Modification of Honeybee Silk by the Addition of Antimicrobial Agents

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ABSTRACT: Honeybee silk proteins can be produced at high levels in recombinant systems, fabricated into materials, and are tolerant of amino acid modifications: properties that make them exciting templates for designing new functional materials. Here, we explore the properties of materials either made from silk-antimicrobial peptide (AMP) fusion proteins or silk containing entrapped AMPs or silver nanoparticles. Inclusion of AMP within the silk protein sequence did not affect our ability to express the proteins or process them into films. When AMP-silk proteins and Escherichia coli cells were coincubated in solution, a reduction in cell numbers was observed after degradation of the chimeric protein to release a truncated version of the AMP. In films, the AMP was retained in the silk with leaching rates of <1% per day. Films containing silver nanoparticles were antimicrobial, with the silk preventing aggregation of nanoparticles and slowing the rate of dissolution of the particles.

INTRODUCTION

Antibiotic resistance is a growing problem in health care. Systemic antibiotics are failing, and few replacements have been found in recent decades. New technologies to control microbial infections are needed. Two antimicrobial ingredients that are currently receiving considerable attention are antimicrobial peptides (AMPs) and silver nanoparticles. AMPs are short cationic peptides that are part of the innate immune system found in living organisms. α-Helical AMPs, which assume an amphipathic α-helical conformation in microbial membranes, are the most abundant group of AMPs. Two of the most studied peptides from this group are magainin, derived from Xenopus, and its synthetic analogue pexiganan. Pexiganan is a 22 amino acid peptide with activity against Gram-positive, Gram-negative, and fungal species, which has been shown to be effective as a topical coating for treating infections associated with diabetic foot ulcers and, when entrapped within collagen matrices as a wound dressing, has been demonstrated to reduce the likelihood of infection.

Silver is an antimicrobial that has been used for more than six millennia to prevent microbial infections, with Herodotus dictating that “no Persian king would drink water that was not transported in silver containers.” Silver is now used widely: silver sulfadiazine is in the WHO Model List of Essential Medicines as an anti-infective medicine. Silver, in various forms, is commercially available for a range of medical conditions from wound dressings to treatments for warts and corns. Silver has also been incorporated into clothing to produce “odor-free clothes”, and its presence in food containers and washing machines is marketed as a way to reduce bacterial numbers.

The use of silver salt preparations remains controversial. The nitrate component of silver nitrate used in burn treatment is toxic to tissues and can decrease wound healing, and a recent Cochrane review has found that silver sulfadiazine applied directly to a burn increases the rate of infection by between 8 and 80%. These findings are prompting a move away from silver-based solutions for wound treatment. Instead, elemental silver preparations are increasingly being used as an alternative, with a myriad of products commercially available that use silver nanoparticles as the active ingredient.

Our laboratory is developing recombinant honeybee silk proteins as templates for designing new materials. The honeybee silk protein, AmelF3, has been expressed in transgenic Escherichia coli at high levels and fabricated into solid-state materials such as fibers, scaffolds, and films. The ability to modify these proteins using molecular biology allows us to insert nonnatural peptide motifs into their primary sequence. The natural sequence variation of homologous silk proteins as templates for designing new materials. The honeybee silk protein, AmelF3, has been expressed in transgenic Escherichia coli at high levels and fabricated into solid-state materials such as fibers, scaffolds, and films. The ability to modify these proteins using molecular biology allows us to insert nonnatural peptide motifs into their primary sequence. The natural sequence variation of homologous silk proteins as templates for designing new materials. The...
indicating that, in addition to adding functionality by modifying the primary sequence, further functionality can be obtained by entrapping bioactive molecules within the silk materials.

In this work, we investigate the antimicrobial activity of several composite recombinant honeybee silk materials: chimeric silk-AMP proteins, silk with entrapped AMPs, and silk with entrapped silver nanoparticles. Previous studies have demonstrated that *E. coli* is susceptible to both AMPs and silver ions; hence, we used a common laboratory strain of *E. coli* to investigate the ability of these compounds to render honeybee silk antimicrobial.

