Antistaphylococcal Nanocomposite Films Based on Enzyme–Nanotube Conjugates

ARTICLE *in* ACS NANO · JULY 2010

Impact Factor: 12.88 · DOI: 10.1021/nn100932t · Source: PubMed

Published in final edited form as:
ACS Nano. 2010 July 27; 4(7): 3993–4000. doi:10.1021/nn100932t.

Antistaphylococcal Nanocomposite Films Based on Enzyme-Nanotube Conjugates

Ravindra C. Pangule[†], **Sarah J. Brooks**[†], **Cerasela Zoica Dinu**[†], **Shyam Sundhar Bale**[†], **Sharon L. Salmon**[‡], **Guangyu Zhu**[†], **Dennis W. Metzger**[‡], **Ravi S. Kane**[†,*], and **Jonathan S. Dordick**[†,*]

[†] Howard P. Isermann Department of Chemical and Biological Engineering, Rensselaer Nanotechnology Center and Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180

[‡] Center for Immunology and Microbial Disease, Albany Medical College, Albany, NY 12208

Abstract

Infection with antibiotic-resistant pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the primary causes of hospitalizations and deaths. To address this issue, we have designed antimicrobial coatings incorporating carbon nanotube-enzyme conjugates that are highly effective against antibiotic–resistant pathogens. Specifically, we incorporated conjugates of carbon nanotubes with lysostaphin, a cell wall degrading enzyme, into films to impart bactericidal properties against *Staphylococcus aureus* and *Staphylococcus epidermidis*. We fabricated and characterized nanocomposites containing different conjugate formulations and enzyme loadings. These enzyme–based composites were highly efficient in killing MRSA (>99% within 2 h) without release of the enzyme into solution. Additionally, these films were reusable and stable under dry storage conditions for a month. Such enzyme–based film formulations may be used to prevent growth of pathogenic and antibiotic-resistant microorganisms on various common surfaces in hospital settings. Polymer and paint films containing such antimicrobial conjugates, in particular, could be advantageous to prevent risk of staphylococcal-specific infection and biofouling.

Keywords

carbon nanotube; lysostaphin; nanocomposite; antimicrobial film; methicillin-resistant Staphylococcus aureus (MRSA)

It is estimated that out of the expected 2 billion people carrying *Staphylococcus aureus* (*S. aureus*) worldwide, up to 53 million individuals are infected with methicillin-resistant *S. aureus* (MRSA).1 Specifically, *S. aureus* – related diseases such as septicemia and pneumonia are responsible for a large number of hospitalizations (ca. 480,000 in 2005) and deaths (nearly 11,000 in 2005) in the U.S., alone.2 Staphylococcal infection caused by *S. aureus* and *Staphylococcus epidermidis* (*S. epidermidis*) is also a major concern in patients with indwelling devices such as catheters, heart valves, and prostheses.3 While medical and surgical equipment, hospital rooms, and various other objects in hospital settings provide an

kaner@rpi.edu, dordick@rpi.edu.

Supporting Information Available: Mechanism of Lst-mediated cell wall degradation, bactericidal activity of films against *S. epidermidis*, bactericidal efficiency of six-times washed films, and SEM characterization of films with different Lst loadings. This material is available free of charge *via* the Internet at http://pubs.acs.org.

easily accessible platform for pathogenic bacteria to settle and proliferate, any surface once touched by an infected person serves as a breeding ground for such pathogens.

Considering the magnitude of pathogen-related complications and the assistance of various surfaces in growth and spread of these pathogens, significant attention has been given to the design and synthesis of effective antimicrobial agents and coatings.[4–7] For example, various microbicidal coating formulations have been developed that are active as a result of either releasing biocides, such as silver, or functionalizing surfaces with amphipathic polycations and antimicrobial peptides that are bactericidal *via* rupturing the bacterial membrane on contact.[4–6,8,9] Specifically, Bagheri *et al.* have shown that surface-immobilized cationic amphipathic antimicrobial peptides cause such bactericidal effect through membrane-permeabilizing mode of action.[8] Although silver-based materials are strongly bactericidal, the activity diminishes over time because the coatings continuously release the biocidal agent.[10] Moreover, concern has been raised recently about the potential health effects of nano-silver and its release in the environment.[11,12] In the case of polycationic polymer-based coatings, the surface may require treatment with cationic surfactant to recover antimicrobial activity.[13,14] Finally, low molecular weight bactericidal agents are often associated with resistance and gradually lose their effectiveness over time. Designing antimicrobial films that are capable of retaining prolonged activity is thus important in solving problems associated with the spread of pathogenic microbes.

Viruses and microorganisms have developed unique survival mechanisms that exploit biocatalytic mechanisms to gain a competitive advantage in their natural niche. For example, bacteriophages code for cell lytic enzymes required to exit the host bacterium and infect neighboring microbial hosts.[15,16] Various bacteriophages from *S. aureus*, *Streptococcus pneumoniae*, *Bacillus anthracis*, and *Listeria monocytogenes* employ amidases, muramidases, and endopeptidases to attack the host cell wall.[17–21] In the microbial kingdom, *S. simulans* biovar *staphylolyticus* and *S. capitis* EPK1 secrete glycyl-glycine endopeptidases, lysostaphin (Lst) and ALE-1, respectively, to kill competing staphylococci such as *S. aureus* and *S. epidermidis*.[22,23] Interestingly, most of these cell lytic enzymes feature a two-domain structure, which is characterized by bacteria-specific cell wall targeting and catalytic domains. In the case of Lst, the C-terminal 92-amino acids bind to a specific molecular target on staphylococcal cell walls, while the N-terminal portion containing the active site is responsible for cleaving the pentaglycine cross-bridges present between the sugar components of the peptidoglycan layer (Figure S1 in the Supporting Information).[24,25]

In general, these cell lytic enzymes serve as potential alternatives to antibiotics in combating resistant pathogens. Specifically, Lst was found to be highly effective in treating *S. aureus* –related neonatal infection and endophthalmitis when tested in animal models.[26,27] Lst has also been shown to disrupt *S. aureus* and *S. epidermidis* biofilms formed on artificial bioimplant surfaces.[28] While these studies demonstrate the efficacy of Lst in conventional *post*-infection treatment, an approach that prohibits proliferation and transmission of staphylococci would be ideal in preventing the broad infectivity associated with these microorganisms.

In the present work, we exploit this approach by preparing enzyme-nanotube conjugates and their composite films to generate active and stable bactericidal paint films. The molecular-level curvature of carbon nanotubes stabilizes a wide range of enzymes,[29] while the high surface area-to-volume ratio enables high catalytic loading without diffusional limitations.[30,31] In addition, the high aspect ratio of the carbon nanotubes leads to efficient entanglement within the solid matrix, thereby allowing enzymes to be retained.[32] As a result, we report here the ability of Lst-containing nanocomposite films (Scheme 1a) to

achieve >99.9% killing of MRSA upon contact within 2 h. These results suggest that such paint composite films with embedded antimicrobial Lst-nanotube conjugates may be effective in preventing the risk of staphylococci-specific infection and biofouling of common surfaces.

