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The interplay between silk fibroin's structure and its adhesive properties

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Abstract

Bombyx mori-derived silk fibroin (SF) is a well-characterized protein employed in numerous biomedical applications. Structurally, SF consists of a heavy chain (HC) and a light chain (LC), connected via a single disulfide bond. The HC sequence is organized into 12 crystalline domains interspersed with amorphous regions that can transition between random coil/alpha helix and beta-sheet configurations, giving silk its hallmark properties. SF has been reported to have adhesive properties and shows promise for development of medical adhesives; however, the mechanism of these interactions and the interplay between SF's structure and adhesion is not understood. In this context, the effects of physical parameters (i.e., concentration, temperature, pH, ionic strength) and protein structural changes on adhesion were investigated in this study. Our results suggest that amino acid side chains that have functionalities capable of coordinate (dative) bond or hydrogen bond formation (such as those of serine and tyrosine), might be important determinants in SF's adhesion to a given substrate. Additionally, the data suggest that fibroin amino acids involved in beta-sheet formation are also important in the protein's adhesion to substrates.

Abstract

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Supporting information

Quantified pull away test data for the effects of solution concentration, temperature, pH, ionic strength, beta-sheet content and lyophilization/reconstitution process on silk adhesion to substrates. Additional structural characterization data for silk solutions and films.

Keywords

silk fibroin; structural changes; adhesion; physical interactions

1. Introduction

Tissue sealants and adhesives have emerged as an attractive alternative for the use of sutures or staples for surgical closure.^{1–4} A variety of natural or synthetic biomaterial-based products are currently available and they all offer advantages such as rapid deployment, reduced surgical complications, bioresorbtion, decreased antigenicity, and in the case of sealants, prevention of body fluid leakage.^{5,6} These commercial products satisfy many of the practical wound closure requirements, but are often associated with adverse effects such as poor adhesion in a wet environment, slow bioresorbtion, suboptimal biocompatibility of degradation products, and high material swelling with adjacent tissue compression. These deficiencies highlight research opportunities aimed at improving overall sealant/adhesive properties. Several research groups have already focused their efforts in this area, and high complexity, chemically engineered tough adhesives, with improved adherence to wet surfaces have been characterized.^{7–9} One medical application that could drastically benefit from a biomimetic adhesive that targets wet surfaces, is post-surgical seroma prevention. Seromas are pockets of serous fluid that accumulate in the dead space between the separated areas of planar tissue as a result of surgical procedures such as hernia repair, abdominoplasty or mastectomy.^{10–12} Seromas occur in 100% of patients post-operatively and is currently treated via surgical drain placement or multiple aspirations.¹³ While medically a seroma is classified as a complication only when it persists over 6 weeks, for the patients, the presence of drains or being subjected to numerous fluid aspirations with a syringe come with pain, discomfort, increased risk of surgical site infection, and prolonged healing time and hospital stay (3–15 days). A biomimetic adhesive that would minimize the dead space between separated tissues by keeping them together could prevent seroma formation and would have the potential to revolutionize the current post-operative standard of care.^{14–16} In this context, silk fibroin (SF), a natural, high molecular weight (MW~416 kDa) polymeric protein commonly extracted from *Bombyx mori* silkworm cocoons, appears suitable as a low complexity alternative for the development of next generation biomimicking sealants and adhesives.^{17–19}

SF-based materials that employed the protein either as an additive to an in situ, chemically crosslinkable system or in a polyethylene glycol and catechol-derivatized format have been shown to yield performant adhesives.^{17,18,20–22} Additionally, it was shown that water-soluble SF-only films were able to adhere to moistened latex substrates.²¹ However, the mechanism of the SF's interactions with substrates and the interplay between the protein's structure and adhesion is not understood.

The SF consists of two distinct subunits, a heavy chain (HC, ~390 kDa) and a light chain (LC, ~26 kDa), which are connected through a single disulfide bond.²³ The mature form of the HC consists of 5242 amino acids while the mature LC that consists of 246 amino acids. The HC is organized into 12 crystalline domains interspersed with amorphous regions that

enable the protein to transition from random coil and alpha helix conformations to antipolar-antiparallel beta-sheet (β -sheets) containing structures.²⁴ The propensity of the HC to form β -sheets under various external stimuli (i.e., temperature, pH, ionic strength, etc.) enable the processing of the protein solution into numerous formats (such as gels, films, sponges, fibers, etc.) with tunable physical and mechanical properties.^{17,22,25} At the primary structure level, each of the HC crystalline domains consists of subdomains of ~70 residues that primarily start with repeat glycine, alanine and serine-rich (GAGAGS) hexapeptides and terminate with a GAAS tetrapeptide. Hydrophobic interactions between the glycine (Gly or G) and alanine (Ala or A) amino acids and intra- and intermolecular hydrogen bonding between the serine (Ser or S) residues are believed to be driving the protein's secondary structure conformations.^{26,27}

