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**SYNTHESIS OF BIOINORGANIC ANTIMICROBIAL
PEPTIDE NANOPARTICLES WITH POTENTIAL
THERAPEUTIC PROPERTIES (POSTPRINT)**

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Synthesis of Bioinorganic Antimicrobial Peptide Nanoparticles with Potential Therapeutic Properties

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Amphiphilicity and cationicity are properties shared between antimicrobial peptides and proteins that catalyze biomineralization reactions. Merging these two functionalities, we demonstrate a reaction where a cationic antimicrobial peptide catalyzes self-biomineralization within inorganic matrices. The resultant antimicrobial peptide nanoparticles retain biocidal activity, protect the peptide from proteolytic degradation, and facilitate a continuous release of the antibiotic over time. Taken together, these properties demonstrate the therapeutic potential of self-synthesizing biomaterials that retain the biocidal properties of antimicrobial peptides.

Introduction

Low molecular weight antimicrobial peptides (AMPs) are present in most living organisms as an integral part of the innate immune system. They function in host defense as antibiotics by providing direct killing through disruption of pathogen membranes. In mammals and other higher organisms, they also function as immunomodulatory agents by interacting with host cells and eliciting a response from the immune system.^{1–4} In many cases, a single peptide sequence has rapid action against a wide range of bacterial and fungal pathogens and, as such, cationic peptides are the focus of a rapidly expanding class of amino acid-based antimicrobial drugs. Their advantage is that they offer a different biocidal mechanism to conventional antibiotics, which are becoming ineffective against the spread of multidrug-resistant pathogens.⁵

Nearly all short peptides containing a considerable portion of hydrophobic residues and carrying a net positive charge will exhibit a direct antimicrobial effect.^{3,6} Building from these characteristics, *de novo* peptide synthesis focusing on cationic and hydrophobic residues have produced an abundance of peptides demonstrating antimicrobial properties.^{7–14} While several hundred peptide sequences have been identified as antibiotics,^{15–17} a considerable amount of research and development into their stability, sustainability, dosage, and means of delivery is needed before they can be used as effective therapeutic agents. For example, the amphiphilic nature of AMPs promote interactions with many salts, as well as with polar and nonpolar molecules, which reduces their effectiveness in saline fluids, such as blood, or when in contact with polyanionic and hydrophobic materials, such as heparin and tissue-culture-treated plastics.^{3,4,18} In addition, inhibitory enzymes present in bodily fluids and expressed extracellularly from pathogenic microorganisms will also decrease effective concentration levels of AMPs.^{19–22} One challenge, therefore, is to develop methods that

will increase the stability of cationic AMPs and protect them from inactivation without repressing their antimicrobial effect.

In many applications in biochemical and biomedical engineering, protein stability and protection is maintained through the use of mineralization or polymerization reagents to encapsulate and retain protein activity (e.g., sol gels, epoxy resins, synthetic phospholipid vesicles, and polymer sponges).^{23–26} Enzyme encapsulation techniques mimic the processes carried out by organisms that biomineralize inorganic macromolecular architecture for protection and structural stability, and the encapsulation methodology draws from the study of biological systems that form these structures. One notable example is a class of biosilica-forming silaffin proteins, which catalyze and direct diatom frustule formation.^{27,28} A synthetic peptide R5 (SSKKSYSYSGSKGSKRRIL), which was derived from a repeat sequence motif within the silaffin precursor protein from the diatom, *Cylindrotheca fusiformis*, mediates the *in vitro* formation of silica nanoparticles. Further investigations using R5 for biosilica formation have led to immobilization of a variety of enzymes for sensor capabilities, catalytic applications, and novel biomaterial production.^{29–39} In a few cases, the applicable enzymes themselves catalyze biomineralization reactions, which result in their self-immobilization and retention of activity within the inorganic matrix without addition of a separate biomineralizing peptide.^{40,41} Whether part of a physiological function or of man-made design, many peptides that catalyze biomineralization reactions (e.g., histidine-rich proteins,^{42–44} noble metal nanoparticle-forming peptides,^{45,46} silaffins, silications, and other lysine-rich proteins⁴⁷) have an overall positive charge and are amphiphilic in nature; characteristics that are also shared with many AMPs. Overlapping traits such as these could be exploited in designing AMPs that mediate their own immobilization, thereby enhancing their effectiveness through increased stability and protection from inactivation.

Materials and Methods

Chemicals and Reagents. KSL (KKVVFVKVFK, C-terminal amide) was synthesized by New England Peptide Co. (Gardner, MA) to 95% purity. Tetramethyl orthosilicate (TMOS), potassium hexafluorotitanate (PHF-Ti), acetic acid, bovine serum albumin (BSA), and type I trypsin from bovine pancreas were all purchased from Sigma Aldrich

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Co. (St Louis, MO). All other reagents were purchased at the highest quality available. Polypropylene 96-well plates for antimicrobial assays were obtained from Corning, Inc. (Corning, NY). Mueller Hinton culture agar and Yeast Mold culture media were from Sigma-Aldrich. Mueller Hinton liquid culture media was from Fluka (Buchs, Switzerland). The following strains were purchased from the American Type Culture Collection (ATCC, Manassas, VA): *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 14990), and *Candida albicans* (ATCC 10231).

