Published in final edited form as:

Biomacromolecules. 2010 December 13; 11(12): 3406–3412. doi:10.1021/bm100925s.

pH sensitive ionomeric particles obtained via chemical conjugation of silk with poly-amino acids

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Abstract

Silk-fibroin based biomaterials have been widely utilized for a range of biomaterial-related systems. For all these previously reported systems, the beta-sheet forming feature of the silk was the key stabilizing element of the final material structure. Herein, we describe a different strategy, based on the engineering of silk-based ionomers that can yield stable colloidal composites or particle suspensions through electrostatic interactions. These silk-based ionomers were obtained by carbodiimide-mediated coupling of silk fibroin with poly-lysine hydrobromide and poly-glutamic acid sodium salts, respectively. Colloidal composites could be obtained by mixing the ionomeric pair at high concentration (i.e., 25% w/v), while combining them at lower concentrations (i.e., 5% w/v) yielded particle suspensions. The assembly of the ionomers was driven by electrostatic interactions, was pH-dependent and reversible. The network assembly appeared to be polarized, with the interacting poly-amino acid chains clustered to the core of the particles and the silk backbone oriented outward. In agreement with this assembly mode, doxorubicin, a hydrophilic anti-tumor drug, could be released at a slow rate, in a pH-dependent manner, indicating that the inside of the ionomeric particles was mainly hydrophilic in nature.

Keywords

silk; poly-amino acids; ionomers; composite; network assembly; cytocompatible

Introduction

Silks have been widely employed for a variety of biomedical and biotechnological applications^{1–4}. Their popularity is due in part to availability and ease of purification^{4–6} but mainly to their attractive mechanical and biological properties^{7–9}. Silks have an unusual amino acid sequence with the bulk of the protein organized into alanine and glycine-rich hydrophobic blocks and large side chain amino acids clustered in chain-end hydrophilic blocks¹⁰. The hydrophobic blocks are further organized into crystalline regions while the hydrophilic blocks are found in the amorphous regions¹¹ of the protein. Under certain conditions, such as non-physiological pH, temperature or ionic strengths, the crystalline regions can fold irreversibly into beta-sheets via intra- and inter- molecular hydrogen bonding and hydrophobic interactions. This conformational transition locks the silk protein into supramolecular structures in the form of hydrogels, films, sponges, fibers, ropes, depending on the processing method.

In this work, we aimed at generating silk-based materials that would assemble through electrostatic interactions and to further emphasize the versatility of silk systems. This approach would confer the material a much desired feature – reversibility – because

electrostatic interactions could be modulated through a variety of factors, such as pH, temperature, ionic strength, electricity or shear stress^{12–13}. The concept for this study was to chemically functionalize silk with charged high molecular weight poly-amino acids to yield ionomeric counterparts that could assemble electrostatically (Figure 1).

Due to its nature, the amino acid composition of silk fibroin (SF) does not offer numerous alternatives for chemical modifications. In the *Bombyx mori* silk worm, SF is comprised of a heavy chain and a light chain, linked together through a single disulfide bond¹⁴ to form the ~420 kDa protein¹¹. The majority of amino acids from the protein backbone are chemically inert, the most attractive targets for chemistry being the sparse carboxyl side chain containing amino acids. The original carboxyl content per molecule of SF provided by the glutamic (E) and aspartic acid (D) residues represents 1.4% of the molecule. In order to achieve a high charge density for the ionomers, our strategy was to first enrich the silk fibroin's (SF) carboxyl content. The most abundant amino acids from SF suitable for chemistry are tyrosine (Y) residues which account for 5.2% of the total amino acids and serine (S) residues that represent 11.9% of the protein^{11–15}. Therefore, successful chemical modification of Y and S would yield a theoretical carboxyl content of 18.5% per SF molecule. Second, to further maximize the charge density on the molecule we covalently attached charged high molecular weight polymeric natural amino acids, specifically poly-lysine hydrobromide and poly-glutamic acid sodium salt.

The data presented here illustrates the synthesis of ionomeric silk-based counterparts and in vitro characterization of the composites formed (colloidal gels and particle suspensions). To our knowledge, this is the first report of a natural and biocompatible ionomeric composite obtained via electrostatic interaction of oppositely charged species with a protein backbone.

Experimental Section

Materials

Para-toluene sulfonic acid, 4-amino benzidine, sodium nitrate, chloroacetic acid, poly-lysine hydrobromide (MW 15,000 Da) and poly-glutamic acid sodium salt (MW 15,000 Da) were purchased from Sigma-Aldrich, St. Louis, MO. The zero-length crosslinker 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) was from Thermo Fisher Scientific, Rockford, IL.

