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Engineered Chimeric Peptides as Antimicrobial Surface Coating Agents toward Infection-Free Implants

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Abstract

Prevention of bacterial colonization and consequent biofilm formation remains a major challenge in implantable medical devices. Implant-associated infections are not only a major cause of implant failures but also their conventional treatment with antibiotics brings further complications due to the escalation in multidrug resistance to a variety of bacterial species. Owing to their unique properties, antimicrobial peptides (AMPs) have gained significant attention as effective agents to combat colonization of microorganisms. These peptides have been shown to exhibit a wide spectrum of activities with specificity to a target cell while having a low tendency for developing bacterial resistance. Engineering biomaterial surfaces that feature AMP properties, therefore, offer a promising approach to prevent implant infections. Here, we engineered a chimeric peptide with bifunctionality that both forms a robust solid-surface coating while presenting antimicrobial property. The individual domains of the chimeric peptides were evaluated for their solid-binding kinetics to titanium substrate as well as for their antimicrobial properties in solution. The antimicrobial efficacy of the chimeric peptide on the implant material was evaluated *in vitro* against infection by a variety of bacteria, including *Streptococcus mutans, Staphylococcus. epidermidis,* and *Escherichia coli,* which are commonly found in oral and orthopedic implant related surgeries. Our results demonstrate significant improvement in reducing bacterial

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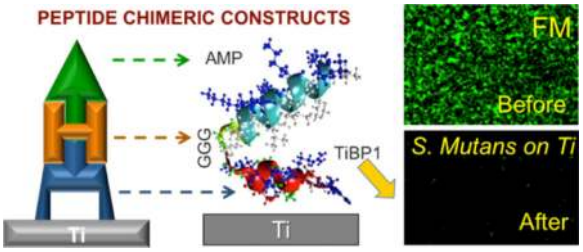
ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.5b03697. Further information including MALDI-TOF analyses of synthesized peptides, calculations for statistical analysis, in solution antimicrobial activity of TiBP1 and TiBP2 and the bifunctional peptides with various concentrations against *S. mutans, S. epidermidis, E. coli,* surface characterization of titanium via using SEM and EDS analysis, and computational model of Ramachandran plots of bifunctional peptides (PDF)

The authors declare no competing financial interest.

colonization onto titanium surfaces below the detectable limit. Engineered chimeric peptides with freely displayed antimicrobial domains could be a potential solution for developing infection-free surfaces by engineering implant interfaces with highly reduced bacterial colonization property.



Keywords

chimeric peptides; antimicrobial peptide (AMP); titanium binding peptides (TiBP); surface functionalization; infection; modular peptides; peptide-based implant coatings

1. INTRODUCTION

Local and systemic infections remain a significant problem in the field of medical device implantations. Such infections have been proven to be difficult to treat at the implant site using systemic antibiotics due to the unique and complex micro-environment within the human body.[1–3] Resistance of implant infections to systemic antibiotics and the host immune response have fueled substantial interest in the research and development of biomaterials to prevent bacterial adhesion or completely eliminating infection.[4] To achieve this, various kinds of coatings have been developed to improve antimicrobial properties of biomaterials surfaces specifically for Ti-based implants including antibiotics loading,[5] covalently attaching antimicrobial peptides,[6–9] doping of inorganic bactericides,[10] polymer-based surface functionalization,[11,12] nonantibiotic organic bactericide-loaded coatings, and antifouling coatings.[13] While these methods have been shown to reduce bacterial adhesion on implant surfaces, existing immobilization strategies often require cumbersome and complex procedures. These include the presence of specific, often covalently bound, functional groups on the solid surface, extensive optimization of the procedures that often have a limited capacity to be used in practice for modification of a wide variety of implant materials.[14] Additionally, the loading of antibiotics and other antimicrobial agents onto biomaterials has raised concern about a possible link to increased bacterial resistance and toxicity.[15]