Synthesis of Antimicrobial Peptide Nanoparticles. For biomineralization reactions, solutions containing 4 mg mL⁻¹ of KSL in 0.1 M KH₂PO₄ (pH 5) were mixed with either 1 M TMOS (prehydrolyzed in 1 mM HCl) or 100 mM PHF-Ti in ultrapure water (0.9 mL peptide: 0.1 mL inorganic precursor). PHF-Ti solution was heated to 50 °C to aid solubility. The pH of the solution was then raised to approximately 8 using 15 N NaOH (10 μL) to induce precipitation of the peptide and inorganic oxide. Precipitates were pelleted by centrifugation (13000g) for 5 min and washed three times using ultrapure water. Between each wash step, the pellet was completely resuspended by vigorous vortexing and repeated pipetting to break up aggregated particles. Particles were stored as a wet pellet at -20 °C until use. Before use, particles were resuspended to the same volume as the initial biomineralization reaction and the concentration of peptide was determined by subtracting the free peptide in the reaction supernatant and the wash steps from the initial concentration of peptide added to the biomineralization reactions. The same procedure was completed for biomineralization reactions containing 0.8 mg mL⁻¹ of KSL, except the starting pH of the buffer was 8 and reactions were initiated at the addition of TMOS and NaOH was not added to reactions.

Characterization of Silica- and Titania-Antimicrobial Nanoparticles (Si-ANPs and Ti-ANPs). Attenuated total reflectance Fourier transform infrared spectroscopy (ATR FT-IR) was recorded on a Nicolet FT-IR 6700 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Samples were scanned in an ATR cell (Smart Miracle, single bounce) containing a zinc selenide crystal. The data collection was completed using OMNIC 2.1 software. For ATR FT-IR, precipitates were reconstituted in methanol and then dried under vacuum at 60 °C before analysis. Field emission scanning electron microscopy and transmission electron microscopy (FESEM and TEM) were completed by Karen Kelly at the University of Florida Interdisciplinary Center for Biotechnology Research, Electron Microscopy Core Laboratory, Gainesville, FL. FESEM samples were aspirated onto graphite adhesive aluminum stubs, sputter coated 100 Å Au/Pb, and imaged with Hitachi S-4000 (Hitachi High Technologies America, Inc., Pleasanton, CA). TEM samples were resuspended in water, deposited onto 400 mesh carbon-coated Formvar grids, air-dried, and examined with Hitachi H-7000. TEM-assisted energy dispersive spectroscopy (EDS) elemental analysis was completed by Pamela Lloyd at Air Force Research Laboratories, Wright-Patterson Air Force Base, OH. Samples were deposited onto lacey carbon grids and analyzed using a Phillips LaB6 CM20 TEM equipped with a RevolutionEDX system (4pi Analysis Corp., Durham, NC).

High Performance Liquid Chromatography (HPLC). The concentrations of soluble peptide in the postreaction and wash supernatants, in diffusion and trypsin digestion assays, and in culture media during incubation with *S. aureus* were measured by separating the peptide from other solution components on an HPLC system (1100 Series, Agilent Technologies, Santa Clara, CA) equipped with a reverse phase column (Chromolith Performance RP-18e, Merck, Darmstadt, Germany). Samples (150 μL) were removed from reaction buffer or assay media, centrifuged for 4 min at 10000g and 50 μL of the supernatant was used for analysis. A gradient mobile phase of methanol and water with 0.1% trifluoroacetic acid (0 min, 95:5; 7.5 min, 67.5:32.5; 8 min, 40:60; water/methanol, percent) was employed and peptide concentration was measured against known standards.

Antimicrobial Assays. Antimicrobial assays to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal

and fungicidal concentration (MBC and MFC) were completed according to the Hancock modified MIC method.¹⁸ KSL in the free form or in ANPs (4 mg protein) was added to 1 mL of dilution buffer (0.01% acetic acid and 0.2% BSA in deionized water) and used as the concentrated stock for serial dilutions. With respect to the ANPs, the final peptide concentration in the assay buffer was calculated from the initial concentration used in the biomineralization reaction, less the amount lost after nanoparticle synthesis and during subsequent washing of the particles. *S. aureus*, *E. coli*, and *C. albicans* cultures were shaken overnight at 200 rpm at 37 °C in either Muller-Hinton broth (M-H) for bacterial strains or Yeast Mold broth for *C. albicans* (*S. epidermidis* was grown at 30 °C in M-H). Each culture was subcultured into fresh media to a concentration of 10⁵ cells mL⁻¹ and then dispensed into polypropylene 96-well microtiter plates. Polypropylene plates were used to reduce KSL binding to the plastic. Viable cell count was confirmed by growing serial dilutions of cultures on solid growth media. Known concentrations of KSL were then added to wells and incubated for 24 h at 37 °C (*S. epidermidis* was incubated at 30 °C). The MIC was recorded as the lowest concentration of peptide in which no visible growth could be observed. For biocidal determination, the contents of the wells of no visible growth were spread onto solid growth media plates and incubated overnight at 37 °C (*S. epidermidis* was grown at 30 °C). The MBC and MFC were determined at the concentration of KSL at which no colonies formed on the plate.

