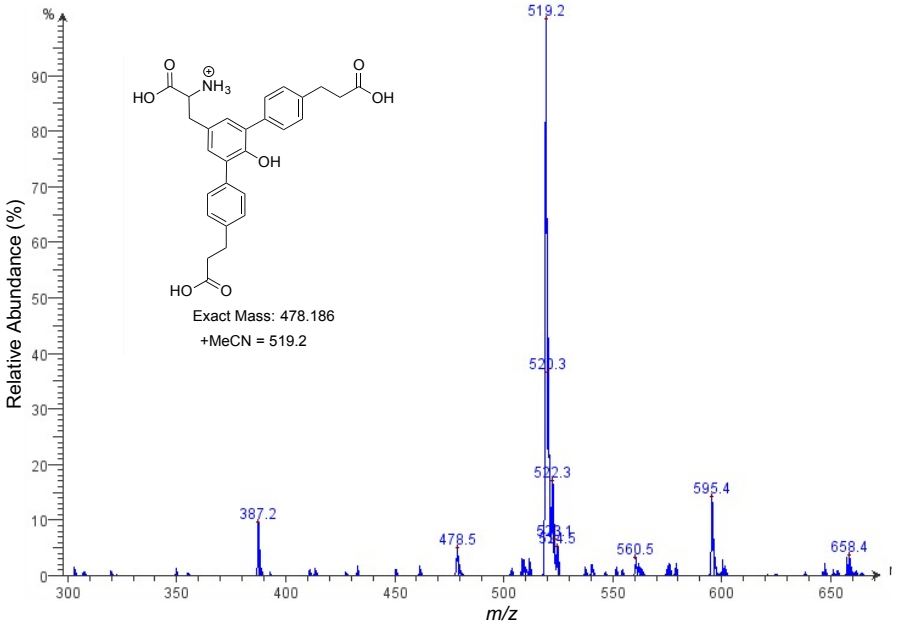
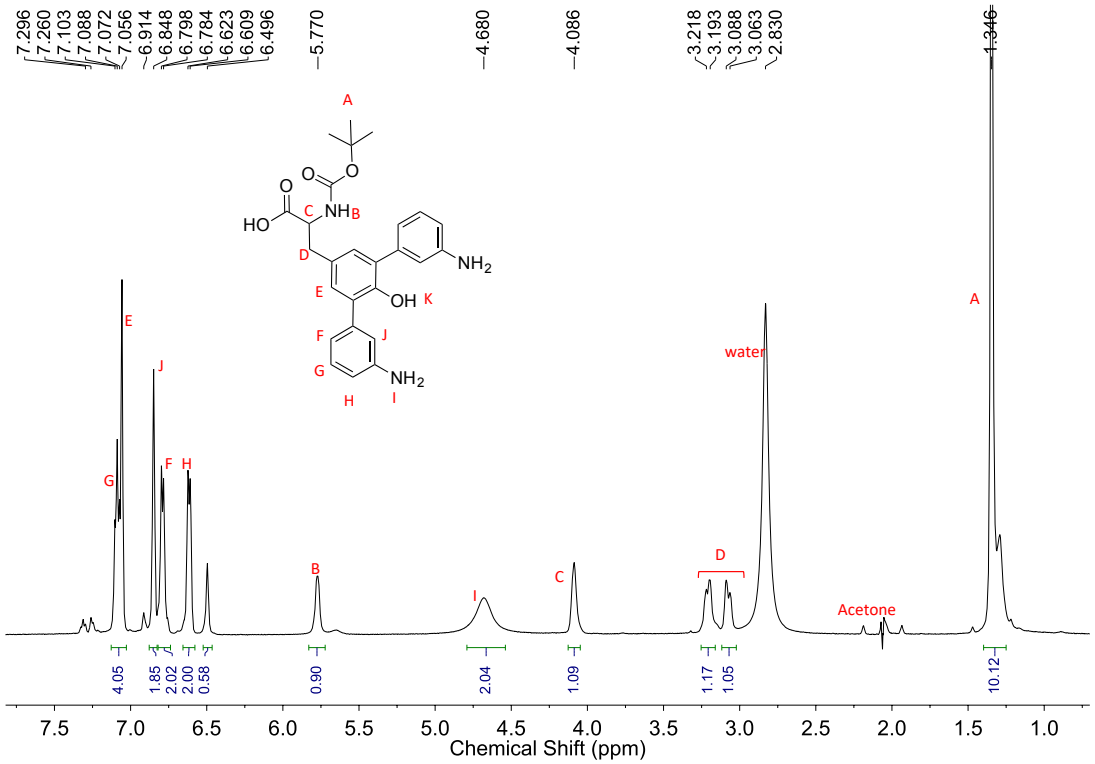


Figure S3. A) ^{1}H NMR spectrum and B) Mass spectrum of the isolated product synthesized from a Suzuki-Miyaura reaction with Boc-DIT and CEBA.

