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COMBINATION ANTIMICROBIAL NANOCOMPOSITE MATERIALS FOR NEUTRALIZATION OF BIOLOGICAL THREAT AGENTS (PREPRINT)

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Through natural selection, microorganisms acquire resistance to conventional antibiotics. Coupled with advances in genomics and genetic engineering, the evolution of highly virulent, multidrug-resistant pathogens provides a resource that could be misused for the design of future biological warfare agents. We are exploring approaches that integrate molecules of the innate immune system and nanoscale inorganic materials in order to create novel antimicrobial composites and self-sterilizing coatings. In vitro biomineralization reactions allow antimicrobial proteins to be incorporated and stabilized within inorganic materials, such as amorphous silica, titania and colloidal silver. The approach yields materials that combine the biocidal properties of different antimicrobial mechanisms. The development of new antimicrobial therapies is an important countermeasure to emerging multidrug-resistant pathogens.

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ABSTRACT

Through natural selection, microorganisms acquire resistance to conventional antibiotics. Coupled with advances in genomics and genetic engineering, the evolution of highly virulent, multidrug-resistant pathogens provides a resource that could be misused for the design of future biological warfare agents. We are exploring approaches that integrate molecules of the innate immune system and nanoscale inorganic materials in order to create novel antimicrobial composites and self-sterilizing coatings. *In vitro* biomineralization reactions allow antimicrobial proteins to be incorporated and stabilized within inorganic materials, such as amorphous silica, titania and colloidal silver. The approach yields materials that combine the biocidal properties of different antimicrobial mechanisms. The development of new antimicrobial therapies is an important countermeasure to emerging multidrug-resistant pathogens.

INTRODUCTION

Throughout the plant and animal kingdoms, organisms possess mechanisms that actively guard against pathogens. Known as innate immunity, it refers to an organism's basic defenses against pathogens. The strategy includes passive physical barriers such as epithelial or mucosal layers as well as active, biochemically-derived components. Innate immunity is distinct from adaptive immunity in higher animals, which generally requires prior exposure to specific antigens to mount effective responses. Innate immunity has a broad, relatively nonspecific mode of action. (1-3). It is remarkably effective as a first line of defense; organisms are constantly exposed to potential pathogens, but only rarely do the invaders slip past the barrier to cause disease or require the adaptive immune response to fend off attack. Seemingly, biomolecules of the innate immune response are an excellent source to exploit for biomedical applications. Unfortunately, many questions still remain unanswered that pertain to the mechanisms and characteristics of innate immunity. Further study is needed to helps us to exploit the biochemical machinery for effective therapeutic treatments and other practical applications.

The focus of the present study is to understand how biochemical mechanisms may be harnessed to design future generations of active materials. In particular, we have used the bacteriolytic enzyme, lysozyme, and antimicrobial peptides (AMPs) as active components in nanocomposite materials. Because of their properties and that they not been overused in the health care community, the biomolecules are interesting candidates for use in next-generation antibiotics and antimicrobial materials. These antimicrobial agents have evolved to function within a biological organism. Consequently, specialized methods are needed to stabilize and

retain their physiological activity, in order to effectively integrate them into practical applications.

The immobilization of enzymes and other biomolecules within and adsorbed to inert supports has been practiced for over 30 years. Porous silica and silica sol gels are often used to support immobilized enzymes (4, 5). The approach can provide stabilized catalysts, but loading capacity is limited and conventional methods for producing the silica involve organic solvents and alcoholic byproducts that can denature the enzyme present during formation. Alternatively, mineralization reactions are wide-spread in biological systems and these biomineralization processes can be mimicked in the laboratory setting. Biomineralization refers to the process that organisms use for generating hard tissues that incorporate inorganic minerals (bone, teeth, shells, and exoskeletons). Biomineralization is typically initiated by a protein that acts as a sort of template or scaffold in order to form a composite material that combines the protein and inorganic components. The process can be mimicked *in vitro* to some degree, providing methods for production of nanometric structures and inspiration for a burgeoning branch of materials science (6-9). For example, peptides based on the silaffin protein from the marine diatom *Cylindrotheca fusiformis* catalyze formation of silica nanospheres *in vitro* (10). The reaction occurs under benign conditions (aqueous solution, neutral pH, ambient temperature), providing an attractive processing technique to form homogenous silica nanoparticles. The reaction also provides a method to entrap additional enzyme in silica matrices. When additional molecules are included in the precipitation reaction mix, they are entrained within the newly-formed material (11, 12). The approach might be broadly applicable to incorporating sensitive molecules within silica frameworks. In addition, biomineralization reactions are not limited to amorphous inorganic matrices. Organic molecule-mediated formation of metal nanoparticles has also been shown in a wide range of studies and is effective in synthesizing hybrid bioinorganic composites that retain properties from the organic and inorganic components (13, 14). In particular, several studies have shown that silver nanoparticles can be easily synthesized from soluble silver salts using biomolecules as reducing agents and/or stabilizing surfactants (e.g. proteins, ribonucleic acids, and extracellular components) (15-17). This phenomenon is of particular interest to our studies, as well as to the larger medical community, as silver inhibits the growth of the wide range of pathogenic bacteria, fungi, and viruses (18).

