Sporicidal/Bactericidal Textiles via the Chlorination of Silk

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S Supporting Information

ABSTRACT: Bacterial spores, such as those of the *Bacillus* genus, are extremely resilient, being able to germinate into metabolically active cells after withstanding harsh environmental conditions or aggressive chemical treatments. The toughness of the bacterial spore in combination with the use of spores, such as those of *Bacillus anthracis*, as a biological warfare agent necessitates the development of new antimicrobial textiles. In this work, a route to the production of fabrics that kill bacterial spores and cells within minutes of exposure is described. Utilizing this facile process, unmodified silk cloth is reacted with a diluted bleach solution, rinsed with water, and dried. The chlorination of silk was explored under basic (pH 11) and slightly acidic (pH 5) conditions. Chloramine-silk textiles prepared in acidified bleach solutions were found to have superior breaking strength and higher oxidative Cl contents than those prepared under caustic conditions. Silk cloth chlorinated for ≥1 h at pH 5 was determined to induce >99.99996% reduction in the colony forming units of *Escherichia coli*, as well as *Bacillus thuringiensis* Al Hakam (*B. anthracis* simulant) spores and cells within 10 min of contact. The processing conditions presented for silk fabric in this study are highly expeditionary, allowing for the on-site production of protein-based antimicrobial materials from a variety of agriculturally produced feed-stocks.

KEYWORDS: silk, halamine, sporicidal, Bacillus anthracis, antibacterial, fibroin
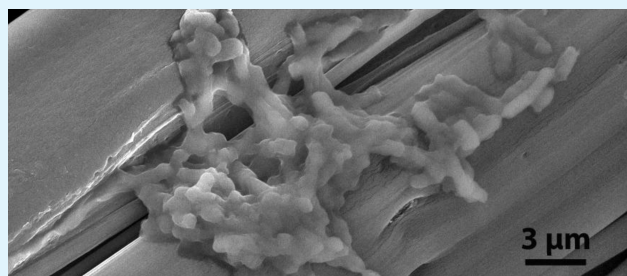
1. INTRODUCTION

Under adverse environmental conditions (e.g., lack of nutrients) *Bacillus* species undergo a specialized transition, known as sporulation, to a metabolically dormant state.[1] Such bacterial spores possesses a multilayered architecture that features a thick protein coat and an inner membrane of limited permeability.[1] This structural "armor", in conjunction with low water content, DNA protecting proteins and small molecules, and postgermination DNA repair mechanisms act in unison to ensure the greatest probability for survival of the bacteria when favorable conditions arise.[1,2] Bacterial spores are incredibly resilient, remaining viable for long periods of time in the environment, with some reports indicating the germination of spores that were ~250 million years old.[1,2]

In addition to their long-term environmental stability, bacterial spores are highly resistant to acute threats, such as those posed by microorganism predation or human decontamination attempts.[2,3] Indeed, the heat, radiation, and chemical treatments that would kill their vegetative counterparts are often ineffective in dispatching spores.[2] A limited number of chemicals agents, including strong acids, alkylating agents, formaldehyde, and oxidizing agents (e.g., sodium hypochlorite, hydrogen peroxide, peroxynitrite) are capable of neutralizing spores.[2] Alkylating agents and formaldehyde act to kill spores by inducing DNA damage, whereas oxidizing agents are likely to cause severe damage to the spore's inner membrane.[2,4,5] Of these sporicidal chemicals, the oxidizing agents, and in particular hypochlorite ions, are of interest as they are the basis for halamine-based self-decontaminating materials.[6]

Halamines are defined as compounds or materials that contain one or more nitrogen−halogen (e.g., chlorine) covalent bond, that liberate oxidizing halogen(s) in water (Scheme 1).[6]

Scheme 1. Chloramine Chemistry; The Loading and Release of Oxidizing Cl from a Nitrogen Moiety

$$R_1R_2NH + HOCl \rightleftharpoons H_2O + R_1R_2NCl$$
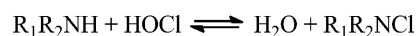
The nitrogen component of the halamine may be an imine, amide, or amine.[6,7] Although the development of halamines initially focused on small molecules for water treatment applications, a broad range of halamine-bearing polymers and fabrics have been developed in the past decade.[8−10] These fabrics include cotton, polyester, Kevlar, and nylon that have been coated or grafted with amine/amide-bearing monomers (e.g., methacrylate-, melamine-, hydantoin-, and imidazolidinone-based monomers) that may be chlorinated to yield

chloramine-functionalized textiles.[11−31] Polyamide textiles (e.g., Nomex) have also been used without modification and directly chlorinated to yield halamine-bearing cloth.[32,33] The halamine-functionalized textiles generated in these previous studies have been demonstrated to possess rapid and highly effective bactericidal activity against clinically relevant bacteria such as *Escherichia coli* and *Staphylococcus aureus*.[11−33] Although halamine fabrics have proven successful in rapidly (e.g., contact times of 0.5−10 min) killing vegetative bacterial cells, these state-of-the-art textiles have been less effective in deactivating bacterial spores.[20,23,34] For example, cotton fabrics functionalized with a chlorinated melamine derivative were found to induce a ∼2 log reduction in the CFU (colony forming units)/mL of *Bacillus atrophaeus* spores (i.e., initial concentration of ∼1 × 10⁶ CFU/mL) following a contact time of 15 min.[20] The total deactivation of *B. atrophaeus* spores by the chloromelamine-cotton fabric in this prior study required a contact time of 6 h.[20] Cotton grafted with methacrylamide and subsequently chlorinated was determined to possess similarly modest sporicidal activity, achieving ∼2 log and ∼6−7 log (i.e., total kill) of *Bacillus subtilis* spores (i.e., initial concentration of ∼1 × 10⁶⁻⁷ CFU/mL) following spore/textile contact times of 1 and 4 h, respectively.[34] Kevlar textiles modified with methacrylamide have achieved the total deactivation of *B. subtilis* spores within 2 h of contact, though the concentration of the spore challenge was comparatively low (i.e., initial concentration of ∼1 × 10⁴⁻⁵ CFU/mL).[23]