### RESULTS AND DISCUSSION

**Antimicrobial Activity of AMP-Silk Chimeric Proteins.**

AMPs are receiving widespread research attention as an alternative to existing antibiotic compounds. However, the use of peptide-based drugs is limited by their rapid degradation which requires the use of very high and therefore toxic concentrations during therapeutic applications. Methods to stabilize or deliver these drugs to their site of action are required before their full potential can be realized. Recent studies have demonstrated that some AMPs retained efficacy despite being attached to fusion partners such as other AMPs, recombinant spider silk proteins, recombinant elastin, or other proteins. Here, we investigated whether inclusion of the AMP, pexiganan, in honeybee silk generates antimicrobial materials and/or stabilizes the peptide.

As with other so-called α-helical AMPs, the pexiganan peptide is unstructured in solution and assumes an antiparallel dimer of amphipathic helices in the presence of microbial membranes, leading to membrane permeabilization, cell lysis, and cell death. In solution, different portions of the honeybee silk protein adopt structures similar to the solution and membrane-bound structures of the pexiganan peptide: the N- and C-termini being unstructured, and the central region adopting a coiled-coil structure comprising multiple amphipathic helices. We designed AMP-silk chimeric proteins in which different regions of the silk protein were replaced or extended with the pexiganan protein sequence: either at the C-terminus of the silk protein (construct T: terminal extension); as an extension of the silk protein’s coiled-coil region (construct E: extension); or as a partial replacement of the silk protein’s coiled-coil region (construct R: replacement) (Figure 1). On the basis of previous examples in the literature, we expected that construct T was most likely to show antimicrobial activity.

Figure 1. Schematic representation of the placement of the 22 amino acid pexiganan AMP in honeybee silk protein (AmelF3). Construct R (replacement) retains the length of the native AmelF3 sequence with the end of the coiled-coil domain being deleted and replaced with the AMP. Construct E (extension) has the AMP added onto the end of the coiled-coil region. Construct T (terminal) has the pexiganan added to the C-terminus of the silk protein.

Consistent with earlier studies that had demonstrated that the expression of AMPs into *E. coli* inclusion bodies masks the toxic effects of the peptides during production (see Ref 33), the presence of the antimicrobial sequences attached to the silk protein did not have a detrimental effect on the high-level expression of E and R constructs into the inclusion body. The T construct, which placed the AMP sequence at the C-terminus, was expressed at a lower rate, although protein expression did not appear to adversely affect the cell growth. After solubilization and purification, all three chimeric proteins were solubilized, purified, and fabricated into transparent films which did not differ in handling ability and visual characteristics from films generated from unmodified proteins. This result showed that large modifications to the silk protein at various locations along the protein backbone do not inhibit our ability to make these proteins into solid-state materials.

Initially, we investigated the antimicrobial activity of silk materials using a simple laboratory assay based on that described by Lok et al. *E. coli* strain K-12 cells [American Type Culture Collection (ATCC) 27325] were a convenient tool for this purpose because their growth rate in medium is reproducible; therefore, differences in population growth rates could be attributed to sample treatments. This method is convenient for comparing treatments that have log-scale effects on cell numbers yet can detect more modest antimicrobial effects than determined from standard minimum inhibitory concentration (MIC) assays. We incubated *E. coli* cells with films generated from unmodified honeybee silk protein in a nutrient-free buffer. After a set incubation period, bacterial growth medium was added and growth of the culture was monitored by measuring the optical density (OD) at 600 nm for up to 20 h (Figure 2). The slope of the linear part of the growth curve did not differ between control and silk-exposed populations (control: $197 \pm 2.2 \times 10^{-5}$; silk: $195 \pm 0.57 \times 10^{-5}$ OD units-min⁻¹), indicating that the recombinant silk film was essentially inert to *E. coli* growth during the growth phase. The population of the cells in the presence of silk achieved log-phase growth earlier than the control population, indicating that a higher proportion of cells survived the incubation in the nutrient-free buffer, suggesting that the silk film protects the cells during this initial incubation.

