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Enzymatic Post-Translational Halogenation for Adding Functionality to Biomaterials

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ENZYMATIC POST-TRANSITIONAL HALOGENATION
FOR ADDING FUNCTIONALITY TO BIOMATERIALS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science

By
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B.S., Kent State University, 2018

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ABSTRACT

Compean, Alexander L. M.S., Department of Biochemistry and Molecular Biology, Wright State University, 2021. Enzymatic Post-Translational Halogenation for Adding Functionality to Biomaterials

Silk fibroin from the silkworm, Bombyx mori, is a unique biomaterial that has been extensively studied for a variety of applications due to its promising properties such as controllable self-assembly, robust mechanical properties, and biological compatibility. Previously, there have been numerous methods describing the chemical modification of silk fibroin that utilize synthetic or enzymatic means that do not use halogens as a means of functionalization. Herein, a halogenation strategy is presented to modify silk fibroin with the aim of developing a novel functional material through the carbon-halogen (C-X) bond. Modification with NaX (X = Cl, Br, and I) salts, hydrogen peroxide (H₂O₂), and a vanadium dependent haloperoxidase (VHPO) from Curvularia inaequalis produced halogenated tyrosine residues along the protein’s amorphous regions. Halogenation was confirmed using various methods including 1D and 2D ¹H NMR and a chymotrypsin digest with LCMS. Secondary structure was analyzed by FTIR-ATR, circular dichroism (CD), and Raman spectroscopy which revealed that halogenated silk fibroin prefers helical conformations in solution and beta sheet structures when made into dried films but still has random coil content. Addition of halogens increased hydrophilicity on silk fibroin films evaluated by contact angle measurements. Finally, to showcase the C-X bond as a route for functionalization, Suzuki-Miyaura coupling was employed to add a
fluorescent molecule, fluorescein, through a palladium catalyzed reaction scheme. Although coupling efficiency was observed to vary for chlorine, bromine, and iodine, the results demonstrate that this strategy can be used to add new functional groups on to silk fibroin which can potentially modulate the material’s characteristics and enhance function.
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I. INTRODUCTION

A. Biomaterials in Synthetic Biology

Technological advancements in biology has emerged a novel field of science that allows for the engineering of biological systems for desired effects, products, and materials. This new area is called synthetic biology due to the inherent nature of taking known biological processes from various organisms and applying them to their non-natural systems which supplies new products (Khalil & Collins, 2010). There are many instances in which synthetic biology has been useful in industry from harnessing metabolic pathways for lower cost metabolites to developing new small molecules for a variety of applications. Due to biology’s fine control over its normal processes, scientists can use those principles to engineer new materials that meet the demands of societal problems (le Feuvre & Scrutton, 2018)(Smanski et al., 2016) (Council, 2015). An example of this is post-translational modification of biomaterials to modulate its properties for further downstream applications.

B. Halogenated Products in Nature and Synthetic Chemistry

Halogens are widely utilized elements in natural molecules for a variety of purposes, such as signaling and antimicrobial activities (M. Harris et al., 2002; W. Gribble, 1998, 2004) along with being found on structural proteins to give them better strength properties, such as spongin A and B from sea sponges or nvjp-1 from the jaw of Nereis virens marine worm (Birkedal et al., 2006; Ueberlein et al., 2014). These elements are prevalent in marine environments due to high concentrations of their salts in sea water.
(Faulkner, 2001). However, they are also found to be part of metabolites in terrestrial organisms. Halogenation of biomolecules is accomplished by various categories of halogenases which primarily use chlorine or bromine for their processes while iodine and fluorine are less common (Gribble, 2003). This catalytic activity, in certain classes, is mediated by utilizing hydrogen peroxide as an oxidant source to halogenate organic molecules, changing the carbon environment from C-H to a C-X (X = halogen). Although halogenated molecules serve natural functions as previously described, that the utilization of C-X bonds opens up new types of functionalization avenues to the molecule not previously allowed through synthetic means. Synthetically derived halogenated chemicals serve various purposes for industry, with their halo intermediates being used to couple new functional groups onto specialty molecules. These cross-coupling reactions (Suzuki-Miyaura, Sonagashira, Stille, etc...) utilize a palladium metal catalyst to drive the reaction to transform the C-X bond to a C-C bond with a new functionality (Johansson Seechurn et al., 2012; Kosugi et al., 1977; Miyaura & Suzuki, 1979; Sonogashira et al., 1975)