Silk fibroin extraction

Silk fibroin was obtained as previously described¹⁶. Briefly, *Bombyx mori* cocoons were cleaned and cut into small pieces. In a subsequent degumming process, sericin was removed by boiling the cocoons for 1 h in 0.2 M Na₂CO₃ solution. The silk fibroin was then dissolved in 9M LiBr at 60°C for 1 h to a concentration of 20% w/v then it was dialyzed against water (MWCO 3,500) for 72 h.

Ionomer synthesis

Poly-amino acid coupled silk fibroin was obtained by first enriching the carboxyl content of the molecule, then coupling poly-lysine hydrobromide and poly-glutamic acid sodium salt, respectively, to silk fibroin via carbodiimide mediated chemistry. Specifically, 6–7% w/v silk fibroin solution in 100 mM borate buffer pH 9.0 was reacted with 40 mM diazonium salt in order to introduce carboxyl groups on the tyrosine residues of silk¹⁶. The reaction product was purified by using NAP-25 (VWR International, West Chester, PA) desalting columns. The resulting solution was then reacted with 1M chloroacetic acid for 1h at room temperature under magnetic stirring to add carboxyl functionalities on the serine residues. The product was purified by dialysis (MWCO 3,500) for 72 h. The resulting solution was

then divided into three equal amounts. To one part 1g of poly-lysine hydrobromide was added, the pH of the solution was adjusted to 6.0, then 30 mg of EDC was added. The reaction was carried out for 4h at room temperature under gentle magnetic stirring then dialyzed (MWCO 20,000) for 72 h. The same protocol was followed for obtaining the poly-glutamate derived silk fibroin. One part of the solution was used as a control reaction, for which the above protocol was followed, without the addition of poly-amino acids.

¹H-NMR

To confirm the structures of our final compounds, solutions were lyophilized, then re-dissolved in deuterated water (D₂O) and analyzed with a 300 MHz Bruker NMR spectrometer. For the azo-SF and COOH-SF, samples were analyzed sequentially as the reactions were run, resulting in a lower concentration of azo-SF and COOH-SF compared to the starting SF, because of solvent dilution and exchange. The SK-PL and SK-PG samples were lyophilized then dissolved to a concentration of 10 mg/ml in deuterated water and compared to SF at the same concentration.

Silk fibroin—¹H-NMR (300 MHz, D₂O): δ 0.72 (br, Valγ), 1.21 (br, Alaβ), 1.90 (br, Valβ), 2.74–2.85 (br, Asp/Tyrβ), 3.77 (m, Serβ/Glyα), 4.14–4.29 (m, Alaα/Serα), 6.61 (m, Tyrφ), 6.90 (m, Tyrφ), 7.03–7.12 (br, Pheφ).

Azo-silk fibroin—¹H-NMR (300 MHz, D₂O): δ 0.74 (br, Valγ), 1.22 (br, Alaβ), 1.88 (br, Valβ), 2.74–2.85 (br, Asp/Tyrβ), 3.78 (m, Serβ/Glyα), 4.16–4.30 (m, Alaα/Serα), 6.63 (m, Tyrφ), 6.90 (m, Tyrφ), 7.03–7.12 (br, Pheφ), 7.05(br, azo), 7.19 (br, azo).

Carboxy-silk fibroin—¹H-NMR (300 MHz, D₂O): δ 0.69 (br, Valγ), 1.21 (br, Alaβ), 1.83 (br, Valβ), 2.54 (br, Asp/Tyrβ), 3.52 (s, -CH₂-COOH), 3.78 (m, Serβ/Glyα), 4.15 (m, Alaα/Serα), 7.06(br, azo), 7.18 (br, azo).

Poly-lysine hydrobromide silk fibroin—¹H-NMR (300 MHz, D₂O): δ 0.73 (br, Valγ), 1.27–1.29 (m, Lysγ), 1.54 (m, Lysβ/Lysδ), 2.84 (m, Lyse), 3.52 (s, -CH₂-COOH), 3.73 (m, Serβ/Glyα), 4.15 (m, Lysα).

Poly-glutamic acid sodium salt silk fibroin—¹H-NMR (300 MHz, D₂O): δ 0.73 (br, Valγ), 1.22 (br, Alaβ), 1.52–2.00 (m, Gluβ), 2.15 (m, Gluγ), 3.52 (s, -CH₂-COOH), 3.78 (m, Serβ/Glyα), 4.16 (m, Gluα).

