Electrodeposited silk coatings for bone implants

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Abstract

The aim of this study was to characterize the mechanical properties and drug elution features of silk protein-based electrodeposited dental implant coatings. Silk processing conditions were modified to obtain coatings with a range of mechanical properties on titanium studs. These coatings were assessed for adhesive strength and dissolution, with properties tuned using water vapor annealing or glycerol incorporation to modulate crystalline content. Coating reproducibility was demonstrated over a range of silk concentrations from 1 to 10%. Surface roughness of titanium substrates was altered using industry relevant acid etching and grit blasting, and the effect of surface topography on silk coating adhesion was assessed. Florescent compounds were incorporated into the silk coatings, which were modulated for crystalline content, to achieve four days of sustained release of the compounds. This silk electrogelation technique offers a safe and relatively simple approach to generate mechanically robust, biocompatible and degradable implant coatings that can also be functionalized with bioactive compounds to modulate the local regenerative tissue environment.

Keywords

electrogelation; electrodeposition; dental implant coating; bioactive coating

Introduction

The mechanical and morphological properties of titanium, along with the biocompatibility and osteo-promoting characteristics have made this the metal of choice for dental implant systems ¹. Unlike other materials titanium can be easily surface treated, chemically modified or coated to improve implant success via introduction of appropriate surface texture or via incorporation of bioactive molecules ^{2–6}.

The performance and success of coated implants is influenced to a large degree by the surface structure and consistency of the applied treatment⁷. It is essential that the coatings and associated deposition techniques are controllable and reproducible. Current coating systems are lacking in terms of this control. For example, coating deposition via plasma spraying or electrodeposition is dictated by numerous parameters (temperature, particle size distribution and voltage), resulting in variable coating thickness, porosity and weight^{7,8}. Further, coating processes with these and other techniques often includes the use of extreme temperatures, pHs, mechanical stress and chemical modifications, thereby limiting or significantly reducing biomolecule incorporation and retention of bioactivity^{1,9–11}.

Recently we reported an electrogelation technique which allows direct deposition of silk protein coatings on surfaces^{12,13}. Electrogels (egel) are formed from regenerated silk protein derived from silkworm *Bombyx mori* cocoons. These gel coatings contour or conform to the topography of the conducting surface. Egel coatings retain robust mechanical and physical properties that are characteristic of silk materials and in part what leads to their utility in a range of biomaterial formats^{12,14–18}. Gel deposition efficiency is driven by factors intrinsic to the silk solution, including concentration and process time, and increasing these parameters results in greater gel deposition¹⁹. Extrinsic factors such as voltage, time and distance between electrodes also directs gelation rate. Higher voltage and longer gelation times result in more gel deposition while increasing the distance between the electrodes decreased gelation rate¹⁹.

The objective of this work was to generate silk egel coatings on titanium implant materials and to characterize the reproducibility, mechanical strength and drug release potential of these coatings. The egel is intended as a cost effective, bioactive dental implant coating aimed at improving osseointegration via the incorporation of therapeutic agents. Silk is already used in some medical devices, thus the biocompatibility and safety of this protein are already established. The system is also anticipated to result in shelf-stable pre-coated implants, in dry coating form, that can be stored at room temperature and used on-demand, with reactivation of the bioactive components upon hydration.

Material and Methods

Silk solution preparation

Silk fibroin protein was purified from *B. mori* silkworm cocoons resulting in an aqueous silk fibroin solution as previously described²⁰. Briefly, cocoons were purchased from Tajima Shoji Co. (Yokohama, Japan), cut into pieces and processed for 5–20 minutes in an aqueous solution of 0.02M sodium carbonate (Sigma-Aldrich, St. Louis, MO). Degummed fibers were rinsed in deionized (DI) water and dissolved in 9.3M lithium bromide (Sigma-Aldrich, St. Louis, MO) solution at 60°C for 3–4 hours. Solution was placed into dialysis cassettes (3.5K kDa MWCO, Thermo Fisher, Rockford, IL), and dialyzed in DI water for 48 hours. The 5–8% w/v silk solution was placed into 3.5K kDa dialysis tubing and allowed to concentrate via air drying. Solutions were stored at 4°C until needed.

Formation of electrogels on titanium studs

A 20 μL of silk solution was dispensed onto a titanium stud (HIMED, Old Bethpage, NY) surface (diameter = 7 mm). Using a dovetail slide micromanipulator (Wagner Instruments, Hamden, CT) a platinum wire or titanium stud of matching diameter was slowly lowered approximately 2 mm from the stud, to contact the surface of the silk solution without touching the underlying titanium stud. The wire/stud was connected to the negative terminal of a 25V power supply and centrally positioned onto the stud to ensure even coating deposition. The stud containing the silk solution was connected to the positive terminal of the same power supply. The power supply was switched on for 20 seconds, resulting in deposition of a silk gel. A second titanium stud was placed onto the newly formed gel, expelling excess solution. This resulted in the adherence of the two studs which were pulled to failure to determine mechanical strength of the coating.