KSL Diffusion Assays. Si-ANPs (3.8 mg mL⁻¹ peptide in dilution buffer) were added (1:1, v/v) to either the antimicrobial assay media (M-H broth) or in phosphate-buffered saline (PBS) and incubated at 37 °C over a period of 5 d in 24 h cycles. For each 24 h cycle, samples were taken from the assay media and diffusion of the peptide was quantitated by HPLC. In between each 24 h cycle, the supernatant was separated from the nanoparticles by centrifugation (4 min at 10000g) and exchanged with fresh media.

Trypsin Digestion Assays. Si-ANPs and KSL in the free form (4 mg mL⁻¹ peptide concentration) were incubated with the protease trypsin (KSL/trypsin molar ratio, 30000:1) at 37 °C in 0.1 M NaHCO₃ buffer (pH = 7) for a total of 20 min. Samples of the supernatant were periodically removed from the assay buffer and the presence of intact KSL and degradation products was measured using HPLC. After incubation, the Si-ANPs were removed from the reaction buffer by centrifugation (4 min at 10000g) and washed once with 0.5% SDS. A sample of the wash supernatant was analyzed using HPLC to identify any peptide remaining with the Si-ANPs following protease treatment.

Monitoring Soluble KSL Concentration during Incubation with *S. aureus*. A series of replicate cultures (200 μL each) were added to wells of polycarbonate microtiter plates. The cultures consisted of either free KSL or Si-ANPs in M-H broth media (25 μg mL⁻¹ protein) and contained between 10⁶ and 10⁷ cells mL⁻¹ of *S. aureus*. Plates were incubated at 37 °C and, at regular time intervals, one replicate culture was removed and centrifuged, and the soluble peptide concentration in the supernatant was determined by HPLC.

Synthesis and Antimicrobial Activity of Silica and Titania Nanoparticle Synthesized Using a Derivative of the R5 Peptide. A derivative of the R5 peptide containing six additional histidines on the C-terminus (SSKKS₆SGSYSGSKGSKRRILHHHHHH) was used to synthesize silica and titania nanoparticles as described previously.³⁵ Nanoparticles were similar in size and morphology to KSL-synthesized nanoparticles. Antimicrobial assays using these nanoparticles with the four bacterial and yeast strains were completed as described above.

Results and Discussion

The cationic decapeptide, KSL (KKVVFVKVFK)¹⁰ was used as a model template molecule to determine whether the peptide will catalyze self-immobilization into bionanocomposites that retain the antimicrobial properties of the peptide. Originally isolated from a combinatorial peptide array, KSL demonstrates activity against a wide range of microorganisms.^{10,48} KSL (4

mg mL⁻¹ final concentration in 0.1 M KH₂PO₄, pH = 5) was mixed with an inorganic precursor, either tetramethyl orthosilicate (TMOS, 100 mM final concentration, prehydrolyzed in 1 mM HCl) or potassium hexafluorotitanate (PHF-Ti, 10 mM final concentration). When the pH of the solutions was raised to 8 using 15 N NaOH, the solutions turned cloudy within seconds after mixing and a precipitate continued to develop over several minutes. After 5 min, precipitates were centrifuged and washed three times with deionized water. Pellets were resuspended during each wash step using extensive vortexing and repeated pipetting to break apart aggregated particles. Field emission scanning electron microscopy (FESEM) and transmission electron microscopy (TEM) of washed silica precipitates show aggregates of nanoparticles less than 100 nm in diameter (Figure 1). Similar morphology was observed in titania precipitates, except TEM images show that the material is more heterogeneous in composition than the silica preparations. Large smooth spheres that consisted of an electron dense material are shown within a larger matrix of less dense material. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy and TEM-coupled energy dispersive spectroscopy (EDS) confirmed that each set of nanoparticles contained both protein and inorganic oxide, respectively (Figure S1 in Supporting Information). The carbonyl stretching and NH bending vibrations of the peptide backbone amide I and II bonds at 1627 cm⁻¹ and 1534 cm⁻¹, respectively, are clearly observed in the ATR-FTIR spectra. EDS analysis indicated that the nanoparticles contained either titania or silica in corresponding precipitates. High performance liquid chromatography (HPLC) analysis was used to monitor the amount of peptide found in the postreaction supernatant and wash steps. The amount of peptide remained in the reaction supernatant after precipitation with TMOS and PHF-Ti was 3.2 ± 0.4 and 0.2 ± 0.04% of the initial concentration, respectively. A further 1.5 ± 0.3 and 1.0 ± 0.2% (reactions containing TMOS and PHF-Ti, respectively) of the peptide was removed in the supernatant after three wash steps. On average, a total of 95 and 99% of the peptide were retained in silica and titania oxides, respectively, after synthesis and three subsequent wash steps.