Increasing the rate at which halamine fabrics inactivate bacterial spores (e.g., the biological warfare agent *Bacillus anthracis*) would be highly advantageous.[35,36] Herein, the development of chloramine textiles that kill bacterial spores within minutes of exposure is described. In contrast to prior research that focused on the chlorination of aramid fabrics or textiles prepared through sophisticated synthesis regimes, the base material explored in this study is the natural polyamide, *Bombyx mori* (i.e., silkworm) silk. The use of silk in this role is advantageous as silk-based materials are highly processable, widely available throughout the world, have excellent mechanical properties, offer good biocompatibility, and have recently been utilized in a number of high tech applications.[37−42] Although previous studies have demonstrated the ability of chloramine-charged proteins to damage biomolecules (e.g., DNA or lipids) in a physiological context, this study represents the first communication of the use of chlorinated proteins (i.e., silk) to kill bacterial cells and spores. Although silk textiles have been previously functionalized (e.g., with Ag nanoparticles or metal ions) to add antimicrobial activity, this study is unique in offering a facile and inexpensive route to materials with both sporicidal and bactericidal activity.[43−49]

2. EXPERIMENTAL PROCEDURE

2.1. Chlorination of Silk Fabrics. Habutae silk fabric (i.e., *Bombyx mori* silk origin) was purchased from Testfabrics Inc. (West Pittiston, PA) and used without further processing or modification. Degumming of this silk fabric in a 100 °C, 0.02 M Na₂CO₃ solution for 30 min indicated that fabric contained a small quantity of residual sericin (1.3 ± 0.4 wt %). Clorox household bleach (Clorox Company, Oakland, CA) was utilized as the chlorination source throughout this study. Unless otherwise stated, the chemical reagents utilized in this study were purchased from Sigma Aldrich (St. Louis, MO). Singly distilled facility water (dH₂O) was used throughout this work. Silk was chlorinated by submerging the fabric in a 10 vol% Clorox bath, maintaining a bath volume to sample weight ratio of ∼167:1. The pH of the chlorination solutions utilized was either unmodified (∼pH 11)

or adjusted to pH 5 through the addition of glacial acetic acid (110 mM final concentration). During the chlorination procedure, the bath was rocked at 15 rpm on a ProBlot 25 rocker (Labnet International Inc., Woodbridge, NJ). After the desired reaction time (varying from 1 min to 16 h) the chlorinated fabric was removed from the bath and extensively rinsed in dH₂O baths (dH₂O bath volume to sample weight ratio of ∼1000:1). The treated silk textiles were subsequently hung and dried at room temperature. Control samples were produced by a nearly identical procedure, where the Clorox solution was replaced by a pH 5, 100 mM sodium acetate buffer.

2.2. Characterization of Silk Fabrics. Scanning electron microscopy (SEM) was conducted on silk fabrics sputter coated with 35 Å of gold (i.e., to minimize sample charging) utilizing a Sirion field-emission gun microscope (FEI Corporation, Hillsboro, OR) operating at an accelerating voltage of 2−3 kV. X-ray photoelectron spectroscopy (XPS) was conducted with an AXIS Ultra DLD spectrometer (Kratos Analytical Inc., Manchester, UK). XPS was conducted utilizing Al Kα monochromatic X-ray irradiation (1486.6 eV) at 120 W (10.0 mA emission current, 12.0 kV accelerating voltage), incident on the sample at 54.7 degrees relative to the sample normal. The spot size was approximately 1 mm². Energy-dispersive X-ray spectroscopy and elemental mapping was conducted utilizing an EDAX (Mahwah, NJ) equipped on a XL-30ESEM (FEI Corporation, Hillsboro, OR). Fluorescence microscopy was conducted with a BX51 (Olympus Corp., Center Valley, PA) microscope utilizing a DAPI filter and UV light source.

The oxidative chlorine content of untreated and chlorinated silk fabrics was assessed by iodometric titration in accordance with standard methods.[14,50] Briefly, ∼0.3 g of unmodified or chlorinated silk fabric was placed into a 50 mL centrifuge tube containing 35 mL of assay solution (350 mM acetic acid, 120 mM KI) and rotated at 30 rpm for 1 h. After 1 h, the fabric sample was removed from the assay solution and discarded. The assay solution was subsequently titrated with standardized 0.1N and 0.01N Na₂S₂O₃ solutions utilizing a starch indicator solution to determine the titration end point.[14,50] All values reported for silk fabric Cl content represent the mean average of at least 3 independently prepared samples, whereas reported error or error bars represent one standard deviation.

The wash durability of silk fabrics chlorinated for 1 h at pH 5 was assessed in the following manner: 3 g samples (n = 4) of silk halamine cloth were washed in 3 L of 24 °C dH₂O containing 0.25 wt % sodium dodecyl sulfate (SDS) agitated by rocking at 30 rpm on a ProBlot 25 rocker for 15 min, after which the samples were removed from the SDS solution and hand wrung. The washed silk samples were rinsed in 3 L of dH₂O agitated by rocking at 30 rpm for 5 min, removed from the rinsewater, and hand wrung. This rinsing procedure was repeated 1 additional time and the chlorinated silk samples were removed from the rinsewater, hand wrung and allowed to drip dry for ≥1 h. Following the end of each complete washing, rinsing, and drying cycle, a ∼60 mg subsample was cut from each chlorinated silk sample and the quantity of Cl contained within the fabric was determined by iodometric titration. Chlorinated silk samples were exposed to a total of 5 complete wash−rinse−dry cycles.

The breaking strength of unmodified and chlorinated silk textiles was determined in accordance with the specifications detailed in ASTM D 5035−06, Standard Test Method for Breaking Force and Elongation of Textile Fabrics (Strip Method). Silk samples, 2.5 cm in width and 15 cm in length were prepared such that the gauge direction of the samples was parallel to the machine direction of the fabric. Mechanical testing was conducted utilizing a H10KS tensile tester (Tinius Olsen Inc., Horsham, PA), equipped with a 500 N load cell. Silk samples were secured with 6.25 cm² rubberized grips pneumatically pressurized to 0.2−0.4 MPa and strained at a rate of 10 mm/min until material failure. The gauge length of the silk samples was 7.5 cm and testing was conducted under ambient laboratory conditions (i.e., 23 °C, 13% relative humidity(RH)). All values reported for the breaking strength of silk fabrics represent the mean average of at least 9 independently prepared samples while reported error or error bars represent one standard deviation.

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The tensile testing of *B. mori* cocoon fibers was conducted in the following manner: *B. mori* cocoons were purchased from Mulberry Farms (Fallbrook, CA), the desiccated silk worms removed, the cocoons were delaminated by hand and the resulting cocoon pieces degummed in a 0.02 M Na_2CO_3, 100 °C solution for 30 min, followed by extensive rinsing in 18.2 MΩ water. Approximately 1/2 of the quantity of degummed fibers were subsequently chlorinated for 1 h in a pH 5 reaction solution as described above. Individual silk fibers were affixed to cardboard mounts with superglue and tensile tested in a TA Instruments RSA3 DMA (New Castle, DE) utilizing a constant extension rate of 0.01 mm/sec under ambient laboratory conditions (21 °C, 25% RH). All values reported from the tensile testing of individual silk fibers were calculated utilizing an assumed fiber diameter of 10 μm and represent the mean average of at least 14 independently prepared samples while reported error or error bars represent one standard deviation.