Characterizing the titanium stud surface

The 7 mm titanium studs were either left untreated, acid etched or grit blasted. Studs to be acid-etched were first rinsed in DI water then placed into individual glass vials. After drying, studs were submerged in freshly prepared 3.5% $\text{HCl}/\text{H}_2\text{SO}_4$ (v/v) acid solution, the vials were sealed and the acid allowed to etch the surface overnight. Following etching the treated studs were rinsed in a series of ultrasonic baths containing water, acetone and water. Microcrystalline diamond (MCD) grit blasting was performed using aluminum oxide particles (particle size 300 – 600 μm)²¹ and then rinsed in DI water.

A stylus profilometer (6M Veeco Dektak, Plainview, NY) was used to characterize the surface roughness of all titanium substrates. Scanning Electron Microscopy (SEM) imaging was used to qualitatively observe the morphology of the titanium studs. A Supra 55VP microscope (Carl Zeiss Inc., Thornwood, NY, USA) at an operating voltage of 5 KV was used to image the stud surfaces. No gold sputtering was applied to the samples.

Mechanical analysis

Uniaxial tensile tests were performed on an Instron 3366 testing frame (Instron, Norwood, MA) equipped with a 100 N capacity load sensor. Egels were formed directly onto the top surface of a titanium stud fixed to a custom Instron adapter. The treated stud (smooth, grit blasted or acid etched) was placed onto the gel and allowed to dry for 12 hours prior to testing. The custom adaptors were mounted directly onto the Instron and the adhered stud was held in place via standard Instron pneumatic clamps or vise clamps. For tensile testing, the loading rate was fixed at 0.5 mm min^{-1} . For shear testing, a blade was positioned perpendicularly to the Ti stud and lowered at a rate of 0.5 mm min^{-1} . Each treatment group was tested for tensile stress at ultimate strain (%). The ultimate strain value (UTS) was taken to be the strain at ultimate failure of the silk gel. Prior to mechanical testing, samples were treated by methanol or water annealing, or were hydrated in 0.1 M phosphate-buffered saline (PBS) for 30 minutes. Hydrated samples remaining submerged in PBS bath for the duration of the mechanical testing.

Silk electrogels containing hydroxyapatite

Hydroxyapatite (HA), 10% w/v HA was added to the 5% silk solution to generate a homogenous silk-HA slurry solution. The silk-HA solution was electrogelled directly onto titanium studs and a second stud adhered as described for electrogels prepared with without HA.

Silk electrogels containing glycerol

Glycerol was added to silk solution prior to electrogelation. A 5% w/v silk solution was used and glycerol was added at 1:1, 1:2 or 1:4 w/w (silk:glycerol). Then 20 μ l of the silk:glycerol solution was dispensed onto the surface of a titanium stud and electrogelled for 20 seconds at 25 volts as described earlier.

Water and methanol annealing of silk gels

Water annealing (WA) and methanol annealing (MA) of the egels was performed prior to mechanical testing. For WA, adhered studs were placed in a 37°C chamber and a ~23 inHg vacuum was applied for 12 hours, crystalizing the silk to make it water/PBS insoluble¹⁸. For MA, adhered studs were dried overnight and then submerged in 80% methanol for 30–45 minutes to ensure crystallization^{17,18,22}

Quantifying gel deposition

Egels were formed on titanium substrates as described above. The coated titanium studs were air dried for 24 hours at room temperature, at which point the coated area was measured using digital calipers (CD-6"CSX, Mitutoyo, Aurora, IL). The combined weight of the 'stud+coating' was obtained using a Mettler AT20 (Mettler-Toledo, Columbus, OH), the studs were then cleaned using phosphate buffered saline (PBS) and the 'uncoated shim' weight was obtained. Coating weight was calculated by subtracting the weight of the 'uncoated stud' from that of the 'stud+coating'. The weight of the deposited coating was normalized over the coating area.

Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy (FTIR) analysis of the silk coatings was performed with a Jasco FT/IR-6200 spectrometer (Easton, MD), equipped with a multiple reflection horizontal Miracle attenuated total reflectance (ATR) attachment (ZnSe crystal, from Pike Tech., Madison, WI). For each measurement, 64 scans were performed each with nominal resolution of 4 cm^{-1} . The secondary structures of the protein samples were identified from the absorption spectra, peak positions of the amide I region (1595–1705 cm^{-1}) were obtained after Fourier self-deconvolution. Curve fitting was performed using Jasco Spectra-Manager Analysis Software (Jasco, Easton MD), as previously described¹⁸.

