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Genetically engineered chimeric silk-silver binding proteins

Heather A. Currie,

Department of Biomedical Engineering, Tufts University, Medford, MA-02155 (USA)

Olivier Deschaume,

School of Science and Technology, Nottingham Trent University, Nottingham, NG11 8NS (UK)

Unité POLY, Université Catholique de Louvain, Boltzman A+2, Croix du Sud 1, B-1348, Louvain-La-Neuve (Belgium)

Rajesh R. Naik,

Materials and Manufacturing Directorate, Air Force Research Laboratory, Wright- Patterson Air Force Base, Dayton, OH-45433 (USA)

Carole C. Perry, and

School of Science and Technology, Nottingham Trent University, Nottingham, NG11 8NS (UK)

David L. Kaplan

Department of Biomedical Engineering, Tufts University, Medford, MA-02155 (USA),
(david.kaplan@tufts.edu)

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Composite or hybrid materials are commonly found in Nature formed through the concentration and subsequent nucleation of ions upon organic templates that are most often protein based. Examples include the deposition of calcium containing salts in bone, teeth and the inner ear and iron oxide structures in magnetotactic bacteria. Biological organisms use a limited number of metal ions, the principal ones being calcium and iron, with lesser amounts of strontium, and barium. The ability to utilize other ions to generate composites offers the possibility of new material properties. New materials incorporating silver would be useful in the context of antimicrobial functions. Therefore, in the present study a new route to such functionalized biomaterials is reported whereby genetically engineered fusion proteins are described whereby nucleotides corresponding to short silver binding peptides identified by a combinatorial biopanning process were incorporated into the consensus sequence of silk from the spider, *Nephila clavipes*. The resulting chimeric silk-silver binding proteins nucleated Ag ions from a solution of silver nitrate while the silk protein provided a stable template material which could be processed into films, fibers and three dimensional scaffolds. The silk films inhibited microbial growth of both Gram positive and Gram negative microorganisms on agar plates and in liquid culture, thus highlighting the potential of these chimeric material systems as anti microbial biomedical coatings.

Correspondence to: David L. Kaplan.

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1. Introduction

Natural silks are produced by silkworms and spiders for the formation of protective cocoons, webs and escape lines. Silks are useful biomaterials which display outstanding mechanical properties such as high strength and toughness.^[1, 2] Silk proteins are high molecular weight with repetitive protein sequences that self assemble to form crystalline regions (beta sheets). Multiple hydrophobic repeats of alanine, glycine-alanine and glycine-alanine-serine result in the formation of these stable β -sheet structures through the formation of physical cross-links, which contribute to the high mechanical strength. In contrast, semi amorphous repeating glycine-glycine-x domains that are hydrophilic contribute to the toughness.^[3, 4] Variations in the size and distribution of these hydrophobic and hydrophilic domains with respect to material properties has been investigated through the generation of silk inspired block copolymer designs.^[5, 6]

The use of silks as biomedical materials is an active area of investigation due to their biocompatibility, slow degradation profile and biodegradability.^[7, 8] In addition, silks can be formed into fibers, films, gels, films and three dimensional scaffolds via all aqueous processing.^[9, 10] Recombinant spider silk which incorporates the consensus repeat units of spidroin 1 from the major ampullate dragline silk (MaSp 1) of the spider *Nephila clavipes*, has potential as a biomedical material due to the self-assembly and material properties outlined above. Spider silk generated by recombinant DNA methods becomes accessible to genetic modifications through the incorporation of additional functional domains. These modifications can result in the formation of new materials with enhanced properties, such as cell binding functions (RGD) and organic- inorganic hybrid materials.^[11–13] Incorporation of the silica nucleating domain R5 into recombinant spider silk generated new mineralized silk hybrid materials, while hydroxyapatite has been produced on recombinant silk incorporating dentin matrix proteins. The resulting composite materials were found to display stiffer, tougher materials properties.