Gelation tests

Poly-amino acid silk fibroin, poly-amino acid only and control silk fibroin solutions 25% w/v in water were prepared at different pH values (4–9). Equal amounts of solutions were mixed together and gelation/aggregation was assessed by a modified test tube inversion method¹⁷. Specifically, if no fluidity or liquid accumulation was observed to the bottom of the inverted vial/tube after ionomer mixing, it was concluded that a gel has formed.

Dynamic oscillatory rheology

A dynamic oscillatory frequency sweep was performed on an ARES strain-controlled rheometer (TA Instruments, New Castle, DE). Parallel, 8-mm diameter stainless steel plates with a 0.5 mm gap were used for the measurement. A wide frequency range ($\omega = 0.1$ –100 rad/s) was used to collect the data and a 0.1 N normal force was applied on the top plate during lowering. A low viscosity mineral oil was applied around the sample to prevent evaporation and sample drying during the measurement.

Confocal microscopy

Colloidal gels cast directly on glass slides were analyzed by confocal laser scanning microscope (TCS SP2, Leica Microsystems, Germany) in reflectance mode. For the 3D images, 51 scans were performed.

Cytocompatibility assay

Primary human cervical fibroblasts passage 6 (2 μ l of 1×10^4 cells/slide), were added to 25 μ l of silk fibroin-poly-glutamate and cast onto a 35 mm glass bottom Petri dish with 14 mm microwell (Mattek, Ashland, MA). To this cell suspension, 25 μ l of silk fibroin poly-lysine was added leading to instant aggregation with cells entrapped into the composite. The composite was allowed to set for 5 more minutes then 2 ml of cell culture media was gently added not to disturb the composite. The plates were incubated for 72 h at 37°C/5% CO₂. Cell viability was assessed by using a Live/Dead viability/cytotoxicity kit (Invitrogen, Carlsbad, CA). Images were collected with a fluorescent microscope (Leica Microsystems, Wetzlar, Germany) at 100X magnification. The viability was estimated by counting the number of viable and dead cells, respectively, in 10 images acquired from different gel sections. The cells were a gift from Dr. Michael House from Tufts Medical Center.

Drug release

Drug loaded particles were prepared by dissolving silk fibroin-poly lysine and silk fibroin-glutamate in doxorubicin solution in 1XPBS, at pH 7.4. The final concentration of each solution was 5% w/v. Solutions were then mixed together in equal volumes, to yield drug-loaded particles. Samples were then washed three times in 1XPBS, pH 7.4, to remove any unbound drug. Particles were then resuspended in 500 μ l 1XPBS (pH 4.0, 7.0 and 10.0, respectively). Vials (triplicates) were then incubated at 37°C 50 rpm. At set time intervals, particles were spun down at 2000 rpm for 5 minutes, 100 μ l supernatant removed for concentration determination and replaced with fresh buffer. The loading capacity of the particles was determined by reading the 585 nm emission (specific for doxorubicin) of the initial solution and of the final solution after particle formation. The drug release profile was obtained by calculating the cumulative doxorubicin release from spectrometric measurements over 10 days. The amounts of released doxorubicin were calculated by using pH - dependent standard curves.

Results and Discussion

SF carboxyl content was increased in a two step procedure. First, Y residues of degummed SF were coupled under alkaline conditions with a carboxyl containing diazonium salt yielding a yellow-orange azo-benzene-SF derivative (azo-SF). Second, S residues of azo-SF were reacted with chloroacetic acid under strong alkaline conditions to yield the carboxy-silk fibroin (carboxy-SF) intermediate (Figure 2A). The synthesis of both the azo-SF and carboxy-SF reaction intermediates was monitored by ¹H-NMR. The diazonium coupling reaction leading to azo-SF introduces additional benzene rings that alter the initial proton signal signature in the aromatic region of the spectrum (7.05–7.19 ppm) and additionally shifts the absorption spectrum of the sample resulting in the development of orange color (in contrast to the colorless starting material). The carboxy-SF intermediate has new peak appearing at 3.52 ppm corresponding to the methylene protons from the reacted chloroacetic acid reagent (Figure 2B).

To further maximize the charge density on the molecule we attached high molecular weight charged polymeric natural amino acids. The positively and negatively charged silk-based ionomers were obtained by carbodiimide-mediated coupling with poly-lysine hydrobromide (PL) and poly-glutamic acid sodium salt (PG), respectively (Figure 3A). The structures of

the two reaction products were confirmed by ^1H -NMR. After poly-amino acid coupling, characteristic signals corresponding to PL and PG spectra in the 1.2–4.2 ppm region were observed (Figure 3B). Passing the samples through gel filtration columns did not change the ^1H -NMR spectra, indicating that the poly-amino acids were covalently attached to SF.