RESULTS AND DISCUSSION

Lst-Nanotube Conjugates

We first wished to understand the effect of conjugation of Lst to multi-walled carbon nanotubes (MWNTs) on the enzyme activity. We therefore examined the steady-state kinetics of Lst in free as well as nanotube-bound form using the colorimetric assay based on *N*-acetylhexaglycine hydrolysis. Both free and nanotube-bound Lst followed Michaelis-Menten kinetics (Figure 1a), and Lst bound to MWNTs retained approximately one quarter of its intrinsic catalytic activity, as reflected in the V_{max} value (Table 1). Similar values of K_M for the bound and free Lst suggested that the apparent binding of Lst towards the hexaglycine substrate was unaffected by immobilization. The relatively high retention of Lst-MWNT conjugate activity on a small molecule substrate is consistent with other reports for enzyme-MWNT conjugates.31 We also tested free Lst and Lst-MWNT conjugates against *S. aureus*. While free Lst (0.5 μg) killed >99% of a 10^6 colony forming units (CFU)/mL suspension of *S. aureus* in 1 min, the same amount of Lst on the MWNT conjugate killed only ~ 35% cells in 1 min, and required nearly 30 min to achieve similar cell-killing activity as that for the free enzyme (Figure 1b).

The peptidoglycan layer of *S. aureus* consists of *N*-acetylglucosamine- and *N*-acetylmuramic acid-containing polysaccharides that are interconnected through tetrapeptide (both L- and D-amino acids) and pentaglycine bridges (Figure S1).33,34 Lst, through its glycylglycine endopeptidase activity, can cleave these pentaglycine bridges, thereby causing weakness in the peptidoglycan layer and resulting in cell wall rupture. Lst takes advantage of its binding domain, presumably to position its catalytic domain properly for its endopeptidase activity. When the enzyme is attached to MWNTs, the nanotube (15 ± 5 nm in diameter) may be able to gain proximity to the peptidoglycan layer; however, the enzyme may lack sufficient flexibility on the nanotube surface to act efficiently. Nevertheless, it was encouraging that some activity was observed, which suggested that bactericidal activity could be improved by providing a flexible spacer between Lst and the MWNT surface. Indeed, we have shown previously that a short poly(ethylene glycol) ($dPEG_{12}$ or PEG) linker was capable of increasing the activity of perhydrolase, an octameric protein with a molecular weight of 184 kDa, when covalently attached to MWNTs.35 In the case of surface-tethered antimicrobial peptides, Glinel *et al.* and Bagheri *et al.* have shown that the attachment of antimicrobial peptides onto a surface using a relatively long, water-soluble, and flexible oligo(ethylene glycol) linker facilitates the interaction of the antimicrobial peptide with the bacterial cell membrane, resulting in membrane permeabilization and hence a bactericidal effect.8,9

Along these lines, we performed covalent attachment of Lst to MWNTs through a $dPEG_{12}$ linker (Scheme 1a). The resulting Lst-PEG-MWNT had only marginally improved observed activity against the *soluble* synthetic substrate (V_{max} ~ 33% of that of native Lst, Table 1). However, the PEG linker showed a two-fold increase in the bactericidal activity during short exposure times (~1 min) compared to Lst-MWNT, and killed >99% of *S. aureus* cells (10^6 CFU/mL suspension) in approximately 10 min (Figure 1b).

Finally, to demonstrate the broad activity of Lst-based conjugates against the staphylococci family, similar cell-based assays were performed with microbial suspensions containing 10^6 CFU/mL of *S. epidermidis*. As shown in Figure S2, the enzyme was highly active against *S. epidermidis*, albeit less active than on *S. aureus*, consistent with the known activity of Lst.36

As with Lst on *S. aureus*, the PEG linker improved activity of the nanotube-bound enzyme against *S. epidermidis* too. The enzyme was not effective against non-staphylococcal cells, including *Escherichia coli* (*E. coli*) and *Bacillus cereus* (*B. cereus*), which is again consistent with the known activity of Lst. Importantly, the lack of activity against *E. coli* and *B. cereus* indicates that the enzyme-MWNT conjugates do not possess indiscriminate cytotoxicity. The aggregate of the aforementioned results, therefore, indicates that Lst-nanotube conjugates show effective and selective bactericidal activity towards staphylococci.

Lst-containing Antimicrobial Nanocomposite Films

The ability of Lst-PEG-MWNT conjugates to retain sufficient bactericidal activity against *S. aureus* enabled us to explore the incorporation of the conjugates into paint. Briefly, latex paint film composites containing varying amounts of conjugates were tested for bactericidal activity against *S. aureus* by incubating the films in a 10^6 CFU/mL microbial suspension for 6 h (Scheme 1b). Paints containing Lst loadings of ≥ 2% (w/w) were found to be highly effective against *S. aureus*, resulting in essentially complete cell killing, as determined by viable cell counts (Figure 2) and fluorescence images of the cells exposed to paints containing Lst-nanotube conjugates (Figure 2 insets). Next, we confirmed that the release of enzyme from the MWNTs or release of the conjugates from the paint did not contribute significantly to the observed bactericidal activity. Specifically, we incubated the paints with aqueous buffer for 6 h, removed the paint films, and then tested the activity of the wash solution by adding *S. aureus* cells (final cell density of 10^6 CFU/ml). The activity of this solution was less than 5% of that of the nanocomposite films at all loadings studied (Figure 2), indicating that leaching of Lst or Lst-nanotube conjugates from the paint films was insignificant.

We also assessed the antimicrobial activity of films washed six times with aqueous buffer as well as the activity of the wash solution collected after the sixth wash, against *S. aureus* cells (final cell density of 10^6 CFU/mL). As shown in Figure S3, no bactericidal activity was observed in the wash solution after the sixth wash, suggesting that there was no loss of Lst or Lst-nanotube conjugates from the paint films. Moreover, the bactericidal activity of the films after six washes was similar to the activity of the films that were washed once (Figure S3). Furthermore, the observed bactericidal activity was solely due to the presence of Lst-nanotube conjugates incorporated into the paint. In the absence of active Lst, *i.e.*, with paint containing either carboxyl-functionalized MWNTs, or bovine serum albumin (BSA)-MWNT conjugates (where BSA is a non-bactericidal protein), or heat-inactivated Lst conjugates, no bactericidal activity was observed. Finally, paint films containing native Lst without nanotubes lost ~ 52% of the enzyme during just the first wash, indicating that *without* the nanotube template, the enzyme rapidly leaches from the paint film. Altogether, these results suggest that use of MWNTs as an Lst support, and incorporation of these conjugates into a paint matrix results in a highly stable, surface-active, and non-leachable antistaphylococcal coating.