To combat the issues of antibiotic resistance and toxicity, we offer a novel surface functionalization technique utilizing antimicrobial peptides (AMPs). As an evolutionary conserved constituent of the innate immune response AMPs are believed to target the negatively charged cell membrane of bacteria and possibly the embedded lipids bearing phospholipid head-groups, mediating killing by membrane disruption or transmembrane pore formation.[16,17] They are effective against a broad range of microorganisms and can work synergistically with classical antibiotics, possibly facilitating the access of antibiotics to the infection site and, thus, providing an avenue for a more aggressive treatment approach

against biofilm formation. Although they can be candidates for clinical applications,[18,19] the major barrier for the use of AMPs as antibiotics or injectable therapeutics is their uncontrolled toxicity or ability to lyse eukaryotic cells.[20] To circumvent these potential harmful effects, the immobilization of AMP on implant surfaces is a practical way to effectively use it as a local therapeutic agent.[8] Antimicrobial peptides have been proposed to covalently attach to the implant materials such as titanium and hydroxyapatite using silane- and thiol-based carbodiimide chemistry or by delivering them via polymer based resins.[11,12,21] Besides not being biofriendly, these approaches are also not adequate to control orientation and structural conformation of the AMPs that could directly affect the function of surface-immobilized antimicrobial peptides.[8,19]

Biomolecular linkers that can induce controlled interactions at the bio/material interface while having high affinity to the implant material could also be a way to immobilize AMPs onto the implant surfaces. During the past decade, the potential utility of the solid binding peptides as surface functionalizing agents has increasingly been demonstrated in a wide range of applications including tissue engineering and regenerative and restorative medicine.[22–25] Specifically, there has been a great deal of interest in identifying and characterizing peptides[26] that bind to various biomaterials and implants, such as Ti, TiO_2, Au, Pt, SiO_2, calcium phosphate minerals, graphite, and structural polymers.[22,24,25,27–29] Using solid binding peptides, desired biomolecules such as AMPs could be immobilized onto the surfaces with orientation control which is critical for their biochemical accessibility.

Building upon the solid-binding peptide approach, we engineered bifunctional peptides that are composed of both a solid-binding peptide with affinity to the implant surface and an AMP motif[8,30,31] (Figure 1). Here, we employed titanium (Ti) as a common material used for a wide variety of implanted devices.[32] The chimeric bifunctional peptide construct features high-affinity Ti-binding property on the one end while exposing the AMP motif on the other and, hence, forming an effective and highly versatile biomolecular tool to bind to the surface while retaining the combating activity against the invading bacteria. In this study, two such bifunctional peptides were synthesized, TiBP1-GGG-AMP and TiBP2-GGG-AMP, by conjugating each of the two previously identified titanium-binding peptides (TiBP1 and TiBP2)[23] with an AMP sequence which features well-known cationic properties displaying an α-helical structure.[33]

The designed bifunctional peptides were characterized both in solution and on the Ti surface to determine their concomitant solid-binding property and antimicrobial efficacy against three bacteria *Streptococcus mutans* and *Staphylococcus epidermidis,* as Gram-positive ones, and *Escherichia coli,* as a Gram-negative one. The principles laid out in this work, e.g., modularity of the component peptides, could be applied to other AMP sequences with a variety of structures and functionalities and expanded to metallic, ceramic, or polymeric biomaterial surfaces by using the solid-binding peptides with specific amino acid sequences resulting in solid-specific affinities.

2. EXPERIMENTAL SECTION

2.1. Peptide Synthesis and Purification

The peptides AMP, TiBP1-GGG-AMP, and TiBP2-GGG-AMP (Table 1) were synthesized by a standard solid phase peptide synthesis technique on Wang resin (Novabiochem, San Diego, CA) using *f-moc* chemistry. A CS Bio Co. CS336S automated peptide synthesizer (Menlo Park, California, USA) and HBTU activation were used for the synthesis. The resulting resin-bound peptides were cleaved and side-chain-deprotected using Reagent K (TFA/thioanisole/H_2O/phenol/ethanedithiol (87.5:5:5:2.5)) and precipitated by cold ether. Crude peptides were purified by RP-HPLC up to >98% purity (Gemini 10u C18 110A column). The purified peptides were confirmed by mass spectroscopy (MS) using a MALDI-TOF mass spectrometer (see Supporting Information Figures S1, S2, and S3). The 4 mM stock solutions of each peptide were made in sterile deionized water by dissolving the peptides. Subsequent dilutions for experiments were carried out with sterile 1X PBS.