The silica- and titania-antimicrobial peptide nanoparticles (Si-ANPs and Ti-ANPs, respectively) formed as a result of a biomineralization reaction between KSL and the inorganic precursor and not solely due to a pH-dependent precipitation of silica or KSL. The KSL concentration used primarily in this study (4 mg mL⁻¹) is not completely soluble at pH 8; an alkalinity which is necessary to induce biomineralization. As a result, KSL was solubilized at pH 5 and NaOH was then added to raise the pH to 8 to catalyze the reaction. In control reactions where TMOS and PHF-Ti were absent, an increase in pH induced precipitation of KSL, but without the inorganic precursor present in the reaction, the pellet was completely resolubilized during subsequent wash steps. Si-ANPs and Ti-ANPs containing protein and the inorganic oxide did not dissolve after repeated washing. To confirm that KSL was essential to biomineralization, a soluble amount of KSL (0.8 mg mL⁻¹) was added to reaction buffer at pH 8. After addition of the precursor, KSL catalyzed inorganic oxide precipitation and nanoparticles did not form when either the precursor or KSL was absent under these reaction conditions (results not shown).

Previous reports indicate that the electrostatic interactions between the positive-charged amide groups of lysine and negatively charged silicate molecules induce nucleation of protein-silica centers, leading to condensation and precipitation of amorphous silica.⁴⁹⁻⁵¹ Because the majority of the charged

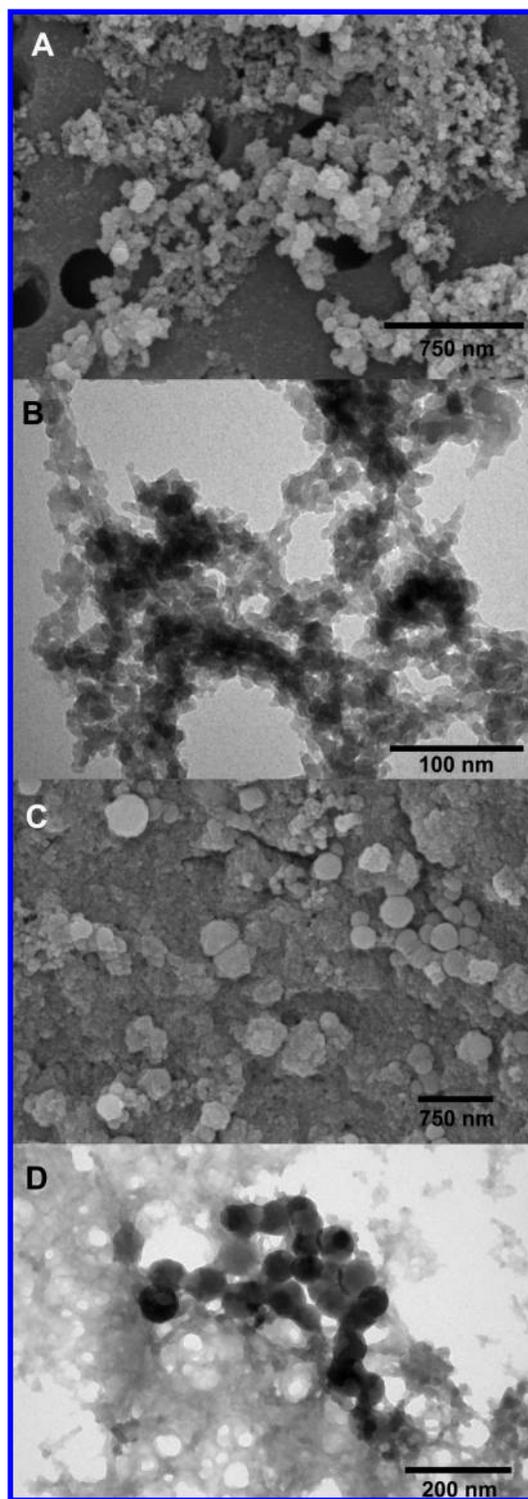


Figure 1. FESEM and TEM micrographs, respectively, of KSL-catalyzed silica (A and B) and titania (C and D) nanoparticles.

species on KSL are the primary amines of lysines, the biomineralization mechanism of KSL most likely follows other polyamine systems. Binding between silica groups and the amide side chains of KSL would leave the remaining phenylalanines and valines exposed to solvent, thereby rendering the surface of peptide-coated composite hydrophobic. The hydrophobic properties of the nanoparticles were evident in the biomineralization reactions containing 0.8 mg mL⁻¹ KSL. In these reactions, Si-ANPs and Ti-ANPs appeared to partition away from the aqueous solvent, adhering to the walls of the plastic tube and migrating to the air-water interface. Nonetheless,