The relationship between SF's primary sequence, secondary structure and its mechanical or physical properties has already been examined and were found to be interconnected.^{26,28,29} Taking a similar approach, herein, the effect of physical parameters (specifically, SF solution concentration, temperature, pH, ionic strength) and protein structure on adhesion was investigated. The results suggest that the adhesion of SF to a substrate involves amino acid side chains with functionalities capable of physical interactions, in the form of coordinate/dative bonding or hydrogen bonding depending on the substrate, and that the same amino acid side chains are involved in structural transitions to β -sheet conformations. Moreover, the data have shown that silk fibroin can be processed into a format suitable for product development (films) while exhibiting performant adhesion to biological substrates under physiological conditions.

2. Materials and Methods

2.1. Materials

Sodium carbonate (Na_2CO_3) was purchased from EMD Chemicals Inc. (Gibbstown, NJ). Polyethylene glycol molecular weight 10,000 Da (PEG 10,000) was purchased from Alfa Aesar (Ward Hill, MA). Methanol (MeOH, HPLC grade), lithium bromide (LiBr), hydrochloric acid (HCl, 6N), phosphate buffered saline (PBS) and dialysis cassettes (Slide-A-Lyzer, MWCO 3500) were purchased from ThermoFisher Scientific (Waltham, MA). Sodium hydroxide (NaOH, pellets) was purchased from VWR International, LLC. (Radnor, PA). Tris-Acetate SDS Running Buffer and NuPAGE™ LDS Sample Buffer was purchased from Novex (Carlsbad, CA). NuPAGE™ 7% Tris-Acetate Gel, Sample Reducing Agent, and HiMark™ Pre-stained HMW Protein Standard were purchased from Invitrogen (Carlsbad, CA).

2.2. Silk Fibroin Solution Preparation (Extracted Silk) and Concentration

Silk fibroin (SF) was extracted from commercial, medical device grade *Bombyx Mori* silk yarn (Bratac, Brazil)³⁰ according to a modified protocol.³¹ Specifically, 7.5 g of yarn was cut with scissors into 1–2-inch pieces and added to 3 L of boiling aqueous solution of 0.02 M Na_2CO_3 or 0.2 M Na_2CO_3 , respectively, to remove sericine. The silk fibers were removed after 30 or 60 minutes, respectively, rinsed three times with deionized water and dried overnight under environmental conditions. The dried fibroin was then dissolved in 9.3 M

LiBr solution at 20% w/v and placed in a 60°C oven for 4 h. The resulting solution was then transferred to dialysis cassettes and dialyzed against deionized water for 48 h. The resulting pure SF solutions had a typical concentration in the 6–8% w/w. For experiments requiring higher protein concentrations, SF solutions were reloaded in dialysis cassettes concentrated against 10% w/v aqueous PEG 10,000 solution until the desired concentration was reached.

2.3. Reconstituted silk solutions

Extracted silk solutions in water were frozen at –80 °C for 4 h then freeze-dried on a lyophilizer (BenchTop Pro XL, SP Scientific, Gardiner, NY) for 24 h. For reconstitution, lyophilized silk was weight out and dissolved in deionized water or PBS with vortexing.

2.4. Silk Film Preparation

Silk films were prepared by casting 0.5 mL of silk solution (8% w/w) into a 24 well plate. Samples were left on the bench overnight at room temperature and humidity. After films have formed they can be used immediately or can be stored in an air tight container for use at a later date. For thickness measurements films were first cut to the desired dimension and then measured using a 0–1” outside micrometer (Chicago Brand, Medford, OR).

2.5. Adhesion Testing

2.5.1. Pull-Away Testing—A Discovery HR2 hybrid rheometer (TA Instruments, New Castle, DE) equipped with a 40 mm parallel plate geometry and a Peltier plate for temperature control was used for pull-away testing of all solutions.³² For all solution tests a geometry gap of 100 μm was set. To ensure complete geometry coverage, the test solution was applied in excess (200 μL /test), the overflow was trimmed and the complete coverage was confirmed visually. Subsequently, after 60 seconds of equilibration the geometry gap was increased a constant linear rate of 600 mm/min for 2 seconds. The adhesiveness of solutions was determined by the axial force measured during this process. Testing was done in triplicate for all samples ($n=3$). For the preparation of β -sheet containing samples, MeOH (80% v/v) was added to silk solutions in a 10:1 v/v ratio, then cast immediately into a 24 well plate and allowed to undergo structural changes in situ, at room temperature for 24 h, to yield gel-like materials. The average thickness of those samples was $355 \pm 23 \mu\text{m}$ ($n=3$) and an 8 mm parallel plate geometry was used to increase the accuracy of measurements for these samples. The adhesion testing procedure was performed with the geometry gap increased to 330–370 μm to accommodate the thickness of the gels.