We repeated the assay using stabilized films generated from the silk proteins modified with AMPs at various positions. None of the films generated from the chimeric proteins led to a decrease in *E. coli* numbers. We also tested the antimicrobial activity of chimeric silk films using zone of inhibition assays, where films were incubated in the presence of a growing lawn of *E. coli* cells, and did not observe any clearing around the films. The lack of antimicrobial activity was not likely due to the inactivation of the AMP by the aqueous methanol treatment used to stabilize the silk films because synthetic pexiganan treated with aqueous methanol in the absence of silk protein retained activity.

To understand the loss of efficacy of the AMP in our silk materials, we investigated the rate of release of synthetic pexiganan entrapped in the silk films using a fluorescently labeled pexiganan variant. Leaching of the labeled peptide from the silk films was slow, with only around 2% of the peptide leaching out of the films containing 25% AMP after 1 week incubation (Figure 3). We used a nonlabeled synthetic pexiganan to investigate the antimicrobial activity of the silk films containing entrapped synthetic pexiganan (Figure 4). Controls without silk film showed antimicrobial activity.
consistent with previous reports, whereas low levels of antimicrobial activity were observed from films containing high levels of AMP (500 μg AMP: 1.5 mg of silk, equivalent to 1 mg·mL⁻¹ in the assay), and no antimicrobial activity was observed with AMP loadings containing the equivalent of 5–25 × MIC (Figure 4).

The reduced antimicrobial activity of the loaded films was consistent with the low levels of leaching of the labeled peptide from the silk films (Figure 3). The low levels of pexiganan leaching from our protein films are in contrast to that observed by Gopinath et al. who reported around 80% release of pexiganan from collagen films containing 1.5% AMP over 30 h. Stabilization of the honeybee silk films using methanol increases the amount of β-sheet structure in the silk materials. Although our results demonstrated that methanol treatment had no effect on the peptide in isolation, the extensive H-bonding network associated with β-sheet structure that arises from this treatment in the silk likely tethers the peptide within the material reducing its release rate. Similarly, a reduction in antimicrobial activity was found to be associated with the formation of β-sheet structure in spider silk-AMP fusion proteins. The association of β-sheet structure and peptide release gives us a starting point to design a material that will allow controlled long-term release of AMP.

To understand if the absence of antimicrobial activity in our chimeric proteins was the result of fusion of the AMP to the silk protein or due to masking of the activity due to the solid-state format of the silk-AMP proteins, we incubated soluble silk-AMP proteins with E. coli cells in solution rather than in solid-state formats. Initial tests using Clinical and Laboratory Standards Institute (CLSI) guidelines M07-A7 and M27-A3 with proteins at 3 and 6 mg·mL⁻¹ did not detect antimicrobial activity against Gram-negative bacteria, Gram-positive bacteria, or fungus. Using an agar plate counting assay, capable of detecting more modest reductions in cell numbers, we found a reduction in E. coli numbers when cells were exposed to 3 mg·mL⁻¹ protein (equivalent to 227 μg·mL⁻¹ AMP; 10 times the published MIC for E. coli) (Figure 5A). Greater activity was observed when either the incubation time or the number of cells in the protein solutions was increased (Figure 5A). Similar reduction in the E. coli cell number was found after incubation of cells with any of the three chimeric constructs.

Figure 2. Growth curves of E. coli populations after incubation in the presence and absence of recombinant honeybee silk films. Error bars indicate the standard error of the mean of measurements from 18 cultures.

Figure 3. Amount of fluorescently labeled pexiganan (AMP) released from silk protein films in solution over time.

