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SYNTHESIS AND CHARACTERIZATION OF ANTIMICROBIAL NANOMATERIALS

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14. ABSTRACT

The work describes how understanding fundamental biological and biophysical phenomena can be used to develop rational biotechnology solutions for Air Force requirements. Particular emphasis throughout is the integration of catalytically active biomolecules with support matrices and the utilization of these hybrid materials for development of decontamination treatments and self-sanitizing materials. Specific objectives are i) to understand the relationship between protein template molecules and metal-oxide nanoparticles, ii) define the bacteriolytic mechanism for enzymes immobilized in metal-oxide nanoparticles and iii) to evaluate antimicrobial activity from combinations of bacteriolytic hydrolases and antimicrobial peptides.

15. SUBJECT TERMS

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1. SUMMARY

This report summarizes research in support of the Air Force Research Laboratory (AFRL) Airbase Technologies Division (RXQ), conducted at Tyndall AFB, Florida, from 10 April 2006 through 31 December 2012. The scope of this task was to perform fundamental and developmental research to understand how biochemical mechanisms may be harnessed to design bio-active materials.

The work describes how understanding fundamental biological and biophysical phenomena can be used to develop rational biotechnology solutions for Air Force requirements. Particular emphasis throughout is the integration of catalytically active biomolecules with support matrices and the utilization of these hybrid materials for development of decontamination treatments and self-sanitizing materials. Specific objectives are i) to understand the relationship between protein template molecules and metal-oxide nanoparticles, ii) define the bacteriolytic mechanism for enzymes immobilized in metal-oxide nanoparticles and iii) to evaluate antimicrobial activity from combinations of bacteriolytic hydrolases and antimicrobial peptides.

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2. INTRODUCTION

Throughout the plant and animal kingdoms, organisms possess mechanisms that actively protect against pathogens. This innate immunity refers to an organism's basic defenses against infection. The strategy includes passive physical barriers such as epithelial or mucosal layers as well as active, biochemically-derived components. In higher animals, innate immunity is distinct from "adaptive" immunity, which generally requires prior exposure to specific antigens for the body to mount an effective response. Innate immunity has a broad, relatively nonspecific mode of action that is remarkably effective as a first line of defense.[1–3] Despite increased understanding of the mechanisms for adaptive immunity, many questions pertaining to the mechanisms and characteristics of innate immunity remain unanswered. Further study is needed to help exploit the biochemical machinery of immunity for effective therapeutic treatments and practical applications.

The focus of the present study is to understand how biochemical mechanisms may be harnessed to design bio-active materials. Bacteriolytic proteins (such as lysozyme) and antimicrobial peptides (AMPs), for example, demonstrate broad-spectrum antimicrobial and antifungal activity but have been underutilized as a treatment mechanism in the healthcare community. These biomolecules are therefore interesting candidates for next-generation antibiotics and antimicrobial materials. One caveat, however, is that many antimicrobial agents have evolved to function within a biological organism. In consequence, effectively integrating them into practical applications requires specialized methods to stabilize and retain their physiological activity.

Immobilization of enzymes and other biomolecules within and adsorbed to inert supports (such as porous silica and silica sol–gels) has been widely practiced.[4, 5] The approach can provide stabilized catalysts, but volumetric loading capacity can be limited and conventional methods for producing silica involve organic solvents and alcoholic byproducts that can denature the enzyme. *Biomineralization* refers to the process that organisms use to generate hard tissues from inorganic minerals (bone, teeth, shells and exoskeletons). Biomineralization is typically initiated by a protein that acts as a template or scaffold to steer formation of a composite material that combines the protein and inorganic components. The process can be mimicked *in vitro*, providing methods for production of nanometric structures and inspiration for a burgeoning branch of materials science.[6–9]

For example, the ability of diatoms and radiolarians, to sequester silicic acid from sea water and then use it to build intricate exoskeletons composed of amorphous silica is well documented. Peptide analogs of the proteins that mediate the diatom's biosilicification reaction can be used *in vitro* to yield silica nanospheres from soluble precursors. That the reaction occurs under benign conditions (aqueous solution, neutral pH, ambient temperature), provides 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.[10, 11] Our work has taken advantage of the *in vitro* reaction for facile immobilization of enzymes and peptides that may be used in biotechnology applications.[12] The approach has now been widely demonstrated as a methodology that is broadly applicable to incorporating sensitive molecules within silica frameworks.[10]