In Nature, hybrid materials incorporating both organic and inorganic phases have evolved for specific biological functions, such as the toughness and strength imparted by the inclusion of calcium salts in bone and nacre and silicon dioxide in diatom and plant cell walls. In these materials, the formation of a mineral phase involves organic templates, which nucleate and control the inorganic growth through non bonded associations such as electrostatic interactions, hydrogen bonding and hydrophobic effects.^[14, 15] The identification and manipulation of peptides involved in such directed assembly is an active area of study in materials science investigating green routes to hybrid material synthesis.^[16–20]

Silver ions released from nanoparticles have demonstrated broad antimicrobial capacity, active against Gram positive and negative bacteria, as well as antibiotic resistant strains, fungi, protozoa and viruses.^[21] A number of modes of bacteriocidal action for silver ions have been proposed, including interactions with bacterial membrane constituents causing structural damage and ultimately cell death, disruption of membrane bound enzymes of the respiratory chain, inhibition of the expression of ribosomal subunit proteins and enzymes essential to ATP production, and affecting DNA replication.^[22–26] Due to this range of toxic effects, the development of resistance to silver ions / nanoparticles appears unlikely and therefore the use of silver as an antimicrobial offers the potential to address issues of antibiotic resistance. Advances in this area have included the incorporation of silver nanoparticles into a number of biomedical materials including dental resin composites and medical device coatings.^[27, 28]

The present contribution describes the formation of new recombinant silk-silver binding chimeric proteins and the formation of silver nanostructures from protein solutions and on silk films. Phage display has been used to identify short peptide sequences capable of directing the formation of silver nanostructures from a solution of AgNO₃ and these peptides were utilized in the present study.^[29, 30] The antimicrobial capacity of the silver treated protein films was also examined by incubation with both Gram positive and Gram negative bacteria. The incorporation of silver nanoparticles through mineralization of a silk based substrate establishes a new material which incorporates the mechanical strength, biocompatibility and aqueous processing of silk with an advantageous antimicrobial functionality.

2. Results and Discussion

2.1 Production of recombinant silk- silver binding fusion proteins

The consensus repeat of spider dragline silk was replicated 6 or 15 times incorporating the nucleotides for a C terminal 12 amino acid sequence identified by phage display found to bind to silver nanoparticles (Figure 1) using techniques we have previously described.^[5, 11–13] The clones were verified by sequencing and expression of the chimeric proteins was carried out in *E. coli*. Expression of the recombinant chimeric proteins was analyzed by SDS-PAGE and purified proteins (6mer Ag-4, 6mer Ag-P35 and 15mer Ag-4 and a control silk 6mer) were pooled and dialyzed (Figure 2 (a–c)). Analysis of 6mer Ag-4 by SDS-PAGE revealed the presence of lower concentrations of additional bands with molecular weights both higher and lower than that of the protein of interest. Western blot analysis utilizing antibodies to the His tag was used to determine whether these additional proteins were related to the fusion protein (Figure 2d). Both SDS and Westerns displayed the same banding pattern indicating that the larger protein was an aggregate of 6-mer Ag4, corroborated by its molecular weight which was comparable to a dimer of 6mer-Ag4. The smaller low intensity bands appear to be early termination products which still contain the N-terminal His tag indicating a small proportion of the protein produced may have terminated before the translation of the C terminal domain.

2.2 Silver precipitation assay

The purified proteins were examined for their ability to form silver nanoparticles from a solution of 0.1 mM Silver nitrate (AgNO₃). UV spectroscopy was used to compare the effects of the chimeric silk-silver binding proteins with the synthetic Ag4 peptide (produced by fmoc chemistry) alone in silver nitrate solution. Figure 3a shows the production of a large broad surface plasmon resonance band centered at $\approx 440\text{nm}$ upon incubation of Ag4 peptide in AgNO₃ solution. This result is comparable with the findings of Naik *et al.*, upon the identification of the Ag4 peptide by biopanning.^[29] Figure 3b which results from the incubation of the chimeric silk-Ag4 peptide, also shows a similar surface plasmon resonance peak centered around 440nm with a lower intensity than produced by the Ag4 peptide alone. This is likely to be due to the overall lower concentration of the Ag4 moiety present in the chimera. The control recombinant silk (no Ag4 domain) did not yield any surface-plasmon absorption band over the course of the incubation. Silver nanoparticles are known to display a characteristic surface plasmon resonance in the region of 400 nm with increased peak width corresponding to a broad size distribution among the particles. The presence of larger particles in solution leads to a shift to longer wavelengths.^[31] These findings therefore indicate the production of silver particles with a diverse size distribution in both the Ag4 and Silk-Ag4 protein solutions.