Figure 4. Growth curves obtained after E. coli cells are incubated with pexiganan alone or pexiganan entrapped in honeybee silk.
After incubation of 10^5 cells degradation products from each protein. (C) Cell numbers remaining populations for different incubation with (+) or without (−) E. coli solutions of the silk protein (AmelF3) or chimeric silk-AMP proteins solutions. (A) Cell survival after inoculation of different incubation periods in the presence of E. coli cells. Arrows indicate major degradation products from each protein. (C) Cell numbers remaining after incubation of 105 cells-mL^-1 E. coli for 1 h with recombinant honeybee silk protein or various silk-AMP proteins.

Figure 5. Activity of chimeric silk-AMP proteins in E. coli-containing solutions. (A) Cell survival after inoculation of different size E. coli populations for different incubation periods in the presence of solutions of the silk protein (AmelF3) or chimeric silk-AMP proteins (construct E). (B) Degradation of silk or silk-AMP proteins after incubation with (+) or without (−) E. coli cells. Arrows indicate major degradation products from each protein. (C) Cell numbers remaining after incubation of 105 cells-mL^-1 E. coli for 1 h with recombinant honeybee silk protein or various silk-AMP proteins.

To understand why incubation in the presence of increased E. coli cell numbers and/or increased incubation time resulted in greater antimicrobial activity, we compared the silk proteins, before and after incubation with E. coli cells, using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 5B). SDS-PAGE showed that the silk-AMP proteins were degraded during incubation with E. coli. We investigated the major degradation products using liquid chromatography/mass spectroscopic analysis and found that the proteins were cleaved between the 4th Lys and 5th Phe of the pexiganan sequence. Zasloff has shown that truncation of magainin 2 in the equivalent position results in an increase in the MIC for E. coli by around 30 times. Given the relationship between incubation time, cell number, and antimicrobial activity, we attribute the antimicrobial activity observed in the silk-AMP chimeric protein study to products that arise after proteolysis of the AMP in the chimeric proteins during the incubation period by exogenous proteases released by E. coli. Similar activity was observed for all three constructs, suggesting that the presence of silk protein sequences at the C-terminus of the AMP did not negatively affect the activity (Figure 5C).

The results presented here demonstrate that we are able to produce silk-AMP chimeric proteins without negatively affecting the protein expression levels or the ability to form into materials. Even when not covalently attached to the silk protein, the AMP remains entrapped within the silk material, indicating that there is a strong interaction between the peptide and the silk material. These properties provide a platform for designing a responsive wound dressing material. Possibly, by controlling the β-sheet content in the materials, we may be able to control peptide leaching rates. Alternatively, signals that trigger dissolution of the silk will allow entrapped AMP to be stably maintained in the silk and then released when required.

Silver Nanoparticles Are Stabilized and Make Silk Antimicrobial When Entrapped in Films. The ability of entrapped silver nanoparticles to render honeybee silk materials antimicrobial was assessed. Silver is a broad-spectrum antimicrobial agent used widely in medical and consumer products. It is the silver ions that are active, and in the metallic form, silver is not antimicrobial. However, metallic silver nanoparticles are frequently used in consumer products because silver ions in gel or liquid form rapidly diffuse and are easily precipitated by a range of counterions, and therefore, local effective concentrations cannot be maintained. When silver nanoparticles are exposed to water and oxygen, the surface dissolves to release silver ions.

Suspensions of E. coli cells were exposed to recombinant honeybee silk protein films containing entrapped silver nanoparticles for 2 h, and the size of the surviving population was compared to that of the control population (Figure 3). Population sizes were back calculated from the bacterial growth curves after the addition of growth media, and based on the time, the cell density reached 0.2 absorption units (600 nm), assuming consistent growth rates across all treatments. This method requires that the toxicity of silver be neutralized by the addition of the chloride ions in the bacterial growth media, and if the silver ions remained present then it would be expected that the growth curves of the microbial population would be affected. We compared the rate of population growth in all treatments and found them to be statistically similar (Supporting Figure 1), thus justifying the use of the growth curve to compare population sizes after treatments.