2.2. Titanium Surface Characterization

Surface properties of 0.5 mm thick 99% titanium foil (Alpha Aesar, Cat# 43677) were determined by scanning electron microscopy (SEM). The SEM images and EDS spectra were recorded at 9 keV accelerating voltage by using a LaB_6 filament as the electron source. The EDS spectra were collected for 100 s at approximately 1,500 counts per second (cps) (see Supporting Information Figure S4).

2.3. Quartz Crystal Microbalance (QCM) Experiments - Determination of Solid-Binding Activity of the Peptides

The QCM was used to quantify the values of the binding strength of the titanium-binding and bifunctional peptides. Five-megahertz quartz crystals (Q-Sense, Linthicum, MD) were coated with 25 nm of titanium via physical vapor deposition, and the coated crystals were used in a KSV QCM-D Z500 parallel flow system, which monitors frequency change over time. Peptides were diluted in PBS buffer at various concentrations and introduced to the crystal surface by a flow cell. The flow was stopped, and the peptides were allowed to bind to the surface until reaching equilibrium. Each concentration was flowed several times to avoid depletion of the peptide in the flow cell. The binding activity of the peptides was observed by the frequency shift, which is directly related to the wet mass of the adsorbed peptide. To determine the dissociation constant (K_D) of each peptide on the titanium substrate, the equilibrium frequency shift caused by peptide binding was measured at several concentrations. These values were then fit using the Langmuir adsorption model.[23,34–36] Initially, peptide concentrations of 0.1 to 2 μM were used. These concentrations were then adjusted as necessary to be in a similar range as the K_D value of each of the peptides. Titanium surfaces were, first, cleaned by using a surfactant solution (1% SDS, 1 N NaOH) for overnight and then washed with DI water for several times, followed by drying with the nitrogen. The crystals were placed under ozone cleaning for 15 min before reuse.

2.4. Modeling Studies via Molecular Dynamics

We modeled AMP, TiBP1-GGG-AMP, and TiBP2-GGG-AMP by first building linear forms using the HyperChem's molecular modeling software (Hyperchem 7.5, USA). The energy minimization of these peptides was then carried out under implicit solvent conditions using the conformational analysis program. In this procedure, the conformational search module finds the minimum-energy structures by varying the chosen dihedral angles toward creating new initial structures. In each round of energy minimization, unique low-energy conformations are stored, and high-energy and duplicate structures are discarded. Using the conformational search module, we found 1,000 different local minima on the potential energy surface, and we chose the lowest one as the global minimum or the lowest-energy conformation.[37,38] Then, the lowest energy conformations are solvated with TIP3P water explicitly. Finally the overall system is energy minimized using the Polak-Ribiere conjugate gradient method until convergence of the gradient (0.01 kJ/mol) was reached using the CHARMM 27 force field.[39] The final configurations and the corresponding Ramachandran Plots were generated using the VMD (Visual Molecular Dynamics) software[40] (see Supporting Information Figure S5).

2.5. Circular Dichroism (CD) Spectroscopy Experiments

A solution containing 50 μM peptide, PBS at pH 7.4 was prepared for circular dichroic analysis. The spectrum, which is the average of 16 scans from 185–260 nm with a 0.5 nm/s scan rate, was collected at 20 °C using an AVIV Stopped Flow 202SF CD spectropolarimeter. The averaged spectrum, which was then subtracted with the appropriate buffer background, was smoothed using the Savitzky-Golay algorithm. A section of the smoothed spectrum (from 190–240 nm) was compared to the five component reference spectra [(1) α helix, (2) β sheet, (3) β turn Type-I, (4) β turn Type-II, and (5) random coil] compiled by Reed et al., using a constrained least-squares fit. Note that the standard spectra do not consider any aromatic nor disulfide dichroic contributions. This is appropriate because the analyzed peptides do not contain significant nonstructural features. (TiBP2 contains only one peptide with an aromatic residue, Y.) The secondary structure estimates are reported as the fractional weight ± the standard deviation. All spectral smoothing and secondary structure estimation were executed using commercial graphing software (IGOR Pro. 6.0). Ellipticity is reported as mean residue ellipticity, θ_M (deg cm^2 dmol^{-1}).