2.5.2. Single Lap Joint Shear (Lap Shear) Testing—A Discovery HR2 hybrid rheometer equipped with a DHR Film/Fiber Tension Accessory (TA Instruments, New Castle, DE) was used for lap shear testing of all samples.³³ Natural Chamois leather (Amazon, Seattle, WA) was washed with a mild detergent followed by 5 deionized water washes then allowed to dry for several days. Once dried, the large Chamois leather sample was cut into individual $10 \times 30 \text{ mm}$ strips for testing. For solution-based testing 100 μL of sample was applied to approximately a $10 \times 10 \text{ mm}$ area (100 mm^2). For film-based testing, the film samples were cut into a $10 \times 10 \text{ mm}$ square and applied to a $10 \times 10 \text{ mm}$ area of the leather wetted with 75 μL of deionized water. The general test procedure was to have the two strips were then pressed together, and allowed to adhere for 3 h. After 3 h, the adhered

samples were subjected to lap joint shear adhesion testing. For the temperature dependence experiments, the 3h adherence step was performed at the indicated temperatures. For pH and ionic strength dependence, the films were cast from solutions at the indicated pH values or in PBS, but tested per the general procedure. For MeOH pre-treatment, films were treated for 1 hour prior to following the general procedure, while for post-treatment, substrates were adhered per the general process then treated for 1 hour prior testing. The grip to grip separation was set at 20 mm, and then the crosshead speed was maintained at 50 mm/min. The shear adhesive bond strength (S) was calculated as the maximum shear force divided by the adhesive area.

2.6. Gel electrophoresis

The molecular weight distribution of extracted SF was determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For each condition, 5 µg/ml of silk protein was reduced with NuPAGE Sample reducing agent and loaded onto a 7% Tris-Acetate gel (NuPAGE, Life Technologies, Grand Island, NY). The gel was run under reducing conditions for 75 min at 150 V, with a high molecular weight ladder as reference (HiMark Prestained, Life Technologies) and stained with a SimplyBlue SafeStain staining solution (Thermo Fisher Scientific, Waltham, MA).

2.7. Circular Dichroism (CD) Spectroscopy

The CD spectra of silk fibroin samples in various solvents were acquired using a Jasco J-820 CD spectropolarimeter (JASCO Corporation, Tokyo, Japan). Samples were prepared in a final concentration of 0.1 mg/ml from either fresh or lyophilized silk solution. For temperature dependence study, samples were dissolved in water and analysis was carried out at 3, 20 or 37 °C. For pH dependence study, samples were dissolved in water adjusted to pH 5.5, 7.0 or 8.5 and analysis was carried out at 20 °C. For ionic strength dependence study, samples were dissolved in water or phosphate buffered saline (PBS) and analysis was carried out at 20 °C. Background scans were obtained for the individual solvents. Measurements were acquired using a quartz cuvette with a 0.1 cm path length. Each spectrum represents the average of ten scans from 190 to 240 nm with a 1 nm resolution, obtained at 200 nm/min with a bandwidth of 1 nm.

2.8. Fourier-Transform Infrared Spectroscopy

The structural conformations of silk solutions were analyzed with a Nicolet iS5 FT-IR equipped with an iD7 diamond attenuated total reflectance (ATR) accessory (Thermo Scientific, Waltham, MA). The absorbance of samples was measured between 4000 – 400 cm⁻¹, with 64 scans, and a resolution of 4 cm⁻¹. Background spectra were collected under the same conditions and subtracted from the sample. For all solutions 50 µL of sample was loaded onto the ATR accessory. For film/gel samples a small amount of film/gel was placed on the ATR accessory.