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Biologically-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.[13–16] 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, 18] This phenomenon is of particular interest, as silver inhibits the growth of a wide range of pathogenic bacteria, fungi and viruses.[19]

Herein, we report synthesis of antimicrobial composites in which biological molecules—inspired by the innate immune system of higher animals—are integrated with inorganic or metallic 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 healthcare community and, therefore, have the potential to be effective countermeasures against pathogens exhibiting multiple-antibiotic resistance.

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3. PREPARATION OF ANTIMICROBIAL COMPOSITE MATERIALS

3.1. Introduction

Numerous biochemical mechanisms protect organisms against potential microbial infection. Materials that incorporate active antimicrobial biomolecules could provide a significant advance for self-sanitizing surfaces and decontamination processes. To achieve this goal, fundamental research is essential to define the interface between biomolecules and a range of potential inorganic support materials. The understanding and discoveries can then be applied to development of a new generation of reactive materials. One approach for *in vitro* immobilization of biomolecules is modeled after the biosynthesis of marine diatom exoskeletons.

The process involves rapid formation of porous nanoparticles, which simultaneously entrap biomolecules that are present in the reaction solution.[20] We have found that the bacteriolytic enzyme hen egg white lysozyme (HEWL) will mediate silica formation from soluble precursors, yielding by its nature, a material with antimicrobial properties.[21] Parallel studies are underway to elucidate the mechanism of lysozyme-mediated silica formation.[22] The biocidal spectrum of the material can be broadened by addition of other molecules such as AMPs or by utilizing an inorganic support with antimicrobial activity.

3.2. Approach

3.2.1. Lysozyme-mediated Silicification

Lysozyme–silica composites were fabricated using methods previously described.[21] Wet lysozyme–silica composites (maintained in an aqueous environment after gelation) were exchanged with D_2O for small-angle neutron scattering (SANS) studies.[22]

3.2.2. Lysozyme/silica Composites on Silk Fabrics

Silk fabrics were functionalized with lysozyme and mineralized to silica and titania *in situ*.[23] Impregnation of the fabric was confirmed by scanning (SEM) and transmission (TEM) electron microscopy, and X-ray photoelectron spectroscopy (XPS). Bactericidal activity was confirmed by radial diffusion assays on swatches of modified silk fabric.[23]

3.2.3. Antimicrobial Peptides

AMP nanoparticles were prepared using biomineralization reactions with tetramethyl orthosilicate (TMOS) as a precursor for silica formation or potassium hexafluorotitanate as a precursor for titania nanoparticles.[24] Nanoparticle size and composition were characterized by TEM, SEM, and attenuated total reflectance Fourier-transformed infrared spectroscopy. Antimicrobial assays were based on minimum inhibitory concentrations and minimum bactericidal and fungicidal concentration using previously described protocols.[25] Assays were performed with the bacterial strains *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) and *S. epidermidis* (ATCC 14990), and the yeast *Candida albicans* (ATCC 10231). Silica and titania nanoparticles were prepared using the R5 peptide as described above and used as a non-antimicrobial control.

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3.3. Results and Discussion

3.3.1. Lysozyme-mediated Silicification

During studies on the biocatalytic condensation of silica sol–gels, lysozyme was identified as a protein that catalyzes and templates the precipitation of silica nanoparticles and produces a composite material that retains the native antimicrobial properties of lysozyme.[21, 26, 27] Despite encapsulation in a silica matrix, the immobilized lysozyme retains its native hydrolase activity; the enzyme will cleave soluble oligosaccharide substrates and lyse the cell wall of the bacterial strain *Micrococcus lysodeikticus*.[21] The discovery that that the lysozyme immobilized within the silica nanoparticles shows effective bacteriolytic activity was unexpected because to exhibit native activity the enzyme must directly interact with the bacterium. The structural properties of the composite material were therefore investigated using a combination of SEM, TEM and SANS.[22] SEM and TEM revealed that the composite has a hierarchical structure composed of quasi-spherical structures, approximately 450 nm in diameter, which are in turn composed of closely packed spherical structures of approximately 8–10 nm in diameter. Using SANS with contrast variation, it was possible to separate the scattering signatures of the lysozyme and silica within the composite. Furthermore, it was determined that the lysozyme molecules are spatially correlated in the material and form clusters with colloidal silica particles. The size of the clusters determined by SANS agrees well with the structural architecture observed by TEM (Figure 1).

