AFRL-AFOSR-VA-TR-2021-0003

Silk Modifications for Tunable Materials

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01/07/2021
Final Technical Report

DISTRIBUTION A: Distribution approved for public release.

Air Force Research Laboratory
Air Force Office of Scientific Research
Arlington, Virginia 22203
Air Force Materiel Command

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)* 07-01-2021	**2. REPORT TYPE** Final	**3. DATES COVERED** *(From - To)* 01 Aug 2017 - 31 Jul 2020

4. TITLE AND SUBTITLE
Silk Modifications for Tunable Materials

5a. CONTRACT NUMBER

5b. GRANT NUMBER
FA9550-17-1-0333

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
David Kaplan

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
TRUSTEES OF TUFTS COLEGE INC
230 W 41ST STREET FL 7
NEW YORK, NY 21442401
US

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
AF Office of Scientific Research
875 N. Randolph St. Room 3112
Arlington, VA 22203

10. SPONSOR/MONITOR'S ACRONYM(S)
AFRL/AFOSR RTB2

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
AFRL-AFOSR-VA-TR-2021-0003

12. DISTRIBUTION/AVAILABILITY STATEMENT
A Distribution Unlimited: PB Public Release

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Naturally-derived silk fibroin holds remarkable potential for use in the field of advanced materials, due to its tough material properties and ability to be processed into a variety of material formats. The project goal is to exploit the function of tyrosines in silk protein assembly by chemically modifying these groups. A versatile and promising processing technique for silk proteins is to covalently crosslink the tyrosine groups, either to create crosslinked silk hydrogel matrices, or to incorporate functional handles into the silk.

Aims
(1) Modulate the degree of tyrosine-tyrosine covalent crosslinks in silk matrices, and to understand the impact on structure-function and material properties. Both enzymatic and chemical crosslinking reactions will be pursued in order to modulate the rate and extent of dityrosine crosslinks formed.
(2) Chemically modify tyrosines to modulate self-assembly and crystallization of silk. The objective is to utilize selective chemical modifications of the tyrosines to systematically introduce new chemistries and functional handles into the silk, and to evaluate the impact on secondary structure and material properties.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			JUNG-HWA GIMM
U	U	U	UU	6	**19b. TELEPHONE NUMBER** *(Include area code)* 426-9542

Standard Form 298 (Rev.8/98)
Prescribed by ANSI Std. Z39.18

Silk Modifications for Tunable Materials [AFOSR Grant FA9550-17-1-0333] - Kaplan
Dec 1, 2020 - Final Report

Overview

Naturally-derived silk fibroin holds remarkable potential for use in the field of advanced materials, due to its tough material properties and ability to be processed into a variety of material formats. The project goal is to exploit the function of tyrosines in silk protein assembly by chemically modifying these groups. A versatile and promising processing technique for silk proteins is to covalently crosslink the tyrosine groups, either to create crosslinked silk hydrogel matrices, or to incorporate functional handles into the silk.

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Results

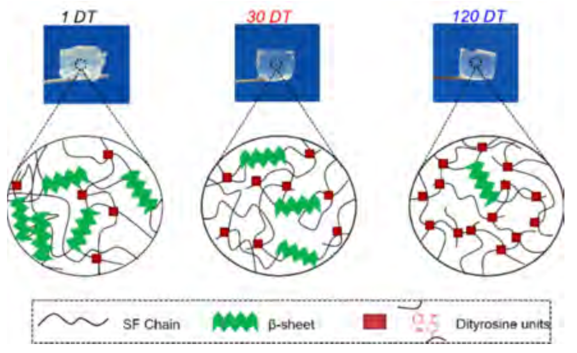


Fig. 1. Impact of molecular weight of silk on hydrogel properties after enzymatic crosslinking. Scheme depicting the effect of DT on the enzymatic crosslinking of SF hydrogels in the presence of HRP and H_2O_2. All MWs formed into hydrogels, while the content of chemical crosslinks and crystals varied, impacting the properties of the different hydrogels formed in these reactions.

ability – The molecular weight (MW) distribution SF chains varies from ~80 to 400 kDa depending the extraction and purification process utilized to pare the protein polymer. The effect of different degumming (extraction) time (DT) on material properties of enzymatically crosslinked rogels (peroxidase, hydrogel peroxide, Figure 1) s addressed, including secondary structure, rosine crosslinks, mechanical stiffness, *in vitro* radation, swelling/contraction and optical sparency. The results indicated that DT (thus, lecular weight) plays a crucial role in determining terial properties of the hydrogels; increase in lecular weight results in increased crystalline β-et formation and mechanical stiffness, while reasing degradation rate and optical sparency. The findings on the relationships ween properties of silk hydrogels and molecular ight should facilitate the rational design of silk-ed hydrogel biomaterials to match properties needed for diverse purpose in biomedical engineering.