A)



B)

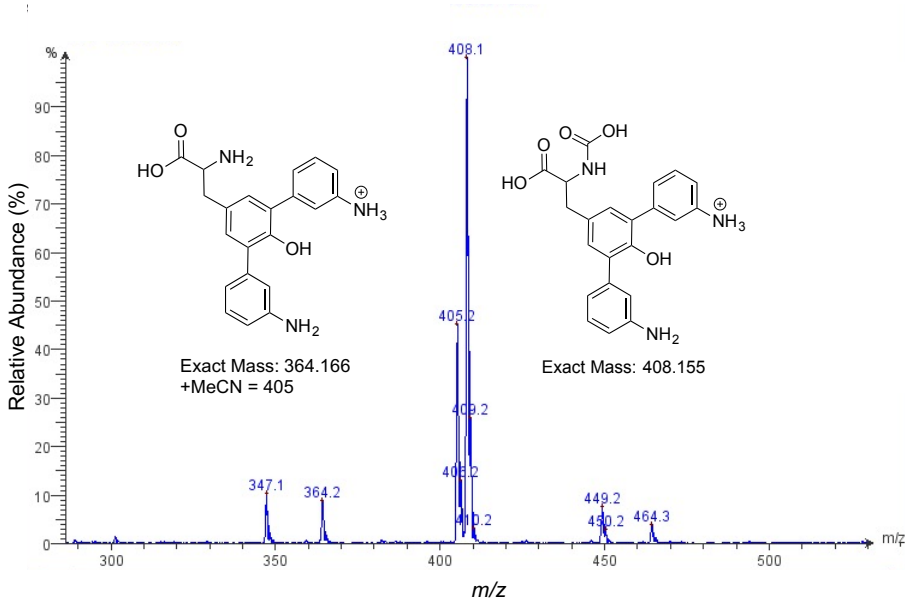


Figure S4. A) ^{1}H NMR spectrum and B) Mass spectrum of the isolated product synthesized from a Suzuki-Miyaura reaction with Boc-DIT and APBA.

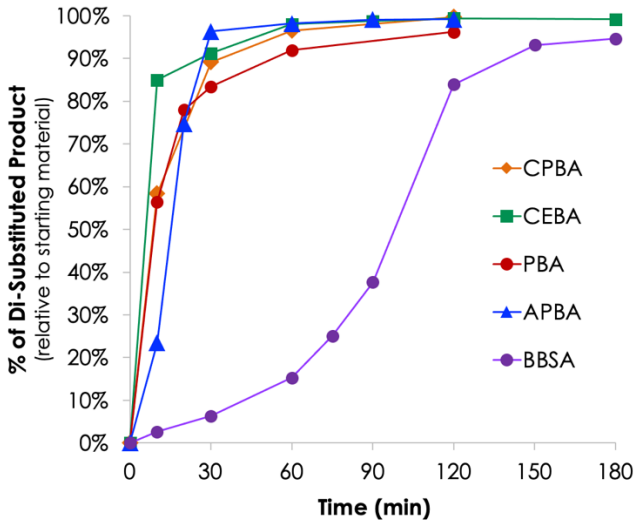


Figure S5. Di-substituted product formation from Boc-DIT in BBS pH 9 at 37 ºC, using 4 molar eq. boronic acid and 2.5 mol% Pd catalyst relative to tyrosine (Figure 1 in the text used 5 mol% Pd relative to tyrosine).