In order to assess the networking properties of the ionomers, solutions of 25% w/v SF-PL and SF-PG, respectively, in water were adjusted to different pH values ranging from 4.0 to 9.0 and mixed in equal volumes. The assembly of the ionomers under these conditions was assessed using a test tube inversion test where the flow (solution)-no flow (gel) transition of the material was monitored. If no fluidity or liquid accumulation to the bottom of the inverted vial/tube was observed it was concluded that a gel has formed. The individual ionomeric solutions have a yellow-orange, clear appearance (Figure 4A). Upon mixing equal volumes of pH 7.0 solutions instant gel-like colloidal composite formation was observed (Figure 4B). At pH 5.0 and 8.0 composites were formed within seconds but did not incorporate water as well as their counterparts formed at neutral pH and small amounts of liquid could be observed at the top of the inverted vial. Under more acidic (pH 4.0) or basic (pH 9.0) conditions, ionomer assembly was more impaired or did not occur at all (Figure 4B). The gel-like behavior of composites formed at neutral pH was also confirmed rheometrically (Figure 4C). Blending of each ionomer solution with control SF solution did not yield composites regardless of pH, nor did mixing 25% w/v solutions of PL and PG. To further test the system, we added small amounts of acid (1N HCl) or base (1M NaOH) to the composites that were formed. In agreement with our model where we assumed that in the absence or alteration of the stimulus (in this case neutral pH) the assembled network would disassemble (Figure 1), the composites transitioned back to a liquid state (data not shown).

By visual assessment, the ionomeric composites appeared to have a granular structure of the composites and this was confirmed by confocal imaging. High magnification imaging confirmed the presence of three-dimensionally distributed particles (Figure 5). Scanning electron microscopy (SEM) images obtained from nickel-spattered samples also confirmed the presence of spherical particles ranging between $1.04 \pm 0.65 \mu\text{m}$ (Figure 6A). The presence of spherical particles was observed in both low (5% w/v) and high (20–30 % w/v) ionomer concentrations, however at higher concentrations particles were less separated (Figure 6A–C).

The cytocompatibility of the colloidal composites was assessed by performing a live/dead cell assay using primary human cervical fibroblasts. Equal volumes of SF-PL pH 7.0 and SF-PG pH 7.0 solutions were mixed in the presence of cells to achieve *in situ* gelation and cell entrapment. After 72 h incubation the cells were stained to assess viability. The images indicated that cells were well distributed in the colloidal composite, but had a rounded morphology (Figure 6D). We suggest that the morphological outcome is caused by the lack of cell adhesion and attachment sites, as the poly-amino acid side chain are likely oriented towards the inside of the ionomeric particles. The cell viability in the colloidal composites was estimated to be in the 90% range. These results suggest that ionomeric composites can sustain cell viability but they do not favor cell attachment or spreading.

The particulate nature of the complexes in conjunction with the *in situ* entrapped cell images showing cellular morphology would indicate that ionomer assembly occurs via differential orientation of the hydrophobic and hydrophilic parts of the molecules. Based on these observations, we hypothesize an assembly model where oppositely charged poly-amino acid side chains on the ionomers network through electrostatic interactions and drive the particle assembly, yielding supramolecular structures with predominantly hydrophilic cores surrounded by a silk backbone-based hydrophobic region. These structures will then be able to disassemble in response to the environmental pH, thus proving the reversibility of the

system. To investigate this model and test the potential applicability of our particles as drug delivery systems, we chose to assess doxorubicin loading and release (Figure 7). Doxorubicin is a fluorescent, hydrophilic chemotherapeutic agent that would be entrapped in the hydrophilic core and slowly released from the particles if their assembly occurs via the aforementioned model. Oppositely, if the particle core would be hydrophobic, the drug would nonspecifically bind to the surface and would be released in burst mode. To this end, particles were formed in 1X PBS, pH 7.4, in the presence of 250 $\mu\text{g/ml}$ doxorubicin. The loading capacity obtained was approximately 4 μg drug/mg of particles (~ 100 μg doxorubicin was loaded per 5% w/v particles, in a volume of 0.5 ml buffer). The drug release profile was calculated relative to the loaded drug amount. The drug release profile for particles incubated in buffers at three different pH values (4, 7 and 10) was monitored daily over a 10 day period. For the pH 7.0 buffer, only 6% of the loaded drug was released over 10 days, corresponding to a release rate of 0.024 μg drug released/day/mg particles. At acidic or basic buffer pH values, the release was 25% (pH 4.0) and 15% (pH 10.0), corresponding to a release of 0.1 μg drug/day/mg particle and 0.06 μg drug/day/mg particle, respectively. This would indicate that the loaded drug would be entirely released in 167 days under physiologic conditions, 40 days in an acidic environment and 67 days under basic conditions.