Herein, we report synthesis of two antimicrobial composites where biological molecules inspired by the innate immune system of higher animals are integrated with inorganic supports to form hybrid bioinorganic materials. The effectiveness of the antimicrobial activity is influenced by properties of both the biochemical and inorganic components. The resulting products offer effective antimicrobial activity and demonstrate the facile integration of biomolecules into devices and instruments. These novel materials exploit natural antimicrobial mechanisms that have not been overused in the health care community and therefore, have the potential to be an effective countermeasure against multi-antibiotic resistant pathogens.

EXPERIMENTAL

Synthesis, physical characterization, and antimicrobial activity of the following composites were completed as reported elsewhere: antimicrobial peptide silica and titania nanocomposites (19), lysozyme-silver nanoparticles (20) and electrophoretic deposited coatings (21). Detailed explanation of synthesis and preparation procedures, physical and chemical analysis, and antimicrobial assays can be found in these manuscripts.

RESULTS

Peptide-based synthesis of silica and titania antimicrobial nanoparticles (Si- and Ti-ANPs)

The antimicrobial peptide, KSL (KKVVFKVKFK) mediated its own immobilization within silica and titania nanoparticles and retained the antimicrobial properties of the free peptide (19). KSL, a highly cationic peptide, not only demonstrates antimicrobial activity against a wide range of microorganisms, but also has inherent ability to mediate biomineralization of silica and titania (Figure 1). When the peptide was added to phosphate buffer and either tetramethyl orthosilicate or potassium hexafluorotitanate, amorphous silica and titania oxide nanospheres form, which retain the antimicrobial properties of antimicrobial peptide and provide inhibitive and biocidal activities that is comparable to the native peptide (Table 1). Furthermore, the novel composites protected much of the peptide from degradation and inactivation and also facilitate a continuous dose of the peptide over time. These protective and time-release properties of the nanocomposite material facilitated a more stable dose of the peptide than the un-immobilized, free form of the peptide when incubated with *Staphylococcus aureus* (Figure 2). Altogether, the composites show promise for use as a potentially effective antibiotics. For example, the material may be included in topical treatments or as components in self-sterilizing coatings.