Dityrosine analysis was conducted in the following manner: unmodified and chlorinated then $Na_2S_2O_3$-neutralized silk fabric samples (~0.2 g) were hydrolyzed in 20 mL of a 6 N HCl solution containing 15% D_2O at 110 °C for 16 h. The hydrolysates were diluted 10-fold in 90:10 H_2O/D_2O and 400 MHz proton NMR spectra were acquired on a Bruker Avance NMR spectrometer using a water suppression pulse sequence.

Solid-state carbon NMR spectra were collected on a Tecmag Apollo NMR spectrometer at 125 MHz for carbon using a 2 mm probe from Revolutions NMR. The spectra were acquired using cross-polarization (1 ms) and magic-angle sample spinning at 15−17 kHz with 50 kHz radio frequency fields for cross-polarization and decoupling. The chemical shifts were referenced to the methyl peak of hexamethyl benzene at 17.35 ppm using an external standard.

2.3. Examination of Bactericidal/Sporocidal Properties of Chlorinated Silk. Antimicrobial testing of chlorinated silk material was conducted using a modified AATCC Test Method 100−1999.[20] Triplicate experiments were conducted for each of the chlorination times, and triplicate samples were collected in each experiment. *Bacillus thuringiensis* var. Al Hakam spore suspensions were prepared by incubating a vegetative culture for three days in 50 mL Nutrient Broth + CCY salts + glutamate at 34 °C with rotation at 220 rpm then washing eight times by centrifuging at 6,000 rpm for 5 min and resuspending the pellet in 4 mL 0.1% Tween-80.[51] Upon the final wash, the pellet was resuspended in 2 mL 0.1% Tween-80, divided into 4 aliquots, and frozen at −80 °C.[51] On each day of experimentation, the *B. thuringiensis* spores were diluted to 10^8 CFU/mL in sterile PBS (Gibco). For vegetative cells, 24 h cultures of *E. coli* K12 and *B. thuringiensis* var. A1 Hakam, were grown at 37 °C with rotation at 220 rpm in LB Broth (BD Diagnostics) and Nutrient Broth (BD Diagnostics), respectively, were washed twice by centrifuging at 10,000 rpm for 5 min and resuspending in sterile PBS before diluting or concentrating to approximately 10^8 CFU/mL. Overnight culture enumerations were estimated using a hemacytometer to determine the appropriate dilution factor for vegetative cell cultures. One mL of each culture was then pipetted onto 1 g stacks of silk coupons, approximately 6 cm × 6 cm, in sterile Petri dishes and stored in the dark during exposure. At 10 min, the silk stacks were transferred into 50 mL sterile PBS + 0.03% $Na_2S_2O_3$ (Sigma) to neutralize the chlorine, shaken vigorously, and sonicated for 5 min.[20] Twenty-five milliliters of each sonicated sample were then filtered through 0.2 μm Nuclepore membranes (Whatman), and the filters were placed into 1 mL of sterile PBS and vortexed for 30 s. After serial dilutions in LB Broth or Nutrient Broth, 100 μL of the samples from *E. coli* K12 and *B. thuringiensis* var. Al Hakam (spores and vegetative cells) were plated onto LB Agar (BD Diagnostics) and Nutrient Agar (Nutrient Broth with 1.5% Granulated Agar, BD Diagnostics), respectively, and incubated overnight at 37 °C. Positive control cultures were tested by placing 1 mL of each culture in 50 mL of sterile PBS + 0.03% $Na_2S_2O_3$ then sonicating and filtering 25 mL before plating on the respective medium. One milliliters of sterile PBS was processed in the same manner in each experiment to serve as a negative control. A pilot study showed no significant difference between positive control

cultures or 1 mL culture samples exposed to unmodified or acetate-buffer treated silk for 10 min (data not shown).

3. RESULTS AND DISCUSSION

3.1. Characterization of Chlorinated Silk Fabrics. The chlorination of silk was explored under slightly acidic (pH 5) and basic (pH 11, the pH of Clorox bleach) pH conditions. During chlorination under basic conditions, the treated fabrics yellowed with time, taking on a bright yellow color within 10 min (see the Supporting Information). Silk soaked in slightly acidic chlorine baths developed a pale yellowish tint within the first minutes of treatment that did not further evolve with time (see the Supporting Information). The initial silky texture of the untreated silk fabric was maintained when samples were chlorinated at pH 5, but changed to a coarse, friable texture when reacted at pH 11. In addition to these differences in color and texture, halogenation under slightly acidic or basic conditions also resulted in significant differences in the weight change of the fabrics. Silk soaked in pH 5 and 11 chlorine solutions for 30 min exhibited mass changes of +9.9 ± 0.4% and −18.4 ± 0.2%, respectively.

X-ray photoelectron spectroscopy (XPS) analysis of silk fabric treated for 30 min in pH 5 or pH 11 diluted Clorox solutions indicated that these samples were composed of carbon, oxygen, nitrogen, and chlorine (Figure 1). The presence of carbon, oxygen, and nitrogen in these spectra are attributable to the proteinaceous chemistry of the silk material and these elements appear in the spectrum of the unmodified fabric (Figure 1A). Chlorine is not present on the surface of unmodified silk fabric and appears only in the spectra of chlorinated samples (Figures 1B and 1C). Analysis of XPS spectra indicate that the chlorine content of samples bleached for 30 min under acidic or basic conditions was fairly close, as the nitrogen to chlorine ratios of these samples was determined to be 5.2:1 and 5.0:1, respectively. The bulk composition of unmodified and chlorinated silk fabrics was conducted by energy-dispersive X-ray spectroscopy (EDS) spot chemical analysis and elemental mapping (see the Supporting Information). Correlating well with the results obtained through XPS analysis, Cl was found to be present in silk reacted in a diluted Clorox solution for 30 min at pH 5 and 11, and absent in unmodified fabrics.