2.3 Silver nanostructure formation on Silk-silver binding films

Recombinant silk-silver binding protein films incubated in 0.4 mM AgNO₃ were analyzed by scanning electron microscopy for the presence of silver nanoparticles/nanostructures (Figure 4). The silk-Ag peptide films developed an orange/brown coloration, indicative of the formation of silver nanoparticles. All of the recombinant silk films, including the control without silver binding sequences, formed particles and/or nanostructures on their surface. However, morphological differences among the various silks were observed with variations in size, shape and aggregation. The silk films lacking sequences designed for silver binding (Figure 4b) resulted in the formation of large particles with spherical, cubical, and triangular morphologies. Mean particle size on the recombinant silk-only films (controls) was broad $173 \text{ nm} \pm 66.7 \text{ nm}$. A similar pattern was observed for the 6mer silk-Ag-P35 film (Figure 4d) which displayed several different morphologies and a broad size distribution ($121.3 \text{ nm} \pm 49.5 \text{ nm}$). The incubation of the 6mer Ag-4 with AgNO₃ (Figure 4c) resulted in the most homogeneous material with aggregated spherical particles of approximately $35.5 \text{ nm} \pm 8.5 \text{ nm}$ in diameter found covering large areas of the films. Incorporation of the Ag-4 sequence onto a longer silk fragment (15mer Ag-4, Figure 4e) resulted in the formation of rod-like morphologies which appeared to be formed from the longitudinal aggregation of spherical particles. The resulting rods had a width comparable to the nanostructures observed for 6mer Ag-4, with diameter at $57.2 \text{ nm} \pm 17 \text{ nm}$ and length $226 \text{ nm} \pm 82 \text{ nm}$. The silk films incorporating a non silver-specific peptide sequence (Figure 4f) produced the least aggregated particles with a morphology similar to the silk only (control) film and particle sizes of $91 \text{ nm} \pm 26 \text{ nm}$. The particles produced by silk-Ag4 chimeric proteins appear altered from those produced by the Ag-4 peptide alone. Previous work using this peptide demonstrated the formation of polyhedral particles with an average diameter of $102 \text{ nm} \pm 28 \text{ nm}$ after incubation.^[29] Incorporation of silk to form this new recombinant protein (6mer silk-Ag4) resulted in the formation of much smaller particles with a narrow size distribution ($35.5 \text{ nm} \pm 8.5 \text{ nm}$), more spherical in appearance. An increase in the length of the silk component of the chimeric protein resulted in a corresponding increase in particle size. Conversely, the particle size produced by the Ag-P35 domain increased upon formation of the silk-Ag-P35 chimera ($121.3 \text{ nm} \pm 49.5 \text{ nm}$) compared with the particles formed in the presence of Ag-P35 peptide only ($52 \pm 13.2 \text{ nm}$).^[30] Previous studies have shown that the antimicrobial properties of silver nanoparticles are dependant on both the size and shape of the particles. Other researchers have shown that reduced particle size led to increased antimicrobial properties of silver nanoparticles due to increased surface area, while shape was also important with a higher number of facets providing increased biocidal effects.^[32, 33]

Examination of the elemental composition at the surface of the mineralized films was carried out by X-ray photoelectron spectroscopy (XPS). The presence of the Ag 3d_{5/2} peak at 370eV was observed on all mineralized samples with the exception of the 15mer non silver specific sequence, which only displayed peaks for carbon and oxygen (Figure 5). The percent atomic concentration of silver on the surface of all other samples was quantified and found to be in the range of 1.2–1.3%, with the exception of 15mer Ag-A, which showed an increase in silver to 1.82%. In all Ag-containing samples the level of Ag detected was greater than the level of chloride, most noticeably in sample 6mer Ag4 where the level of silver was three-fold greater than chlorine. These data on elemental composition, together with the coloration of the films after incubation with AgNO₃, indicates that silver is present in the form of silver nanoparticles rather than as silver chloride. Note, every effort was made to prevent the addition of chloride ions to any of the samples, however, it was impossible to completely eliminate this element due to its presence as a trace contaminant in most reagents.

2.4 Antimicrobial activity of AgNO₃ treated recombinant silk-silver binding proteins

Silver treated recombinant silk films were examined for their potential as antimicrobial coatings through incubation on Luria- Bertani agar plates seeded with either the Gram negative bacterium *E. coli* or Gram positive *S. aureus* (Figure 6). Growth of both *E. coli* and *S. aureus* was inhibited at the edge of the mineralized silk films while the zone of inhibition did not extend far from the edges, indicating the silver nanostructures do not diffuse far from the films during incubation. This suggests the silver particles are tightly bound to the films. Non- mineralized control films showed dense growth around the films.