2.9. Wide-angle X-ray scattering (WAXS) measurement

Synchrotron WAXS measurements were conducted at the BL45XU beamline at SPring-8, Harima, Japan, according to a previous report.³⁴ The X-ray energy was 12.4 keV at a

wavelength of 0.1 nm, the sample-to-detector distance for the WAXS measurements was 258 mm, and the exposure time for each diffraction pattern was 10 s. The obtained diffraction data were converted into one-dimensional profiles using the software Fit2D.³⁵

2.10. Statistical analyses

Values, represented as mean $\pm$ standard deviation (S.D.) were compared either with Student's *t* test (2-tailed, type 3) (for data groups of two) or Single Factor ANOVA (for data groups of three) with *p* = 0.01 considered statistically significant.

3. Results and Discussion

Silk fibroin solutions

Intrinsic adhesion of SF.—To assess the intrinsic adhesiveness of the protein, freshly prepared protein solutions, as described under Materials and Methods, were evaluated with two different methods. The first one is a pull away test method (Figure 1A) that measures the peak normal force needed to break the seal between the two parallel fixtures (steel substrate) and the sample tested, and is a representation of the sample's actual 'stickiness'.

This test allows for the determination of a sample solutions stickiness (by measuring the peak normal force) as well as the adhesive and cohesive strength (by measuring the area under the force-time curve, with a larger area indicative of a stronger adhesive) as the two parallel plates are separated (Figure 1B).³² The second method used to assess our materials is an adhesive lap joint shear test (Figure 1C) with a biological substrate (chamois leather) and was used to determine the shear strength of the tested adhesive.³³

Based on previously published data on SF adhesiveness and the interdependence of protein's properties on the extraction conditions^{20,21,36}, the intrinsic adhesiveness of protein extracted in solutions of different alkalinity (0.2 M versus 0.02 M Na₂CO₃) and different boiling times (30 min versus 60 min) was investigated. When evaluated via the pull away test method, SF solutions (20% w/w) extracted under lower alkalinity and shorter boiling time (SF1) elicited superior adhesiveness, to the stainless-steel substrates, as illustrated by higher peak normal force values and higher area under force-time curve values, compared to their counterparts extracted with longer boiling times and higher alkalinity (SF2–4) (Figure 2A, Table 1).

In addition, SF1 showed significantly higher adhesiveness, as illustrated by higher peak normal force and area under force-time curve, compared to New Skin® Liquid Bandage, a commercially available tissue sealant control that, similarly to SF, interacts with substrates via non-covalent, physical interaction (Figure 2A, Table 1). When the differently extracted SF solutions were evaluated via gel electrophoresis, the data indicate that higher alkalinity and longer boiling times clearly alter the molecular weight (MW) distribution of the protein, with more drastic effects associated with increased alkalinity (Figure 2B). Typically for proteins, a decrease in molecular weight and chain length, is associated with a different amino acid distribution per peptide chain, which in turn would translate to different overall peptide charge, different folding patterns or different ability to interact with substrates. Although we did not specifically assess the amino acid composition of our degraded samples, given the polymeric, highly repetitive nature of the silk protein, we postulate that

the observed differences in adhesion between SF1 and SF2-SF4 are predominantly reflective of changes in the polymeric chain length and ensuing secondary structures.

The silk solutions obtained via different extraction conditions exhibited different adhesive properties even on biological substrates (Figure 3). Specifically, in lap shear tests with leather substrates, SF1 appeared to be almost twice as strong as SF3 and approximately 13 times stronger than the Liquid Bandage control.

Effects of physical parameters on solution adhesiveness.—In order to better understand the mechanism of interaction between SF and substrates, the effects of solution concentration, temperature, pH and ionic strength on protein adhesiveness was interrogated (Figure 4). In pull away tests and on stainless steel substrates, our results indicate that increasing the concentration of the protein solution results in an increase in adhesiveness (Figure 4A, Table S1). Specifically, a 20% w/w SF exerts 11 times higher peak normal force values than an 8 % w/w solution and has a 2 times higher area under force-time curve (Table S1). When SF solutions were tested at different temperatures, the pull away test data show that the protein's adhesiveness decreases with increasing temperatures (Figure 4B, Table S2). Notably, under physiological temperatures (37 °C), SF normal peak force values were approximately 3 times lower than at 3°C and correspondingly, the area under force-time curve values were 2 times lower (Table S2). The pH effects on adhesiveness were also significant (Figure 4C, Table S3). Solutions with alkaline pH values (pH = 8.5) elicited approximately 1.5 times decrease in peak normal force and area under force-time curve values compared to their neutral (pH = 7.0) or acidic (pH = 5.5) counterparts (Table S3). Lastly, our data show that differences in solvent ionic strength (deionized water – conductivity of 0.06 mS/cm at 25°C, versus 1X phosphate buffered saline (PBS) – conductivity of 15–20 mS/cm at 25°C) on protein-substrate interaction are negligible (Figure 4D, Table S4), with comparable peak normal force and area under force-time curve values obtained for SF in deionized water and SF in 1X PBS (Table S4).