Although XPS and EDS characterization indicated the presence of Cl in the treated silk samples, these methods cannot be used to quantify the amount of oxidizing halogens that may be available for release from the chlorinated textiles. Instead, the active chlorine content of the treated silk samples as a function of preparative bleaching pH and time was determined by iodometric titration (Figure 2). As seen in Figure 2A, the active Cl content of samples produced by chlorination for 30 min at pH 5 or 11 is nearly equivalent, a result that is consistent with close N:Cl ratios obtained from XPS analysis of these textiles. Further inspection of Figure 2A reveals that at times less than 30 min, the active Cl content of samples chlorinated at pH 5 is greater than that of silk treated at pH 11. Indeed, greater efficacies of chlorination at acidic pH values have been reported in the synthesis of several halamine polymers.[15,30,32,34] The greater chlorine loading of these materials under low pH conditions was attributed to the presence of direct chlorinating species (Cl^+) under acidic conditions that react with nitrogen atoms through more efficient mechanisms than the active species (ClO^-) present in basic environments.[15,30,32,34] As seen in Figure 2B, the
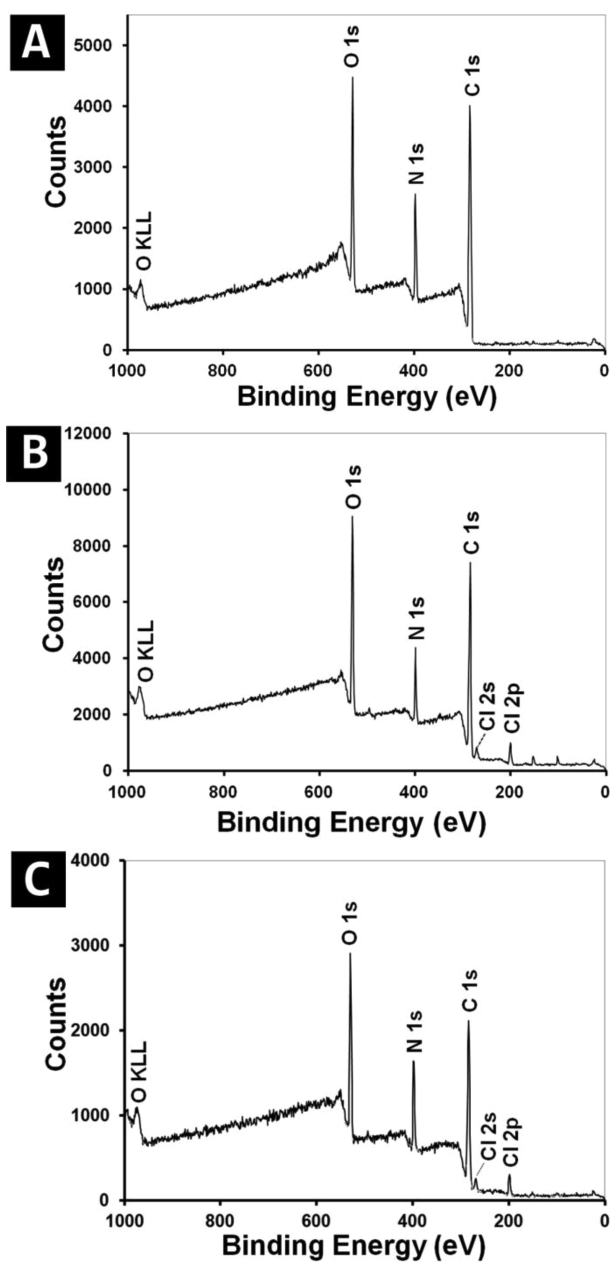
Figure 1. XPS analysis of (A) unmodified silk fabric, (B) silk fabric chlorinated at pH 11 for 30 min, and (C) silk fabric chlorinated at pH 5 for 30 min.

Figure 2. Oxidizing Cl content of silk fabrics, as a function of preparative chlorination time and pH, as determined by iodometric titration. Silk samples treated at pH 11 are represented as a dashed gray line with gray triangles and those chlorinated at pH 5 are represented by a black line with black diamonds. For clarity, the available active Cl content of fabrics chlorinated for short (min) and long (h) periods of times are highlighted in A and B, respectively.

oxidative Cl content of silk chlorinated under acidic conditions increases with increasing preparative reaction times, with levels up to 3.51 ± 0.02 mmol Cl/g silk achievable for textiles incubated for 16 h (the longest time tested). In order to assess the stability of the halamine compounds formed by the chlorination reaction, Cl loaded silk samples were subjected to several rounds of wash testing. Although active chlorine may be lost during the washing process due to the hydrolysis of halamine bonds (Scheme 1) and interactions with detergents,[52] the Cl content of chlorinated silk remained unchanged through 5 washing cycles (see the Supporting Information). Although chlorinated silk does possess good wash durability, the ultimate service life of these fabrics is likely to be less than that of chlorinated Nomex. As a proteinaceous material, silk possesses α-hydrogens in its backbone that may participate in the
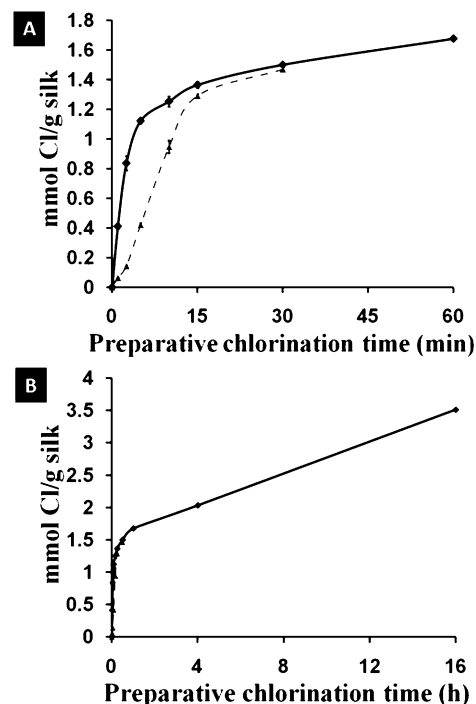
hydrolysis of N−Cl bonds.[32] Such α-hydrogens are absent in the aromatic structure of the Nomex backbone.[32] The amount of Cl loaded onto silk treated at pH 11 for preparative times beyond 30 min was not assessed as textiles began to lose structural integrity when incubated for long periods of time under basic chlorinating conditions.