The antimicrobial activity of the silver nanoparticles entrapped in the silk was compared to positive control treatments containing only nanoparticles (Figure 6). The positive controls demonstrated expected antimicrobial activity, with 10 nm nanoparticles showing greater activity compared to 100 nm nanoparticles (Figure 6). The greater activity of the smaller nanoparticles can be attributed to their larger surface area/weight ratio, leading to the release of more silver ions. As with controls, when entrapped within the silk, again the antimicrobial effect was correlated to the surface area of the entrapped silver. The activity conferred by the entrapped silver particles was lower than the activity from the control samples.
Without silk, with nearly 10 times as much as entrapped silver nanoparticles required to give antimicrobial activity similar to controls containing silver alone (Figure 6).

To understand the activity of the silver nanoparticles when entrapped in silk, we investigated the stability of the particles with and without entrapment. We compared the stability of free particles or particles entrapped in silk films, by monitoring their absorbance at 390 nm over time in aqueous buffer. The absorbance of the free particles rapidly declined in the first 100 h of incubation (Figure 7). No shift in absorbance to higher wavelengths was observed, indicating that the silver nanoparticles were dissolving rather than aggregating. In comparison, only a relatively modest loss of absorption was observed when the silver was entrapped within the silk films (Figure 7). We conclude from this that entrapment of silver nanoparticles within the silk films reduces the rate of dissolution of the particles. Similarly, Wen et al.37 report a significant reduction of silver ion release from silver nanoparticles in the presence of cytoskeletal proteins. This stabilization of the silver within the silk protein solution provides a method to deliver silver ions and hence antimicrobial activity, over a sustained period of time.

**CONCLUSIONS**

Recombinant AMP, pexiganan, can be produced within honeybee silk proteins as inclusion bodies in *E. coli*, and this modification does not affect the ability of the host silk proteins to form films. A simple antimicrobial assay was developed that used the growth kinetics of laboratory strain *E. coli* to assess the efficacy of known antimicrobial agents. The recombinant silk materials themselves do not affect the growth kinetics of *E. coli* cells. AMP efficacy required the AMP to be released from the silk proteins. Although this technique does not generate an antimicrobial material per se, it provides a method for protein designers to present a bioactive peptide in an inactive form that can be activated upon proteolytic release. In solid-state materials, free AMP was retained within the recombinant silk material, leaching at low rates, thus providing a platform for designing a responsive wound dressing. Recombinant silk protein films entrapped silver nanoparticles, preventing aggregation and reducing their rate of dissolution. The silk silver material had antimicrobial activity correlating to the amount and morphology of the entrapped silver nanoparticles. These results contribute to our understanding of how we can fabricate a responsive antimicrobial material in the future.

**EXPERIMENTAL SECTION**

**Design and Construction of DNA Encoding Silk-AMP Chimeric Proteins.** The following DNA sequence encoding the AMP, pexiganan (accession number AAC93471), was designed from the peptide’s amino acid sequence using EMBOSS Backtranseq optimized for *E. coli* expression:38