Further analysis of antimicrobial activity was carried out in liquid LB medium with the incubation of silver treated films with a log phase cultures of *E. coli* or *S. aureus*. All silver treated silk films inhibited the growth of both bacterial species over 24 hours (Figure 7). Untreated recombinant silk 15mer films were also examined in each study alongside a positive control culture. Both the *E. coli* and *S. aureus* cultures grew well in the presence of the untreated silk films demonstrating that microbial inhibition was not due to the silk alone, but required the presence of the silver particles. Silver ions and nanoparticles have been extensively studied for their antimicrobial effects and have shown potential as long lasting biocidal agents. However, due to their low colloidal solubility silver nanoparticles have limited effects when free in solution.^[22] Therefore, in order to exploit the full potential of silver nanoparticles in antimicrobial uses, a matrix capable of encapsulating nanoparticles or nucleating silver ions from solution would be advantageous. To this end, recombinant spider silks with robust mechanical properties and modified through recombinant techniques to incorporate silver binding peptides were pursued. Phage display techniques were previously employed to identify short, 12 amino acid peptide sequences (Ag4 and Ag-P35) which were capable of binding to silver nanoparticles and comparison of these sequences revealed a preference for proline and hydroxyl containing amino acids.^[29, 30] Incubation of these short peptides with AgNO₃ resulted in the formation of silver nanoparticles which were found to differ in both size and morphology depending upon the peptide sequence, demonstrating the ability of these sequences to nucleate the resulting nanoparticles. By incorporating the silver binding peptides at the C terminus of recombinant spider silk it was possible to produce silver nanoparticles in the recombinant silk solution upon incubation with AgNO₃. The formation of nanoparticles from a recombinant silver binding fusion protein was previously observed using light chain ferritin as the protein component.^[34] This resulted in the formation of small (<10 nm) silver particles which were produced inside the recombinant light chain ferritin cages after incubation in AgNO₃. By creating silk-silver binding chimeric proteins, silver nanoparticles with variations in size (35–120nm) and morphology (spheres and rods) have been produced on a stable protein substrate which had antimicrobial functionality.

3. Conclusions

This study demonstrates the synthesis of new silk-silver binding recombinant proteins through the bioengineering of silver binding peptide sequences to the C-terminus of spider silk repeat sequences. The resulting proteins regulate the formation of a variety of silver nanostructures both in solution and on films upon incubation with silver nitrate solution. The antimicrobial action of the silver nanostructures highlights the potential of these chimeric proteins in biomedical applications, by exploiting the combined features of robust material features from the silk component with the antimicrobial features of the silver nanoparticles bound to the silver-binding component.

4. Experimental

Materials

All cloning work was carried out using a pET30a vector obtained from Novagen (Madison, WI). Restriction enzymes, T4 DNA ligase, calf intestinal phosphatase (CIP), *Taq* DNA polymerase, and dNTPs were purchased from New England Biolabs (Beverly, MA). Synthetic nucleotides for the vector insert and the metal binding sequences along with vector derived PCR primers, NuPAGE™ 4–12% Bis-tris acrylamide gels, SeeBlue Plus 2 protein standards, 1kbp DNA ladder and DH5α *E. coli* were obtained from Invitrogen (Carlsbad, CA). QIAquick gel extraction kit, QIAprep spin miniprep and nickel-Nitrilotriacetic acid (Ni-NTA) were purchased from Qiagen (Valencia, CA). Kanamycin, Isopropyl β-D-1-thiogalactopyranoside (IPTG) and Silver nitrate (AgNO₃) were purchased from Sigma-Aldrich (St. Louis, MO). Western blot reagents (Perfect Protein AP Western Blot Kit) were purchased from Novagen (Madison, WI) along with the anti-His mouse monoclonal antibody. SpectraPor dialysis membrane (500 MWCO) and all other chemicals were purchased from Fisher (Pittsburgh, PA). Bacterial strains *E. coli* (25922) and *S. aureus* (25923) were obtained from ATCC (Manassas, VA).