The observed activity of the Lst-containing paints and the minimal leaching of free Lst or Lst-PEG-MWNT conjugates into the cell suspension suggest that the observed bactericidal activity is due to contact-mediated events. We speculate that single or multiple contact of an *S. aureus* cell with Lst-containing paints leads to hydrolysis of a sufficient number of pentaglycine bridges within the surface peptidoglycan layer. As a result, weak spots are created in the cell wall that can no longer withstand the internal osmotic pressure, leading to weakening of the structure and hence cell lysis or rupture.[37,38] Accessibility of the Lst to *S. aureus* is facilitated by the PEG linker between the enzyme and MWNT, and perhaps further facilitated by the presence of MWNTs that are likely to protrude from the paint surface,

thereby enhancing the accessibility of the active surface with the cells and access of the Lst to the peptidoglycan layer.

To further understand how surface-embedded Lst-PEG-MWNT conjugates can access and kill *S. aureus* cells, we used scanning electron microscopy (SEM) to estimate surface loading of the conjugates in the paint. As depicted in Figure S4, the conjugates and paint components form a homogeneous mixture, which in turn suggests uniform distribution of enzyme on the film surface. The surface coverage was assessed using ImageJ software. Surface coverage of conjugates ranged from 0 to 60% for films with Lst weight fractions varying from 0 to 4% (w/w), respectively (Figure S4f). Essentially, an increase in surface concentration of conjugates and hence an increase in the surface concentration of enzyme molecules would lead to a higher number of Lst-mediated cell wall hydrolysis events. As a result we expected an enhancement in the bactericidal activity with an increase in Lst loading, which was confirmed by the experimental results.

Effectiveness against MRSA

Having established that Lst-based nanocomposite paints are highly effective against a nonpathogenic strain of *S. aureus*, the paints were then tested against four methicillin-resistant pathogenic strains. These strains differ from each other primarily in the type of gene complex known as staphylococcal cassette chromosome *mec* (*SCCmec*), which is responsible for the resistance to multiple antimicrobial agents, as well as in the presence or absence of genes corresponding to various toxins.39 Critically, these strains are responsible for *S. aureus*-related infectious diseases in community and hospital settings. Lst-based nanocomposite paints were highly effective against various strains of MRSA (Figure 3), which suggests the potential application of these films in community and hospital settings as an alternative to biocides. Interestingly, while MRSA has evolved to be resistant to numerous antibiotics, they remain highly sensitive to Lst, perhaps due to the fact that antibiotic-resistance is associated with the presence of pentaglycine bridges in the peptidoglycan layer.40 The pentaglycine interpeptides are known to provide strength and stability to the staphylococcal cell wall, and also act as sites for anchoring various surface proteins responsible for pathogenicity and adhesion. While the possibility of Lst-resistance through mutations in interpeptide linkages cannot be neglected, various studies have revealed that such mutants have diminished fitness and are susceptible to methicillin.40,41

Operational and Storage Stability

A major challenge of biocatalytic materials is to make them stable and reusable. To assess these outcomes, we determined the bactericidal activity of the nanocomposite paints (containing 4% w/w Lst in the form of Lst-PEG-MWNT) on multiple assays of the same set of paints over a period of 30 days, with paints being stored dry at room temperature in between two use cycles. The paints retained a high fraction of the initial bactericidal activity; after seven sequential 6-h assays the residual activity was ca. 50%, representing ca. 5×10^5 *S. aureus* cells killed in 6 h during the 7^{th} use (circles, Figure 4). Moreover, tests of storage stability revealed that films which were stored dry at room temperature showed >99% bactericidal activity against *S. aureus* after 30 days (squares, Figure 4). In fact, extended storage stability for six months was achieved where >99% bactericidal activity was observed. These results demonstrate that the films are highly stable and reusable at room temperature.

CONCLUSIONS

In conclusion, we have designed highly effective antistaphylococcal nanocomposite paints based on nanotube conjugates of a cell wall degrading enzyme, Lst. These paints were

capable of killing staphylococci, including methicillin-resistant strains of *S. aureus*, on contact and without release of antimicrobial agents. Conjugate formulations containing a PEG-based linker between the nanotube and enzyme resulted in enhanced bactericidal activity. Enzyme-containing nanocomposite paints represent a facile route to the generation of highly efficient bactericidal surfaces. This approach may be extended to other antimicrobial mechanisms involving proteases, muramidases, and other cell lytic enzymes, the specificity and bacteriolytic efficiency of which can be improved by protein engineering and domain swapping. As a result, highly active, stable, and environmentally benign surface treatments can be envisioned with broad activity against pathogenic bacteria.

MATERIALS AND METHODS

Functionalization of Carbon Nanotubes

Acid-treated multi-walled carbon nanotubes (MWNTs, NanoLab, Inc., Newton, MA) were used in preparing enzyme-nanotube conjugates.31 Specifically, MWNTs (50 mg) were first suspended in 200 mL of acid mixture containing concentrated H_2SO_4 and HNO_3 in a volumetric ratio of 3:1. The nanotube-acid mixture was sonicated for 6 h in an ice bath. This mixture was then diluted with 800 mL deionized (DI) water and filtered through 0.2 μm polycarbonate membrane filter (Millipore, Billerica, MA). The nanotubes were subsequently washed three times with 1000 mL DI water to obtain an acid-free nanotube suspension. Finally, a dry powder of oxidized MWNTs was obtained by filtering the nanotube suspension through 0.2 μm filter paper followed by drying under vacuum overnight.

Enzyme Immobilization

Lst (AMBI Products LLC, NY) was covalently attached to MWNTs by following a four-step coupling reaction protocol (Scheme 1a).35 First, 2 mg oxidized MWNTs were dispersed in 2 mL 2-(*N*-morpholino)ethanesulfonic acid sodium (MES) buffer solution (50 mM, pH 4.7, Sigma, St. Louis, MO) by 30 min sonication. To this dispersion, a dry powder of *N*-hydroxysuccinimide (NHS, Pierce, Rockford, IL) was added to obtain 80 mM NHS. In the final step of activation, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (160 mM) (EDC, Acros Organics, Morris Plains, NJ) was added and the mixture was stirred for 30 min. The activated MWNTs were then filtered, washed with MES, and used immediately for the attachment of polyethylene glycol (PEG). To that end, 10 mg of amino-dPEG$_{12}$-COOH (Quanta Biodesign, Powell, OH) dissolved in 2 mL DI water was added to the activated MWNTs. The mixture was shaken at room temperature (RT) for 3 h and at 200 rpm. Subsequently, the PEG-based conjugates were filtered and washed extensively with DI water to remove unbound PEG. The COOH-PEG moieties attached to MWNTs were then activated using the EDC-NHS chemistry as previously described (Scheme 1a). Finally, enzyme attachment was performed by dispersing 2 mg of activated PEG-MWNTs in 2 mL of 2 mg/mL Lst solution prepared in DI water, followed by 3 h incubation at 4°C and 200 rpm. The resulting Lst-PEG-MWNT conjugates were filtered and washed extensively to remove free enzyme. The conjugates were then washed with 1% triton to remove loosely bound enzyme molecules.