Optimization of Fenton Reactions Related to Tunability – The oxidation of tyrosine residues of silk fibroin involves the generation of dityrosine and 3,4-dihydroxyphenylalanine (DOPA). However, it remains a challenge to selectively control the reaction pathway to produce dityrosine or DOPA in a selective fashion. Here, silk hydrogels with controllable formation of dityrosine, DOPA and DOPA-Fe^{3+} complexes, within the crosslinked networks were developed (Figure 2).

By adsorbing Fe^{3+} ions onto the chitosan particles in the chitosan particle-assisted Fenton reaction, the interaction of Fe^{3+} ions with silk fibroin could be reduced. This process resulted in significant suppression of the premature formation of *β*-sheet structures that cause steric hindrance to the collision between tyrosyl radicals, allowing the resulting hydrogels to favor the formation of dityrosine over DOPA. In this case, however, DOPA-Fe^{3+} complexes were not observed since most Fe^{3+} ions were already bound to silk fibroin and used for inducing premature *β*-sheet structures, as well as adsorbed to chitosan particles at the beginning of the reaction, thereby limiting Fe^{3+} ions available for the complexation with DOPA. The chitosan particle/EDTA-assisted Fenton reaction enabled the formation of small-sized and uniformly distributed *β*-sheet structures as well as large abundance of DOPA in the hydrogel networks, making non-EDTA-chelated Fe^{3+} ions more accessible to the complexation with DOPA. Our findings have important implications for understanding the oxidation of tyrosine residues of silk fibroin through metal-catalyzed oxidation reactions (i.e., Fenton reaction). The crosslinking strategies demonstrated here also show promise for the preparation of catalyst/enzyme-free silk hydrogels.