Table 1. Minimum Inhibitory and Biocidal Concentrations of Free KSL, Si-ANPs, and Ti-ANPs

strain	cells ^a	peptide form ^c	total peptide ^d	
			MIC ^e	MBC/MFC ^f
<i>E. coli</i> ATCC 25922	5.27 (2.65) ^b	free	0.8 ± 0.2	0.9 ± 0
		Si-ANPs	3 ± 2	5 ± 2
		Ti-ANPs	2 ± 0.9	6 ± 0.2
<i>S. aureus</i> ATCC 25923	2.71 (2.25)	free	18 ± 8	> 225 ± 0
		Si-ANPs	24 ± 1	80 ± 25
		Ti-ANPs	62 ± 41	91 ± 22
<i>S. epidermidis</i> ATCC 14990	1.64 (1.58)	free	0.6 ± 0.2	1 ± 0.5
		Si-ANPs	1 ± 0.4	3 ± 2
		Ti-ANPs	2 ± 0	3 ± 0
<i>C. albicans</i> ATCC 10231	1.50 (0.58)	free	0.7 ± 0.2	1 ± 0.4
		Si-ANPs	2 ± 1	5 ± 2
		Ti-ANPs	4 ± 1	5 ± 2

^a 10⁵ colony forming units (CFU) mL⁻¹. ^b Standard deviations are representative of at least three assays. ^c KSL added to cell cultures either as the nonbiomineralized, soluble 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 bactericidal or fungicidal concentration.

biomineralization reactions were completed at a higher KSL concentration (4 mg mL⁻¹) for the remainder of the study, which facilitated a greater loading capacity of peptide within the bioinorganic composites. Using KSL concentrations in the reaction in excess of its solubility most likely resulted in nanoparticles consisting of not only a mixture of biomineralizing peptide and inorganic oxide, but also excess peptide that coprecipitated with the solid matrix. The latter fraction of KSL is nonspecifically bound to the inorganic oxide, which should retain the amphiphilic properties of the peptide on nanoparticle surfaces. True to form, nanoparticles synthesized at the higher KSL concentration did not exhibit the same hydrophobic properties as those made at the lower concentration (e.g., mostly adhering to the walls of plastic tubes and floating on the water surface). While it is unknown how the excess peptide is specifically associated to the nanoparticles, it is tightly bound to the inorganic matrix to an extent that it is not readily solubilized because very little is removed during wash steps.

Antimicrobial Activity of ANPs. The antimicrobial activity of Si-ANPs, Ti-ANPs, and soluble KSL without inorganic oxide (free form) was determined using a microtiter broth dilution method modified for cationic AMPs (Table 1).¹⁸ The concentration of KSL retained in the nanoparticles was calculated by subtracting the amount removed during nanoparticle preparation (measured in the postreaction supernatant and wash steps) from the total amount added to synthesis reactions. In most cases, the nanoparticle preparations exhibited a modest increase in the MIC and either the MBC or the MFC against the strains tested when compared to the free form. In a few cases, the nanoparticles provided an equal or greater antimicrobial effect toward bacterial cells. There was no statistical difference between the MIC of free KSL and Si-ANPs determined for *S. epidermidis*. In addition, nanoparticles exhibited an enhanced biocidal activity toward *S. aureus*. An MBC of 80 ± 25 μg mL⁻¹ and 91 ± 22 μg mL⁻¹ was measured for Si-ANPs and Ti-ANPs, respectively, while the maximum concentration of the free form (225 μg mL⁻¹) had little effect on the survival of cells. These results contrast the MIC values determined for *S. aureus*, as the biomineralized forms of KSL were less effective than the free form (18 ± 8 μg mL⁻¹ versus 24 ± 1 μg mL⁻¹ and 62 ± 41 μg mL⁻¹ for the free, Si-ANPs, and Ti-ANPs, respectively). Control experiments show that the inhibitive effect of nanoparticles originates exclusively from KSL and not from the inorganic oxide. Similar silica and titania nanoparticles synthesized using a derivative of the R5 peptide³⁵ did not exert a biocidal effect under the same assay conditions (MIC > 225 μg mL⁻¹ in each assay; see Table S1 in Supporting Information).

The effect of ANPs toward *S. aureus* was surprising; the contrast in the MIC and MBC ratios between the free form and the bioinorganic composites suggests that Si-ANPs and Ti-ANPs exerts a stronger biocidal effect than the free form, yet requires a higher concentration to obtain an equal inhibitory effect. Assuming KSL is not altered in the biomineralization reaction and functions identically to the free form, the differences in the MIC and MBC values likely originate from the effective concentration of peptide that interacts with cells. Because amorphous silica and titania do not inhibit microbial growth under our assay conditions, the antimicrobial activity must be attributed to interactions between the cell membrane and the KSL-bound on the particle surface and the release of soluble peptide from nanoparticles. In models proposing the antimicrobial mechanism of cationic peptides (e.g., toroidal pore and barrel-stave), a conformationally free form of the peptide is required for insertion and orientation across membrane barriers.⁵² While surface-immobilized AMPs have shown to bind to cell surfaces and reduce viability,^{53,54} it is likely a fraction of the encapsulated peptide diffused from the particles and this free form of the peptide contributed to the biocidal effect in the assays.