Lst-MWNT conjugates were prepared by direct covalent attachment of the enzyme to MWNTs by following the aforementioned EDC-NHS coupling process, but in the absence of PEG. Similarly, nanotube conjugates of bovine serum albumin (BSA) (Fisher Scientific, NH) were prepared by following the direct attachment protocol. Protein loading values were determined by elemental analysis (Galbraith Laboratories Inc., Knoxville, TN), yielding 0.41, 0.30 and 0.25 mg protein/mg of MWNT for Lst-MWNT, Lst-PEG-MWNT, and BSA-MWNT, respectively.

Determination of Kinetic Parameters

The activity of Lst conjugates was determined by using a colorimetric assay with *N*-acetylhexaglycine as the substrate.42 HPLC purified *N*-acetylhexaglycine was purchased from Genemed Synthesis, Inc. (San Antonio, TX). A stock solution of 100 mM *N*-acetylhexaglycine was prepared by suspending the substrate in DI water and by adding NaOH (Fisher Scientific) until the solid dissolves. For determination of kinetic parameters, substrate solutions of 1.25, 2.5, 5, 10, 20 and 30 mM *N*-acetylhexaglycine concentration were prepared by diluting the stock solution in assay buffer containing 5 mM trisodium citrate, 1 mM disodium EDTA, and 100 mM sodium borate (all salts were from Sigma) and adjusting to pH 8.0 by dropwise addition of NaOH.

Lst solutions containing 10 μg/mL free or conjugated Lst were prepared by diluting the stock solutions in 0.5% (v/v) Tween 20 (Sigma). Enzyme assay solutions were prepared in duplicate, in a flat-bottom 96 well plate (NUNC brand, Thermo Fisher Scientific, NH), with each well containing 50 μL of enzyme solution of each type (free and conjugated) and 50 μL of substrate solution. The plate was covered to prevent evaporation of solution and was incubated at 37 °C for 60 min. The release of primary amine as result of hydrolysis of *N*-acetylhexaglycine by Lst was detected by initiating color development on addition of 10 μL of 5 mg/mL 2, 4, 6-trinitrobenzene-sulfonic acid-3-hydrate (TNBS) (Sigma) prepared in 100 mM sodium bicarbonate (Sigma) solution; the liberated primary amine reacts with TNBS to give a chromophore that can be detected by measuring absorbance at 405 nm. The reaction between the amine and TNBS was allowed to proceed for 20 min, and was subsequently quenched by addition of 50 μL of 1 M sodium acetate (pH 4.5) to each well. The absorbance was measured at 405 nm after 10 min of incubation. To compare the reaction kinetics of Lst-nanotube conjugates prepared with and without the PEG linker against free Lst, enzyme solutions were incubated with substrate solutions of different concentrations for time periods up to 6 h. Reaction rates were calculated using an experimentally determined molar extinction coefficient of 1.55 AU_{405} μmol^{-1} cm^2 for the chromophore (TNBS derivative of glycine).

Enzyme–containing Nanocomposite Films

Lst-nanotube based composite films were prepared with a diluted paint solution prepared by mixing 0.1 mL latex enamel (gloss white, Yenkin-Majestic Paint Corporation, Columbus, OH) in 9.9 mL DI water. Thin Lst-nanotube based composite films containing different Lst weight fractions (0, 1, 2, 3, and 4%, w/w) were prepared by filtering a mixture of conjugates and diluted latex paint through 0.2 μm polycarbonate membrane filter. The membrane-supported films were dried overnight under vacuum, and then adhered onto cover slides (Corning Inc., NY) using double-sided adhesive tape for the ease of handling. A set of control samples were prepared, which included films containing neither MWNTs nor conjugates, films containing MWNTs and BSA-MWNTs (in which the amount of MWNTs was equal to that present in the samples containing 4% w/w Lst), and films containing heat-inactivated Lst-PEG-MWNT conjugates. The films were washed with phosphate buffered saline (PBS, 10 mM, pH 7.5, Dulbecco's Phosphate Buffered Saline, Sigma) solution to remove loosely entrapped conjugates before testing them against the microbial suspension. The films were then vacuum-dried and stored at room temperature. Another set of films was prepared, which contained native Lst without nanotubes (in which the amount of Lst was equal to that present in 4% (w/w) Lst nanocomposite films). To determine the amount of Lst released from these films, the films were washed with PBS solution and the amount of Lst in the wash solution was measured using the micro bicinchoninic acid (Micro BCA) assay (Pierce Biotechnology, Rockford, IL).

Characterization of Nanocomposite Films by Scanning Electron Microscopy (SEM)

To study surface topography and conjugate surface coverage, films were characterized *via* SEM using a JEOL-JSM 840 Scanning Electron Microscope with the field emission gun operated at 10.0 kV. Films containing Lst-PEG-MWNT conjugates were dried overnight under vacuum. These dried samples were mounted on an SEM sample holder using double-sided carbon tape and imaged. A semi-quantitative analysis for determining conjugate surface coverage was performed by processing SEM images with NIH ImageJ software (http://rsb.info.nih.gov/ij).

Microorganisms and Growth Conditions

Microbial cultures (*Bacillus cereus* (*B. cereus*) – 14737, *Escherichia coli* (*E. coli*) – 53323, *S. aureus* – 33807, *S. epidermidis* – 14990, ATCC, Manassas, VA) were grown at 37°C for 16 h at 200 rpm in nutrient broth (3 g/L beef extract, 5 g/L peptone) (Difco, Detroit, MI) prepared in DI water. A 100 μL sample from this culture was then added to 4 mL of fresh nutrient broth which was allowed to grow for 6 h under the same conditions. From this growing culture, 1 mL was centrifuged at 10,000 rpm for 5 min at RT to obtain a pellet which was then washed twice with PBS solution to remove excess medium. Bacteria were then reconstituted in PBS. In order to obtain an approximate measure of cell density in terms of colony forming units (CFU)/mL, the optical density (OD) of the microbial suspension was measured at 540 nm (ca. 10^9 CFU/mL/A.U.43). The bactericidal efficiency of composite films was determined by using a diluted suspension containing ca. 10^6 CFU/mL.