2.6. Bacterial Maintenance and Culturing

Three bacteria species - *Escherichia coli* ATCC 25922, *Streptococcus mutans* ATCC 25175, and *Staphylococcus epidermidis* ATCC 29886 - were used in the present study. All of them were cultured according to ATCC protocol using the following media: Tripticase Soy Broth (TSB) (Fluka, 22092) for *E. coli,* Brain Heart Infusion (BHI) Broth (Sigma-Aldrich, 53286) for *S. mutans,* and Nutrient Broth (NB) (Difco 0003) for *S. epidermidis*. For all three bacterial species, the bacterial pellet obtained from ATCC was rehydrated in 0.5 mL of the above-specified media, and several drops of the suspension were immediately placed and streaked on an agar slant of the specified media. The agar-plate was then incubated aerobically at 37 °C for 24 h (and in the presence of 5% CO_2 in the case of *S. mutans*). *S. mutans* overnight cultures were made by aseptically transferring a single-colony forming

unit into 10 mL of BHI, followed by aerobic incubation at 37 °C in the presence of 5% CO_2 for 16 h under static conditions. Overnight cultures of *S. epidermidis* and *E. coli* were made by aseptically transferring a single-colony forming unit into 10 mL of NB or TSB (respectively), followed by aerobic incubation at 37 °C with constant agitation (200 rpm) for 16 h.

2.7. In-Solution Antimicrobial Activity of Bifunctional Peptides

The in-solution antimicrobial activities of the peptides were analyzed for *S. mutans, S. epidermidis,* and *E. coli* spectrophotometrically. For each bacteria species, peptide solutions (AMP, TiBP1-GGG-AMP, and TiBP2-GGG-AMP) were added in specified media to reach different final concentrations from 10 μM to 200 μM and inoculated with the bacteria to a final concentration of 10^7 cells/mL. Bacterial growth at 37 °C was monitored over the course of 24 h by optical density measurements at 600 nm on a Tecan Safire Spectrophotometer No I 112 913. For each experiment, a positive control consisting of solely 10^7 cells/mL of bacteria in the specified media and another negative control consisting of only media were monitored as well. Additionally, bacterial growth of the three bacteria species in the presence of TiBP1 and TiBP2 was also monitored to determine if the titanium-binding peptides exhibited any antimicrobial features.

2.8. Titanium Substrate Preparation and Peptide Immobilization

0.5 mm thick, 99% Titanium Foil (Alfa Aesar 43677) was cut into 1 cm × 1 cm squares. The titanium substrates were cleaned by sonicating them for 15 min each in a 1:1 acetone:methanol mixture, then isopropyl alcohol, and finally deionized water. Before proceeding any cell adhesion experiment, surface properties and substrate purity were examined with SEM and EDX analysis (see Supporting Information, Figure S4).

Following all bacterial-adhesion experiments, substrates were first soaked overnight in a 1:1 mixture of 20% bleach:70% ethanol before being cleaned by the above regimen. Cleaned 1 cm^2 titanium foil substrates were transferred into a presterilized 24-well plate. The substrates were then sterilized under UV light for 15 min on each side. Substrates were subsequently incubated aerobically at 37 °C under constant agitation (200 rpm) with 500 μL of 1X PBS (positive control), AMP, TiBP1-GGG-AMP, or TiBP2-GGG-AMP solutions for 4 h. The used peptide concentrations were 200 μM for *S. mutans* and 50 μM for *S. epidermidis* and *E. coli* experiments determined by minimal inhibitory peptide concentration for each bacteria. Following the 4 h of incubation with peptides, the peptide solutions were removed from each well. Twice 1 mL of sterile 1X PBS was then added to each well, pipetted up-and-down twice, and removed from the well. Using sterile forceps, each titanium substrate was moved to a clean well, free of any peptides.