Diffusion of KSL from Si-ANPs. Analysis of the supernatants following successive washes of the nanoparticles after synthesis showed that the concentration of peptide did not decrease in subsequent wash steps (Figure S2 of the Supporting Information section). The results suggest that KSL will diffuse slowly from nanoparticles and provides a continuous release of free peptide in solution. To gain further insight into the extent of peptide diffusion, Si-ANPs were incubated at 37 °C in either the antimicrobial assay medium or in PBS. Diffusion of the peptide (1.9 mg mL⁻¹ initial concentration) was measured over a 24 h cycle and reached a maximum concentration of 205 μg mL⁻¹ and 633 μg mL⁻¹ in the antimicrobial assay media and PBS, respectively (Figure 2 and Figure S3). KSL released from silica retained antimicrobial activity and was identical to the free form, as determined by antimicrobial assays, UV/vis spectroscopy, HPLC retention, and MS analysis (results not shown). The results confirmed that KSL is not altered in the immobilization reaction. The diffusion assay was extended for four additional 24 h cycles. Between incubations, nanoparticles were collected by centrifugation and the supernatant was exchanged with fresh media. KSL continued to diffuse from the nanoparticles over the length of the experiment. Ultimately, KSL diffusion did diminish and, during the final cycle, the concentration of soluble peptide in the supernatant was minimal. Over the 5 day period, the diffusion assay revealed that an

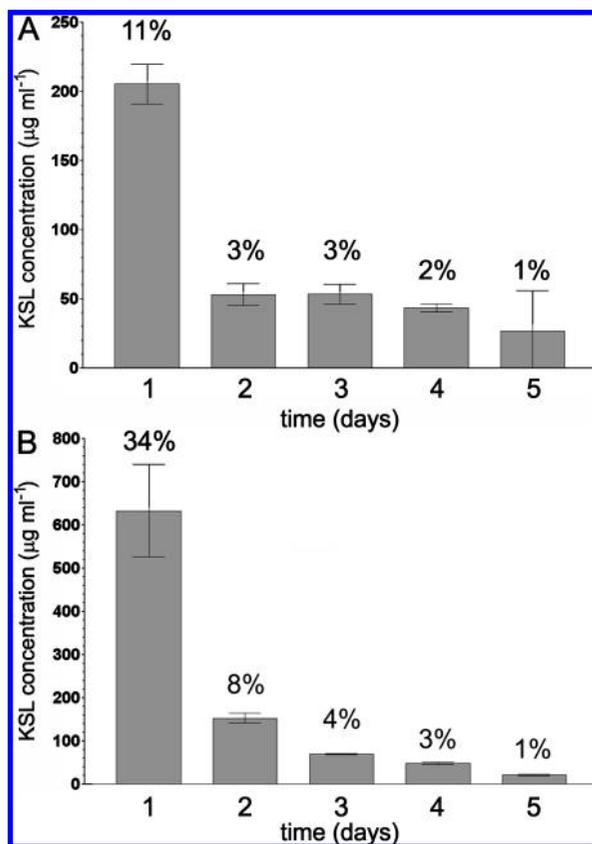


Figure 2. Diffusion of antimicrobial peptide from silica nanoparticles following incubation in antimicrobial assay buffer (A) or phosphate buffered saline (B). The fraction of total peptide (1.9 mg mL^{-1} , final concentration) that diffused within each 24 h time period is listed as a percentage above each bar (see also Figure S3 for more detailed diffusion rates). Standard deviations are calculated from at least three separate assays.

appreciable amount of KSL is bound to silica in such a way that it is released slowly from the particle, yet a significant amount of peptide also remains associated to the nanoparticle. It was important to find whether the peptide that remained entrained in silica following the 5 day diffusion assay was still active and available to provide an antimicrobial effect. Another series of antimicrobial assays was completed using silica nanoparticles recovered after the diffusion experiment. The KSL concentration remaining with the silica was recalculated and added to bacterial cultures to determine the new inhibitory concentrations. No antimicrobial effect was observed when silica nanoparticles that had undergone diffusion were added to *S. aureus* and *S. epidermidis* cultures ($\text{MIC} > 225 \text{ } \mu\text{g mL}^{-1}$, Table S1). An effect was detected in assays containing *E. coli* and *C. albicans*, but MIC values were more than 1 order of magnitude greater than concentrations measured with Si-ANPs before the diffusion assay. The remaining KSL appears to be tightly bound to the silica and this association severely inhibited the function of the peptide. As discussed above, these observations are consistent with the presumption that when nanoparticles are synthesized in excess KSL, a fraction of the peptide is sequestered within the inorganic matrix during the mineralization reaction and the remaining peptide is not covalently associated to the composite. The later fraction is most likely responsible for the continuous-release capacity of the nanoparticles and the fraction of KSL that is sequestered for biomineralization is immobilized within the inorganic matrix to an extent that it does not diffuse from the matrix and effectively interact with cells.