Determination of Bactericidal Efficiency

Antimicrobial activity of native and nanotube-interfaced Lst in free solution was determined by testing 0.5 μg of Lst against 1 mL of a microbial suspension containing 10^6 CFU/mL. Colony counts, and hence number of live cells, were determined by removing aliquots from the microbial suspension at regular intervals and spreading them on nutrient agar. To evaluate whether the Lst-based film can kill *S. aureus* on direct contact, a film was exposed to 5 mL microbial suspension of 10^6 CFU/mL in a petri dish, which was shaken on an orbital shaker at 60 rpm and RT for 6 h. Aliquots from the microbial suspension were removed to determine bactericidal efficiency. To assess whether the bactericidal effect is a result of leaching of conjugates, a film was immersed in 5 mL PBS and was shaken at 60 rpm and room temperature for 6 h. The film was then removed, air dried for 2 min, and stored at room temperature for further use. Microbes were then added to the wash solution so as to attain final concentration of 10^6 CFU/mL. The resulting microbial suspension was then shaken on an orbital shaker at 60 rpm for 6 h.

Aliquots from the microbial suspension exposed to a film or from the suspension used in a control leaching test were removed, diluted in PBS, spread onto nutrient agar, and incubated at 37 °C for 18 h. To determine the bactericidal efficiency, bacterial colonies grown on the agar plate were counted. Bactericidal efficiency of Lst-containing film was determined by comparing corresponding colony counts with those obtained for the control films (prepared without addition of conjugates). After 6 h incubation in microbial suspension, the films were removed and washed with PBS to remove any non-specifically bound microbes. Films were then air-dried for 2 min and stored at RT in a vacuum desiccator until further use.

Antimicrobial Tests against MRSA

The effectiveness of the Lst-containing composite films against pathogenic strains of *S. aureus* was tested with four methicillin-resistant *S. aureus* strains; BAA 1556 (USA 300), BAA 1692 (USA100), BAA 1695, and BAA 1696 (USA 400) (ATCC, Manassas, VA). The

same procedures as used for non-pathogenic *S. aureus* strains were followed to study the bactericidal activity of the Lst on these strains.

Fluorescence Imaging of Microbes *via* Confocal Microscopy

Following the 6 h incubation of the control and Lst-based nanocomposite films in 5 mL microbial suspension of 1×10^6 CFU/mL, 1 mL from each suspension was removed in a microcentrifuge tube. Commercially available two component LIVE/DEAD Bac*Light* bacterial viability kit (Molecular Probes) was used to stain the microbes for fluorescence imaging. To the 1 mL microbial suspension two nucleic acid stains, namely SYTO 9 and propidium iodide, were added to obtain a resulting concentration of 5 μM and 45 μM for SYTO 9 and propidium iodide, respectively. The resulting mixture was incubated for 15 min at room temperature and was protected from light. After staining, the cells were imaged using a Zeiss LSM 510/Meta laser scanning confocal microscope at 40× magnification. When excited at 488 nm, live cells show green fluorescence (Emission wavelength = 500 nm) and lysed cells display red fluorescence (Emission wavelength = 635 nm).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We acknowledge the financial support from NIH (DE017213) and DTRA (HDTRA1-08-1-0022). Discussions with Dr. P. Asuri and Dr. I. V. Borkar are gratefully acknowledged.

References

1. Grundmann H, Aires-de-Sousa M, Boyce J, Tiemersma E. Emergence and Resurgence of Methicillin-Resistant *Staphylococcus aureus* as a Public-Health Threat. Lancet. 2006; 368:874–885. [PubMed: 16950365]
2. Klein E, Smith DL, Laxminarayan R. Hospitalizations and Deaths Caused by Methicillin-Resistant *Staphylococcus aureus*, United States, 1999–2005. Emerging Infect Dis. 2007; 13:1840–1846. [PubMed: 18258033]
3. Donlan RM. Biofilm Formation: a Clinically Relevant Microbiological Process. Clin Infect Dis. 2001; 33:1387–1392. [PubMed: 11565080]
4. Kumar A, Vemula PK, Ajayan PM, John G. Silver-Nanoparticle-Embedded Antimicrobial Paints Based on Vegetable Oil. Nat Mater. 2008; 7:236–241. [PubMed: 18204453]
5. Tiller JC, Liao CJ, Lewis K, Klibanov AM. Designing Surfaces that Kill Bacteria on Contact. Proc Natl Acad Sci US A. 2001; 98:5981–5985.
6. Haldar J, An DQ, de Cienfuegos LA, Chen JZ, Klibanov AM. Polymeric Coatings that Inactivate Both Influenza Virus and Pathogenic Bacteria. Proc Natl Acad Sci US A. 2006; 103:17667–17671.
7. Chapman RG, Ostuni E, Liang MN, Meluleni G, Kim E, Yan L, Pier G, Warren HS, Whitesides GM. Polymeric Thin Films that Resist the Adsorption of Proteins and the Adhesion of Bacteria. Langmuir. 2001; 17:1225–1233.
8. Bagheri M, Beyermann M, Dathe M. Immobilization Reduces the Activity of Surface-Bound Cationic Antimicrobial Peptides with No Influence upon the Activity Spectrum. Antimicrob Agents Chemother. 2009; 53:1132–1141. [PubMed: 19104020]
9. Glinel K, Jonas AM, Jouenne T, Leprince J, Galas L, Huck WTS. Antibacterial and Antifouling Polymer Brushes Incorporating Antimicrobial Peptide. Bioconjugate Chem. 2009; 20:71–77.
10. Akhavan O. Lasting Antibacterial Activities of Ag-TiO_2/Ag/a-TiO_2 Nanocomposite Thin Film Photocatalysts under Solar Light Irradiation. J Colloid Interface Sci. 2009; 336:117–124. [PubMed: 19394952]
11. AshaRani PV, Mun GLK, Hande MP, Valiyaveettil S. Cytotoxicity and Genotoxicity of Silver Nanoparticles in Human Cells. ACS Nano. 2009; 3:279–290. [PubMed: 19236062]