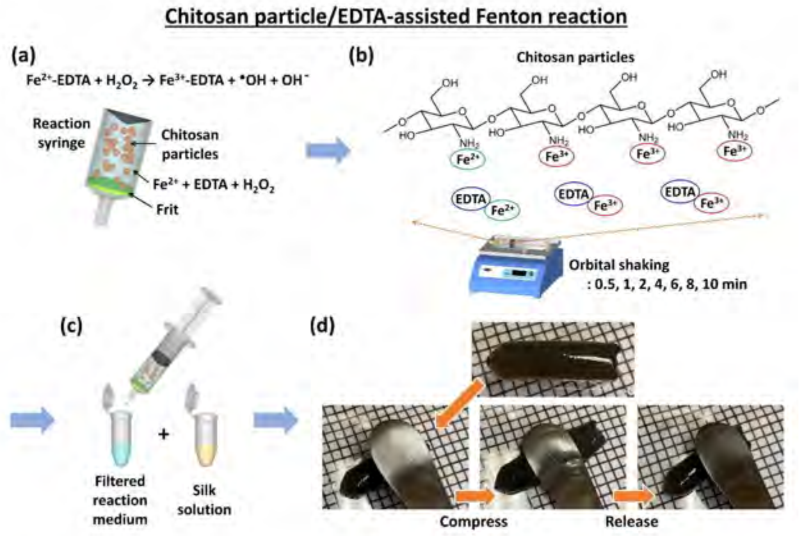


Fig. 2. Preparation of silk hydrogels using chitosan particle/EDTA-assisted Fenton reaction. (a) Graphical illustration of chitosan particle/EDTA-assisted Fenton reaction initiating in the reaction syringe having a porous frit at the bottom, where Fe^{2+}-EDTA complex reacts with H_2O_2 in the presence of chitosan particles, generating Fe^{3+}-EDTA complex and $^{\bullet}OH$ radical. It was hypothesized that the synergistic combination of the Fe^{3+}-EDTA complexation and adsorption of non-EDTA-cheated Fe^{3+} ions onto chitosan particles could more effectively limit the formation of premature β-sheet aggregates in the hydrogel networks. (b) Proposed mechanism of the adsorption of non-EDTA-chelated Fe^{3+} ions onto chitosan particles in the reaction syringe during the orbital shaking. After initiation of the reaction in (a), the syringe was immediately placed on the orbital shaker to vary contact time (0.5, 1, 2, 4, 6, 8, and 10 min) between the reaction medium and chitosan particles. (c) Filtering of chitosan particles by the frit and the initiation of the crosslinking of silk fibroin using the filtered reaction medium. (d) Photographs showing the elasticity of a silk hydrogel with 0.5 min contact time. The hydrogel compressed by the stainless steel spatula was recovered to its original shape instantaneously when the load was released.

The use of chitosan particles in the Fenton reaction allowed the interaction of Fe^{3+} ions with silk fibroin to be limited through the adsorption of Fe^{3+} ions onto chitosan particles by manipulating contact time between the reaction medium and chitosan particles. This led to significant suppression of the premature formation of *β*-sheet structures that cause steric hindrance to the collisions between tyrosyl radicals, and thus, enabled higher selectivity towards the formation of dityrosine than DOPA. The addition of ethylenediaminetetraacetic acid (EDTA) to the chitosan particle-assisted Fenton reactions resulted in hydrogels that significantly favored the formation of DOPA over dityrosine due to the increase in the hydroxylation of phenol in the presence of EDTA (Figure 3). Despite the existence of Fe^{3+}-EDTA complexes, Raman spectra indicated the DOPA-Fe^{3+} complexation in the hydrogels. Mechanistically, the hydrogel networks with small-sized and uniformly distributed *β*-sheet structures, as well as the abundance of DOPA, appear to make non-EDTA-chelated Fe^{3+} ions more accessible to complexation with DOPA.

These findings have important implications for understanding the oxidation of tyrosine residues of silk future studies on silk protein-based hydrogels capable of generating intrinsic adhesive features, as well as for exploring dual-crosslinked silk hydrogels constructed by chemical crosslinking and metal-coordinate complexation.

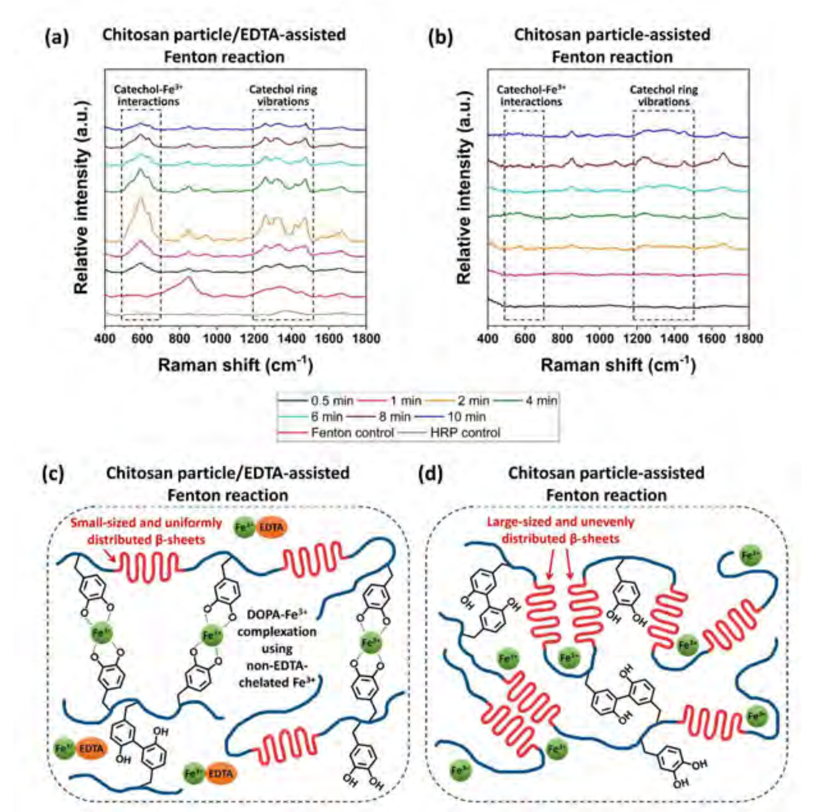
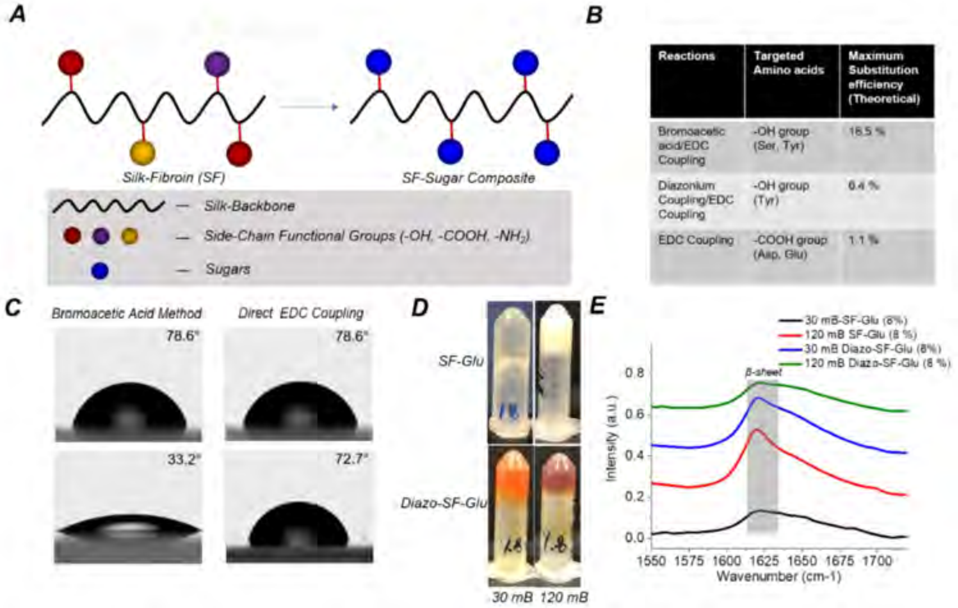