The results of the diffusion and subsequent antimicrobial assays are consistent with the hypothesis that KSL released from the ANPs provides the antimicrobial activity. The remaining KSL that is tightly associated to silica does not contribute to the antimicrobial effect. Therefore, the difference in MIC values between the free and composite forms (Table 1) is most likely a result of a lower soluble KSL concentration in the media containing the ANPs. However, ANPs provided more potent biocidal activity toward *S. aureus* than the free form, even though the concentration of active peptide is arguably lower in assays containing ANPs. The anomaly may be due to a gradual release of KSL from ANPs, which is providing a sustained and biologically available concentration of peptide. In this case, a lower, albeit, more stable and time-dependent dose of KSL may be providing a more effective kill (i.e., lower MBC). In addition, association to the inorganic matrix may protect the peptide from other factors that could otherwise limit its activity. For example, *Staphylococcus* sp. and other microorganisms release degradative proteases and other inactivating proteins as a defense mechanism against AMPs.^{55–60} KSL encapsulated within nanoparticles or adsorbed on their surfaces may be protected against inactivation and its gradual release from the nanoparticle may be facilitating a more sustained and, hence, a more effective dose over time.

Protective Properties of Si-ANPs against Proteolytic Degradation. The degree of protection provided to KSL by the inorganic matrix was analyzed by incubating Si-ANPs and free KSL in the presence of trypsin. When free KSL was incubated with the protease, the peptide was completely degraded within minutes of incubation (Figure 3A,B). No intact KSL was detected and HPLC absorbance peaks corresponding to fragments of hydrolyzed KSL were evident at earlier retention times (Figure 3B). The results for the Si-ANPs treated with trypsin were much different (Figures 3C,D). Here, a fraction of the KSL associated with silica was protected from degradation, as intact KSL was identified in the supernatant after incubation with trypsin (Figure 3C). After washing the trypsin-exposed Si-ANPs with 0.5% SDS to disassociate additional peptide from nanoparticles, HPLC analysis of the wash supernatant proved moreover that a significant amount of KSL was protected by the nanoparticle (Figure 3D). Coupled with a sustained rate of peptide diffusion from the inorganic matrix, the findings were consistent with a mechanism in which nanoparticles can replenish and maintain an intact and active peptide in the presence of proteases and other inhibiting agents.

Monitoring Soluble KSL Concentrations during Incubation with *S. aureus*. Diffusion of antimicrobial peptide from nanoparticles and its persistence in solution over time was determined in the presence of *S. aureus* cells. Results in Figure 4 demonstrate the initial concentration of free KSL is higher in cultures at the start of the antimicrobial assays, in comparison to the soluble concentration released from silica. Within minutes after exposure to cells, the concentration of the free peptide decreases as peptide is sequestered from the supernatant through binding to cell surfaces and other organic material and due to degradation by proteases. In contrast, the soluble concentration of KSL in cultures containing Si-ANPs increases as the peptide diffuses from the silica, overcoming losses resulting from incubation with cells. Regression analysis of the soluble peptide concentrations over the 24 h assay revealed that Si-ANPs maintained a more stable concentration of peptide in the growth medium. The variation in soluble peptide concentrations between the free and the silica peptide preparations during the course of the assay will most certainly affect the culture viability in

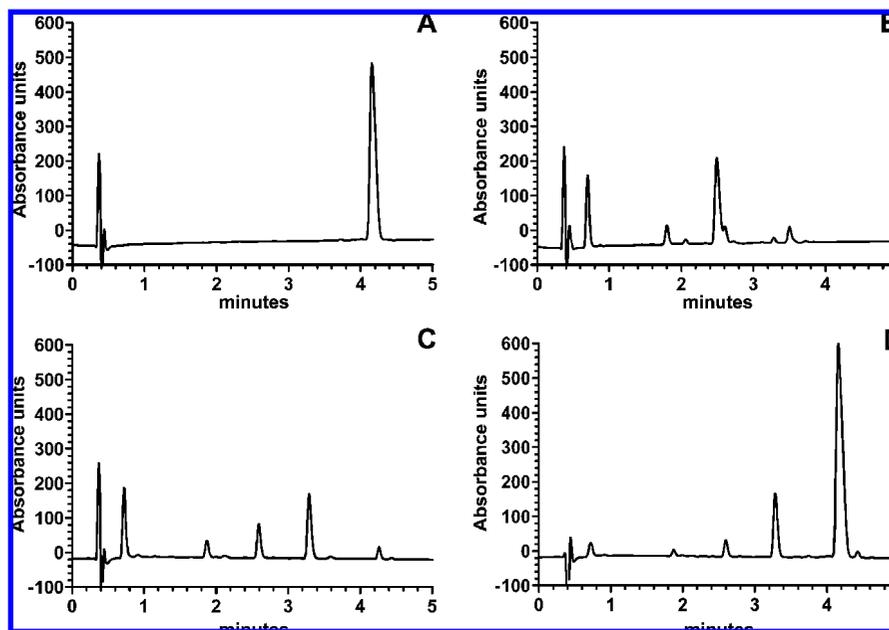


Figure 3. Protease treatment of free KSL and Si-ANPs analyzed using reversed phase HPLC. Free KSL prior to trypsin treatment (A) and the hydrolysate product after incubation with trypsin (B). Soluble hydrolysate product following trypsin treatment of Si-ANPs (C) and KSL peptide and hydrolysate products retained with Si-ANPs after incubation with trypsin and washing the nanoparticles with 0.5% SDS (D). The retention time of intact KSL is at approximately 4.2 min.