C. Halogenases in Nature

With the discovery and application of novel halogenating enzymes (Ernie Liu et al., 1987), enzymatic halogenation of compounds is rivaling that by synthetic means due to nature’s ability to yield useful products in aqueous environments, at high quantities, and at a moderate pH. Multiple classes of halogenases have been identified and are categorized based on the cofactor needed to create the hypohalous acid (HOX) oxidant
needed for halogenation. Vanadium-dependent haloperoxidases (VHPOs) are a particular class of halogenases that use an orthovanadate cofactor (VO$_4^{3-}$), hydrogen peroxide (H$_2$O$_2$), and a halogen salt (NaX, X = Cl, Br, I) for their mechanism of halogenation (Littlechild, 1999). HOX will then nonspecifically add a halogen (Cl, Br, I) through electrophilic substitution to vinyl and/or aromatic containing compounds. VHPOs have been found in both marine and terrestrial environments, and the VHPO used in this study is from *Curvularia inaequalis*, a terrestrial fungus. Traditionally, VHPOs are used by organisms for either microbial defense or stress signaling, however, *C. inaequalis* is a parasite and uses halogenation for attacking plant roots to break open cells walls. Applying the halogenating function of this VHPO has led to an increase of use in the biotechnological, industrial, and pharmaceutical base due to their practicality and biological properties needed for some of the commercially relevant chemicals in those specific industries (Smith et al., 2013; Wischang et al., 2011).

**D. Silk Fibroin as a Biomaterial**

Silk fibroin, from the *Bombyx mori* silkworm, is a robust biopolymer used in a variety of applications, such as biomedical, electrochemical, optical materials (Altman et al., 2003; Meinel et al., 2006; Z. Zhou et al., 2017), and as a fiber for various textile applications. Fibers of silk fibroin cocoons have been used for centuries to create strong and versatile textiles because of its structure. Silk fibroin has a repeating sequence of glycines, alanines, and serines that helps gives the biomaterial its strength, biocompatibility, and reinforced mechanical properties through interactions of the
antiparallel β-sheet structures formed by these repeats (Heslot, 1998). Since these amino acids make up >85% of the protein (C. Z. Zhou et al., 2001), modification of the sequence is a difficult task due to the low reactivity of these residues. However, there have been several (Lefèvre et al., 2007) methods developed aimed at the modification of reactive amino acids that aid in the tuneability of this biopolymer for increased functionality (Chen et al., 2018; Gotoh et al., 1993; Murphy et al., 2008; Murphy & Kaplan, 2009). Tyrosine is a suitable amino acid for modification due its reactivity and distribution along the protein, making up ~5% of the silk fibroin sequence (C. Z. Zhou et al., 2001), with most of the tyrosines being in the amorphous regions of silk fibroin as outline in Figure 1.

**Figure 1.** Representation of the primary structure of silk fibroin from *Bombyx mori*’s heavy chain. R01-R12 denotes the repetitive regions of the sequence and A01-A11 are the amorphous regions. An approximate primary sequence of the R10 region is outlined by i, ii, and iii while iv illustrates the sequence for the amorphous region. Reprinted with permission from Asakura et al., 2005 (Copyright 2005 American Chemical Society)
The function for tyrosine has been studied (Asakura et al., 2004) and shown to support the formation of β-sheet structures through enhancing hydrogen bonding either intra- or inter-molecularly as well as π-stacking of the aromatic rings for further stabilization which adds to the overall crystalline formation. With this knowledge, modifications of this residue have been used to change the properties of silk fibroin materials through induced β-sheet formation, increased or decreased hydrophilicity, and the creation of an interchain network to form hydrogels (Murphy et al., 2008; Murphy & Kaplan, 2009; Partlow et al., 2014).

This study will investigate the modification of silk fibroin using a halogenating enzyme to understand how halogens change protein self-assembly as a whole as well as the material properties. Furthermore, the halogen bond will be used to synthetically attach a fluorophore for demonstration of increased functionality to silk fibroin.
II. MATERIALS AND METHODS

All chemicals, unless noted, were purchased from Sigma-Aldrich, Saint Louis, MO.

A. Expression of VClPO from *Curvalaria inaequalis*

Vanadium dependent chloroperoxidase (VClPO) synthetic gene was bought from Integrated DNA Technologies (Coralville, IA) and cloned into a pET15b vector, using the Gibson Assembly protocol provided by NEB Labs. First, PCR was performed with Phusion High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA) to add the 5’ and 3’ overhang sequences to the VClPO gene in order to assemble properly into the vector. Table 1 provides the forward and reverse primers used for PCR. After, following the double digest protocol also from NEB Labs using the restriction enzymes NdeI and XhoI (New England BioLabs, Ipswich, MA), the gene was inserted into the digested site to create a N-terminus HisTag on the enzyme for purification. Next, a 2 uL aliquot of the Gibson Assembly mixture was transformed into NEB 5-alpha competent *E. coli* cells (New England BioLabs, Ipswich, MA) and was heat shocked following the provided protocol from NEB Labs. 100 uL of the cell solution were then transferred to an LB ampicillin (Gold Biotechnology Inc., St. Louis, MO) agar selection plate and allowed to grow overnight at 37°C. Colonies were then picked from the selection plate and transferred into 7 mL of LB broth with ampicillin to be grown overnight at 37°C. Miniprep procedures were then followed from Promega’s quick protocol and eluted in
Nuclease Free water (New England BioLabs, Ipswich, MA). Samples were then stored at -20°C until used.