The pH-dependent drug release profile observed could potentially make this system useful for cancer therapy by circumventing many of the classical chemotherapy issues^{18–19}. Evidence suggests that solid tumor extracellular pH values are in the 5.7 – 7.8 range²⁰. Doxorubicin is typically administered as a single intravenous injection (40 to 60 mg/m^2) every 21 to 28 days to minimize side effects. Injection of drug-loaded ionomeric particles into the tumor could provide enhanced permeability and retention, and would ensure a consistent, localized and controlled drug dosage. Moreover, the differential drug release rate of the ionomeric particles would decrease the cytotoxic effect on normal tissues, but would locally deliver a high enough drug dose to impact malignant cells.

The use of silk-based systems in the form of nano- and microspheres has been previously reported, with particle sizes ranging between 35 nm–440 μm and drug release times between 8 h – 4 weeks^{21–25}. In contrast to these systems, the ionomeric particles described herein are formed through a radically different stabilization mechanism that is driven by electrostatic interactions rather than beta-sheet formation. For doxorubicin, the observed drug release rates are much slower and would ensure a more prolonged drug delivery timeline. We anticipate that a “library” of drugs and release profiles could be constructed based on the hydrophathy and molecular size of the therapeutic agents. To our knowledge the system described here represents the first report of silk-based composites formed via electrostatic interactions. Moreover, this system is cytocompatible, pH-responsive, reversible and, based on our preliminary results, competitive as pH-sensitive drug delivery vehicle.

Conclusions

Simple chemistry was used to synthesize pH sensitive ionomers by covalently linking SF to PL and PG, respectively. Depending on the ionomer concentration, the charged counterparts could assemble into stable colloidal gels (20–30% w/v) or particle suspensions (i.e., 5% w/v). These composites sustained cell viability and released doxorubicin at different rates, depending on the pH of the aqueous solution. These results would indicate that these new materials could be suitable as cancer- directed drug release vehicles. Further studies are currently underway to assess the material performances in in vivo systems.

Acknowledgments

We thank Dr. Bill Rice for his help with confocal microscopy data acquisition and Dr. Aneta Mieszawska for her assistance with SEM image acquisition. Funding for this project was provided by the NIH (P41 EB002520).

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Figure 1.
Illustration of concept for the ionomeric silk-poly-amino acid based materials.

A

B

Figure 2.

A - Synthetic scheme for the synthesis of the carboxy-silk intermediary. B - ¹H-NMR spectral regions illustrating the successful sequential conversion of silk fibroin to azosilk fibroin to carboxy silk fibroin.

A

B

Figure 3.

A -Synthetic scheme for the synthesis of the silk-poly-lysine hydrobromide and silk-poly-glutamic acid sodium salt ionomers. B - ¹H-NMR spectral regions illustrating the successful coupling of carboxy silk fibroin with poly-lysine hydrobromide and poly-glutamic acid sodium salt respectively

Figure 4.

A – Appearance of 25% w/v silk-poly-lysine hydrobromide (left) and silk-poly-glutamic acid sodium salt solutions. B - Solution (flow)-gel (no flow) transition of mixtures of equal volumes of ionomer solutions by test tube inversion. Ionomer solution concentrations were 25% w/v in water. C - Dynamic oscillatory rheology measurement of colloidal ionomeric sample obtained from 25% w/v ionomeric solutions at pH 7.0. $G' > G''$ indicating that the sample behaves like a gel. The slight frequency dependence is most likely due to the particulate nature of the material.

Figure 5. Confocal microscopic analysis of colloidal gels formed at pH 7.0. Right – 2D image of gel section. Left – 3D imaging of particle distribution in the gel (the axis in the x, y, z directions are 7 μm).

Figure 6.

Particulate structure and cytocompatibility of ionomeric composites. Panels A Scanning electron microscope (SEM) image of 5% w/v composites, showing the morphology and size distribution of constituent particles. B. SEM image of 20% w/v and C. 30% w/v composites illustrating clustered, less differentiated particles. D - Cytocompatibility of ionomeric colloidal composites. This image is an overlay of green-fluorescence (live cells) and red-fluorescence (dead cells) images.

Figure 7.
pH-dependent release profile of doxorubicin from silk-fibroin based ionomeric particles
(inset – chemical structure of doxorubicin).