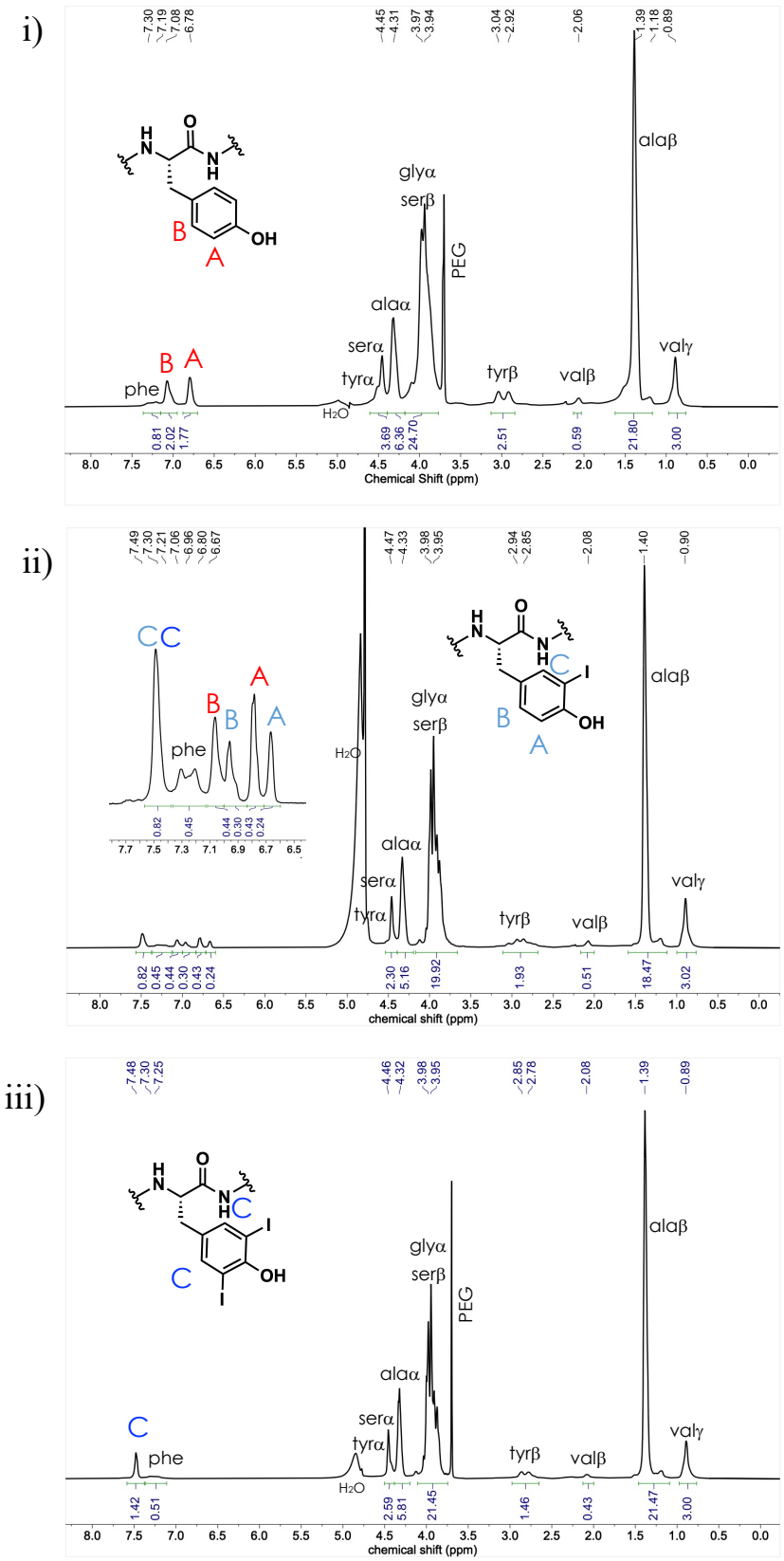


Figure S6. ^{1}H NMR spectra of (i) unmodified silk; (ii) partially iodinated silk produced with 2 equiv. NaI and chloramine-T; (iii) fully iodinated silk after reaction with 3 equiv. NaI and chloramine-T. All samples are ~1-2 wt% silk in D_2O. Peak integrations are normalized to the valine methyl peak at 0.9 ppm. Spectra have varying degrees of water suppression at 4.79 ppm.