<table>
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<td>Forward</td>
<td>5′-AAACTGCAAGCATTTAAGCGTACAATGGGATCAGTGCACCCCATCCC-3′</td>
</tr>
<tr>
<td>Reverse</td>
<td>5′-CAGTGGTGGTGGTGGTGGTGGTGGTGGCTCGAGTTATGGGGCTTTCTTTACGACTGG-3′</td>
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**Table 1.** Forward and reverse primers sequences used for PCR.

Next, BL21 DE3 *E. coli* (New England BioLabs, Ipswich, MA) were transformed with the pET15b-VCIP0 by electroporation and 700 uL of SOC media (New England BioLabs, Ipswich, MA) was added to incubate. Cell solution was then transferred into a 1.5 mL Eppendorf tube and incubated at 37°C, 220 RPM for 1 hr. 100 uL of cell solution was then added to an LB agar selection plate with ampicillin and left in a 37°C oven overnight. Four colonies from the selection plate were then transferred to a 7 mL LB broth tube with ampicillin and left to grow overnight, shaking at 220 RPM in a 37°C shaker. Each LB broth solution was then added to a larger 1 L LB broth solution with ampicillin and allowed to grow until 0.6 OD<sub>600</sub> in a 37°C shaker at 220 RPMs. At that point, IPTG (0.5 mM) (Gold Biotechnology Inc., St. Louis, MO) was added to induce protein expression and the temperature was reduced to 18°C and allowed to shake for 16 hrs. The 1L culture was then centrifuged at 8,000xg and a reduced temperature of 4°C for 10 minutes. Pelleted cells were added to the lysis buffer (50mM HEPES, 250 mM NaCl, 10% glycerol pH 7.4) and lysed by sonication. The solution was then added to a 50 mL
conical tube and centrifuged at 10,000xg with a reduced temperature of 4°C to remove cell debris. FPLC (Bio-Rad NGC Quest 10 Chromatography, Hercules, CA) fitted with an IMAC HisTag column (HisTrap FF Crude, 5 mL) (Cytiva Life Sciences, Malborough, MA) was used to purify the supernatant. The column was equilibrated with 0.22 um filtered 18.2 MΩ water to remove the storage solution (20% ethanol) for 10 column volumes. Next, the sample was applied to the column and was washed with the wash buffer (50 mM HEPES, 250 mM NaCl 30 mM imidazole, 10% glycerol pH 7.4) for 5 column volumes. Protein was eluted from the column using the wash buffer plus 500 mM imidazole for 10 column volumes in 5 mL fractions and was concentrated by centrifugation using Amicon Ultra-15 Centrifugal Filter Units (10,000 NMWL) (EMD Millipore, Burlington, MA) spun at 5700 RPMs for 10 minutes. The resulting concentration was ~8 mg/mL. Protein was flash frozen in liquid nitrogen and stored at -80°C until used. A 12% Tris-glycine PAGE gel was performed to investigate the purified fraction that was collected from the FPLC purification and was able to validate the protein collected was the VCIPO from *C. inaequalis* and is shown in Figure 2.
Figure 2. PAGE gel of purified VClPO from *C. inaequalis* in lane 3. Other lanes are different halogenases not used in this study.

Phenol red activity assays were performed as published (de Boer et al., 1987) (Hasan et al., 2006). Briefly, 50 uM phenol red was added to a solution of 10 mM NaBr, 0.1 mM sodium vanadate, 10 mM H₂O₂, 100 mM sodium acetate pH 5.0 and 20 uL of purified enzyme. Assays were allowed to go to presumed completion when the assay solution turned from yellow to blue signifying all phenol red was brominated.

**B. Preparation of Aqueous Silk Fibroin**

Aqueous solutions of silk fibroin have been established in other publications (Rockwood et al., 2011). *Bombyx mori* cocoons were processed and weighed out to be 7.5 grams and boiled for 30 minutes in 3 L of 0.02 M sodium carbonate solution. The degummed silk fibers were then rinsed in 18.2 MΩ water three times and then dried overnight. Silk fibers were then dissolved in 9.3M LiBr in a 20% w/v solution and heated to 60°C for 2 hours. The solution was then dialyzed (Spectrum Repligen Spectra/Por Dialysis Membrane Tubing, 12-14 kDa MWCO, New Brunswick, NJ) against 2 liters of...
18.2 MΩ water at 4°C for two days with six water changes. Silk solutions were then transferred into a 50 mL conical tube and centrifuged twice at 10,000 x g for 20 minutes to remove insoluble impurities. Concentrations of silk solutions were determined by comparing aqueous silk mass to dried silk mass. The resulting concentration was 6.5% w/v silk.