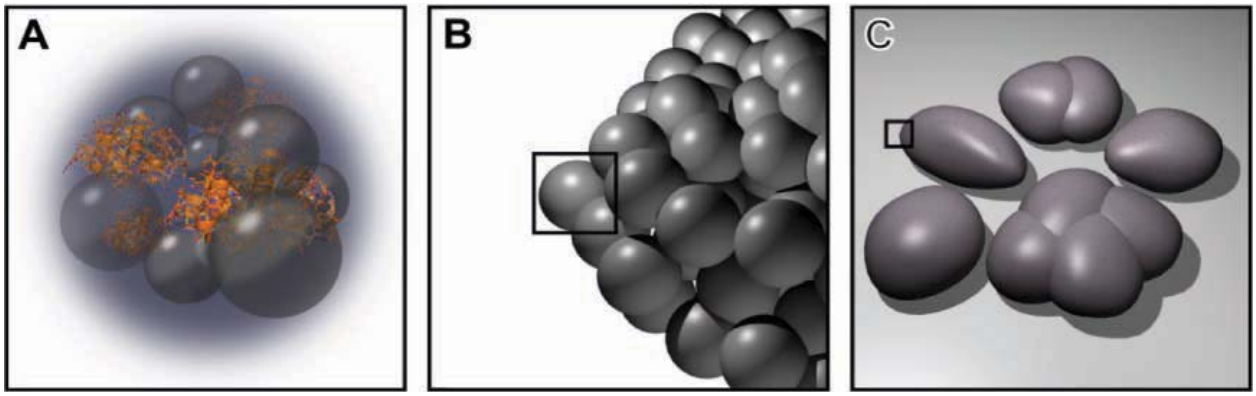

Figure 1. Schematic Representation of the Hierarchical Structure for the Silica–lysozyme Composite Material: (A) Schematic Drawing of Fundamental Silica-lysozyme Cluster (Silica Particles are Shown as Gray Spheres while Lysozyme Molecules are Presented in Orange. Length scales are Based on SEM, TEM and SANS Analysis); (B) Aggregates of Fundamental Clusters [Black square in B Corresponds to Panel A]. (C) Quasi-spherical and Polylobular Representative Structures [Black Square in C Corresponds to Panel B]

We postulate that the enzyme must be able to diffuse from the composite material because it must directly interact with a bacterium to disrupt the cell wall structure. Although our structural analysis demonstrated a well organized sol–gel synthesis that generates a functional material, it did not provide insight into the dynamic properties of the entrapped protein that result in its antimicrobial activity. This information is critical for understanding the biomineralization process as well as optimizing the reaction for biomolecule immobilization in practical applications.

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3.3.2. Lysozyme–silica Composites on Silk Fabrics

Characterization and testing antimicrobial efficacy of the silica–lysozyme composites was investigated by integrating with silk fibers using an electrospinning technique to entwine the reactive composite materials within the textile swatches.[23] Such functionalized textiles may find application in protective wear for medical and military personnel and provide functional wound dressings that reduce infection *in situ*. Silk textiles were functionalized by the surface adsorption of lysozyme; exposure of such lysozyme-conjugated fabrics to mineralizing solutions enabled the self-directed immobilization of the enzyme in a subsequent protective matrix of amorphous silica or titania. Silk-immobilized lysozyme was also utilized to adsorb nanocrystalline TiO_2 from solution onto the fabric surface; a subsequent layer of enzyme served to entrap the ceramic particles under a layer of biomimetically mineralized titania (Figure 2). The multiplicity of antimicrobial activities derived from this approach resulted in bactericidal properties against *S. aureus* and *E. coli* and included a photocatalytic bactericidal response of TiO_2 under UV illumination.[23]