Fig. 3. Raman spectra of: (a) hydrogels crosslinked by chitosan particle/EDTA-assisted Fenton reaction and (b) hydrogels crosslinked by chitosan particle-assisted Fenton reaction. DOPA-Fe^{3+} complexes were observed only in the hydrogels crosslinked by chitosan particle/EDTA-assisted Fenton reaction and did not occur in the hydrogels crosslinked by chitosan particle-assisted Fenton reaction. (c) Proposed network structure for hydrogels crosslinked by chitosan particle/EDTA-assisted Fenton reaction. Small-sized and uniformly distributed *β*-sheet structures as well as large abundance of DOPA would make non-EDTA-chelated Fe^{3+} ions more accessible to the complexation with DOPA. (d) Proposed network structure for hydrogels crosslinked by chitosan particle-assisted Fenton reaction. There is likely limitation of free Fe^{3+} ions available for DOPA-Fe^{3+} complexation because most Fe^{3+} ions are already bound to silk fibroin and used for triggering premature *β*-sheet structures, as well as adsorbed to chitosan particles at the initial stage of the reaction. Moreover, even with available Fe^{3+} ions, large-sized and unevenly distributed β-sheet aggregates may sterically hinder the accessibility of Fe^{3+} ions to DOPA.

Chemical methods for sugar substitution onto silk fibroin - Glucosamine was used as the simple sugar for conjugation onto silk fibroin (Figure 4). Three different chemical reaction pathways were explored to modify silk fibroin biomaterials with glucosamine that can yield different substitution efficiencies based on the amino acid functional groups present in each protein chain: (1) serines (12.1%) were carboxylated by bromoacetic acid to install carboxylic acid groups which further react with the primary amine of glucosamine *via* EDC coupling to generate silk-glucosamine conjugates. (2) Tyrosines (5.3%) were carboxylated by diazonium coupling to incorporate acid groups for further conjugation with primary amines to form silk-glucosamine composites. (3) Direct EDC coupling onto the SF with glucosamine by targeting the aspartic and glutamic acids residues (1.1%). In the above three reactions, the substitution efficiency is controlled with a maximum of 18.5% (Serine and Tyrosine), 6.4% and 1.1%, respectively. These new silk-Glucosamine (SF-Glu) biomaterials were structurally characterized with ^{1}H-NMR spectroscopy and water contact angle measurements to verify the sugar substitution onto the SF. The new SF-Glu that were synthesized were more hydrophilic than the original silk fibroin. The emergent biomaterials had different material properties depending on the chemistry utilized. Higher sugar substitution led to more hydrophilic materials whereas lower substitutions led to more hydrophobic biomaterials based on the water contact angle measurements.