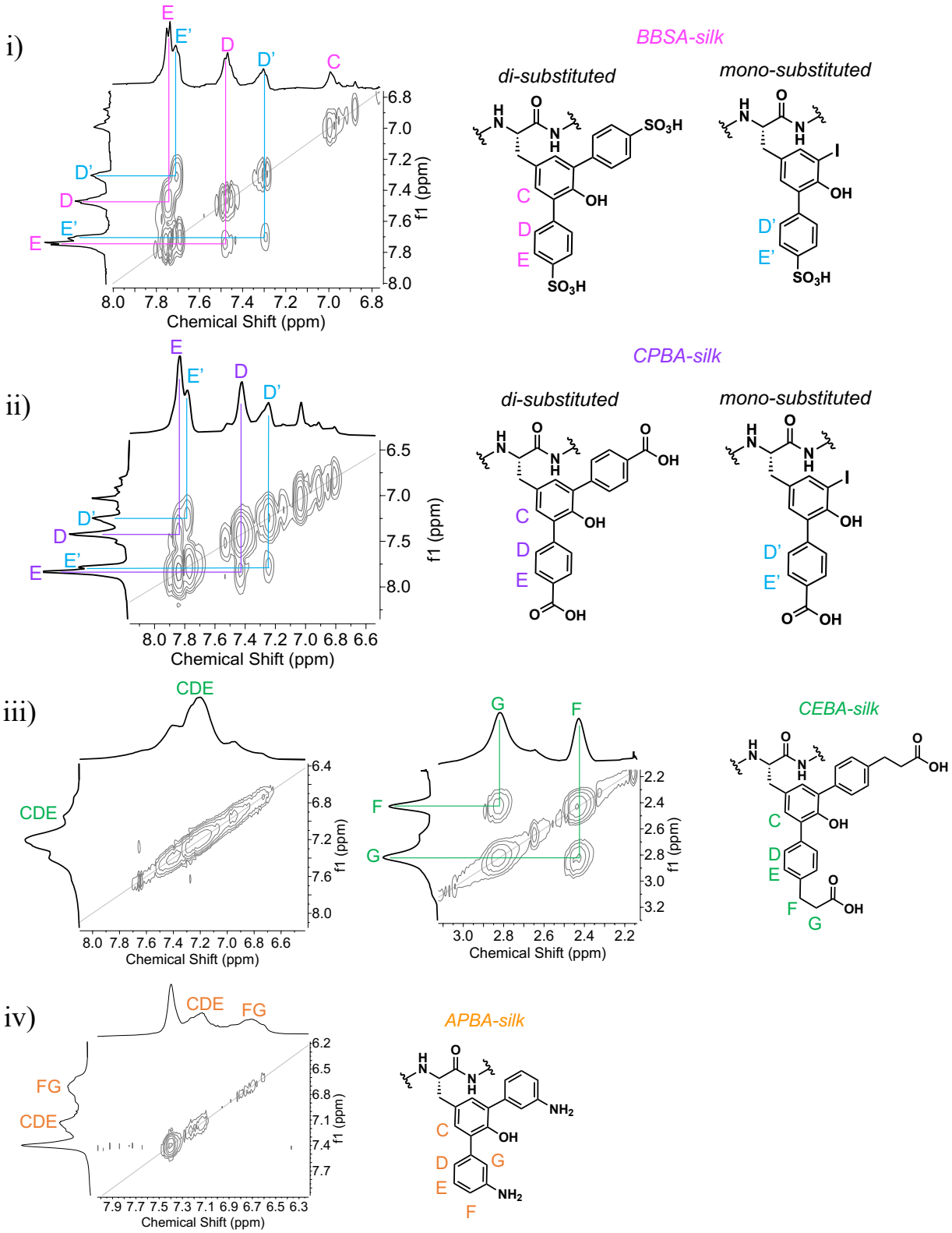


Figure S7. 2D COSY NMR spectra of the Suzuki-Miyuara silk derivatives. BBSA-silk (i) and CPBA-silk (ii) both have distinct cross-peaks suggesting the presence of two unequal para-substituted benzene rings. These have been assigned to the di-substituted and mono-substituted products. The aromatic peaks are broad and ill-defined for the CEBA-silk (iii) and APBA-silk (iv) derivatives, so no cross-peaks can be resolved. However, distinct cross-peaks are apparent that correspond to the methylene groups in the CEBA-silk (iii).