Effects of secondary structure changes on SF solution adhesiveness.—One of the hallmarks of SF is its ability to undergo secondary structural changes leading to β -sheet formation. These secondary structure transitions impart the protein's excellent processability (i.e., into water insoluble films, gels, sponges, etc.). Therefore the effects of such structural changes on SF solution-substrate interactions were evaluated. To induce β -sheet formation, solutions were treated with 80% v/v methanol (MeOH) in a 10:1 v/v ratio for different amounts of time (5 h and 24 h) (Figure 5). The extent of β -sheet formation in the MeOH treated SF samples was assessed by FTIR for the presence of the β -sheet characteristic peak at 1625 cm^{-1} . The 24 h MeOH treatment resulted in SF solution gelation. In agreement with this empirical observation, the spectra indicate the presence of a strong β -sheet peak in the SF gels, while only a slight pattern change was observed in the corresponding region of the 5h treated sample compared to the untreated control (Figure 5A). When tested for adhesion to steel substrates, the 5 h MeOH treated samples, showed lower, but statistically comparable, peak normal force and area under force-time curve values (Table S5). The 5 h time point was randomly selected as an early time point. At 24 h, samples appeared gelled by visual analysis, therefore we considered this to be our end time point. Earlier time points

(<5 h) and intermediate time points (specifically 8 to 20 h) were monitored, but no significant structural changes were detected in the β -sheets region by FTIR. Similarly, no significant differences in pull away test results were recorded.

The gelled, β -sheet containing samples, showed no adhesive properties. The peak normal force was observed at time zero due to the contact of the geometry with the solid gel samples, however upon separating the fixtures the measured normal force immediately reduced to zero.

Physical parameters such as temperature, pH or ionic strength did not induce any immediate structural changes in the protein, and all the samples displayed disordered structures (Figure 6). It is interesting to note however, that the protein structure in PBS was different than in water. Based on the aforementioned results, all subsequent experimental characterization of the protein was conducted with SF1 solutions, extracted under low alkalinity and shortest time (0.02 M Na₂CO₃, 30 min boil).

Reconstituted silk solutions

Adhesion strength.—A requirement for biomedical adhesive products is ease of storage and handling properties. In that context, the adhesive properties of reconstituted lyophilized SF samples were assessed. One of our first observation was that lyophilized samples could only be reconstituted to very low concentration solutions (0.1% w/w). Based on the data presented in Figure 4A and Table S1, the adhesion of such low concentration solutions was expected to be suboptimal. This assumption was confirmed by lap shear test data with fresh and reconstituted silk solutions of identical concentrations (0.1% w/w, Figure S1). The determined baseline adhesive strength levels made it difficult to objectively assess any adhesive strength differences that might have been induced by the processing of the two samples.

Effects of physical parameters on reconstituted protein structure.—Structural analyses were employed to understand how the lyophilization and reconstitution process, and physical parameters such as temperature, pH and ionic strength affect the structure of the protein (Figure 7).

All lyophilized samples were prepared from aqueous silk solutions, then reconstituted in DI H₂O (or PBS, for the assessment of ionic strength effects). In the lyophilized and DI H₂O reconstituted samples, the CD data obtained indicate the presence of characteristic features of β -sheets. This is contrasting with to fresh extracted solutions (Figure 6) and explains the limitations in resolubilizing the lyophilized material, as β -sheet containing structures are intrinsically insoluble.³⁷ Increasing or decreasing the temperature of the reconstituted solutions did not seem to alter the β -sheet content of the samples (Figure 7A), however, acidic pH values appeared to increase sample crystallinity (Figure 7B). Significant structural differences were observed between solutions reconstituted in water versus PBS (Figure 7C), with the data showing disordered protein structure, with negative bands at 195 nm, indicating that the presence of ions in the sample impacts the protein's ability to form β -sheets. Overall these results indicate that the lyophilization and subsequent reconstitution of the protein is not a suitable process for SF adhesive development.

Silk films

Film adhesion.—Given our findings with reconstituted silk, a different approach was employed to impart stability and ease of handling to samples, by using SF films. No adherence of films was observed to dry substrates (both steel and leather), but the films adhered to moist, pre-soaked leather substrates (Figure 8).