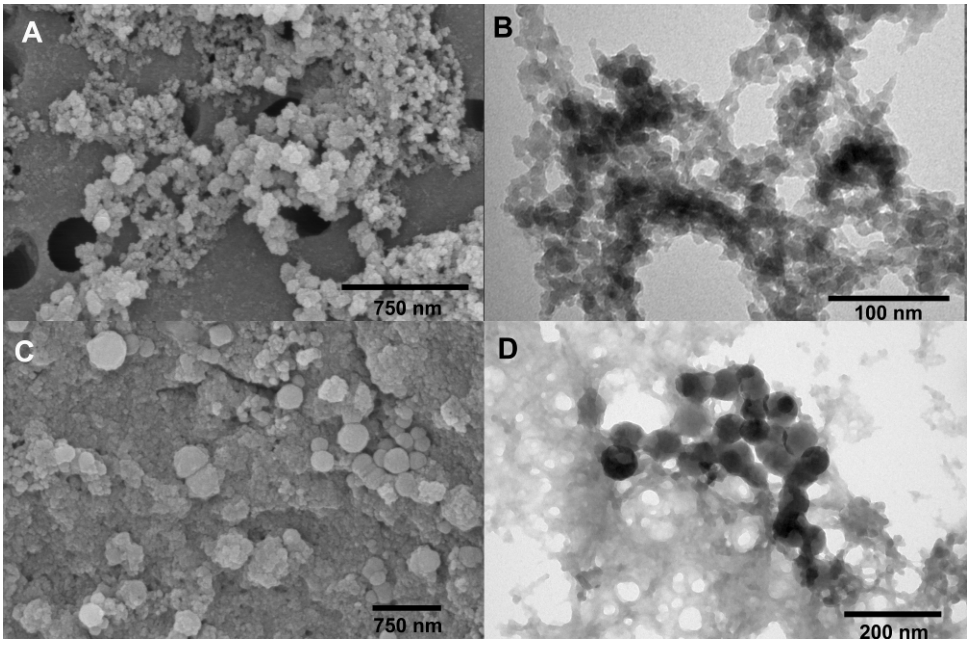

Figure 1. Scanning and transmission electron micrographs (TEM and SEM), respectively, of KSL-catalyzed silica (A and B) and titania (C and D) nanoparticles (19).

Table 1. Minimum Inhibitory and Biocidal Concentrations of native KSL, Si-ANPs, and Ti-ANPs (19).

Strain	cells[a]	peptide form[c]	total peptide[d]	
			MIC[e]	MBC[f]
E. coli ATCC 25922	5.27 ±2.65[b]	free	0.8 ± 0.2	0.9 ± 0
		Si-ANPs	3.0 ± 2.0	5.0 ± 2.0
		Ti-ANPs	2.0 ± 0.9	6.0 ± 0.2
S. aureus ATCC 25923	2.71 ±2.25	free	18.0 ± 8.0	>225.0 ± 0
		Si-ANPs	24.0 ± 1.0	80.0 ± 25.0
		Ti-ANPs	62.0 ± 41.0	91.0 ± 22.0
S. epidermidis ATCC 14990	1.64 ±1.58	free	0.6 ± 0.2	1.0 ± 0.5
		Si-ANPs	1.0 ± 0.4	3.0 ± 2.0
		Ti-ANPs	2.0 ± 0	3.0 ± 0
C. albicans ATCC 10231	1.50 ±0.58	free	0.7 ± 0.2	1.0 ± 0.4
		Si-ANPs	2.0 ± 1.0	5.0 ± 2.0
		Ti-ANPs	4.0 ± 1.0	5.0 ± 2.0

[a] Number (x10^5) of colony forming units (CFU) ml^{-1}at start of assay. [b]Standard deviations are representative of at least three assays. [c] KSL added to cell cultures either as the non-biomineralized, native form (free) or in silica and titania nanoparticles (Si-ANPs and Ti-ANPs, respectively). [d] The total amount of peptide added to cultures in μg ml^{-1}. [e] minimum inhibitory concentration. [f] minimum biocidal concentration.