2.9. Bacterial Adhesion on Titanium Surface

To proceed with bacterial adhesion experiments, overnight cultures for each bacterium were prepared as described above. Bacteria from the overnight cultures were used to inoculate fresh media to a final concentration of 10^7 cells/mL. Cultures were then incubated in the same manner as the overnight cultures were (see Bacterial Maintenance and Culturing), until they reached the mid log phase as determined by optical density measurement at 600 nm (*S.*

mutans O.D. ≈ 0.4, *S. epidermidis* O.D. ≈ 0.25, and *E. coli* O.D. ≈ 1.0). At the mid log phase, the cultures were centrifuged at 4000 rpm for 5 min in a Sorvall RC 5B Plus Centrifuge. The supernatant was removed, and the bacterial pellet was resuspended in 500 μL of specified media. This suspension was then transferred to a 2 mL centrifuge tube and centrifuged at 5500 rpm for 3 min in a Fischer Scientific accuSpin Micro Centrifuge. The supernatant was carefully removed, and the bacterial pellet was resuspended in sterile 1X PBS to a final concentration of 10^8 cells/mL. Then, 1 mL of the 10^8 cells/mL cell suspension was added to each well containing a peptide-modified titanium substrate and incubated for 2 h. For *S. mutans* experiments, incubation was carried out aerobically at 37 °C in the presence of 5% CO_2 under static conditions; for *S. epidermidis* and *E. coli* experiments, incubation was carried out aerobically at 37 °C under constant agitation (200 rpm). After 2 h incubation, first the bacterial suspension was removed, and then the surfaces were washed two times with 1 mL of 1X PBS by pipetting. At the end of the experiment, adhered cells to titanium substrates were fixed with 500 μL of 2% glutaldehyde (cat # 18432, Ted Pella, Redding, CA) (in 50 mM Tris Buffer, pH 7.4) for 30 min, followed by dehydration in a series of increasing alcohol baths (50% ethanol for 10 min, 70% ethanol for 10 min, 90% ethanol for 10 min, followed by a 1 mL wash with 100% ethanol).

2.10. Characterization of Bacterial Adhesion on Bifunctional Peptide Modified Titanium Substrates

500 μL of 5 μM SYTO9 Green Fluorescent Nucleic acid stain (Invitrogen, S3485) was added to each well containing a substrate, protected from light, and incubated for 20 min. Substrates were then washed 3 times with 1 mL of 1X PBS by pipetting the PBS up-and-down two times. Following, the substrates were secured onto a clean microscope slide and viewed under a Nikon Eclipse TE2000-U Fluorescent Microscope. Five random images of each surface were taken and analyzed for percent surface coverage using Meta Morph (Version 6.r6) software. Two independent analyses were conducted and averaged for each image. For each substrate, the averaged value for each of the five images that were taken from different regions were averaged together and subjected to statistical analysis.

2.11. μ-Contact Printing of TiBP1-GGG-AMP with *S. mutans* and *E. coli*

The engineered peptide, TiBP1-GGG-AMP, was stamped onto a clean titanium-substrate in a manner previously described.[41] The patterned side of the polydimethylsiloxane (PDMS) stamp was incubated in 200 μM and 50 μM of TiBP1-GGG-AMP for 5 min, and then the peptide solution was removed by pipet from the surface of the stamp to further incubate with *S. mutans* and *E. coli*, respectively. The stamp was dried with inert gas, followed by a brief washing with 1X PBS. The titanium substrate was then applied to the surface of the stamp and pressed using force for 10 s and left on the stamp for 1 min. Then the substrate was removed from the stamp and washed twice with 1X PBS. The peptide-modified substrates were first subjected to the bacterial adhesion and next FM labeling as we described above.

2.12. Statistical Analysis

The surface coverage data of FM was analyzed using SPSS 15.0 software. Statistically significant values were defined as $p < 0.001$ based on Kruskal–Wallis Test, which is the one-

way analysis of variance. The Mann-Whitney U test was used for multiple comparisons (see Supporting Information Tables S1, S2, and S3).