The degradation of silk fabric in caustic bleach solutions for times in excess of 30 min clearly indicates that the mechanical properties of the treated textiles are affected by the chlorination process. In order to quantify the possible extent of this degradation, as well as the impact of chlorination at pH 5, the breaking strength of silk cloth as a function of preparative chlorination time was explored (Figure 3). As seen in Figure 3A, the exposure of silk fabric to a pH 11, diluted Clorox solution results the extremely rapid degradation of breaking strength, with over 85% of the initial fabric strength lost within 5 min of chlorination under these conditions. Such degradation at high pH values is not unexpected, as previous studies have demonstrated that silk fibers exhibit reduced strength and fibroin is broken down into a number of lower-molecular-weight polypeptides as a result of caustic sericin degumming processes (i.e., 0.05% Na_2CO_3, 100 °C, 1 h).[53,54] In contrast, silk treated with slightly acidic bleach solutions exhibits remarkable mechanical stability, as cloth chlorinated under these conditions loses only 20−30% of its initial breaking strength within reaction periods up to 1 h (Figure 3A). Silk fabric samples treated for 1 h in a pH 5 sodium acetate buffer (i.e., control samples) were not noted to have diminished breaking strengths compared to the as received cloth (data not shown). This preservation of silk's mechanical strength correlates well with the known behavior of silk fibers
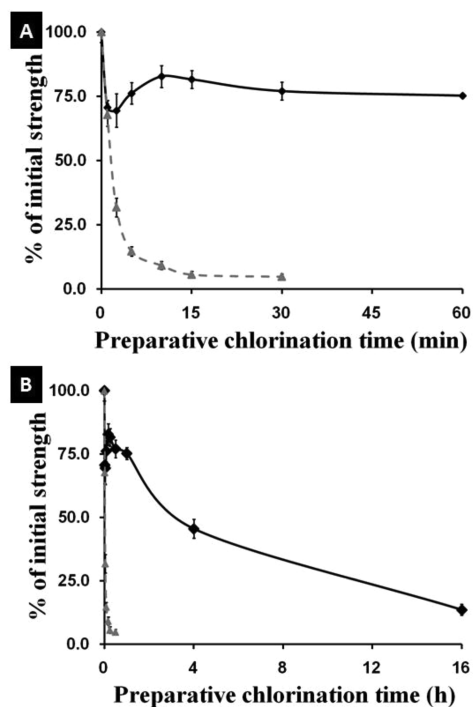
Figure 3. Breaking strength of silk cloth, expressed as % of the untreated silk fabric strength, as a function of preparative chlorination time and pH. Silk samples treated at pH 11 are represented as a dashed gray line with gray triangles and those chlorinated at pH 5 are represented by a black line with black diamonds. For clarity, the breaking strength of fabrics chlorinated for short (min) and long (h) periods of times are highlighted in A and B, respectively.

Figure 4. Representative stress−strain curves for individual degummed (black line) and degummed then chlorinated at pH 5 for 1 h (gray line) *B. mori* silk cocoon fibers.

degummed in organic acid-based solutions.[54] Although pH 5 chlorination conditions are clearly less damaging to the strength of silk than those of pH 11, the mechanical robustness of silk fabric is eventually degraded when incubated for long periods of time under acidic conditions (e.g., 86.4 ± 2.2% loss of initial breaking strength when chlorinated for 16 h) (Figure 3B). Comprehensively comparing the mechanical performance of chlorinated silk fabric to the behavior of other chloramine functionalized textiles is difficult; as such characterization has not been as widely reported as the antimicrobial activity of these materials. A survey of the literature indicates that the reduction in breaking strength observed for silk chlorinated for ≤1 h at pH 5 is comparable to losses in mechanical strength associated with the chemical modification of textiles required to add amine functionality. For example, the grafting of a melamine derivative onto cotton cloth or the coating of a hydantoin-based silane onto polyester fabrics resulted in a 13% loss of the bursting strength and 20−25% loss of the tensile strength of the original textiles, respectively.[17,24] Differing from silk, the tensile strength of hydantoin-silane modified polyester was not observed to decrease following chlorination.[24]

To assess the effects of chlorination on the mechanical properties of silk in greater detail, we conducted tensile testing on individual silk fibers isolated from *B. mori* cocoons. Representative stress−strain curves for degummed fibers and degummed fibers chlorinated at pH 5 for 1 h are presented in Figure 4. Inspection of Figure 4 and analysis of multiple stress−strain curves reveals that the elastic modulus of silk fibroin fibers chlorinated at pH 5 for 1 h (12.7 ± 1.0 GPa) differs little from that of degummed fibers (14.0 ± 2.6 GPa). Although
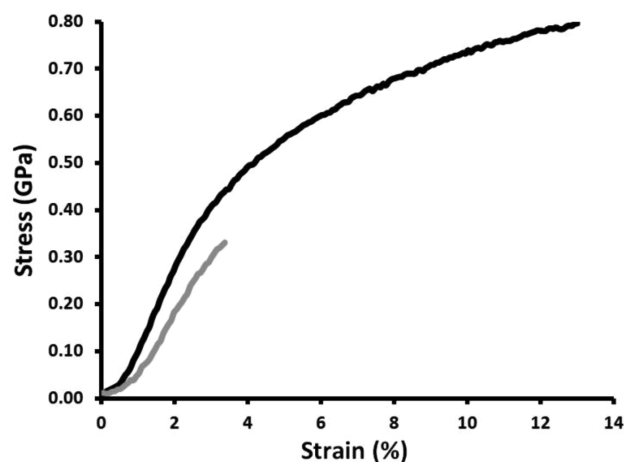
chlorination has a modest impact on the initial modulus of the fibroin fibers, Figure 4 shows that the breaking elongation and thus toughness of chlorinated fibers is reduced considerably from their unreacted counterparts. The average strain to failure and toughness of unchlorinated fibers is 13.3 ± 5.3% and 72.9 ± 39.7 MJ/m^3, respectively. The average strain to failure and toughness of the pH 5 chlorinated fibers is 2.6 ± 0.6% and 2.6 ± 1.2 MJ/m^3, respectively. The reduced ductility of chlorinated silk is comparable with that observed for chemically modified silk fibers in previous studies.[55] The mechanism by which this chlorination-induced loss of ductility occurs is currently unknown, but may be speculatively attributed to the dityrosine cross-linking of fibroin (see discussion below), protein damage, or the interruption of fibroin hydrogen bonds.

SEM characterization of silk fabric chlorinated under acidic or basic conditions was conducted in order to explore the differences detected in the breaking strength these materials and is presented in Figure 5. As seen in Figure 5B, the chlorination of samples for 30 min in caustic bleach solutions induced damage to the silk fabric, as indicated by the presence of numerous broken threads in the weave of the cloth. This large scale damage to the silk textile sharply contrasts with the structure of samples reacted at pH 5 for 30 min (Figure 5C), which exhibit an appearance that differs little from the unmodified cloth (Figure 5A). Damage to the fibers of silk cloth chlorinated at pH 5 does, however, become obvious when the fabrics were reacted for long periods of time (i.e., 16 h), correlating well with the observed drop in breaking strength of these materials (Figure 5D).