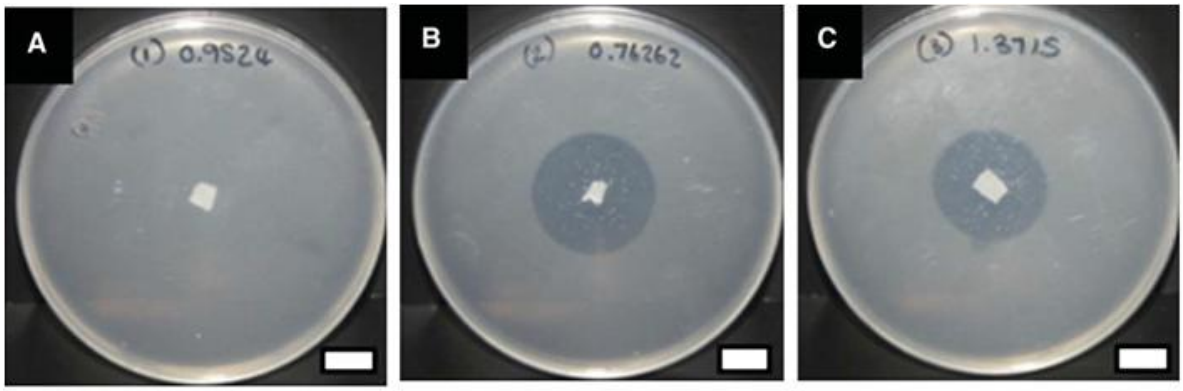

Figure 2 Radial Diffusion Assay of A) Unmodified, B) Physically Adsorbed Lysozyme, C) Silica-encapsulated Lysozyme on Silk Cloth Swatches (Scale Bars Represent 10 mm)

3.3.3. Antimicrobial Peptides

Amphiphilicity and cationicity are properties shared between AMPs and proteins that catalyze biomineralization reactions. Biosilicification catalysts all share a common feature: a high isoelectric point (pI). Certain AMPs have pI values equal to or greater to lysozyme, leading us to investigate whether specific AMPs may also catalyze silicification reactions. Originally identified as an AMP, KSL (KKVVFKVKFK) also directs the formation of biologically derived silica. By merging these two functionalities, KSL catalyzes self-biomineralization within inorganic matrices. The resultant AMP nanoparticles retain biocidal activity, protect the peptide from proteolytic degradation, and facilitate continuous and sustained release of antimicrobial activity over time. As a result, KSL mediates its own immobilization within silica and titania nanoparticles and retains the antimicrobial properties of the free peptide.[24]

KSL, a highly cationic peptide, not only demonstrates inherent ability to mediate biomineralization of silica and titania (Figure 3) but also exhibits antimicrobial activity against a wide range of microorganisms, retaining the antimicrobial properties of AMP, and provides inhibitive and biocidal activities that are comparable to the native peptide.[24] Furthermore, the composite protects the peptide from degradation and inactivation, and facilitates a continuous

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release of the peptide over time. Altogether, the composites show promise for use as potentially effective antibiotics. For example, the material may be included in topical treatments or as components in self-sterilizing coatings.