Cloning of silk-silver binding chimeric proteins

The cloning and expression vector pET30a was modified by the insertion of a linker sequence containing the restriction sites *NheI* and *SpeI* as previously described^[5, 13]. In summary, the multiple cloning site of pET30a was removed by digestion with the restriction enzymes *NcoI* and *XhoI*. Synthetic nucleotide strands composed of *NheI* and *SpeI* restriction sites and terminating with *NcoI* and *XhoI* cohesive ends were annealed by heating to 95°C and cooling to 20°C at a rate of 0.1°C/s. Further denaturation at 70°C and cooling to 20°C was carried out as a means of preventing mismatched double stands. The modified pET30 vector was created by the ligation of this nucleotide cassette into the digested vector in the presence of T4 DNA ligase. The incorporation of the nucleotide sequence coding for either 6 or 15 spider silk repeat units of the major ampullate dragline silk from *Nephila clavipes*, spidroin I (-SGRGGLGGQGAGAAAAAGGAGQGGYGGGLGSQGT-) was previously described^[11]. Synthetic nucleotides (Figure 1) for silver binding peptides as identified by phage display^[29, 30] were designed using codon usage optimized for *E. coli* incorporating the cohesive ends for *NheI* and *SpeI* were annealed in the same manner. Another, sequence of the same size (12 amino acids) which has not been found to influence the formation of silver nanostructures but capable of binding to FePt nanoparticles^[35] was also cloned into the pET30 silk vector as a non Ag control. The modified pET30 vector was digested with *SpeI* and treated with CIP followed by purification using the Qiagen gel extraction kit. Ligation of the vector to incorporate the silver binding nucleotide sequence was carried out using T4 DNA ligase. The ligated plasmids were transformed into DH5α cells and the presence of the vector was verified by growth on plates containing 50 µg/ml kanamycin. Clones containing successful ligation products were confirmed to code for silk-silver binding proteins by DNA sequencing carried out at Tufts Core Facility (TUCF) using the T7 and T7 term promoters.

Expression of the Silk-Metal Binding proteins

Successful clones for the silk-silver binding domain were transformed into the *E. coli* strain RY-3041 (a mutant strain of BL21 defective in the production of the SlyD protein) and grown in LB medium containing 50 µg/ml kanamycin at 37°C to mid-log phase (OD 600 nm ≈ 0.6). Protein expression was induced by the addition of IPTG (isopropyl β-D-thiogalactoside) to a final concentration of 0.5 mM and the cells harvested 4 hours after induction by centrifugation at 10,000g (Sorvall RC-5B centrifuge).

Purification of His tagged proteins

Purification of the recombinant chimeric proteins was carried out utilizing metal affinity chromatography with Ni-NTA which binds to the 6x Histidine tag encoded on the pET30 plasmid. Cell pellets were resuspended in lysis buffer (100 mM sodium phosphate monobasic, 10 mM Tris chloride, 8M Urea, pH 8.0) at 5 ml/g wet weight and stirred for 30 minutes. After lysis, cells were centrifuged at 10,000g for 20 minutes and the supernatant was mixed with Ni-NTA slurry at a ratio of 4:1. Binding of the proteins was allowed to proceed for 16 hours before loading onto a column and washing with lysis buffer with pH decreasing from pH 8.0 to 6.4 and 5.4 prior to eluting the His tagged silk-silver binding proteins in lysis buffer at pH 4.5. Samples were dialysed (500 MWCO) against acetate buffer (10 mM pH 4.5) to promote protein folding followed by dialysis against water after which samples were lyophilized. Expression yields were typically in the region of 20–25 mg/ liter of culture.

Gel electrophoresis and Western blot

Confirmation of successful purification of the silk-silver binding chimeric protein was carried out by running individual fractions eluted from the Ni-NTA column on a 4–12% Bis-Tris SDS gel to confirm the presence of a pure protein. Western blot analysis was used to further verify the production of the recombinant fusion. After electrophoretic transfer of the SDS gel to nitrocellulose, the blot was blocked for 1 hour with 3% bovine serum albumin (BSA) before incubation with anti-His mouse IgG (diluted 1:1000). Colorimetric detection was carried out using horseradish peroxidase conjugated goat-anti mouse IgG (1:5000 dilution) to visualize the protein.

Mineralization of silk-silver binding proteins

The capacity of the recombinant silk protein solution to nucleate silver nanostructures was analyzed by a modification of our prior method^[30]. The recombinant protein (0.4 mg/ml) was dissolved in 0.1M phosphate buffer pH adjusted to 7.4 with KOH, and incubated with 0.1mM AgNO₃ for up to 72 hours. Regular scans between 200–1000 nm were taken to monitor the formation of silver nanoparticles. For mineralization of recombinant silk films, protein was dissolved in ultra pure water to a final concentration of 5% (w/v) and 100 µl aliquots were cast onto polystyrene dishes and allowed to dry. β-sheet formation was induced by treating the films with 70% ethanol then left to air dry. The films formed were incubated in 0.1M phosphate buffer with 0.4 mM AgNO₃ pH 7.4 for up to 72 hours. Non silver treated control films were incubated for the same period of time in 0.1M phosphate buffer pH 7.4. Silver nanostructures on the silver treated and untreated silk films were coated with Pt-Pd using a Cressington 208HR sputter coater before SEM imaging using a Zeiss Ultra55 scanning electron microscope (Harvard University Center for Nanoscale Systems). Elemental analysis was performed using an X-ray photoelectron spectrometer equipped with an Al K_α radiation source (Harvard University Center for Nanoscale Systems).