C. Silk Fibroin Halogenation Assays

Halogenation assays, as shown in Figure 3, were developed from published protocols (Dong et al., 2017). 40 nM of VClPO was added to a 2% w/v silk solution with either 2 mM of NaCl, NaBr or NaI, 0.1 mM sodium vanadate, 213 mM H₂O₂, and 25 mM sodium acetate at pH 5.0. Blank assays included all materials without the enzyme and assays were left overnight. The next day, assay solutions were added to Slide-A-Lyzer MINI Dialysis Device, 20K MWCO, (Thermo Scientific) and dialyzed against 18.2 MΩ water for 1 day with two water changes. Dialyzed solutions were then removed from devices and added to 1.5 mL Eppendorf tubes. Samples were then stored at 4°C until used.

Figure 3. Schematic of halogenase assay.
D. 1D $^1$H on Silk Fibroin

Silk solutions were subject to $^1$H NMR to investigate the aromatic regions from tyrosine. 500 uL of protein samples were added to coaxial tubes (Wilmad-LabGlass) and inserted into 500 uL of D2O in a 5 mm NMR tube. Samples were analyzed on a TecMag Redstone NMR operating at 600MHz with a water suppression method. $^1$H NMR (600 MHz): 6.75 ppm (br s, 2H, Tyrφ), 7.03 ppm (br s, 2H Tyrφ) in unmodified samples.

E. X-Ray Photoelectron Spectroscopy of Silk Films

Films were prepared on silicon wafers (University Wafer, Mechanical Grade, South Boston, MA) and subjected to UV/ozone treatment (Novascan UV Ozone Cleaner, Novascan Technologies, Boone, IA) for 10 minutes. After, silk solutions were drop-casted and allowed to dry overnight with no further treatment. High resolution XPS spectra were collected on a M-Probe Surface Science XPS spectrometer. Spectra were collected using a spot size of 800 mm, 0.01 eV step, and averaged over 75 scans.

F. Circular Dichroism of Modified Silk Fibroin

Circular dichroism spectra were collected on a Jasco J-815 CD spectrometer (Jasco, Easton, MD) using a quartz cuvette with a 1 cm path length from 300 to 190 nm at a scan rate of 20 nm/min and averaged over three repetitive scans. Unmodified and halogenated samples were diluted to 0.5 mg/ml with 18.2 MΩ water. Spectra were plotted in OriginLab Pro 2020 software.
G. Fourier Transform Infrared analysis of Silk Films

Silk films were prepared on silicon wafers that were treated with UV/Ozone for 10 minutes. Next, silk solutions were drop casted onto silicon wafers and allowed to dry overnight. Two films were made per sample with one film being treated with methanol for 10 minutes to induce crystallization. FTIR analysis was performed on a Bruker Lumos Spectrometer using the ATR attachment. Liquid nitrogen was added to the system to provide nitrogen gas for reduction of the water vapor contribution from the atmosphere. Each measurement had 64 scans co-added with 4 cm⁻¹ resolution and the wave number ranged from 600 to 4000 cm⁻¹. Sample’s spectra were plotted in OriginLab Pro 2020 software and were normalized. Peak deconvolution of the Amide I region (1600-1700 cm⁻¹) was also performed on OriginLab Pro 2020 software using Fourier Self-Deconvolution v1.00 and Peak Deconvolution v1.50 application available from the app center. Fourier Self-Deconvolution (FSD) was done by plotting the Amide I region individually from the whole spectrum and applying the application with a smoothing factor of 0.15. A Lorentzian function was applied to each curve to distinguish the contribution of each element (beta-sheets, beta-turns, side chains, etc...) to the Amide I peak. Each spectrum had 7 peaks individually picked based on FSD and were plotted underneath the deconvoluted spectrum. Peak assignments were given based on previous data for silk fibroin (Hu et al., 2006) (Krimm & Bandekar, 1986).
H. Raman Spectroscopy of Silk Films

Raman spectra were obtained on a Renishaw inVia Raman Microscope (Renishaw, Gloucestershire, United Kingdom) with a 785 nm laser and a 100x objective lens. Laser power was set to 10% with 6 reads per sample. Each sample (unmodified, chlorinated, brominate, and iodinated) was drop casted on gold microscope slides (Gold Seal Microscope Slides, Clay Adams) because silicon wafers had interference in the Raman spectra (data not shown) and were tested in three different areas on the slide. The wavenumber range was set to 800 – 1800 cm\(^{-1}\) to collect all necessary peaks related to proteins (Krimm & Bandekar, 1986).