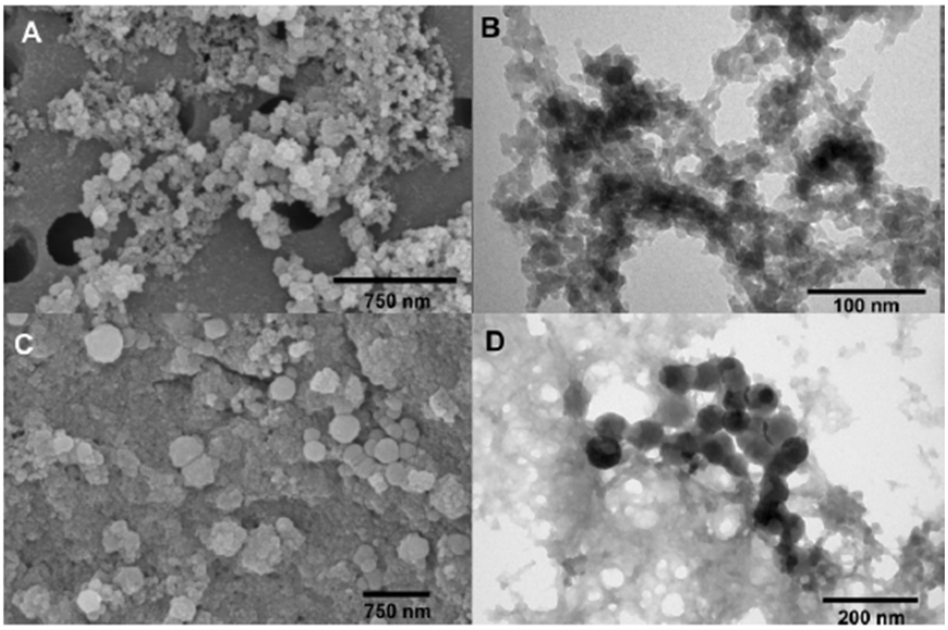

Figure 3. Scanning (l) and Transmission Electron Micrographs (TEM and SEM, Respectively) (r) of KSL-catalyzed Silica (A and B) and Titania (C and D) Nanoparticles

Monte Carlo simulations were used to model the self-organizing behavior of KSL in the presence of phosphate.[28] Specificity of each residue and the interactions between the peptide and phosphate were considered in a coarse-grained model. Results demonstrate that interactions between the lysines and phosphate drive self-organization into lower energy conformations of interconnected peptide scaffolds that resemble the supramolecular structures of polypeptide- and polyamine-mediated silica condensation systems. Furthermore, the specific phosphate–peptide organization appears to mimic the zwitterionic structure of native silaffins (scaffold proteins of diatom shells), suggesting a similar template organization for silica deposition between the *in vitro* KSL and silaffin systems.[28]

Additional physical characterization of silica microspheres derived from the KSL oligopeptide was investigated by using XPS to ascertain intermolecular bonding between the KSL peptide and the silica materials. Additional characterization of the composite material was obtained using Fourier transform infrared spectroscopy and nuclear magnetic resonance spectroscopy to define structure of peptide and its interaction with silica in the materials. Specifically there was strong evidence showing formation of imide bonds between the peptide backbone and silica.[28]

4. PROTEINS AS SCAFFOLDS FOR METAL REDUCTION

4.1. Introduction

Silver nanoparticle synthesis is mediated by a diverse range of biosynthetic mechanisms,

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including several examples of fortuitous silver nanoparticle formation by various species of bacteria, fungi, and plant extracts. [29] Biosynthesis of this type expands the utility of materials by associating useful biomolecules with inorganic matrices to merge the functional properties of each component into one composite. For example, lysozyme will catalyze reduction of silver anions to yield composites of silver nanoparticles and active lysozyme. The composite material can take advantage of the antimicrobial effect of the silver metal as well as the biochemical activity of the template protein. The noble metal support also provides a convenient means for electrodeposition of the complex to create antimicrobial coatings.

4.2. Approach

4.2.1. Protein-catalyzed Metal Reduction

Lysozyme–silver nanoparticles were prepared by reaction of lysozyme and silver acetate in 100 % methanol in the absence of light.[13] Stable silver colloids formed after mixing of lysozyme and silver acetate in methanol, and the resulting nanoparticles were concentrated and transferred into aqueous solution without any significant changes in physical properties.[13]

Nanomaterials formed under a series of reaction conditions were characterized using SEM and spectrophotometric techniques. Antimicrobial assays were conducted with *S. aureus* (ATCC 25923), *C. albicans* (ATCC 10231), *Bacillus anthracis* Sterne strain 34F2, *E. coli* strains J53 and J53(pMG101), and *P. mirabilis* strains LST149 and LST169A.[13] Minimum inhibitory concentrations were recorded as the lowest concentration of silver in which no visible growth could be observed. The cytotoxicity of silver nanoparticles was measured by monitoring the number of viable human keratinocyte cells after incubation n the presence of lysozyme–silver preparations.[13]