12. Chen X, Schluesener HJ. Nanosilver: A Nanoproduct in Medical Application. Toxicol Lett. 2008; 176:1–12. [PubMed: 18022772]
13. Lin J, Tiller JC, Lee SB, Lewis K, Klibanov AM. Insights into Bactericidal Action of Surface-Attached Poly(Vinyl-*N*-Hexylpyridinium) Chains. Biotechnol Lett. 2002; 24:801–805.
14. Klibanov AM. Permanently Microbicidal Materials Coatings. J Mater Chem. 2007; 17:2479–2482.
15. Fischetti VA. Bacteriophage Lytic Enzymes: Novel Anti-Infectives. Trends Microbiol. 2005; 13:491–496. [PubMed: 16125935]
16. Borysowski J, Weber-Dabrowska B, Gorski A. Bacteriophage Endolysins as a Novel Class of Antibacterial Agents. Exp Biol Med. 2006; 231:366–377.
17. Navarre WW, Ton-That H, Faull KF, Schneewind O. Multiple Enzymatic Activities of the Murein Hydrolase from Staphylococcal Phage ϕ11 - Identification of a D-Alanyl-Glycine Endopeptidase Activity. J Biol Chem. 1999; 274:15847–15856. [PubMed: 10336488]
18. Loeffler JM, Nelson D, Fischetti VA. Rapid Killing of *Streptococcus pneumoniae* with a Bacteriophage Cell Wall Hydrolase. Science. 2001; 294:2170–2172. [PubMed: 11739958]
19. Schuch R, Nelson D, Fischetti VA. A Bacteriolytic Agent that Detects and Kills *Bacillus anthracis*. Nature. 2002; 418:884–889. [PubMed: 12192412]
20. Loeffler JM, Djurkovic S, Fischetti VA. Phage Lytic Enzyme Cpl-1 as a Novel Antimicrobial for Pneumococcal Bacteremia. Infect Immun. 2003; 71:6199–6204. [PubMed: 14573637]
21. Loessner MJ, Wendlinger G, Scherer S. Heterogeneous Endolysins in *Listeria monocytogenes* Bacteriophages - a New Class of Enzymes and Evidence for Conserved Holin Genes within the Siphoviral Lysis Cassettes. Mol Microbiol. 1995; 16:1231–1241. [PubMed: 8577256]
22. Schindler CA, Schuhardt VT. Lysostaphin - New Bacteriolytic Agent for Staphylococcus. Proc Natl Acad Sci US A. 1964; 51:414–421.
23. Sugai M, Fujiwara T, Akiyama T, Ohara M, Komatsuzawa H, Inoue S, Suginaka H. Purification and Molecular Characterization of Glycylglycine Endopeptidase Produced by *Staphylococcus capitis* EPK1. J Bacteriol. 1997; 179:1193–1202. [PubMed: 9023202]
24. Baba T, Schneewind O. Target Cell Specificity of a Bacteriocin Molecule: a C-Terminal Signal Directs Lysostaphin to the Cell Wall of *Staphylococcus aureus*. EMBO J. 1996; 15:4789–4797. [PubMed: 8890152]
25. Grundling A, Schneewind O. Cross-Linked Peptidoglycan Mediates Lysostaphin Binding to the Cell Wall Envelope of *Staphylococcus aureus*. J Bacteriol. 2006; 188:2463–2472. [PubMed: 16547033]
26. Oluola O, Kong LK, Fein M, Weisman LE. Lysostaphin in Treatment of Neonatal *Staphylococcus aureus* Infection. Antimicrob Agents Chemother. 2007; 51:2198–2200. [PubMed: 17420212]
27. Dajcs JJ, Thibodeaux BA, Hume EBH, Zheng XD, Sloop GD, O'Callaghan RJ. Lysostaphin is Effective in Treating Methicillin-Resistant *Staphylococcus aureus* Endophthalmitis in the Rabbit. Curr Eye Res. 2001; 22:451–457. [PubMed: 11584345]
28. Wu JA, Kusuma C, Mond JJ, Kokai-Kun JF. Lysostaphin Disrupts *Staphylococcus aureus* and *Staphylococcus epidermidis* Biofilms on Artificial Surfaces. Antimicrob Agents Chemother. 2003; 47:3407–3414. [PubMed: 14576095]
29. Asuri P, Karajanagi SS, Yang HC, Yim TJ, Kane RS, Dordick JS. Increasing Protein Stability through Control of the Nanoscale Environment. Langmuir. 2006; 22:5833–5836. [PubMed: 16768515]
30. Karajanagi SS, Vertegel AA, Kane RS, Dordick JS. Structure and Function of Enzymes Adsorbed onto Single-Walled Carbon Nanotubes. Langmuir. 2004; 20:11594–11599. [PubMed: 15595788]
31. Asuri P, Karajanagi SS, Sellitto E, Kim DY, Kane RS, Dordick JS. Water-Soluble Carbon Nanotube-Enzyme Conjugates as Functional Biocatalytic Formulations. Biotechnol Bioeng. 2006; 95:804–811. [PubMed: 16933322]
32. Asuri P, Karajanagi SS, Kane RS, Dordick JS. Polymer-Nanotube-Enzyme Composites as Active Antifouling Films. Small. 2007; 3:50–53. [PubMed: 17294467]
33. Voet, D.; Voet, JG. Biochemistry. 3. John Wiley and Sons Inc; 1995.
34. Madigan, MT.; Martinko, JM.; Parker, J. Brock Biology of Microorganisms. 11. Prentice Hall; 2006.

35. Dinu CZ, Zhu G, Bale SS, Anand G, Reeder PJ, Sanford K, Whited G, Kane RS, Dordick JS. Enzyme-Based Nanoscale Composites for Use as Active Decontamination Surfaces. Adv Func Mater. 2010; 20:392–398.
36. Kiri N, Archer G, Climo MW. Combinations of Lysostaphin with *β*-Lactams are Synergistic against Oxacillin-Resistant *Staphylococcus epidermidis*. Antimicrob Agents Chemother. 2002; 46:2017–2020. [PubMed: 12019130]
37. Shockman, GD.; Chu, C-P.; Kariyama, R.; Tepper, LK.; Daneo-Moore, L. Peptidoglycan (Murein) Hydrolases: Unusual Enzymes for Unusual Substrates. In: De Pedro, MA.; Höltje, J-V.; Löffelhardt, W., editors. Bacterial Growth and Lysis: Metabolism and Structure of the Bacterial Sacculus. 1. Plenum Press; New York: 1993. p. 213-227.
38. Shockman GD, Daneo-Moore L, Kariyama R, Massidda O. Bacterial Walls, Peptidoglycan Hydrolases, Autolysins, and Autolysis. Microb Drug Resist. 1996; 2:95–98. [PubMed: 9158729]
39. Diep BA, Carleton HA, Chang RF, Sensabaugh GF, Perdreau-Remington F. Roles of 34 Virulence Genes in the Evolution of Hospital- and Community-Associated Strains of Methicillin-Resistant *Staphylococcus aureus*. J Infect Dis. 2006; 193:1495–1503. [PubMed: 16652276]
40. Rohrer S, Ehlert K, Tschierske M, Labischinski H, Berger-Bachi B. The Essential *Staphylococcus aureus* Gene fmhB is Involved in the First Step of Peptidoglycan Pentaglycine Interpeptide Formation. Proc Natl Acad Sci US A. 1999; 96:9351–9356.
41. Kusuma C, Jadanova A, Chanturiya T, Kokai-Kun JF. Lysostaphin-Resistant Variants of *Staphylococcus aureus* Demonstrate Reduced Fitness *in vitro* and *in vivo*. Antimicrob Agents Chemother. 2007; 51:475–482. [PubMed: 17101683]
42. Kline SA, Delaharpe J, Blackburn P. A Colorimetric Microtiter Plate Assay for Lysostaphin Using a Hexaglycine Substrate. Anal Biochem. 1994; 217:329–331. [PubMed: 8203764]
43. Hogt AH, Dankert J, Feijen J. Adhesion of Coagulase-Negative Staphylococci to Methacrylate Polymers and Copolymers. J Biomed Mater Res. 1986; 20:533–545. [PubMed: 3700446]