I. Contact Angle Measurements of Silk Fibroin Films

Silk samples were spin coated on silicon wafers that were treated as described previously. The spin coater system used was a Spincoater Model P6700 (Specialty Coating Systems, Indianapolis, IN) and done by the following procedure: silicon wafer was placed on spinning mechanism and had 60 uL of silk solution added to the middle of the wafer, films were spun by initially increasing spin rate to 1000 RPMs for 30 seconds and a 30 second hold at 1000 RPMs. Films were allowed to dry for 1 hour and then were treated with methanol for 1 hour, subsequently were dried overnight. Next, silk films were analyzed on a Biolin Scientific Attension Theta (Biolin Scientific, Gothenburg, Sweden) with oneAttension software using the sessile drop technique with a 2 uL drop size of distilled water. Two films from each sample were made and analyzed. Each film
had three measurements performed and the data points were averaged together for a final measurement.

**J. Silk Fibroin Film Fabrication**

Silk fibroin aqueous samples were casted into 5 mm diameter polypropylene molds and allowed to air dry overnight. Dry films were then removed from the molds and dissolved in 200 µL of either ddH$_2$O (control), 30% ammonium sulfate, acetone, or methanol for 20 minutes to induce crystallite formation. Films were then washed twice in ddH$_2$O for 5 minutes. ddH$_2$O was removed and the films were allowed to air dry overnight. Pictures of films were taken to compare the treatments of different solvents to unmodified and halogenated samples.

**K. Suzuki-Miyaura Coupling Procedure of Modified Silk Fibroin**

Suzuki-Miyaura coupling assays were developed from numerous sources (Chalker et al., 2009; Spicer et al., 2012). In short, fluorescein boronic acid was made following the procedure published by (Spicer et al., 2012) and was made into a stock solution with 18.2 MΩ water at 166 mM. Initial experiments of coupling were done with monoiodotyrosine (0.4 mmol), fluorescein boronic acid (0.11 mmol), palladium-pyrimidine catalyst (0.86 umol), and 50 mM phosphate buffer pH 7.5 in a 2 mL assay. After an overnight reaction, 30 mL of 1M HCl was added to stop the coupling. Next, 30 mL of ethyl acetate was added twice and was separated by a separatory funnel. The organic phase was collected and concentrated by rotary evaporation. Column chromatography was performed with silica gel, standard grade (60Å, 230 x 400 mesh,
Sorbtech, Norcross, GA) with 15% methanol and 85% dichloromethane. The resulting product was a yellow-orange solid. The solid was dissolved in water to study the max absorbance of the mixture.

A standard curve was made on a SpectraMax M5e Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA) with SoftMax Pro 7 software at known concentrations of 0 mM, 0.001 mM, 0.01 mM, 0.05 mM, and 0.1 mM in a 96 well plate to determine the concentration of fluorescein in solution. Each measurement was performed three times, averaged, and plotted in an excel spreadsheet. A linear fit was used to determine the linear regression and $R^2$ value.

Coupling assays with unmodified and halogenated silk were performed with 63 ug of silk fibroin for each sample, fluorescein boronic acid (0.17 umol), palladium-pyrimidine catalyst (0.4 umol), and 50 mM phosphate buffer pH 7.5 in 100 uL. Assays were allowed to react overnight in a 37°C oven rotating at 200 RPM. Next, assays were diluted with 18.2 MΩ water to 0.5 mL and added to a 0.5 mL centrifugal unit (Amicon Ultra, 30K MWCO). Tubes were centrifuged for 10 minutes at max speed and room temperature five times until all fluorescein boronic acid was removed. Samples were measured in a 96 well plate on the microplate reader described before. Fluorescein concentrations were determined by the linear regression equation developed previously. Overall tyrosine in silk was found by using the hypothetical amount of tyrosine residues in silk fibroin’s heavy and light chains (~288) (C. Z. Zhou et al., 2001) and calculating the molar concentration of tyrosine in solution.
III. RESULTS

A. Characterization of Halogenation on Tyrosine Residues

After purification of the halogenase (VCiPO) from an E. coli vector, an activity assay was performed to assess the activity of the enzyme. The bromination of phenol red signifies that the available sites on phenol red were brominated with at least 4 bromine molecules as shown in Figure 4A (de Boer et al., 1987) (Hasan et al., 2006) and that the purification of this enzyme was sufficient. This change at pH 5 was visually monitored by observing the solution’s color change from yellow to dark blue as shown in Figure 4B, it was noted that in <5 min the conversion took place.

![Reaction scheme of activity assay](image)

**Figure 4. A)** Reaction scheme of activity assay. Due to electrophilic aromatic substitution, halogenation at the *ortho-* positions on the phenol rings is preferred. **B.)** Assay solutions without (left) and with (right) VCiPO.