4.3. Results and Discussion

4.3.1. Protein-catalyzed Metal Reduction

HEWL was demonstrated to catalyze the formation of silver nanoparticles in the presence of light, with lysozyme being the sole reducing agent (Figure 4). Activity and antimicrobial assays demonstrated that the lysozyme–silver nanoparticles retained the hydrolase function of the native enzyme and were effective in inhibiting growth of *E. coli*, *S. aureus*, *B. anthracis* and *C. albicans*.[13] Remarkably, lysozyme–silver nanoparticles demonstrate a strong antimicrobial effect against silver-resistant *P. mirabilis* strains and a recombinant *E. coli* strain containing the multiple antibiotic- and silver-resistant plasmid pMG101. Results of toxicological studies using human epidermal keratinocytes revealed that lysozyme–silver nanoparticles are nontoxic at concentrations sufficient to inhibit microbial growth. Overall, the ability of lysozyme to assemble silver nanoparticles in a single-step reaction offers a simple and environmentally friendly approach to form stable colloids of nontoxic silver nanoparticles that combine the antimicrobial properties of lysozyme and silver. The results expand the functionality of nanomaterials for biological systems and represent a novel antimicrobial composite for potential aseptics and therapeutic use in the future.[13]

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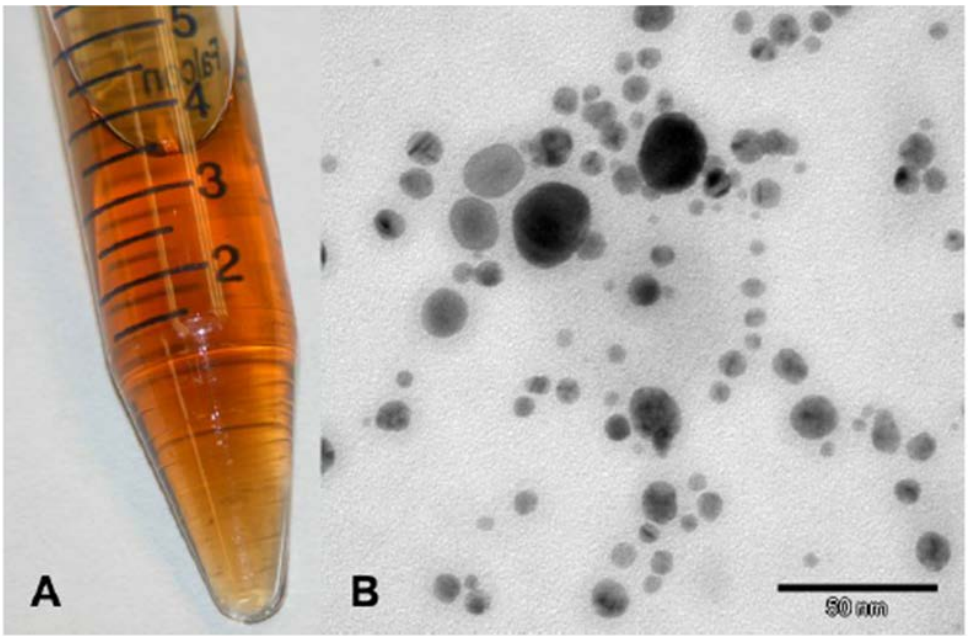

Figure 4. (A) Suspension of Silver Nanoparticles Formed in Lysozyme-catalyzed Process; (B) TEM Image of Silver Nanoparticles

4.3.1.1. Coating Lysozyme–silver Composites to Surgical Surfaces

When exposed to silver ions in methanol, lysozyme acted as the primary reducing agent and formed stable colloidal suspensions of silver (Figure 4). 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.[30] Uniform antimicrobial coatings were deposited on surgical stainless steel blades and needles using an electrophoretic deposition technique. Electrodeposited films firmly adhered to stainless steel surfaces even after extensive washing and retained the hydrolytic properties of lysozyme.