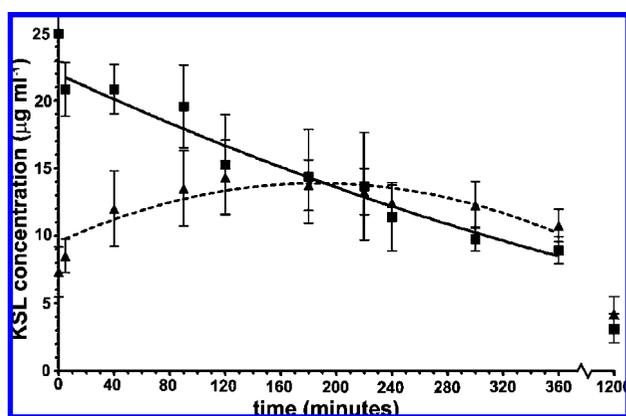


Figure 4. Changes in KSL concentrations during incubation with *S. aureus*. Using HPLC, soluble concentrations of KSL in the free form (squares, solid line) and diffusing from Si-ANPs (triangles, dashed line) were measured during incubation with *S. aureus* cells ($25 \mu\text{g mL}^{-1}$ total peptide with 10^6 and 10^7 viable 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.

different ways. A higher soluble concentration at the start of the assay will exert a greater inhibitive effect and the difference in MIC values of the free and silica-associated forms of KSL is most likely attributed to disparity in the initial concentrations. Alternatively, a more sustained concentration of KSL from silica particles over a longer period of time is likely providing an enhanced biocidal effect on cells, resulting in a lower MBC than measured for the free form.

Conclusion

Recent reviews of antimicrobial and host-defense peptides have delineated the obstacles that must be overcome before use of this emerging class of antibiotics can be fully realized in the therapeutic drug arena.^{3,4} Of these limitations, the development of methods that will deliver an effective dose

of AMPs is of major concern. To inhibit pathogen growth, the mechanism must not only provide a sustained biocidal dose, but must also preserve the active peptide in serum, provide resistance to protease degradation and other inactivating proteins and maintain the AMP concentration at levels that will not promote unintended host toxicity. The self-encapsulating method described here provides a mechanism, *in vitro*, where sustained diffusion of active peptide can be achieved to deliver a controlled dose over an extended period of time. The composite material allows for a release of peptide to replenish concentrations reduced by proteolytic degradation, attachment to bacterial membranes, and other forms of inactivation through nonspecific interactions with salts and other organic material. The ability of ANPs to provide a prolonged and stable concentration of soluble peptide in the presence of cells and proteins is essential to treatments that need to maintain an antibiotic dose that is toxic to pathogens, but nontoxic to the host. In addition to these properties, the ease and versatility of materials synthesis makes the approach a potentially attractive means of drug delivery in a variety of applications. For example, integrating these antimicrobial bionanocomposites with subcutaneous medical devices could result in successful active-release strategies for reducing implant-related infections. As shown in the diffusion assays, an appreciable amount of KSL is released from the nanoparticles during the first 6 h in solution. This rate is applicable to the design of antimicrobial coatings for implants, as a 6 h period after implantation surgery has been identified as a “decisive period” during which inhibition of bacterial growth on the device surface is crucial to the success of the implant.⁶¹ Our results also show that continuous release of peptide in PBS suggests the bionanocomposites may act efficiently in isotonic solutions commonly used in medical practice. It is important to note that the toxicological effects are also of substantial importance to the potential of novel therapeutic agents. While separate studies have shown nanoparticulate silica and KSL are nontoxic,^{48,62} the composites presented here will need to undergo extensive

toxicological analysis before they are to be considered as therapeutic drugs.

The field of study represented here holds the promise for developing a wide range of nanomaterials that integrate AMPs with inorganic composites. Combinatorial peptide arrays have been used successfully in distinct studies to identify peptides for each respective function: those with inherent antimicrobial activity^{8–10} and those that perform biomineralization reactions.^{63,64} By tailoring selection and isolation methods, existing arrays can be screened for peptides that perform both functions, thereby generating antibiotics that self-assemble into specialized inorganic materials. Future work in this direction may provide multiple classes of antimicrobial biomaterials for specific applications, not only for drug delivery, but also to expand the repertoire of self-sterilizing materials for the food, cosmetic, and medical industries.

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Supporting Information Available. FT-IR spectra and elemental analysis of Si- and Ti-ANPs; measurement of peptide loss during nanoparticle synthesis and preparation; detailed peptide diffusion rates from nanoparticles during incubation in media for 5 days; MIC determination of silica nanoparticles synthesized from a derivative of the R5 peptide and Si-ANPs after the 5 day diffusion assay. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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