Compound incorporation and release from electrogel coatings

For the incorporation and release of compounds from the coatings, to simulate drug release, fluorescein isothiocyanate-dextran samples of molecular weight 70,000 and 250,000 Da (FITC-dextran; Sigma Aldrich, MO) were incorporated into the silk solution at 500 $\mu\text{g/ml}$. Gel coatings containing FITC-dextran were formed on titanium studs for 45 seconds and

allowed to air dry for 2 hours prior to WA treatment at 60°C for 1 hour. WA coatings were then placed into individual wells of a 24 well plate containing 2 mL of PBS. One mL of PBS supernatant was replaced daily and used for spectrophotometric analysis.

Spectrophotometric measurements of the supernatant were used to quantify drug release (SpectraMax M2e Molecular Devices, Sunnyvale, CA), with 200 μ L aliquots placed into wells of a 96 well plate for fluorescence measurements (Exc. 490nm/Emi. 520nm). Drug concentration was determined based on standard curves obtained using matching molecular weight FITC-dextran.

Statistical Analysis

All experiments were performed with a minimum of $N = 3$ for each data point. Statistical analysis was performed by one-way analysis of variance (ANOVA) and Tukey's post-hoc test. Differences were considered significant when $p < 0.05$.

Results

Effects of silk solution processing conditions on egel adhesive strength

Egels formed using silk processed for 10 minutes had higher adhesive strength compared to other processing conditions (Figure 1A). A trend toward decreased adhesive strength with longer silk processing time was noted above 15 minutes with 20 minute process times resulting in significantly weaker gels compared to the other process condition tested here ($p < 0.05$). There was a significant increase in adhesive strength corresponding to increased silk concentration from 5 to 10%, with 0.40 ± 0.10 and 1.18 ± 0.32 MPa, respectively ($p < 0.015$) (Figure 1B). Similar changes in adhesion strength corresponding to increased silk content have been reported for other silk formats^{14,23}. The average coating thickness for 1%, 2.5% and 5% coating across all samples was 55.1 ± 28.2 μ g/mm², 84.7 ± 20.0 μ g/mm² and 177.0 ± 34.9 μ g/mm² respectively. From Figure 1C there was no difference in gel deposition for each respective concentration and the corresponding average thickness.

Effect of saline and annealing treatments on egel coating strength

Figure 2 illustrates adhesive strength in dry conditions generally decreased with WA and MA treatments. Both the air dried and WA treatments displayed significantly greater dry adhesive strength compared to the MA samples ($p < 0.05$). In wet conditions, strength was significantly higher for the WA treatments ($p < 0.05$), with no observed differences between air dried and MA coatings. The MA was the only treatment that displayed no difference in dry vs. wet adhesion.

Figure 2B illustrates that glycerol incorporation decreased adhesive strength under dry testing conditions. Hydrated testing revealed that incorporation of 1:1 glycerol:silk significantly increased coating adhesive strength from 0.10 ± 0.03 to 0.30 ± 0.13 MPa ($p < 0.05$). Similarly, 2:1 and 4:1 glycerol:silk ratios increased coating adhesive strength to 0.35 ± 0.07 and 0.33 ± 0.14 MPa, respectively ($p < 0.05$). The mechanical strength of the 4:1 glycerol:silk maintained the same dry and wet adhesive strength (Figure 2B).

Secondary structure in terms of higher β -sheet content was directly related to an increased adhesive strength (Figure 2C)²². Although β -sheet structure increased with annealing treatment, the differences were not statistically significant.

Adhesive strength of eegls adhered on treated titanium substrates and HA incorporation

Figure 3A depicts the rougher surface of the acid etched and the grit blasted titanium studs compared to the smoother, untreated studs. The grit blasted treatment resulted in the roughest surface, followed by the acid etched, both of which were rougher than the untreated studs (Table 1). MCD treatment led to tensile and shear strengths that were significantly higher than those of the smooth studs ($p < 0.02$).

HA incorporation resulted in coatings with significantly higher tensile strengths for both smooth and acid etched titanium stud treatments ($p < 0.03$), but no difference in shear strength compared to gels without HA (Table 1). For the roughest grit blasted substrates, HA incorporation resulted in significantly reduced adhesive strength, from 0.82 ± 0.12 MPa to 0.64 ± 0.04 MPa ($p < 0.03$).