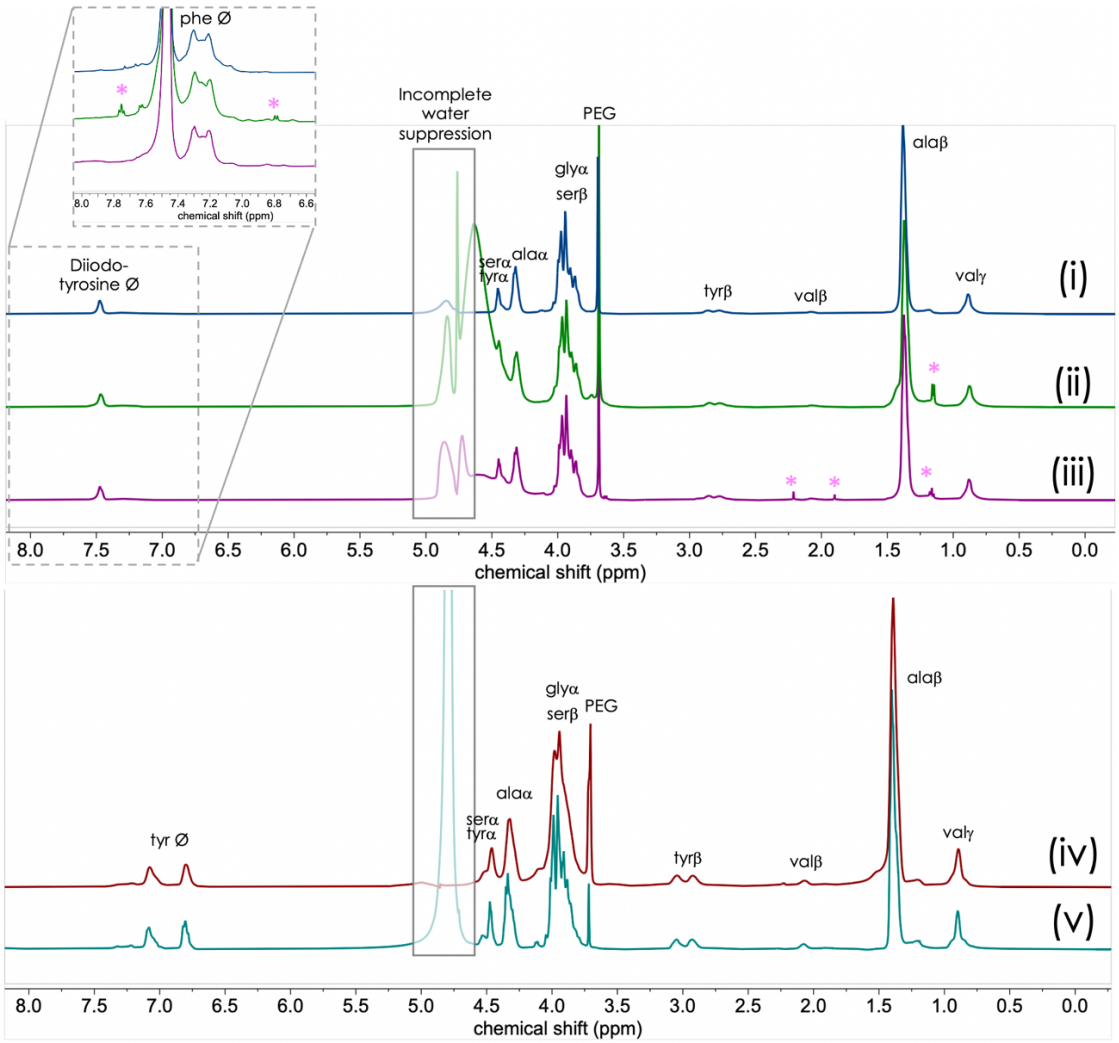


Figure S8. ^{1}H NMR spectra of samples after the following reagents were combined, reacted for 1 h, and then purified using NAP-5 size exclusion columns. (i) iodo-silk; (ii) iodo-silk + 8 equiv. CPBA; (iii) iodo-silk + 14 mol% Pd catalyst; (iv) unmodified silk + 14 mol% Pd catalyst + 8 equiv. CPBA; (v) unmodified silk. Asterisks denote small impurities that remain after size exclusion. All samples are ~1-2 wt% silk in D_2O. Peak integrations are normalized to the valine methyl peak at 0.9 ppm. Spectra have varying degrees of water suppression at 4.79 ppm.