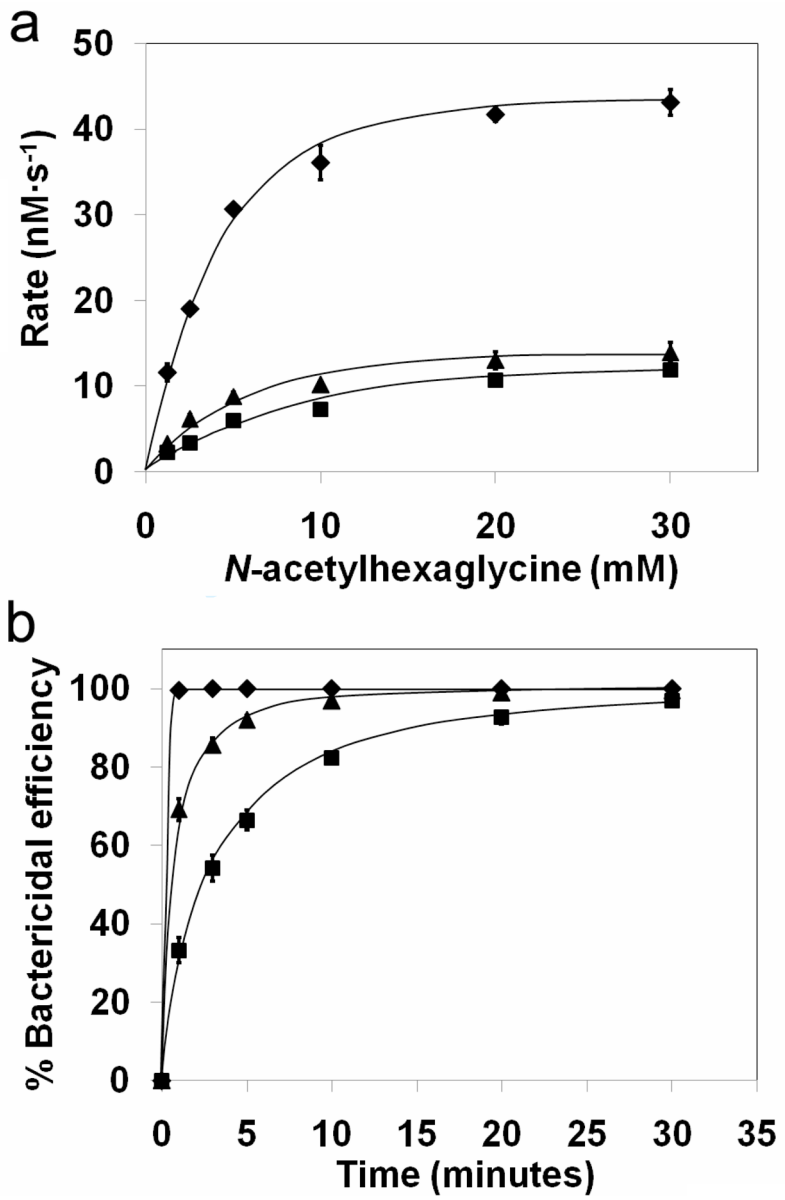


Figure 1.

Kinetics of Lst activity against *N*-acetylhexaglycine and microbes (*S. aureus*). (a) Michaelis-Menten kinetics of Lst activity based on the colorimetric assay. Rates in $nM \cdot s^{-1}$ for native Lst (◆), Lst-MWNT (■) and Lst-PEG-MWNT (▲) are plotted as a function of substrate concentrations. (b) Comparison of bactericidal effect of Lst-MWNT (■) and Lst-PEG-MWNT (▲) with the native enzyme (◆), determined by using a cell-based assay.

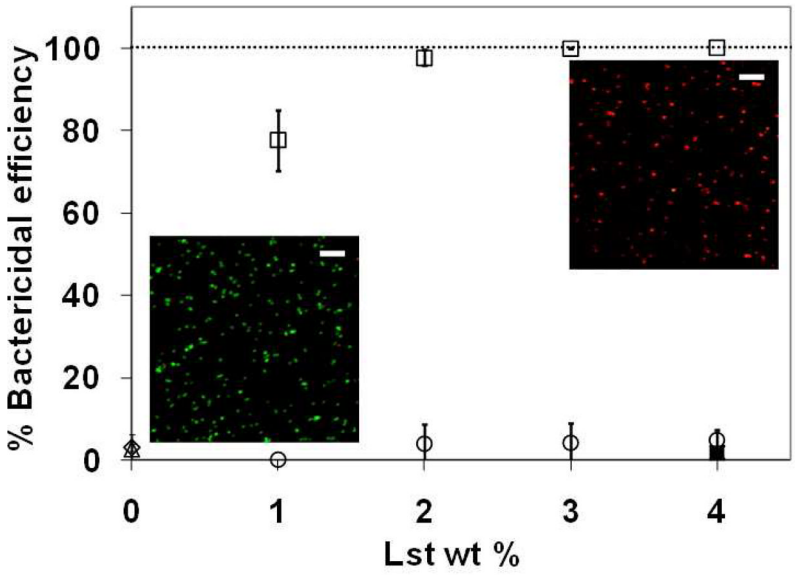


Figure 2.

Effect of Lst loading on the bactericidal activity shown by composite films containing Lst-PEG-MWNT conjugates (□). Controls for these experiments consisted of paints with heat-inactivated conjugates (■), paints with MWNT (◇) and BSA-MWNT (△). Data from leaching tests (○) suggest minimal bactericidal effect due to release of the enzyme or conjugates from films. Inset pictures show confocal images of *S. aureus* cells exposed to films containing 0% w/w and 4% w/w Lst, confirming the bactericidal effect of Lst. The scale bar corresponds to 10 μm.