Next, reconstituted silk fibroin was exposed to VCiPO as described above and was analyzed by 1D $^1$H NMR to understand how the proton environment on the aromatic
rings changed from unmodified to halogenated silk fibroin samples. During this assay, there was also a notable color change from a clear aqueous solution to a yellow and slightly opaque color. The $^1$H NMR spectrum in Figure 5 shows unmodified silk fibroin aromatic peaks at 6.75 ppm and 7.03 ppm corresponding to protons at positions 3,5 and 2,6, respectively. Silk fibroin that has been exposed to VClPO shows a complete loss in proton signal from those aromatic peaks at their respective positions and an increase in intensity in the amide proton region of the spectrum (8.0 ppm – 8.6 ppm) suggesting a downfield shift in the aromatic peak and a widespread amount of halogenation.

![Figure 5](image)

**Figure 5.** $^1$H NMR spectra of unmodified (black) and halogenated samples (red = chlorinated, blue = brominated, and green = iodinated) silk fibroin. Tyrosine’s structure is depicted in the top left with the hydrogens positions along the phenol ring displayed.

Finally, X-ray photoelectron spectroscopy (XPS) was employed to determine the elemental composition of silk fibroin films. XPS is a technique used to analyze the surface chemistry of a material by exposing the surface to X-rays and measuring the
electron energy coming off the sample with each element having a specific electron energy signature. In comparison of the unmodified and halogenated samples in Figure 6, it is clear that halogenation is occurring since chlorine (203 eV), bromine (70 eV), and iodine (631 eV) are all detected in their corresponding samples. Halogen peaks are individually plotted for clarification of binding energy in Figure 7. Chlorinated and iodinated samples had low signal/noise ratios presumably due to the low abundance of potential sites for halogenation on silk fibroin (~5.3% tyrosine) and is competing with small traces of bromine ions due to the 9.3 M LiBr processing step needed to solubilize the fibers.

Figure 6. XPS analysis of unmodified and halogenated silk fibroin films.
B. Secondary Structure Characterization of Halogenated Silk Fibroin

Due to silk fibroin’s polymeric and repetitive nature, an effort to characterize how halogenation changes the secondary structure of silk fibroin was done using multiple techniques. Secondary structures are mostly known as helices or β-sheets, however, silk fibroin is normally found to be in a random coil conformation in aqueous solutions. First, Circular Dichroism (CD) spectroscopy was done to understand which secondary structure is most abundant in solution. From Figure 8, we can see that the unmodified spectra verifies that silk fibroin is in the random coil conformation. However, as halogenation occurs, the structure takes on an α-helical conformation as an intermediate for the crystalline structure shown in the methanol treated unmodified sample. Interestingly, a trend to increasing change in secondary structure from random coil to α-helix to β-sheet formation occurs as the atomic radius of halogen increases. As shown in Ha et al., 2006, silk fibroin in halogenated solvents (HFIP, HFA) prefer a helical structure presumably due to the interaction between the solvents and the hydroxyl groups on various residues of the amorphous regions (serine and tyrosine) that stabilize this conformation change.

Figure 7. XPS analysis of individual halogen peaks
The addition of methanol to silk solutions is often used to induce $\beta$-sheet crystallites which is similar to what is formed in spun silk by the silkworm, *Bombyx mori*.

![Figure 8](image_url)

**Figure 8.** CD spectra of unmodified silk without methanol (black), unmodified silk with methanol (pink), chlorinated silk (red), brominated silk (blue), and iodinated silk (green).