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\text{GGC ATT GGC AAA TTT CTG AAA AAA GCG AAA AAA TTT GGC AAA GCG TTT GTG AAA ATT CTG AAA AAA.}
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Oligonucleotide gBlocks primers containing the designed sequence (listed below) and relevant honeybee silk protein (AmelF3, accession number ACI49702) backbone primers (listed below) were purchased from Integrated DNA Technologies, and the Gibson cloning method39 using the Gibson Assembly cloning kit (New England Biolabs) was used to introduce the pexiganan peptide DNA into one of the three different positions (Figure 1) along the honeybee silk DNA contained within a pET-14b plasmid (Novagen) with a 6-histidine tag encoded at the N-terminal end of the encoded protein. The gBlocks sequences used to generate constructs include E gBlocks: AAG ATC GAG AGT GTG GCA GCC GCC GAT TGC AAA TTT CTG AAA AAA GCG AAA AAA TTT GGC AAA GCG TTT GTG AAA ATT CTG AAA AAA. Oligonucleotide gBlocks primers containing the designed sequence (listed below) and relevant honeybee silk protein (AmelF3, accession number ACI49702) backbone primers (listed below) were purchased from Integrated DNA Technologies, and the Gibson cloning method39 using the Gibson Assembly cloning kit (New England Biolabs) was used to introduce the pexiganan peptide DNA into one of the three different positions (Figure 1) along the honeybee silk DNA contained within a pET-14b plasmid (Novagen) with a 6-histidine tag encoded at the N-terminal end of the encoded protein. The gBlocks sequences used to generate constructs include E gBlocks: AAG ATC GAG AGT GTG GCA GCC GCC GAT TGC AAA TTT CTG AAA AAA GCG AAA AAA TTT GGC AAA GCG TTT GTG AAA ATT CTG AAA AAA.
GAA ATC CGA AAA TAT TGA GGA TAA AAA ATT TGG CAT TGG CAA ATT TCT GAA AAA AGC GAA AAA ATT TGG CAA AGC GTT TGT GAA AAT TCT GAA AAA ATA ATA AAG ATC CGG CTG CTA ACA AAG CCC GAA AGG AAG CTG AGT TGG CTG CTG CCA CCG CTG AGC AAT AAC TAG C; and R gBlocks: CCA TCA GCT GCG AAG ATC GAG AGT GTG GCA GCC GCC GAG GAG TCC GGC ATT GGA AAA TTT CTG AAA AAA GCG AAA AAA TTT GGC GCC GCA GCT CGC GGG GAT GGA GCG ATT ATA GGA GCT TGA GAG GAT GGA GCA ATT GTA GGA.

The silk protein DNA backbone primers used to generate constructs include E construct: forward ATG GAG CTA TAG GAC TTG, reverse GCT GCC ACA CTC TCG ATC TT; T construct: forward CAC CGC TGA GCA ATA ACT GGC, reverse GGA TTT CGA GCT AAC TTC GGC; and R construct: forward GGA GCG ATT ATA GGA ATT CTT GGA, reverse CTC GAT CTT CGC AGC TTA. Ligated constructs: forward GGA GCG ATT ATA GGA CTT GGA, reverse CTC GAT CTT CGC AGC TTA. Ligated gBlocks and backbone DNA sequences were extracted using the QIAprep Spin Miniprep kit (Qiagen) following the manufacturer’s instructions and Sanger-sequenced to confirm the correct mutations.

Expression and Purification of Silk-AMP Chimeric Proteins. The silk-AMP chimeric proteins were produced in the inclusion bodies of E. coli cells using previously described methods developed for the high-level expression of the silk protein. Briefly, the silk-AMP expression plasmids were transformed into E. coli Rosetta 2 (DE3) competent cells (Novagen), plated onto Luria-Bertani (LB) agar containing 100 μg/mL ampicillin, and then incubated at 37 °C for 18 h. A starter culture from a single colony was grown at 37 °C for 1 h, transferred into a 1 L Overnight Express Instant Terrific Broth (Novagen) containing 100 μg/mL ampicillin and 10 mL of glycerol in shaking flasks, and then incubated at 30 °C for 24 h. Recombinant E. coli cells were collected by centrifugation at 5000g for 15 min, and inclusion bodies were purified after the cells were lysed in BugBuster Master Mix (Novagen), containing 1% Benzonase Nuclease (Novagen) according to the manufacturer’s protocol. Washed inclusion bodies were solubilized in 6 M guanidine hydrochloride (Sigma) in 20 mM phosphate buffer containing 40 mM imidazole (His buffer kit; GE Healthcare) by incubation at 4 °C for 24 h. Nonsolubilized protein and particulate matter were removed by centrifugation at 5000g for 30 min. Supernatant was filtered through a 0.22 μm Millex syringe filter unit (Merck). The silk-AMP protein was purified by binding the supernatant to a HisTrap HP column (GE Healthcare), washing the bound protein in 20 mM buffer containing 40 mM imidazole, and then eluting the proteins in 20 mM buffer containing 250 mM imidazole. The salt levels were reduced, and imidazole was removed from the protein solutions using a desalting column (GE Healthcare) with the proteins being eluted into 25 mM NaCl. Proteins were concentrated using Vivaspin 15R centrifugal concentrators (Sartorius), and protein concentrations were quantified using a Direct Detect spectrophotometer (Merck Millipore) according to the manufacturer’s protocol.