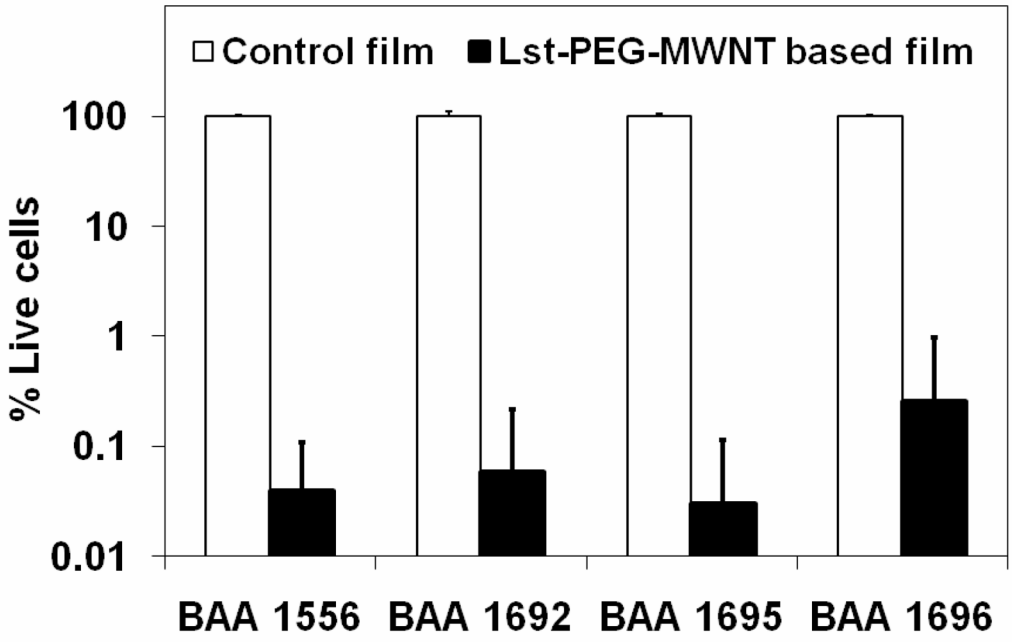


Figure 3.

Percent viable cells obtained after exposing the microbial suspension containing 10^6 CFU/mL MRSA to control and Lst-containing films (4% w/w Lst in the form of Lst-PEG-MWNT) for 2 h. The results are normalized with respect to the colony counts obtained for control samples.

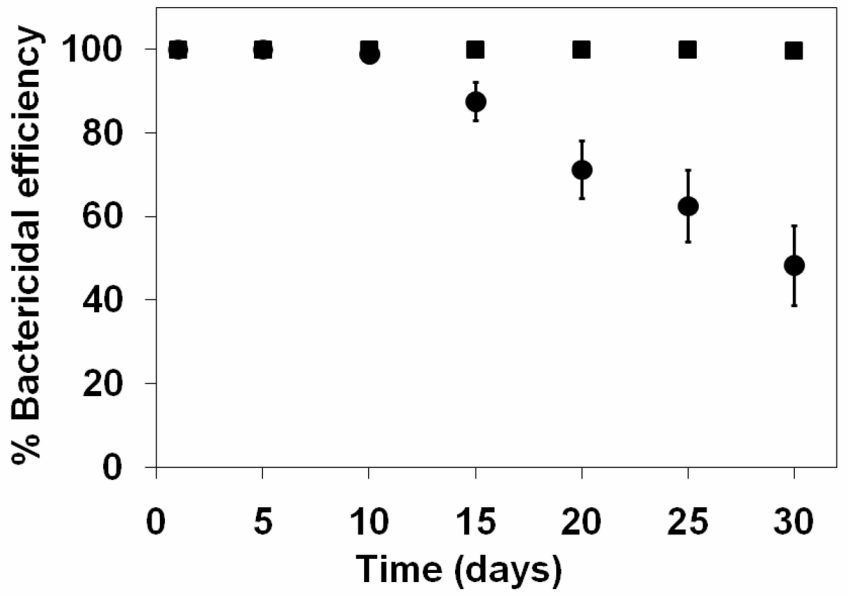
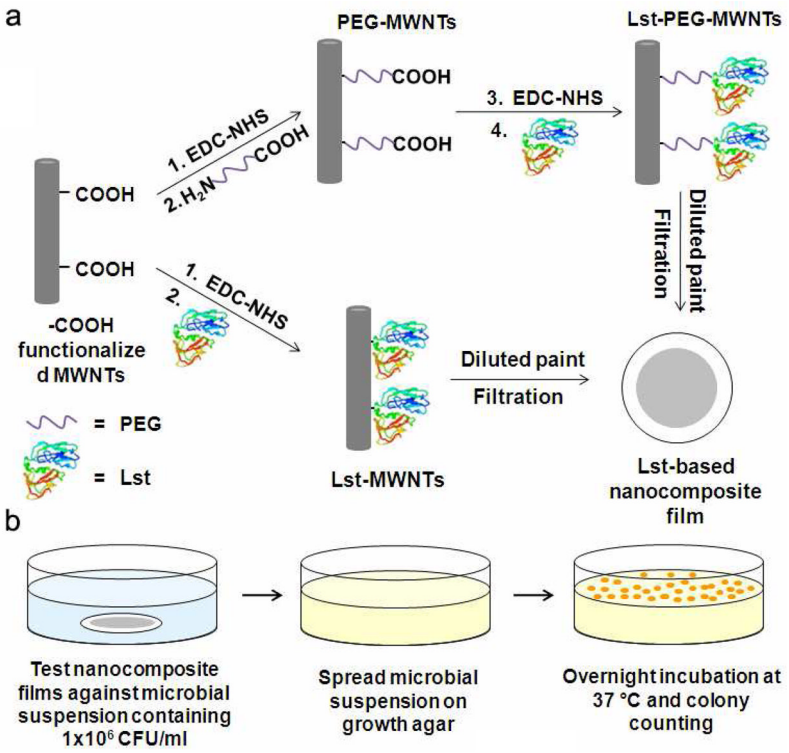


Figure 4.

Operational (●) and storage stability (■) of films containing 4% w/w Lst in the form of Lst-PEG-MWNT. The films were stored in dry conditions, and at room temperature in between the two use cycles.



Scheme 1.

Antimicrobial nanocomposite films containing Lst-nanotube conjugates (not drawn to scale): (a) Preparation of Lst-nanotube conjugates *via* two (no PEG linker) and four (with PEG linker) step EDC-NHS coupling reactions. Nanocomposite films were prepared by filtering a mixture of diluted paint and conjugates onto a polycarbonate membrane. (b) Cell-based assay to determine bactericidal activity of Lst-containing nanocomposite films.

Table 1

Kinetic parameters for native Lst, Lst-MWNT, and Lst-PEG-MWNT based on colorimetric assay

	V_{max} (μM/mg.s)	K_M (mM)	$V_{max}/K_M \times 10^3$ ($mg^{-1}s^{-1}$)
Native Lst	105	4.37	24.1
Lst-MWNT	25.6	6.00	4.27
Lst-PEG-MWNT	33.9	5.05	6.71