3. RESULTS AND DISCUSSION

Figure 1 demonstrates a chimeric peptide based approach where a resulting peptide having a modular structure incorporates functionally different amino acid domains that can be synthesized as a single peptide.

3.1. Design of TiBP-AMP Bifunctional Peptides To Functionalize the Titanium Surface

There are multiple factors that may affect the antimicrobial activity of the bound peptides. Parameters such as surface concentration of bound AMPs as part of chimeric peptides, spacer length to keep bifunctionality of the peptides intact, structural flexibility/rigidity of the spacer, molecular conformation, and hydrophobicity of the peptides need to be investigated. These properties are interrelated and, which, all together dictate the efficacy of AMPs as a domain in a chimeric construct.[8,20,42–45]

Herein, we demonstrate the use of solid binding peptides as a simple biomolecule for the immobilization of AMPs onto the desired biomaterial surface. The solid binding peptides offer several advantages, one of which is the biomolecular self-assembly retained in the chimeric forms as well as the ease of manipulation via biochemical procedures. Thus, based on these promises, we designed bifunctional peptide constructs containing both solid-binding property to a titanium implant surface and antimicrobial activity against the bacteria. Two sets of bifunctional peptides, named, TiBP1-AMP and TiBP2-AMP, were designed and synthesized. The conjugation was accomplished using a flexible triple glycine (G) linker, which does not interfere with peptide activity in either of the terminals (Table 1).

The main reason for choosing GGG as a flexible spacer is that it is hypothesized to allow lateral mobility of the AMP on the solid surface, maintaining its antimicrobial activity. Besides the preference of the linker, physicochemical properties of chosen AMPs such as its charge and 3D molecular structure also need to be considered for efficiency of the antimicrobial activity.[8,44] The AMP used herein has an α helical structure and a +6 cationic charge. It was conjugated with TiBP1, which is Arg rich and has a +3 cationic charge. In the second chimeric system, the AMP was also combined with TiBP2, which is Ser rich with an overall neutral charge. The designed bifunctional peptides (TiBP1-GGG-AMP and TiBP2-GGG-AMP) and AMP alone have different physicochemical properties as listed in Table 1 in terms of charge, pI, and gravity.

In the design of the bifunctional peptide, the main objective is to retain the functionality of each domain, and consequently each domain's capability should display its desired functionality and bioactivity. To assess these functionalities quantitatively, two separate assays were developed: First, the binding affinity of the peptide constructs to the titanium surface was evaluated using QCM. Next, the effect of the overall molecular structure related parameters on the antimicrobial activity was analyzed by a combination of techniques including CD, molecular modeling, and quantitative imaging studies.

3.2. Adsorption Behavior of Bifunctional Peptides on the Titanium Surface

Depending on the immobilization strategy, the coverage of the adsorbed peptide on the surface affects the degree to which the AMP is effective. This clearly depends on the accessibility of the reactive groups of the peptide to the bacterial membrane.[8,42,43] There is a need to determine critical AMP concentration on the surface or surface coverage for suffcient antimicrobial protection.[8,42] For this purpose, we used quartz crystal microbalance spectroscopy (QCM) to obtain quantitative peptide binding analysis. The adsorption behavior of both bifunctional peptides was analyzed by following the frequency shift data obtained from the QCM measurements. By converting the frequency shift to the wet-mass data and applying the Langmuir adsorption model to the adsorption profiles, we obtained K_D (dissociation constant) and ΔG (the free energy of adsorption) for each peptide. In these analyses, the lower the K_D value, the more strongly the peptide binds to the solid surface.