Antimicrobial Activity of Solid Materials. To assess the antimicrobial activity of materials, films were cast in wells of 24-well tissue culture plates. For studies involving silver nanoparticles, silk films (1.5 mg of dry weight silk protein) containing 0, 0.01, 0.1, or 1% of 10 nm or 100 nm diameter silver nanoparticles (nanoComposix) were formed by mixing 1% silk protein solutions with either 2 mM sodium citrate buffer (no silver control) or the appropriate amount of silver nanoparticles in 2 mM sodium citrate buffer. Four films were prepared for each condition. The absorbance spectra of silver nanoparticles in silk films (Supporting Figure 1) were similar to the spectra obtained in dispersed solutions. Controls included silver nanoparticles in equivalent amounts of water and silk protein without silver nanoparticles. Mixtures were cast into plastic trays, and water was allowed to evaporate. The resultant films were transparent (controls), with yellow (10 nm particles) or pink (100 nm particles) coloration. The films were stabilized by overnight treatment with aqueous (70%) methanol, according to the method of Huson et al. The loss of silver nanoparticles during the stabilization treatment was around 10%, as determined from the OD at 390 nm of the aqueous methanol solution after stabilization treatment compared with a standard curve from silver nanoparticles in 70% methanol. Consistent with this, inductively coupled plasma-atomic emission spectroscopy of films showed around 9% loss of silver from the material after methanol treatment.

E. coli cells grown to log phase (3 × 10^5 cfu·mL^-1) in 100 μL N-(2-hydroxyethyl)piperazine-N’-ethanesulfonic acid (HEPES) were plated on top of the stabilized silk films or controls and then incubated in a shaker at 75 rpm for 2 h at room temperature. HEPES buffer does not chelate metal ions. After this treatment, the antimicrobial effect of silver was neutralized by the addition of an equivalent volume of ×2 LB to each well, with the chloride ions in the LB precipitating silver ions from the solution. The proportion of surviving E. coli cell numbers relative to controls was determined from the time it took for cultures to reach an OD at 600 nm of 0.2, assuming a doubling time of 20 min, at 37 °C.

The antimicrobial properties of films containing entrapped pexiganan were examined similarly. Silk films (1.5 mg of silk) containing 0, 50, 100, or 500 μg synthetic pexiganan, controls containing 10 and 50 μg synthetic pexiganan, or silk alone were assayed in 1 mL of solution. These amounts of pexiganan were chosen based on published MIC of the peptide against E. coli ATCC 25922 (16 μg·mL^-1).

For films generated from the chimeric silk-AMP protein, a zone of inhibition assay was also used. Films, stabilized by overnight treatment in aqueous methanol, were placed on LB agar plates seeded with a lawn of E. coli cells. After overnight incubation, the plates were inspected for clearing zones around the silk films.