The antimicrobial efficacy of coatings was tested by using blades and needles in an *in vitro* lytic assay designed to mimic the normal application of the instruments. Coated blades and needles were used to make incisions and punctures, respectively, into agarose infused with bacterial cells. Cell lysis was seen as cleared zones at the contact sites, demonstrating that antimicrobial activity is transferred into the media, as well as retained on the surface of the blades and needles. Blade coatings exhibited antimicrobial activity against a range of bacterial species. In particular, coated blades demonstrated potent bactericidal activity, reducing cell viability by at least 3 log within 1.5 h for *Klebsiella pneumoniae*, *B. anthracis* Sterne, and *B. subtilis* and within 3 h for *S. aureus* and *Acinetobacter baylyi*. The results confirmed that complex antimicrobial coatings can be created using facile methods for silver nanoparticle synthesis and electrodeposition, demonstrating not only that the coatings are a self-cleaning surface, but that they can also transfer antimicrobial activity into a subject during use (Figure 5).

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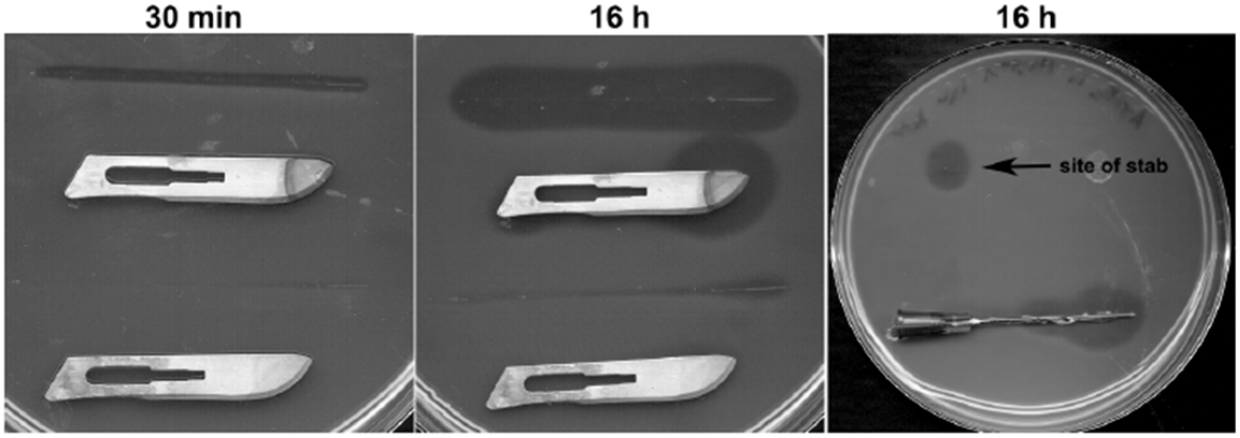

Figure 5. Cell Lysis Assay Measuring Antimicrobial Activity of Coated Blades and Needles: Surgical Blades (left) and Needles (right) were Coated with Lysozyme (top) and Lysozyme–Silver (bottom) and Used to Make Incisions and Punctures, Respectively.

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5. CONCLUSIONS

The rise in multi-resistant pathogens, along with rapid advances in microbial genomics and genetic engineering, raises preventative, therapeutic and decontamination concerns. Because microbes have evolved to overcome present antimicrobial therapies, conventional antibiotics may be ineffective against multi-resistant pathogenic organisms. Consequently, novel methods of materials design and the effective combination of different antimicrobial mechanisms are compelling approaches to counteract resistance to commonly used antibiotics.

Microbicidal materials and coatings have broad application in medical and food processing fields. Additional potential exists for active disinfection/decontamination processes as well as modified materials for personal hygiene to limit biofilm formation on materials exposed to the environment. Two approaches explored within are the fabrication of AMP–inorganic composites and formation of nanoparticulate silver. While these concepts show promise, further study in respect to stability, sustainability, dosage and means of delivery would still be needed before practical applications could be realized. The results of the study will assist in accelerating these types of materials to commercial production and ultimately contribute to mitigation of microbial threats to human health in the future.

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

AMP	antimicrobial peptide
AFRL	Air Force Research Laboratory
ATCC	American Type Culture Collection
HEWL	hen egg white lysozyme
KSL	a synthetic AMP: KKVVFKVKFK
MABL	Microbiology and Applied Biochemistry Team
RXQ	Airbase Technologies Division
RXQL	Airbase Sciences Branch
SANS	small-angle neutron scattering
SEM	scanning electron microscopy
TEM	transmission electron microscopy
TMOS	tetramethyl orthosilicate
XPS	X-ray photoelectron spectroscopy

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