Compound release – FITC dextran

Release of FITC-dextran from egel coatings was monitored across 4 days. Over the first 24 hours the release profile was dictated by the molecular weight of the compound rather than the annealing treatment (Figure 4), with significantly more 250kDa (68–84%) FITC-dextran released than the 70kDa (48–52%) compound. By day 4 FITC-dextran release was impacted more by the annealing treatment than the size of the model compound (Figure 4). The air dried silk released approximately 20–30% more FITC-dextran compared to the corresponding WA groups, demonstrating 100% cumulative release with ~ 275 $\mu\text{g/ml}$ FITC-dextran. FITC-dextran recovery was between 70–80% for the WA treatments, with approximately 200 $\mu\text{g/ml}$ FITC-dextran released over the 4 day period. Upon inspection a green/yellow film residue was noted on the titanium stud which could account for the lower percent recovery.

Discussion

Electrodeposition of silk gels with target applications for coating implant systems of varying topographies was described along with the mechanical properties and compound release potential of these new coatings. While these studies focused on egel use on planer stud surfaces, the technique has been successfully used on metal substrates of varying geometries. Concentration trends of increasing strength with increasing protein content were observed (Figure 1) and this tend have been noted in other coating systems. For example, Lui et al. demonstrated that increasing bovine serum albumin concentrations significantly increased the strength of implant coatings²⁴, and incorporation of chitosan into calcium phosphate coatings also demonstrated higher delamination forces with increased chitosan content². Process time was linked to average molecular weight of the protein solution, with higher extraction times resulting in lower molecular weight of the silk²⁰. The lower molecular weight proteins likely accounted for the decreased strength²⁵ observed with increased processing time (Figure 1A).

Annealing treatments have been used to control the solubility and enzymatic degradation of silk biomaterials^{17,22}. The adhesive strength for all treatment groups in this study exhibited decreased strength in PBS compared to dry testing (Figure 2). This decrease was likely due to dissolution of the silk in contact with PBS. After water annealing the coating maintained flexibility and comparable adhesive strength to non-annealed treatments, demonstrating that silk egel coatings can be adjusted for strength¹⁷. In addition to WA and MA, glycerol has been used to increase the flexibility of silk films and alter both dissolution and degradation rates^{26,27}. The changes caused by glycerol addition have been correlated to the crystallinity content of silk and associated to the mechanical properties^{26,27}. Glycerol acted as a stabilizer in aqueous environments via the formation hydration shells around proteins²⁸. The glycerol may be stabilizing the gel structure by limiting exposure of the gel to the bulk solution including salts, which destabilize egels (Figure 2B). As the glycerol dissolved out of the gel the salts present in solution were free to disrupt the ionic interactions holding the gel together. It follows that gels prepared with higher glycerol content displayed longer dissolution times.

HA incorporation is widely used in the dental field to aid implant osseointegration^{10,29}. In this work we observed that incorporation of HA increased adhesion to smoother surfaces (Table 1). It is possible that the incorporation of the HA powder integrated into the smaller pits and imperfections of the smooth and acid etched surfaces, acting as anchor sites for the silk and ultimately improving adherence. Conversely, for the rougher substrates, the HA could be serving as a filler which occludes the larger pits, resulting in a relatively smoother surface and lowering the adhesive strength (Table 1). Other biomaterials such as chitosan³⁰, collagen^{11,31} and alginates¹⁰ have also been used as vehicles for hydroxyapatite delivery and bone formation. The mechanical properties of these materials varied based on the format and processing techniques used for HA incorporation. In general, the presence of HA improved the overall mechanical strength of the composite material^{10,11,30,31}. Similarly, numerous studies performed with titanium alloys as well as ceramics have noted a correlation between substrate roughness and coating adhesive strength^{6,32,33}, matching the results obtained for egels on modified titanium surfaces in the present study.

The adhesive strength of other biologically derived coatings, such as collagen, chitosan and hydroxyapatite alone, ranged from 0.07–4.83 MPa^{34–37}. The adhesion strength obtained for hydrated silk coatings, ranged from 0.1 – 0.88 MPa depending on concentration and surface topography. These values fell within the range of implant coatings produced with collagen, chitosan or hydroxyapatite. It is important to note that the highest adhesion obtained for many of the other coatings required co-deposition or chemical bonding and that none of these coating were produced using electrodeposition techniques^{36,37}. These coating systems require harsh, expensive or otherwise difficult processing conditions in order to deposit and maintain mechanical robustness³⁸. A 10% solution of silk processed for 10 minutes allowed the production of coatings that were comparable in strength and thickness to clinically relevant implant coatings^{39,40}.