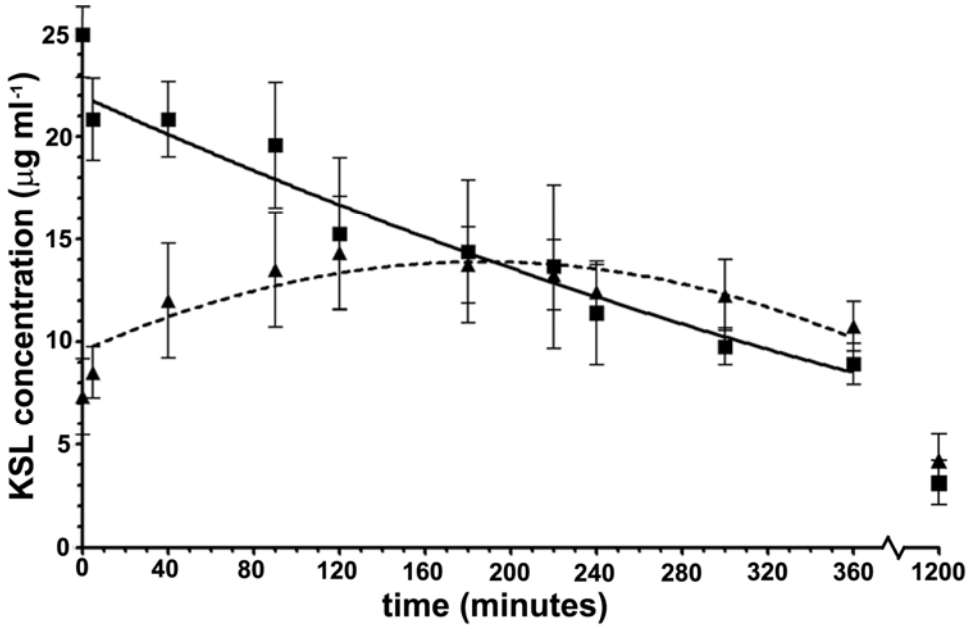

Figure 2. Measurement of antimicrobial peptide over time during incubation with *S. aureus* cells (19). Soluble concentrations were monitored of KSL in the free form (squares, solid line) and Si-ANPs (triangles, dashed line) after addition of peptide (25 μg ml^{-1}) to between 10^6 and 10^7 viable *S. aureus* cells ml^{-1}. Standard error mean represents six individual assays. Regression analysis was calculated using second order polynomial curve fit for measurements between 5 and 360 min.

Synthesis of lysozyme-silver antimicrobial nanoparticles and their deposition into medical instruments

Lysozyme catalyzed silver reduction to form antimicrobial silver nanoparticles (20). When exposed to silver ions in methanol, lysozyme acted as the primary reducing agent and formed stable colloidal suspensions of silver (Figure 3). The enzyme also acted as an effective colloidal stabilizer and solutions can be stored in a concentrated form in methanol or water for months without significant change in physical or chemical properties. Furthermore, the colloid solutions could be used to form homogeneous enzyme and silver coatings on surgical steel (21). Uniform antimicrobial coatings were deposited on surgical stainless steel blades and needles using an electrophoretic deposition technique. The antimicrobial activity of lysozyme and biocidal properties of colloidal silver were retained in the coatings and antibacterial assays demonstrate the coatings have potent biocidal activity over several strains (Table 2). When used in an assay designed to mimic the standard use of the instrument, clearings of cell lysis formed in bacteria-infused agarose around incisions and stab sites, demonstrating not only the coatings were a self-cleaning surface, but also would transfer antimicrobial activity into a subject during use (Figure 4). The findings show that a one-pot method and simple electrophoretic deposition can be used to generate antimicrobial coatings that combine two different antimicrobial mechanisms.

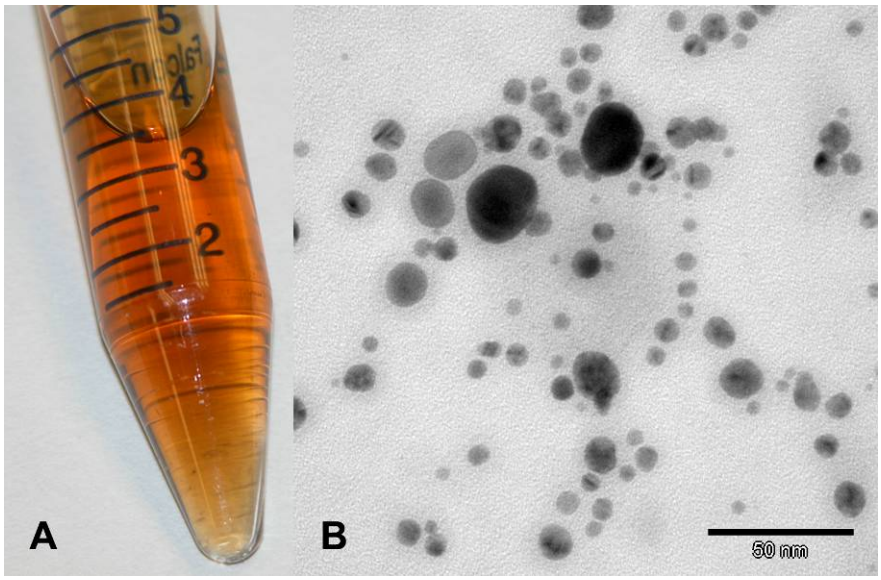

Figure 3. Suspension of silver nanoparticles formed in lysozyme-catalyzed process (A) TEM image of silver nanoparticles (B) (21).