Similarly, to the solutions, SF films obtained from SF1 versus SF3 displayed significantly different adhesive strengths (Figure 8A). Unlike adhesion of solutions to steel, with biological substrates, SF1 films showed 2 times higher adhesion at 37 °C than at 20 °C, and 37 times higher adhesion than at 4 °C (Figure 8B). When SF1 solutions were adjusted to slightly acidic (pH 5.5) or alkaline (pH 8.5) then cast into films, the adhesive strength of the samples was compromised with the acidic samples showing no adherence and the alkaline samples exhibiting 4.3 times lower strength (Figure 8C). Taken together the temperature and pH dependence results would suggest that SF1 films would be suitable for biomedical applications under physiological conditions. In terms of solvent effects on SF1 film adhesiveness, films cast from SF1 in water showed 3.3 times higher adhesion than their counterparts cast from SF1 in PBS (Figure 8D).

Effects of secondary structure changes on SF film adhesiveness.—Similar to SF solutions, an understanding of the effect of secondary structure changes, specifically β -sheet presence on the adhesive strength of silk films was sought. For this, SF1 films were pre-treated for 1 hour with MeOH either before adhering leather strips or after adhering leather strips, then lap shear tested (Figure 9).

Specifically, the pre-treatment was intended to induce β -sheet formation in the films prior to adhesion/interaction with the substrate and assess whether such films are capable of adhesive interactions with a biological substrate. The post-treatment was aimed at assessing if β -sheet formation was still possible in a film after the initial adhesion to a biological substrate, and the effects of such structural changes. The data indicate that MeOH pretreatment negatively impacted the adhesive strength (10.5 times lower), while MeOH post-treatment significantly increased the film's adhesive strength (1.9 times higher). The protein structure in the pre- and post-MeOH treated films was investigated via FTIR and confirmed the presence of β -sheets in both the pre-treated sample and the post-treated samples analyzed after lap shear testing (Figure S2). The observed differences in adhesive strength between the untreated and post-treated samples would suggest that further substrate-silk interaction stabilization can be achieved through β -sheet induction after the initial adhesion.

Effects of physical parameters on protein structure in films.—Two different analytical methods were employed to assess the secondary structure of the silk fibroin in films at different temperatures or cast from different pH, ionic strengths or extraction process solutions. The first method was WAXS and the results are summarized in Figure 10. The peaks with d -spacing values of 0.43 and 0.37 nm are originated from β -sheet structures.^{38,39} According to this assignment, treatment with MeOH and decreasing the solution pH to 5.5 induced β -sheet formation (peaks at 0.43, 0.37 nm), whereas having the protein in PBS

or under mild alkaline conditions (pH 8.5) appeared to decrease the β -sheet content of the sample. The FTIR results are in agreement with the presented data (Figure S3). Overall, the data generated with SF films suggest that this format would be the most suitable for further development as biomedical adhesive, as it is highly adhesive under physiological conditions and because of its physical presentation, would be convenient to manufacture, package, store and use.

Potential mechanisms of interaction between SF adhesives and substrates.—

The adhesive properties of SF were previously highlighted by several publications that focused either on the protein alone or with the protein as part of a multi-component system.^{18,20,21} Our initial adhesion testing experiments were designed to use biological membranes (i.e., porcine intestines) as substrates. However, membrane thickness and tissue homogeneity variations posed issues related to data reproducibility and interpretation. In this context, for simplicity, reproducibility and to generate a fundamental understanding of the interplay between the silk protein's structure and adhesion to substrates, stainless steel and chamois leather were used as substrates. The initial focus was on understanding the factors that impact the intrinsic adhesiveness of SF. As illustrated by our results, the selection of the mildest extraction/purification process is essential in maintaining the polymeric protein's adhesiveness. Additionally, factors such as solution concentration, pH and temperature appear to impact the extent of interactions with substrates, while films appear to be superior to their solution counterparts in terms of adhesion. Interestingly, our data indicates that the adhesive strength between a film and a biological substrate, can be further increased via post-adhesion β -sheet induction. A possible explanation of this observation would be an increase the adhesive/cohesive forces within the film via post-adhesion β -sheet formation, which in addition to the existing adhesion forces between the substrate and film would translate to a larger force to induce the failure of the adhesive.