The release of FITC-dextran was likely dominated by diffusion through the silk-protein matrix⁴¹. Hines et al. demonstrated increased drug retention with exposure to annealing conditions, or greater crystallinity. The annealing process increased beta-sheet crosslinks

and other associations between the protein chains, increasing tortuosity and effectively reducing diffusion (Figure 2C) ⁴¹. The observed lower cumulative release and drug recovery in the annealed treatments could be attributed to incomplete dissolution of the WA gels (Figure 4). Upon inspection a green/yellow film residue was noted on the titanium studs exposed to WA. Further increasing exposure to annealing conditions increased beta sheet content and delayed drug release from egels (unpublished data).

Other coating systems have demonstrated the release of model drugs from coated titanium alloy studs. Cheng et al. showed that electrochemical calcium phosphate-BSA coating deposition on titanium alloys produced ~15% release of incorporated drug over 70 hours, of which 85% was released within 24 hours ⁴². Direct comparisons with electrodeposited coatings produced with collagen, chitosan, alginate or other biomaterials is not possible due to the inability to form such coatings with these polymers. The results from the FITC-dextran release studies here demonstrated that the egel coatings functioned as a reservoir for the storage and release of model compounds and the amount of drug released was in line with other traditional systems. In addition to FITC-dextran, small molecule drugs such as antibiotics have been successfully incorporated and released from egels (unpublished data). In addition, bone morphogenic protein-7 released from egel coatings stimulated osteogenic differentiation by human mesenchymal stem cells.⁴³

Conclusions

A clinically useful implant coating must be biocompatible, degradable over the appropriate time scale, be uniform in coverage, be reproducible in thickness and have the capability to incorporate therapeutic agents ⁴⁴. The silk coatings described here are aqueous-based, biocompatible, controllably degradable and the coating deposition requires no chemical modifications ^{17,18,45,46}. Coating deposition was controlled via time and applied voltage, while physical and mechanical properties were modulated by varying solution conditions and annealing parameters to modulate crystalline content. This silk based electrodeposition system allows for simplified implant functionalization and the incorporation of therapeutics and their release over controllable time frames ^{13,47,48}.

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Figure 1.

Tensile strength of surface treated titanium studs adhered using silk egel determined using an Instron uniaxial mechanical tester. 5% (w/v) silk processed for (A) 5, 10, 15 or 20 min was used to determine effect of process time on coating adherence. Silk processed for 10 min was used to determine the effect of (B) silk solution concentration 5% vs 10% w/v. Reproducibility of coatings formed for 75 seconds at 20 volts (C) was assessed over 5 independent batches of silk solution and at 1, 2.5 and 5% w/w silk concentrations. (n=4) Mean $\pm$ standard deviation.

Figure 2.

Dry and wet (A) adhesive strengths of smooth titanium studs adhered using 5% (w/v) silk egels untreated (air dry) or annealed via exposure to water vapor to methanol or (B) with varying glycerol content. FTIR analysis (C) showing the secondary structure composition of the silk egels after annealing treatments. (n=4) Mean $\pm$ Standard deviation

Figure 3. Topography of (A) untreated, (B) grit blasted and (C) acid etched titanium studs. Left images depict light microscopy of the stud surface, scale bars = 1.5 mm. Right images illustrate SEMs of the titanium surfaces, scale bars = 20 μm .

Figure 4. Cumulative release of 70 kDa and 250 kDa FITC-dextran into saline solution from untreated (air dried) and annealed (water vapor) egel coatings applied directly onto smooth titanium studs. (n=4) Mean $\pm$ Standard deviation

Table 1

Surface roughness and tensile and shear strength of egel coatings deposited on the surface of treated titanium studs in the presence or absence of hydroxyapatite. Numbers represent mean $\pm$ standard deviation (n=4)

Surface (RA value)	Silk Solution (5% w/v)	Tensile Strength (MPa)	Shear Strength (MPa)
No Treatment (0.08μm)	Only Silk	0.34 $\pm$ 0.11	0.61 $\pm$ 0.14
	Silk/Hydroxyapatite	0.54 $\pm$ 0.04	0.75 $\pm$ 0.18
MCD grit blasted (1.07μm)	Only Silk	0.82 $\pm$ 0.12	1.37 $\pm$ 0.32
	Silk/Hydroxyapatite	0.64 $\pm$ 0.04	1.07 $\pm$ 0.34
HCl/H₂SO₄ acid etched (0.46μm)	Only Silk	0.43 $\pm$ 0.18	0.76 $\pm$ 0.52
	Silk/Hydroxyapatite	0.88 $\pm$ 0.05	0.97 $\pm$ 0.52