Antimicrobial Adhesion Activity of Silk-AMP Chimeric Proteins in Solutions. The antimicrobial activities of the silk-AMP chimeric proteins in solution were determined initially using a standard microbroth dilution assay in accordance with the CLSI guideline M07-A7. Briefly, frozen (−80 °C) aliquots of Staphylococcus epidermidis RP62A (ATCC 39584), Staphylococcus aureus (ATCC 29213), E. coli (ATCC 25922), or Pseudomonas aeruginosa (ATCC 27853) were plated on agar plates and incubated overnight at 37 °C, and then a single colony was transferred into 5 mL of nutrient broth (NB) and incubated at 37 °C for 5–6 h to reach log phase. The log-phase bacterial solutions were diluted with fresh NB at OD_600 = 0.1 (approximately 10^8 cfu·mL^-1) and then further diluted 100-fold into Mueller Hinton Broth (MHB) for testing. The chimeric silk-AMP proteins E2 (3 mg·mL^-1) and T2 (6 mg·mL^-1) were prepared as twofold serial dilutions in MHB. Equal volumes of bacterial suspension and protein dilutions were mixed in wells of a 96-well microplate and then incubated at 35 °C for 18 h. The presence or absence of bacterial growth was examined.
visually with the aid of a mirror reader. The activity against fungi was assessed using the wild-type Candida albicans strain DAY185 according to CLSI guidelines M27-A3. Log-growth-phase fungi were diluted to ~1-5 × 10^4 cfu·mL^-1 in a Roswell Park Memorial Institute 1640 medium, then equal volumes of fungal suspension and protein dilutions (as above) were mixed in wells of a 96-well microplate and then incubated at 37 °C for 48 h. The presence or absence of fungal growth was examined visually with the aid of a mirror reader.

To measure the lower levels of antimicrobial activity that were not apparent using the above method, a protocol similar to that used to demonstrate the antimicrobial activity of lacritin was developed. The antimicrobial activity was assessed by incubating 3 mg·mL^-1 recombinant silk-AMP protein with 10^5 to 10^8 E. coli (ATCC 27325) cells in LB for 0, 20, or 60 min, at 37 °C, with shaking. After treatment, the cells were recovered by centrifugation, resuspended in LB, and the number of remaining live cells was determined by plating the equivalent of 400 cells before treatment onto LB agar plates containing 100 mg·mL^-1 ampicillin. The plates were then incubated overnight at 37 °C, and colony-forming units (cfu) were manually counted. The extent of proteolysis of the silk-AMP protein during this treatment was assessed by SDS-PAGE of the supernatant, following the removal of the cells. Degradation products were identified by liquid chromatography/mass spectrometry analysis of the protein bands separated by SDS-PAGE.

**Rate of Release of Pexiganan from Silk Films.** The rate of release of pexiganan from silk films was monitored using pexiganan N-terminal labeled with the fluorescent group, 5-carboxyfluorescein (95% purity; Mimotopes). A standard curve of peptide in 100 mM HEPES buffer (pH 7.6) was prepared by measuring fluorescence at 515 nm using an excitation wavelength of 475 nm of peptide solutions ranging from 0.1 to 8 μM. Good proportionality was observed between the fluorescence signal and peptide concentrations between 0 and 0.5 μM peptide. Silk (1.5 mg)-labeled AMP (25, 125, 250, or 500 μg) films were stabilized according to Huson et al., and then rates of leaching of the peptide from the films into HEPES buffer were determined by measuring the fluorescence of the buffer solution over time.

**Stability of Silver Nanoparticles Entrapped in Silk Films.** The stability of silver nanoparticles entrapped within the silk films compared to the stability of silver nanoparticles by monitoring the changes in the Ultraviolet--visible spectrum of samples in the HEPES buffer is as follows: silver nanoparticles (1 mg·mL^-1) were added to the recombinant honeybee silk protein solution (1% protein w/v) to give a final ratio of 1% silver per dry weight of silk protein. Aliquots (100 μL) were dried in wells of a 24-well tissue culture plate and treated with aqueous 70% methanol overnight to stabilize the films; the methanol solution was removed, and films were allowed to dry. HEPES buffer (pH 7.6, 400 μL) was added to the films, and the plate was incubated at room temperature with shaking at 500 rpm. At regular time intervals, the absorption spectra from 300 to 600 nm from each film were collected using a SpectraMax M3 ( Molecular Devices) spectrophotometer. Between sample times, the sample volume was retained at 1 mL through the addition of water, and the plate was covered to prevent sample drying. Control samples contained equivalent amounts of silver nanoparticles in the same amount of HEPES buffer.

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