Mechanistically, considering the structure of the silk fibroin, the adhesive interactions with the substrate could be approached as hydrophobic (the majority of the constituent amino acids are non-polar) and/or hydrophilic (mediated by polar side chains such as those of serine or tyrosine residues, which constitute ~ 18% of the amino acid sequence of the protein). With silk solutions and metallic substrates, stronger adhesion was observed with decreasing temperatures, and neutral or acidic pH. The structural data in Figure 6 suggest hydrophobic interactions to be unlikely, therefore we hypothesize that coordinate and hydrogen-bonding act as driving force for adhesion.⁴⁰ With biological substrates, physiological temperature seems to yield the highest adhesive strength. Considering the data in Figure S3, the structure of the film does not show noticeable structure changes at 37 °C, suggesting that the temperature effects might be due to changes in substrate surface (prehydrated leather), probably with more functionalities capable of hydrogen bonding exposed. The adhesion between film and leather also seems to be affected by pH and solvent used for film casting. In the case of low pH solutions used for casting, FTIR and CD data indicate that the resulting films contain β -sheets. The lack of adhesion with β -sheet containing films is in agreement with data obtained with solutions, and further supports the conclusion that amino acids involved in β -sheet formation are also involved in adhesive interactions with substrates. The decrease in adhesion with films cast from pH 8.5 solutions

would indicate that protonated side chains are needed for increased adhesion to leather. This extrapolation is further supported by the adhesion of PBS cast films, where the presence of PBS specific ions interacts with ionizable amino acid side chains.

Based on the above, we propose that the mechanism of adhesive SF interaction is mediated by the serine side chains (the most abundant polar amino acid of fibroin) and are predominantly driven by dative bonds with metallic surfaces, and by hydrogen bonds with biological substrates (Figure 11).

Tyrosine residues might also be contributing to the adhesive effects. However, chemical modification of tyrosine in silk does not appear to impair the protein's β -sheet forming capability and the amino acid has relatively low abundance in the protein sequence compared to serines (5.3% Tyr versus 12.1% Ser).⁴¹

Overall, the results indicate that the adherence of SF to substrates, (biological or metallic) is mediated by ionizable amino acid side chains capable of physical interactions such as coordinate and hydrogen bonds. Interestingly, it appears that formation of β -sheets after the initial adhesive interaction with the biological substrate further strengthens the adhesive bond between SF and biological substrates. Even without the use of secondary structure inducers, β -sheets would be expected to form naturally in silk, thus leading to further consolidation of adhesion.²⁰ This finding opens opportunities for customized, substrate-specific engineering of SF-based adhesives and sealants, and highlights the importance of understanding the nature and composition of a given substrate in the context of developing maximally efficient interaction partners. In this context, our immediate studies are directed towards testing the proposed mechanisms with biological substrates and by chemically modifying SF with functionalities with different physical bonding abilities.

Conclusion

The research described herein is focused on the investigation of the nature of the interactions between the SF protein and metallic and biological substrates. Our findings suggest that, in agreement with silk's primary structure, physical interactions mediated by amino acid side chains capable of physical bonding (such as H-bonding for biological substrates or predominantly dative bonding for metallic substrates), are participating in adhesive interactions with the substrate. Moreover, our data suggests that the involvement of such amino acids in β -sheet formation leads to loss of protein adhesiveness. These findings are important as they open opportunities to custom design substrate specific silks that can intimately interact with organic or inorganic substrates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Adhesion test methods used. A – set-up for pull away test, showing the two parallel plates in-between which thin layers of solutions are sandwiched; B – profile of the data generated by the pull away test with the recorded peak force corresponding to the tack or stickiness of the sample and the area under the force-time curve being indicative of the strength of the adhesive. C – set-up for the lap shear test, showing the upper and lower clamps that affix the lap joint specimen (two rectangular pieces of leather adhered together over a 1 cm² surface). For the shear test a data profile similar to the pull away test is generated, and the data are presented as peak force normalized per adhered surface, indicative of the strength of the sample.

Figure 2.

A – Representative pull away test results illustrating the effect of silk fibroin (SF) extraction parameters on the protein's intrinsic adhesion to steel fixtures (SF solutions used at concentrations of 20% w/w); B – SDS-PAGE results illustrating the effect of SF extraction parameters (Na_2CO_3 concentration and boiling time) on the protein's molecular weight (MW) distribution.

Figure 3. Adhesion strength of SF1 (0.02 M, 30 min), SF3 (0.2 M, 30 min) and Liquid Bandage determined with a lap shear test ($n=5$). The ANOVA analysis indicates a significant difference in adhesion between the groups at the $p<0.01$ level ($F(2, 8) = 56.06$, $p = 2E-05$). Student t-test compared to control $p = 0.01$.

Figure 4.