***Enzymatic hydrogel formation of SF-Glu composites and emergent material properties* -** The ability of the SF-Glu composites synthesized using different chemistries to form hydrogels was investigated. Silk-sugar composites prepared from direct EDC coupling and diazonium coupling readily form hydrogels in the presence of horseradish peroxidase (HRP) and H_2O_2, while Silk-sugar prepared by carboxylation and EDC coupling did not form hydrogels, but did form hydrogels by dityrosine crosslinking and β-sheet formation. The effect of glucosamine introduction onto silk is being investigated for emergent material properties, such as crosslink density, β-sheet formation, degradation profile and mechanical stiffness.



Reactions	Targeted Amino acids	Maximum Substitution efficiency (Theoretical)
Bromoacetic acid/EDC Coupling	-OH group (Ser, Tyr)	16.5 %
Diazonium Coupling/EDC Coupling	-OH group (Tyr)	6.4 %
EDC Coupling	-COOH group (Asp, Glu)	1.1 %

Figure 4: (A) Schematic of sugar substitution onto silk-fibroin solution. The color spheres in the scheme indicate targeted functional groups in silk-fibroin used to introduce sugars. (B) Different chemistries used to incorporate sugar moieties onto silk; different chemical methods have different reactive sites required for sugar substitution. (C) Water contact angle measurements of 2% water annealed 30 mB (minute extracted) silk fibroin (SF) and SF-Glu composite prepared by the different methods; top panel: 30 mB SF; bottom panel: 30 mB SF-Glu. (D) Self-supporting hydrogel formation of SF-Glu (30 and 120 mB) composite prepared in presence of HRP/H_2O_2 for 8 wt %. (E) FTIR spectroscopy of SF-Glu hydrogels to determine the secondary structures.

Impact & Future Directions

A fundamental understanding of the role that tyrosine interactions play in silk self-assembly was developed, covering issues of chemical and biochemical crosslinking, the role of metal ions, and the impact of sugar modifications. A new technique for crosslinking tyrosine groups in silk utilizing the Fenton reaction expanded our synthetic toolbox and provided comparisons to the biochemical (enzymatic reactions in terms of control of hydrogel properties. Developing a LC-MS/MS method to assess the crosslinks provided quantitative, molecular-level information on the tyrosine crosslinking reactions, and provided clear evidence that the crosslinking density can be modulated in this system. Selective and systematic modifications of tyrosine residues have produced new silk variants and has allowed us to change the secondary structure and self-assembly, as well as incorporate new functional moieties. Ultimately, the results of this study enable us to finely control silk material properties and add functionality, further expanding the library of materials with exceptional properties that can be generated from silks.

Collaborations initiated via the AFOSR program over the 3 years

Adam Braunschweig, CUNY – characterization of mucin mimics

Doug Clark, Berkeley – formation of composite hydrogels combining the tunable silk hydrogel approach in the report with Doug's foldin proteins – to improve mechanical toughness and utility of his systems.

Patrick Dennis – Air Force Labs - Materials Methods and Processing Strategies – to continue to pursue new materials based on silks, new modes of processing and new characterization tools – included a visit to Tufts by the Air Force team to update on methods, projects and plans, and a reciprocal visit by one of our PhD students (Nicole Raia) to present a seminar and discuss ongoing collaborations at the Air Force Labs.

Maneesh Gupta (AFRL) – project focus on adapting the tunable silk hydrogels in the report towards oxygen sensing systems (via coupling in oxygen-responsive chromophores) into the hydrogels, for physiological monitoring of pilot status.

Greg Holland, San Diego State University – solid state NMR to study silk structure

Murugappan Muthukumar, U. Massachusetts Amherst –solution behavior of modified silk materials

Carol Perry – silk-inorganic composite materials

Vladimir Tsukruk, Georgia Tech – new silk material assemblies

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