In addition to SEM observable damage to the fabric, it is likely that the reduced mechanical strength of chlorinated materials may be due to the deleterious modification or fragmentation of the fibroin proteins that comprise the silk fibers.[53,56−58] Unfortunately, the possible reduction in the molecular weight of the silk could not be assessed (i.e., by SDS-PAGE (polyacrylamide gel electrophoresis)) as chlorinated silk samples, even those treated for short periods of times, proved resistant to conditions typically utilized to dissolve degummed *B. mori* silk (i.e., 60 °C, 9.3 M LiBr).[37] Previous reports indicate that hypochlorite solutions may induce the covalent cross-linking of proteins through the oxidative formation of di- and trityrosine linkages.[56,59] The presence of dityrosine in a protein sample may be qualitatively confirmed by fluorescence
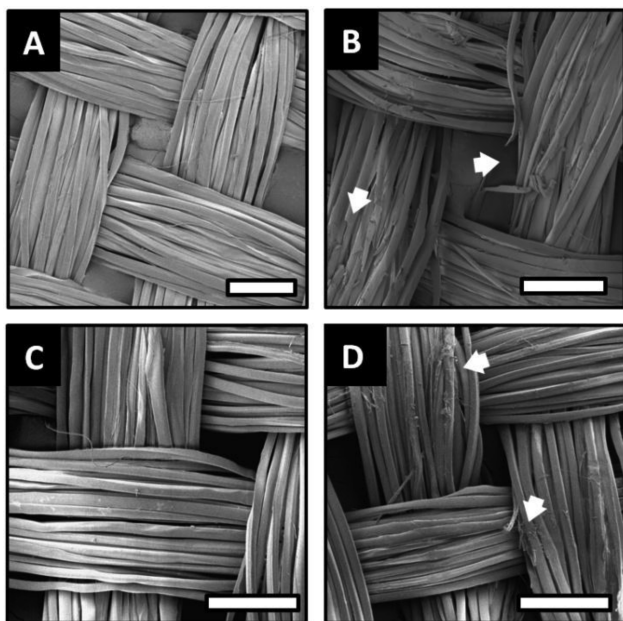
Figure 5. Morphology of silk fabric as a function of chlorination and reaction pH conditions. SEM images of (A) unmodified silk cloth, (B) silk fabric chlorinated for 30 min at pH 11, (C) silk cloth chlorinated for 30 min at pH 5, and (D) silk chlorinated for 16 h at pH 5. Arrows in B and D designate visible damage caused to the silk fabric during chlorination. Scale bars represent 100 μm.

microscopy, as this modified residue possesses pH dependent fluorescence.[60,61] Consistent with the known behavior of dityrosine, silk samples exposed to chlorinating conditions (i.e., both pH 5 and 11) were noted to exhibit blue fluorescence under basic conditions that was eliminated at low pH (see the Supporting Information).[60,61] This pH-linked fluorescent behavior was absent in unmodified silk cloth and indicates that the dityrosine cross-linking of silk is occurring during chlorination.

The possible presence of dityrosine in chlorinated silk was further assessed through the NMR-based characterization of acid-hydrolyzed fabric samples. The aromatic region of the NMR spectrum for silk/HCl and silk-Cl/HCl in 90:10 $H_2O:D_2O$ using the W5 water suppression pulse sequence is presented in Figure 6. This sample preparation was chosen to minimize the signals from the NH and NH_2 protons so the aromatic could be easily distinguished. The spectra were

normalized to the Gly Hα peak at 3.7 ppm, so the intensities are directly comparable. The peaks from the aromatic protons are observed between 6.6 and 7.2 ppm and a broad peak from the NH/NH_2 protons is observed at 7.9 ppm. The two large peaks in the acid-hydrolyzed silk spectrum are assigned to the Tyr $H_{2,6}$ and $H_{3,5}$ protons at 6.95 and 6.6 ppm, whereas the peaks at 7.1 and 7.2 are not assigned. Silk fibroin is known to contain 4.8% Tyr and much lower amounts of and Phe (0.7%) and His (0.2%). Silk chlorination leads to significant changes in the aromatic spectrum, including a decrease in the intensity of the Tyr peaks, and the appearance of two doublets at 6.7 and 6.8 ppm and a sharper peak near 7.1 ppm. The appearance of the new peaks is consistent with the formation of dityrosine, because the cross-linking at the $C_{3,5}$ leads to the loss of one proton. The peak at 7.1 ppm appears sharper because of the reduced J coupling due to the lack of a 3-bond neighboring proton. Although the NMR spectrum of the chlorinated silk fabric acid hydrolysate is indicative of dityrosine, similar peaks may be assigned to chlorotyrosine, a modified amino acid that may also form during the chlorination.[56]

In addition to the solution-based NMR analysis of acid-hydrolyzed silk samples, solid-state NMR spectroscopy was utilized to characterize silk modifications that arise as a result of the chlorination process. NMR is a powerful tool for the study of polypeptide conformation, crystallinity and reactivity because the NMR spectra are sensitive to the local structure. NMR has been extensively used to study the structure of silk, where it has been reported that the chemical shifts and line shapes are sensitive to both conformation and crystallinity.[62] The NMR spectra of *B. mori* fibroin is considerably simplified compared to other proteins by the fact silk is composed predominantly composed of four amino acids, Gly (42.9%), Ala (30.0%), Ser (12.2%), and Tyr (4.8%). The 125 MHz carbon NMR spectra of silk acquired with cross-polarization and magic-angle sample spinning (CPMAS) is presented in the Supporting Information of this paper as well as overlays in Figures 7 and 8. The resolved signals include the peaks from the Ala and Gly carbonyls (173.0 and 170.7 ppm), the Tyr aromatics (C_4, 156.0; C_1 and $C_{2,6}$, 131.3; and $C_{3,5}$, 116.1 ppm), the Ser $C\beta/C\alpha$ peaks (64.7 and
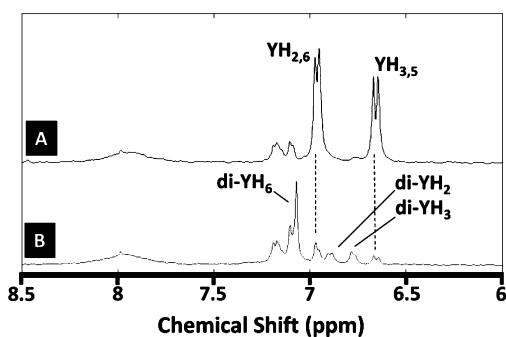