Next, Fourier-transform infrared spectroscopy (FTIR) was used to further characterize the secondary structure of films since CD can only detect the overall secondary structure in solution. This technique uses infrared light to detect how a sample absorbs photons in that region and is able to display different amide peaks that
correspond to multiple positions along the peptide backbone. In the Amide I band (1600 cm\(^{-1}\) – 1700 cm\(^{-1}\)), which mostly corresponds to the stretching of the C=O on the peptide bond (Barth, 2007), it clearly shows the unmodified silk fibroin film in a random coil conformation. In comparison to the halogenated silk fibroin films, except for brominated sample, the backbone begins to arrange in the beta sheet conformation. The same trend can be seen in the Amide II peak (1500 cm\(^{-1}\) – 1600 cm\(^{-1}\)) as well. This peak corresponds to the N-H bend of the in-plane amide group on the peptide bond (Barth, 2007). Figure 9 shows the FTIR spectra for the different silk fibroin films. The Amide I peak can be further deconvoluted to separate the different components contributing to this peak. In the unmodified spectrum, it is clear that the random coil (R) is the major contributor to the Amide I peak with very little contribution from the side chain residues which mostly comes from tyrosine (Hu et al., 2006). However, in the chlorinated and iodinated samples, the influence from the beta sheet structure increases significantly. For all halogenated samples, the contribution from the side chain residues increases in comparison to the unmodified samples which seems to suggest that the halogens either contributes to the crystalline formation or forces stacking of tyrosine residues. Figure 10 shows the deconvoluted Amide I peaks for all samples.
Figure 9. FTIR spectra of unmodified and halogenated silks.
Finally, in an effort to complement the CD and FTIR data, Raman spectroscopy was used to further understand the secondary structure. Raman spectroscopy measures a chemical bond’s vibrational effects by scattering photons at a specific wavelength. Combining these techniques provides a better understanding of the secondary structure’s preference for folding. From the Raman spectra, the peaks corresponding to the Fermi resonance of tyrosine’s ring breathing vibrations and out-of-plane ring-bending vibrations (853 cm$^{-1}$ and 830 cm$^{-1}$) can be compared in a relative intensity ratio ($R_{\text{Tyr}} = I_{853}/I_{830}$) to give an idea of tyrosine’s hydrogen bonding activity as it pertains to the side chain’s -OH group (Monti et al., 1998; Siamwiza et al., 1975). If the phenol ring is buried in the amorphous region, the -OH acts as a strong hydrogen bond donor, which is reflected in
the decrease in intensity of the 853 cm\(^{-1}\) band. In Figure 11, the unmodified silk fibroin shows a higher \(R_{\text{Tyr}}\) of 1.26. While in the chlorinated, brominated, and iodinated silks, the \(R_{\text{Tyr}}\) is 1.13, 1.09, and 1.15, respectively. This decrease in \(R_{\text{Tyr}}\) is due to tyrosine’s buried position within the polypeptide’s hydrophobic core, making the -OH on the side chain more available to be a hydrogen bond donor and acceptor.

![Raman spectra of unmodified and halogenated silk samples. Unmodified silk is represented by the black line. Chlorinated silk is represented by the red solid line. Brominated silk is the blue solid line. Iodinated silk is represented by the green solid line.](image)

**Figure 11.** Raman spectra of unmodified and halogenated silk samples. Unmodified silk is represented by the black line. Chlorinated silk is represented by the red solid line. Brominated silk is the blue solid line. Iodinated silk is represented by the green solid line.
C. Material Properties of Halogenated Silk Fibroin

Halogenation was detected and influences secondary structure of silk fibroin to varying degrees. This strategy, combined with modifications of tyrosines, leads to the expectation that there will be alterations in silk fibroin’s material properties as well. Insight into how halogenation changes the material properties of this biomaterial was explored. Contact angle measurements were performed to assess the hydrophilic or hydrophobic nature of halogenated silk fibroin by measuring the angle of the interface between a water droplet on a silk fibroin film surface. Table 2 summarizes the results by showing that the angle changes significantly from unmodified (~68°) to chlorinated (~59°), brominated (~36°), and iodinated (~45°) silk fibroin films. The brominated silk fibroin sample has the lowest contact angle measurement which is in agreement with the \( R_{\text{Tyr}} \) from the Raman spectroscopy data. This seems to suggest that bromination is either better at hydrogen bonding or has tyrosine’s -OH on the phenol ring in the most optimal position for hydrogen bonding. More investigation will be needed to confirm this.
Figure 12. Contact angle measurements on silk films. Top left corner is unmodified silk, top right is chlorinated silk, bottom left is brominated silk, and bottom right is iodinated silk.

Table 2. Contact angle measurements of halogenated silks compared to unmodified silk.
Other methods of analyzing silk as a material, such as an enzymatic or secondary structure hydrogel formation through horseradish peroxidase (HRP) or sonication were unable to be produced (data not shown). This indicates that HRP derived hydrogels are not possible due to the mechanism of crosslinking involving the binding of multiple tyrosine residues on the same carbon as halogenation is taking place and sonication is not possible due to the increase in stability from the halogens.

However, silk fibroin films treated with 30% ammonium sulfate, acetone, and methanol were able to crystallize and became water insoluble compared to films treated with water. This material fabrication process has the potential to be used for downstream applications that require further investigation. Figure 13 shows the comparison of solvent fabricated films from the unmodified to halogenated samples treated with different solvents.

Figure 13. Silk fibroin films treated with different solvents for material fabrication unmodified and halogenated films. Water (1), 30% ammonium sulfate (2), acetone (3), and methanol (4).
D. Halotyrosine Functionalization on Silk Fibroin

To demonstrate that halogen bonds can be used as a way of adding functionalization to silk fibroin, Suzuki-Miyaura coupling was used to link a fluorescein molecule to the halogenated tyrosine residues. This technique includes a palladium catalyst, a boronic acid, and a halogenated species for coupling to occur. In these experiments, unmodified, chlorinated, brominated, and iodinated silk fibroin were subject to coupling to determine the degree to which halogenation occurs following the scheme shown in Figure 14.