The effect of physical SF solution parameters on adhesion. A – Representative sample results illustrating the effect of SF solution concentration of the pull away force needed to separate steel fixtures, showing increasing adhesion forces with increasing fibroin concentrations; B – Representative sample results illustrating the effect of temperature on the pull away force needed to separate steel fixtures showing an inversely proportional relationship between solution adhesion and temperature; C – Representative sample results illustrating the effect of SF solution pH on the pull away force needed to separate steel fixtures indicating maximum adhesion values for neutral and slightly acidic fibroin solutions; D – Representative sample results illustrating the effect of SF ionic strength on the pull away force indicating no effect of solution conductivity on adhesion to steel fixtures.

Figure 5.

Effect of β -sheet induction on SF adhesion. A – Evaluation of methanol (MeOH) induced β -sheet formation by Fourier-transform infrared spectroscopy (FTIR), illustrating a slight change in the sample pattern in the 1625 cm^{-1} region after 5 h of treatment and the presence of a strong β -sheet peak in the gelled sample (24 h MeOH treatment); B – Representative sample results illustrating the effect of β -sheet induction via MeOH treatment on the pull away force needed to separate steel fixtures showing a slight decrease in force values (the values were not statistically different) for 5 h treated samples and no adhesion for 24 h MeOH treated samples.

Figure 6.

Secondary structure of extracted, fresh silk fibroin solutions. A – at various temperatures (37, 20 and 3 °C); B – at different pH (5.5, 7.0 and 8.5); and C – at different ionic strengths (in water and in PBS, respectively), indicating disordered structures for all conditions.

Figure 7.

Secondary structure of reconstituted silk fibroin solutions. A – at various temperatures (37, 20 and 3 °C); B – at different pH (5.5, 7.0 and 8.5); and C – at different ionic strengths (in water and in PBS, respectively). The CD spectra of samples exhibited characteristic features of a β -sheet structure (minimum at approximately 217 nm) in the range of temperatures and pH tested. Significant differences in secondary structures were seen between water (β -sheet) and PBS (disordered) samples.

Figure 8.

The effect of physical parameters on SF film adhesion strength to biological substrates. A – the effect of SF solution extraction parameters of lap shear test force needed to separate leather substrates, showing higher adhesion strength for SF1 compared to SF2 films (t-test $p = 0.01$); B – the effect of temperature on the adhesion strength needed to separate adhered leather strips showing maximum adhesive efficacy at 37 °C (ANOVA $F(2, 11) = 114.5$, $p = 4.3E-08$; Student t-test compared to 20 °C $p = 0.01$); C – the correlation between the pH of SF solutions used to cast films and film adhesive strength, indicating significant differences between the adhesion of different pH solutions (ANOVA $F(2, 12) = 57.4$, $p = 7.2E-07$; Student t-test compared to pH 7.0 $p = 0.01$); D – the impact of SF solution ionic strength on the adhesive strength of generated films, indicating that the presence of ions negatively impacts the adhesive strength of films (t-test $p = 0.01$) ($n=5$).

Figure 9.

Lap shear test of MeOH untreated, pre-treated and post-treated films with biological substrates. The pre-treatment of the films with MeOH drastically decreased their adhesive capacity, while post-treatment had the opposite effect and appeared to further consolidate film-substrate interactions. The observed differences in adhesion values are statistically significant as indicated by ANOVA ($F(2, 7) = 43.6$, $p = 1.1E-04$). Student t-test compared to untreated control $p \leq 0.01$.

Figure 10.

One-dimensional WAXS data of films prepared from solutions obtained through different extraction processes (SF1 and SF3), films obtained from SF1 solutions with or without MeOH treatment, films cast from SF1 in PBS or deionized water, and films obtained from SF1 solutions at different pH values ($n=3$).

Figure 11. Proposed mechanisms of adhesion of silk fibroin to metallic (top) and biological (bottom) substrates.

Table 1.

Effect of silk fibroin (SF) extraction parameters on the protein's intrinsic adhesion properties.

Sample identity	Extraction parameters	Peak normal force (N) (n=3)	Area under force-time curve (Ns) (n=3)
Silk fibroin 1 (SF1)	0.02 M Na ₂ CO ₃ 30 min boil	54.9 ± 0.7	3.9 ± 0.6
Silk fibroin 2 (SF2)	0.02 M Na ₂ CO ₃ 60 min boil	17.7 ± 1.1	1.3 ± 0.1
Silk fibroin 3 (SF3)	0.2 M Na ₂ CO ₃ 30 min boil	4.4 ± 0.0	0.4 ± 0.0
Silk fibroin 4 (SF4)	0.2 M Na ₂ CO ₃ 60 min boil	2.9 ± 0.8	0.3 ± 0.0
New-Skin® liquid bandage (control)	NA	31.5 ± 2.9	1.7 ± 0.1