Figure 6. 400 MHz NMR spectrum of (A) silk fabric acid hydrolysate and (B) chlorinated silk fabric acid hydrolysate. Peaks corresponding to aromatic protons from tyrosine and dityrosine (putative) are labeled in A and B, respectively.
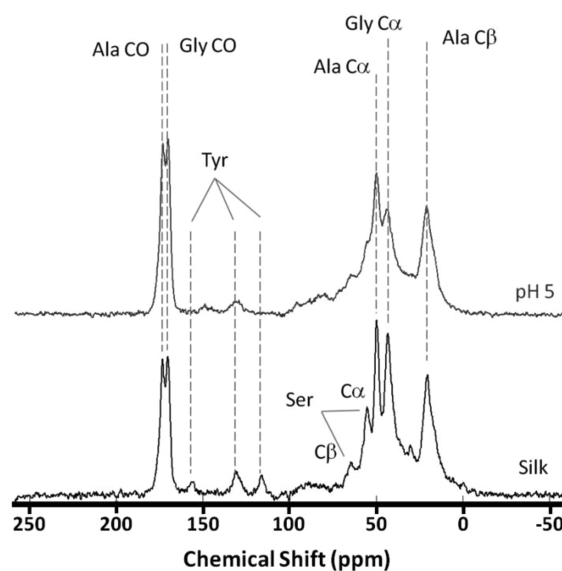

Figure 7. 125 MHz carbon CPMAS spectra of unmodified silk fabric (black) and silk chlorinated at pH 5 for 30 min (gray).
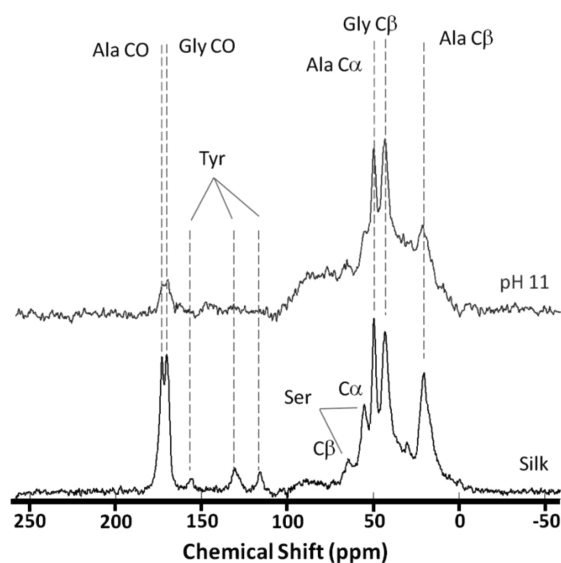
Figure 8. 125 MHz carbon CPMAS spectra of unmodified silk fabric (black) and silk chlorinated at pH 11 for 30 min (gray).

55.3 ppm), the Ala and Gly Cα (49.7 and 43.9 ppm) and the Ala Cβ methyl (21.1 ppm). The relatively sharp peaks for silk are a consequence of the well-ordered (crystalline) structure of silk fibers. Furthermore, the chemical shifts are substantially different from the random-coil conformation.[63] Broadening of the Ala Cβ peak has been attributed to partial resolution of the peaks from disordered β-turns (16.5 ppm) and two packing geometries for antiparallel β-sheets (19.6 and 21.9 ppm).[64]

Changes are observed in the CPMAS spectra of silk with exposure to chlorinating agents. Figure 7 compares the CPMAS spectra of unmodified silk fabric with that obtained from silk chlorinated for 30 min at pH 5. For comparison, the spectra have been normalized to the intensity of the Gly carbonyl peak. Althouh similar peaks are observed for the Ala and Gly carbonyls, the other peaks are reduced in intensity. We note that the Tyr C$_4$ aromatic peak (156.0 ppm) and the C$_{3,5}$ peak (116.1 ppm) are greatly diminished, whereas the overlapping peak from C$_1$ and C$_{2,6}$ carbons is broadened and a new weak peak appears at 149 ppm. The Ser Cα/Cβ peaks are difficult to observe in the reacted silk and the peaks from Gly and Ala are reduced in intensity, relative to the carbonyl. Although the Ala Cβ peak is reduced in intensity, a high field shoulder from the disordered β-turns is still visible. The exposure of silk at pH 11 has more dramatic effect on the carbon NMR spectrum as shown in Figure 8. The most notable feature is the loss of intensity for the Ala/Gly carbonyl carbons at 173 and 170 ppm in addition to the broadening and loss of intensity for the side chain carbons. There is a greater loss in intensity for the Ala peaks relative to Gly, particularly for the Ala Cβ. In addition, there is an increase in intensity between 60 and 100 ppm that presumably arises from carbons attached to oxygen or nitrogen atoms with one or more chlorines. Considering these results, the chlorination of silk at pH 11 appears to affect the polypeptide main chain, which may be associated with the loss of mechanical strength. In contrast, NMR spectra reveal that chlorination at pH 5 produces changes predominantly to the Ser and Tyr residues, with smaller effects on the Ala and Gly peaks.

3.2. Sporicidal/Bactericidal Efficacy of Chlorinated Silk Fabrics.
The primary aim of this study was to utilize the

chloramine forming propensity of proteins to create silk-based textiles that exhibit rapid and potent sporicidal and bactericidal activity.[56] The sporicidal activity of the silk materials produced in this study was assessed utilizing the spores of the bacteria *B. thuringiensis* Al Hakam, which is highly homologous to and used as a nonpathogenic simulant of *B. anthracis*.[65] In addition to testing against spores and vegetative cells of Gram positive *B. thuringiensis*, the bactericidal efficacy of chlorinated fabrics was also assessed against Gram negative *Escherichia coli* K12 cells. *E. coli* K12 was selected for this study as a nonpathogenic analog for *E. coli* O157:H7, which is a common source of infections and food or water-borne disease.[66,67]

Considering the active chlorine content and mechanical strength of silk fabrics chlorinated at pH 5, silk halogenated for 16 h (i.e., highest available Cl), 1 h (i.e., high residual strength), and 4 h (i.e., intermediate Cl and breaking strength to 1 and 16 h samples) were selected for antimicrobial testing. The bactericidal and sporicidal efficacy of these fabrics was assessed by exposing textiles to an aqueous suspension of bacterial spores or cells for contact times of 10 min.[20] The results of this study are presented in Table 1. Unmodified silk or textiles