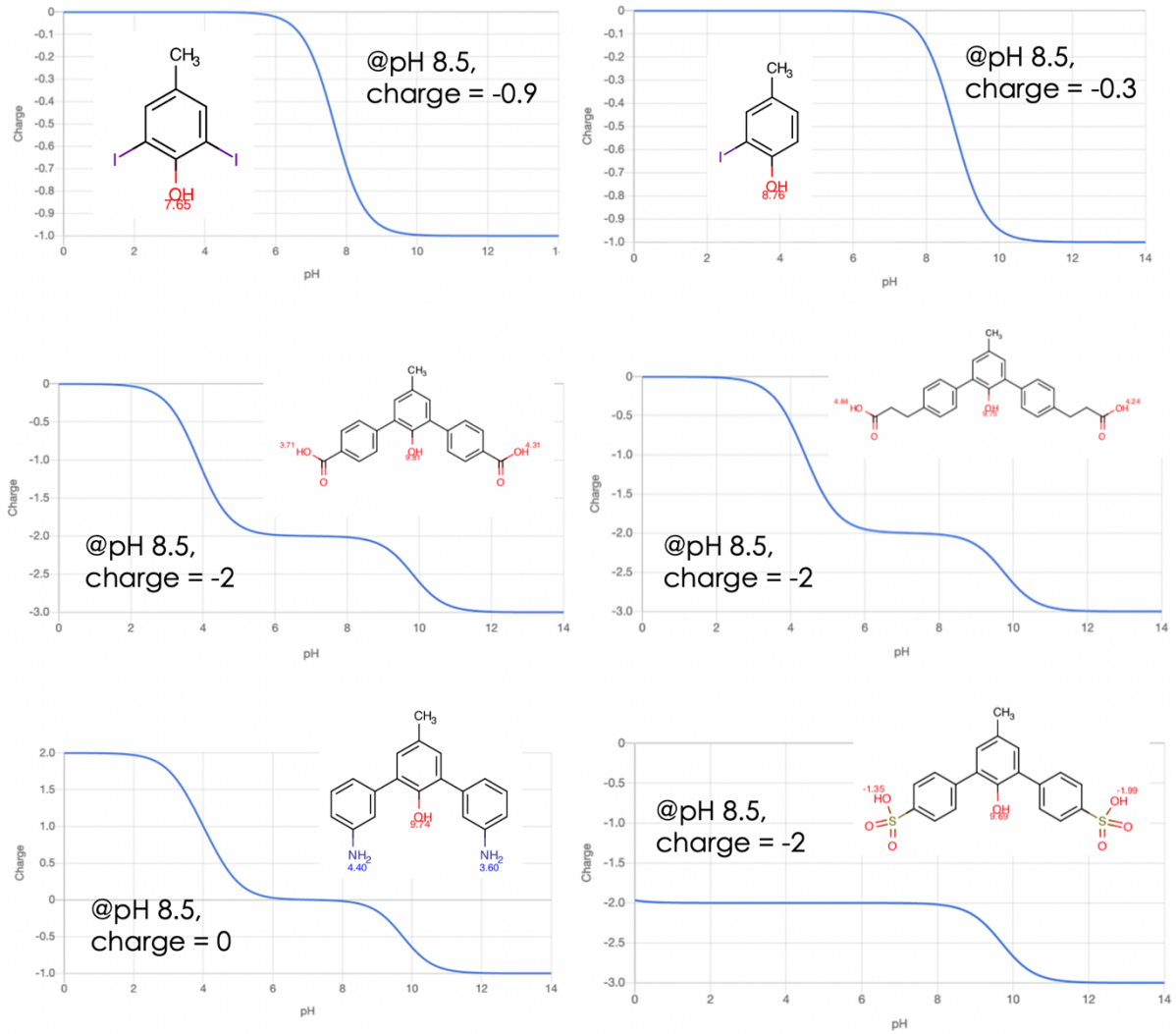


Figure S9. *Chemicalize - Instant Cheminformatics Solutions* software was used to predict the pKa (listed next to the corresponding acidic proton) and the net charge at increasing pH was predicted for model compounds that mimic the structure of our modified silk proteins.

Table S1. Average elemental compositions (n > 3; given in atomic %) of the sample types noted as measured by EDS. Representative spectra are given below in Figure S10.