![Suzuki-Miyaura coupling reaction scheme](image)

**Figure 14.** Suzuki-Miyaura coupling reaction scheme developed from (Chalker et al., 2009)

First, fluorescein boronic acid was coupled to 3-monoiodotyrosine to create a standard curve based on fluorescein content in solution. 3-monoiodotyrosine was chosen as a standard based of its efficiency in coupling (Chalker et al., 2009) and its cost. Next, the absorbance of fluorescein appended tyrosine was determined to peak at 451 nm and was used to build the standard curve. Several dilutions were made to determine the lower
limit of detection of our system as compared to water. Figure 15 shows the standard curve with the linear curve fit equation that was used for the remainder of experiments.

![Standard curve of fluorescein coupled to monoiiodotyrosine.](image)

**Figure 15.** Standard curve of fluorescein coupled to monoiiodotyrosine.

Suzuki coupling reactions were performed next with unmodified (control) as well as chlorinated, brominated, and iodinated aqueous silk fibroin samples. The molar ratio of fluorescein boronic acid to the theoretical tyrosine content in silk fibroin solution was kept at 1:1 (0.166 μmol) to understand the amount of halogen modification to the tyrosine residues. Assays were developed from other sources as described above and were allowed to react overnight. Satisfyingly, the halogenated silks showed addition of fluorescein (Figure 16). Table 3 outlines the amount of fluorescein detected in solution as compared to the standard curve. Chlorinated silks showed the lowest amount of coupling.
with 27% modification of tyrosines. Brominated silks showed an increase of modification with 44%. Finally, iodinated silks showed the largest amount of modification with 55%.

<table>
<thead>
<tr>
<th>Silk Type</th>
<th>Calculated amount of Coupled Fluorescein(μmol)</th>
<th>Percentage of Tyr modified (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cl</td>
<td>0.05</td>
<td>27</td>
</tr>
<tr>
<td>Br</td>
<td>0.07</td>
<td>44</td>
</tr>
<tr>
<td>I</td>
<td>0.09</td>
<td>55</td>
</tr>
</tbody>
</table>

Table 3. Amount of fluorescein coupled to halogenated silks through Suzuki-Miyaura coupling assays.

Figure 16. Image of fluorescently labelled silk fibroin. Left to right: H2O, unmodified not treated in fluorescein boronic acid, unmodified with boronic acid, chlorinated silk, brominated silk, and iodinated silk.
IV. Discussion

Here, a novel method of modifying the structural biomaterial, silk fibroin, through halogenation is presented. Although there are many strategies that have been demonstrated to modify silk (Chen et al., 2018; Gotoh et al., 1993; Murphy et al., 2008), halogenation can be used as a more versatile method due to the limitless possibilities of new chemistries being introduced to silk. These new interactions can have a wide array of applications that will make the silks more functional as a biomaterial, since tyrosine is well distributed along the sequence and is mainly found in the amorphous regions. The degree of halogenation after being treated with the enzyme was difficult to quantify due to silk’s size (417 kDa) and low reactivity. However, Suzuki-Miyaura coupling is an excellent way to quantify the amount of halogenation for two reasons. 1.) Suzuki coupling is specific only for the C-X bond (Johansson Seechurn et al., 2012), 2.) there is a catalog of boronic acids for this type of chemistry that can be employed while also determining silk’s amenability to these new groups. Halogenation was found to change many aspects of silk fibroin’s structure, mostly an α-helical secondary structure in solution and induction of β-sheet formation when dried, which has been seen in other modifying techniques. The Raman spectroscopy data coupled with the contact angle measurements show the increase in hydrophilicity of the silk material as a whole, which suggests that after halogenation, the tyrosine ring tends to bury itself in the hydrophobic core to shield the hydrophobic halogens from the aqueous solution. Thus, stretching the -OH from the phenyl ring and makes it more available for hydrogen bonding. This
structuring might also explain the tendency of the halogenated silks to have more $\alpha$-helical formation in the CD and have more contribution from the tyrosine residues in the deconvoluted Amide I peak in FTIR. This work was looking at fluorescently labeling silk as a proof-of-concept by exploiting the halogen bond. Some further work into controlling the amount of halogenation would be necessary if the desired outcome is to use this strategy specifically for the halogen as a material. However, this strategy can be used to couple more complex chemistries to enhance the functionality of silk fibroin. There is a growing interest in surface modification of materials and with literature showing tyrosines on the outside of silk fibers, this approach is ideal for adding functionality to those materials.
V. References


Miyaura, N., & Suzuki, A. (1979). Stereoselective synthesis of arylated (E)-alkenes by the reaction of alk-1-enylboranes with aryl halides in the presence of palladium