Table 1. Sporicidal/Bactericidal Activity of Functionalized Silk Fabrics[a]

preparative chlorination time (h)	percent reduction in CFU/g of treated fabric		
	B. thuringiensis spores	*B. thuringiensis* cells	*E. coli* cells
1	>99.99998	>99.99999	>99.99996
4	>99.99998	>99.99998	>99.99999
16	>99.99999	>99.999996	>99.999998

[a]Fabric-organism contact time was 10 min. All data represent percent reduction in CFU/g of chlorinated fabric as compared to positive controls.

treated with pH 5 sodium acetate buffer did not result in a reduction of the average CFU/mL of either bacterial cells or spores (data not shown). Conversely, silk fabrics that had been chlorinated for 1, 4, or 16 h at pH 5 were found to have a significant bactericidal effect on *E. coli* and *B. thuringiensis* vegetative cells as well as, *B. thuringiensis* spores (Table 1). The values presented in Table 1 are the average level of the bacterial spore and cell killing activity for the 1, 4, and 16 h chlorinated silk fabrics. Because of a lack of microbial growth, statistical analysis of the sporicidal and bactericidal activity of the halamine fabrics was not possible. Nevertheless, all samples studied achieved a minimum 99.99996% average reduction in CFU/mL, which exceeded our experimental requirements (i.e., >99.999% reduction of both vegetative cells and spores within 15 min). While the use of higher starting inocula (i.e., upward of 1×10^{10} CFU/mL) may have resulted in calculable reductions in CFU/ml (i.e., resulting in countable plates at the 10^0 to 1×10^{-2} dilution), such concentrated cultures vastly exceed the bacterial concentration that the material is likely to encounter in a contaminated environment. Taking into consideration the bacterial inocula and exposure time, the levels of inhibition reported here align with or greatly exceed other published data on antimicrobial halamine-textile composites tested against vegetative cells.[29,32,68−72] The rates of sporicidal activity reported in the current study exceed other reports on the effect of antimicrobial textile composites on spores by several hours and/or log reductions.[20,23,34,51] It should be noted, however, that previous studies have used the

spores of *Bacillus subtilis* or *Bacillus atrophaeus* (formerly *B. subtilis*), which are more closely related to *Bacillus megaterium* than the *Bacillus cereus* group that includes *B. thuringiensis* (used in this study) and *B. anthracis*.[20,23,34,51] Collectively, the data presented in Table 1 clearly demonstrate that chlorine-loaded silk textiles are very effective in killing bacterial cells (i.e., both Gram positive and Gram negative) and spores.

The textile nature and high sporicidal activity of the silk-Cl materials prepared in this work may make these fabrics attractive for the augmentation of personal protection equipment.[33] Although previous studies have indicated that mammalian skin cell lines show excellent viability when in contact with chloramine-bearing fibers, the high levels of active Cl available from chlorinated silk may require an impermeable barrier layer be worn between the fabric and human skin.[73] The use of chlorinated silk in any real-world application, will of course, require extensive additional research and development work, testing, and validation.

4. CONCLUSIONS

A route to the production of silk-based textiles exhibiting rapid and potent sporicidal and bactericidal activity has been developed. In this simple process, silk fabric is reacted with a diluted bleach solution, rinsed with dH$_2$O, and dried. Although effective in producing chloramine-functionalized silk, caustic chlorination conditions were found to be overly severe and induced a significant reduction in the strength of the treated fabric within minutes. The adjustment of the chlorination solution to a slightly acidic pH greatly increased the active chlorine content and residual breaking strength of the treated silk cloth. Silk textiles chlorinated at pH 5 for ≥1 h proved effective in the rapid (10 min) reduction (i.e., >99.99996% reduction in CFU/g fabric) of *E. coli* cells, as well as *B. thuringiensis* Al Hakam spores and vegetative cells. Of the chlorine loading conditions explored, chlorination at pH 5 for 1 h produced textiles with the best combination of properties (i.e., modest loss of breaking strength and high antimicrobial activity). Despite the minor degradation in mechanical strength observed for silk halogenated for ≤1 h at pH 5, the fabric is likely to be sufficiently robust material for all but the most demanding applications. Althoug the chlorination of silk fabrics at pH 5 for 4 or 16 h resulted in textiles with greater available Cl, the biocidal activity and breaking strength of these fabrics was equivalent to and less than those chlorinated for 1 h, respectively. It is expected that the bacterial spore and cell killing efficacy of chlorinated silk may be further enhanced through additional refinements in the processing of these materials. For example, previous studies suggest that more acidic pH conditions, the addition of NaCl to chlorination baths, or the reduction of polymer crystallinity may increase the rate at which polyamides are halogenated.[30,33] Beyond the further optimization of chlorine loading conditions, modest losses in the mechanical strength of chlorinated silk fabrics may be offset through the reinforcement of silk fibers with inorganic materials or the backing of silk cloth with a more bleach stable textile (e.g., cotton).[74,75]

Given the potent bactericidal and sporicidal activity of the chlorinated silk fabrics prepared in this study, silk-Cl materials may find use in a variety of applications. For example, chloramine-loaded silk may be prepared on-site by humanitarian relief workers and utilized as an active filter material for the treatment of contaminated water.[6,8,67] Silk-Cl textiles may additionally find use in the filtration and deactivation of

pathogenic spores (e.g., bacterial or mold) from the air, as components of filter cartridges or as make-shift curtains (i.e., for use in civil defense emergencies). In addition to the antimicrobial activity demonstrated for chlorinated silk, this material may also find use in the mitigation of toxic chemicals, as several prior studies have determined that halamine-bearing textiles can degrade chemical warfare agent simulants and pesticides.[76−78] It is anticipated that the processing parameters presented in this research for silk may also be extended to produce antimicrobial products through the chlorination of other protein-based materials, such as wool cloth or plastics derived from plant proteins.[79]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information
Optical images depicting the development of color during chlorination. EDS analysis and SEM images of silk fabrics subjected to chlorination at pH 11 and pH 5. Cl contents of silk fabric subjected to wash cycles. Fluorescent and optical microscope images of unmodified and chlorinated silk depicting dityrosine fluorescence. NMR spectrum of unmodified silk cloth. This material cleared for public release (88ABW-2012-1048). This material is available free of charge via the Internet at http://pubs.acs.org/

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Notes
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