	Plain silk	Iodo-silk	CPBA-silk	BBSA-silk	APBA-silk
C	66.55	60.37	57.24	57.09	64.87
N	21.03	16.60	17.53	19.05	13.77
O	12.43	20.73	22.53	21.52	19.36
Na	-	0.63	2.07	0.62	0.18
Si	-	-	0.10	0.26	0.16
S	0.02	0.17	0.04	0.27	0.04
Cl	-	-	0.22	0.09	0.07
Pd	-	-	0.13	0.10	0.11
I	-	1.43	0.13	0.87	0.89
Mg	-	-	0.04	0.15	-
Fe	-	-	-	-	0.52

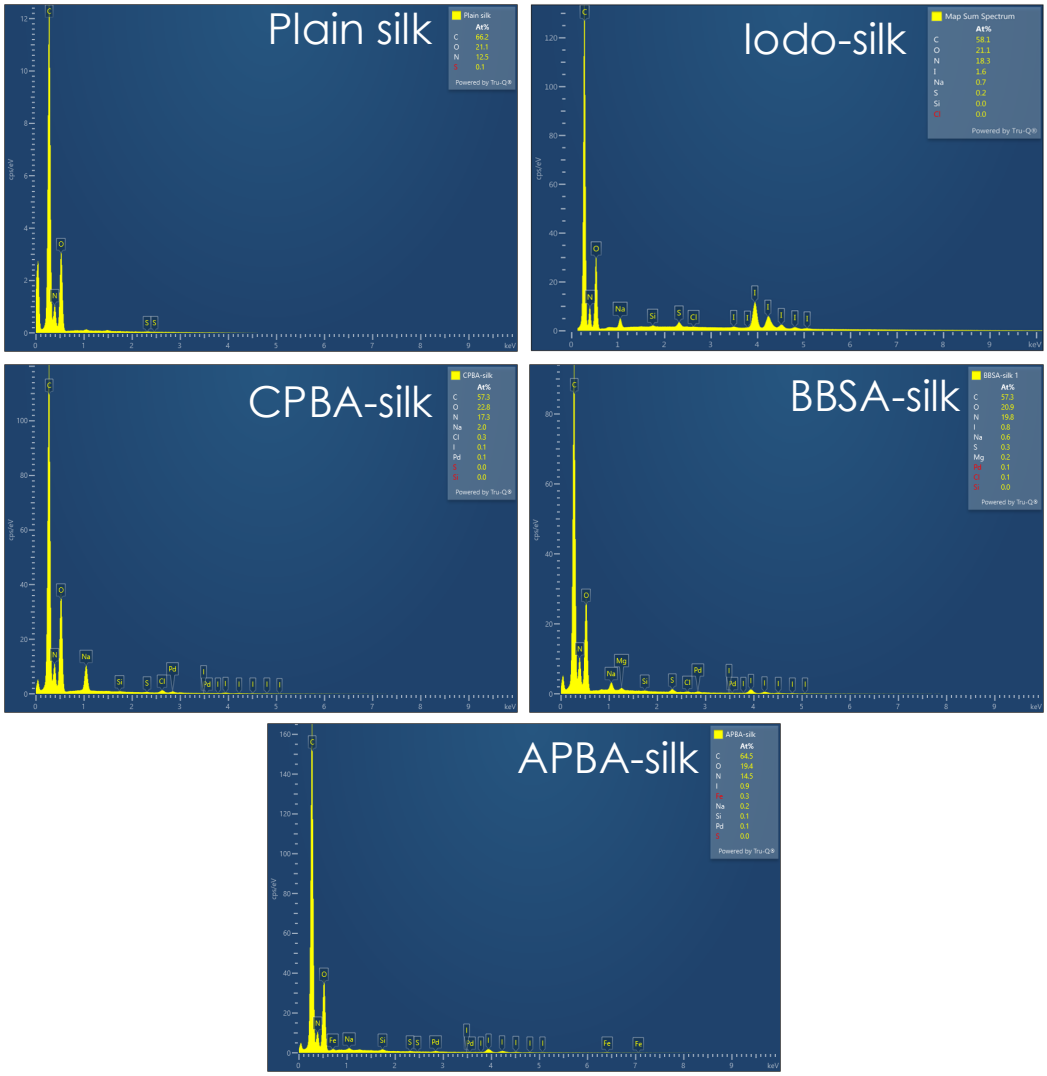


Figure S10. Representative EDS spectra the silk derivatives noted. Suzuki-Miyaura modified silks were reacted with 3 equiv. boronic acid and 12 mol% Pd for 3 h, purified and then dried into a film prior to analysis.