Examination of antimicrobial capacity of AgNO₃ treated silk- silver binding films

Recombinant silk films with silver nanostructures were washed in distilled water and placed on LB agar plates seeded with a log phase culture of Gram negative *E. coli* (ATCC 25922) or Gram positive *S. aureus* (ATCC 25923) then incubated overnight at 37°C. Films were also incubated in 2 ml of LB media inoculated with 2 µl of log phase culture of *E. coli* or *S. aureus*. Cultures were incubated at 37°C and 200 rpm and bacterial growth progression monitored by optical density (OD_{600nm}). The 15mer silk films produced were observed to be significantly less fragile both before and after silver treatment than the 6mer silk films which were difficult to handle.

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A**6mer silk**

Met H H H H H H S S G L V P R G S G **Met** K E T A A A K F E R Q H **Met** D S P D L G T
 D D D D K A **Met** A A S G R G G L G G Q G A G A A A A A G G A G Q G G Y G G L G
 S Q G T S G R G G L G G Q G A G A A A A A G G A G Q G G Y G G L G S Q G T S G R
 G G L G G Q G A G A A A A A G G A G Q G G Y G G L G S Q G T S G R G G L G G Q
 G A G A A A A A G G A G Q G G Y G G L G S Q G T S G R G G L G G Q G A G A A A
 A A G G A G Q G G Y G G L G S Q G T S G R G G L G G Q G A G A A A A A G G A G
 Q G G Y G G L G S Q G T S **Stop**

B**Ag-4**

5' CTAGC AAC TTT AGC TCT CTG TTT CGC TAT CTG CCG AGC GAT A 3'
 N P S S L F R Y L P S D

Ag-P35

5' CTAGC TGG AGC TGG CGC AGC CCG ACC CCG CAT GTG GTT ACC A 3'
 W S W R S P T P H V V T

Non silver binding

5' CTAGC AAA AGC CTG AGC CGC CAT GAT CAT ATT CAT CAC CAT A 3'
 K S L S R H D H I H H H

Figure 1.

A) Protein sequence of the recombinant silk 6mer with N terminal His tag B) Design of the synthetic oligonucleotides for the silver binding sequences Ag-4 and Ag-P35 and the non silver binding sequence which incorporate the *NheI* cohesive end at the N terminal and a C terminal cohesive end for *SpeI* (shown underlined).

Figure 2. SDS-PAGE of purified recombinant proteins: a) 6mer Ag-P35, b) 15mer Ag-4, c) silk 6mer-Ag-4, d) Western blot of duplicate 6mer Ag-4 gel showing higher and lower molecular weight bands are present at low concentration but also contain the His tag.

Figure 3.

UV spectroscopy showing the development with time of the characteristic surface plasmon resonance at 400 nm arising from silver nanoparticles: a. synthetic Ag-4 peptide, b. Recombinant 6mer silk- Ag-4 chimera, c. Recombinant silk alone (control).

Figure 4.

a) Recombinant silk non mineralized, b) recombinant silk mineralized, c) recombinant 6mer Ag 4, d) recombinant 6mer Ag-P35, e) recombinant 15mer Ag 4, f) recombinant 15mer non silver binding sequence.

Figure 5.
XPS results showing Ag 3d_{5/2} and 3d_{3/2} peaks present in the silver treated samples.

Figure 6.

Antimicrobial studies of AgNO_3 treated silk-Ag films: a) *E. coli* coated plate with AgNO_3 treated films of 15mer silk Ag 4 (left) and 15mer silk non Ag sequence (right), b) treated 15mer silk Ag 4 on *E. coli*, c) treated 15mer silk non Ag sequence on *E. coli*, d) treated 15mer silk Ag 4 on *S. aureus*, e) 15mer silk Ag 4 untreated on *E. coli*, f) 15mer silk Ag 4 untreated on *S. aureus*. White arrows indicating the zones of clearance on the Ag treated films and black arrows show the edge of untreated films with no impact on microbial growth.

Figure 7.
Growth curves of A) *E. coli* and B) *S. aureus* over 24 hours in the presence of silver treated silk films