Table 2. Antimicrobial activity of coated blades towards bacterial and yeast strains (21).

Strain	Decreased viability (%)		Inhibition type
	1.5 h†	3 h†	
Acinetobacter baylyi	98	99	bactericidal
Bacillus anthracis Sterne	99	>99.99	bactericidal
Bacillus subtilis	>99.99	>99.99	bactericidal
Klebsiella pneumoniae	99	99.9	bactericidal
Staphylococcus aureus	99	99.9	bactericidal
Staphylococcus epidermidis	4	42	bacteriostatic

† Contact time

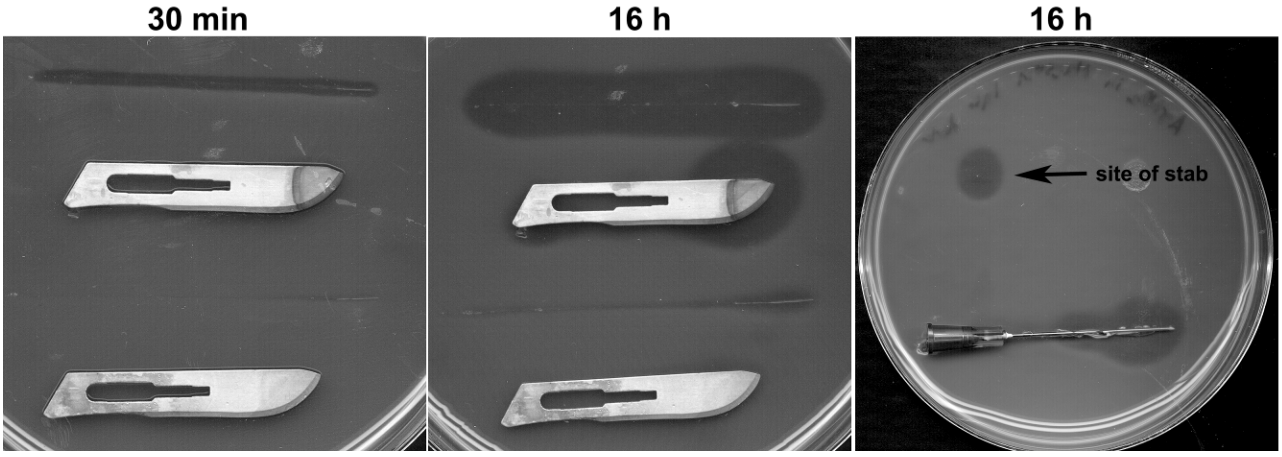

Figure 4. Cell lysis assay measuring antimicrobial activity of coated blades and needles (21). Coated blades (left) and needles (right) were used to make incisions and punctures, respectively, and then placed on top of agarose infused with *M. lysodeikticus* cells. The top blade contains a coating of lysozyme and silver nanoparticles, while the lower blade has a coating of lysozyme only. Zones of cell lysis are seen at the incision and puncture site, as well as surrounding the blades and needles after incubation at 37°C for 30 min and 16 h (labeled as shown).

CONCLUSIONS

The rise in multi-resistant pathogens, along with rapid advances in microbial genomics and genetic engineering affords the opportunity for malicious design of biowarfare agents (22). Because microbes have evolved to overcome present antimicrobial therapies, conventional antibiotics may be useless against a terrorist attack that involves multi-resistant pathogenic agents. Consequently, novel methods of materials design and the effective combination of different antimicrobial mechanisms are compelling approaches to counteract resistance to commonly-used antibiotics (23). Two approaches explored for application are antimicrobial peptides and nanoparticulate silver. While the concept shows promise, a substantial amount of study into their stability, sustainability, dosage, and means of delivery is still needed before the applications can be fully realized. In general, establishing effective methods to combine multiple antimicrobial agents into one application are critical to defense against pathogenic resistance that will eventually arise to new antibiotics in the future, whether through natural evolution or by man-made design. The results of the study will assist in accelerating these types of materials to commercial production and ultimately contribute to biological warfare mitigation in the future.

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