Not surprisingly, both of the bifunctional peptides were found to have stronger binding affinities to titanium than the AMP, the control peptide, alone (Table 2). TiBP1-GGG-AMP exhibits a 2.85-fold lower K_D value than AMP, while TiBP2-GGG-AMP exhibits a 1.5-fold lower K_D value than AMP. Therefore, TiBP1-GGG-AMP has a stronger binding affinity to the Ti surface than the construct using TiBP2. The observed molecular adsorption of the AMP alone to the Ti surface may be due to its nonspecific binding affinity. We observe a notable degree of nonspecific binding of the AMP to the titanium surface. This may be due to the electrostatic interaction between highly charged AMP and the negatively charged titanium surface. Regarding peptide-titanium surface interactions, polar and positively charged amino acids (Lys, Arg, His, Asp, and Glu) have been considered to have interplay at the solid interfaces.[46–50] Additionally, the role of metal surface charge on peptide binding was also investigated specifically on gold surfaces.

The results reveal that excess negative charge on the gold surface plays an important role on the peptide binding properties under nonpolarized conditions.[51] In addition to the degree of surface binding, the other design criteria, such as total charge, charge distribution, and structural folding, and molecular architectural characteristics of peptides, may also play a critical role on the retained antimicrobial activity of the chimeric peptide when adsorbed on the surface.[23,34] In the following section, we provide structural properties for the three peptides that have been evaluated for their interactions with the surfaces.

3.3. Molecular Conformation of Bifunctional Peptides

Determining the molecular structure of a novel protein or a peptide is critical to relate to its function. The bifunctional peptide constructs are comprised of 29 amino acids and, therefore, are amenable to performing CD spectroscopy experiments to analyze their molecular conformational structures. Figure 2 (a) shows the CD spectra of the three peptides (AMP, TiBP1-GGG-AMP, TiBP2-GGG-AMP) under experimental buffer conditions (PBS buffer, see the Experimental Section). Among the three peptides, there are differences in terms of their secondary structures. Because of the short sequences, all three peptides exhibit mostly unfolded or intrinsically disordered structures, in general, but they also have some difference in the degree of beta turn content, a common feature of many short peptides. To further analyze the structures and predict ternary conformations, we used computational

modeling methods as shown in Figure 2 (b-d). The structure of the AMP (Figure 2 (b)) appears to have an α-helical structure, in accord with the literature.[33] We next predicted the bifunctional peptide structures for both TiBP1-GGG-AMP and TiBP2-GGG-AMP as shown in Figure 2 (c) and (d). In these figures, we used two different coloring modes: one for the backbone to differentiate the AMP (cyan), linker (yellow), and the TiBP1 (red) and the TiBP2 (blue) domains and the other for the side chains to differentiate the polar (green), nonpolar (gray), acidic (red), and basic (blue) residues.

These results indicate that the AMP domain preserves its 3-D structure, while linked to the solid binding terminal via GGG linkers in both chimera. It is predicted that AMP will preserve its function within the context of the dual-functionality. In terms of the interdomain interactions between the AMP and the TiBPs, TiBP2 has more interactions compared to TiBP1 with the AMP (Figure 2 (c,d)). This may lend that AMP has more functionality while linked to TiBP1 compared to TiBP2, the extent of which is also explored and characterized by experiments as discussed below. These structural differences may be the result of both the amino acid content and the amino acid order of each peptide. Both TiBP1 and AMP contain a high number of positively charged residues (Arg, Lys) where TiBP2 has more polar (Ser) amino acid content. The models of TiBP1-GGG-AMP indicate each peptide domain poses apart from each other (Figure 2 (c)). It has more open structural conformation (i.e., the AMP and the TiBP1 domains have more independence from each other) that can be the result of the repulsion between the positively charged residues such as Arg in TiBP1 and Lys in AMP. This structural feature may improve accessibility and functionality of each peptide domain that may affect the antimicrobial properties. TiBP2-GGG-AMP as another designed peptide as shown in Figure 2 (d) has more compact and structured features. The reason for this compact structure may be due to the attraction force between Ser and Arg.

3.4. Antimicrobial Activity of Bifunctional Peptides in Solution

The antimicrobial efficiency of TiBP-AMP bifunctional peptides was evaluated in solution against three species of bacteria *S. mutans*, *S. epidermidis*, and *E. coli*. These chosen bacterial species are common in oral and orthopedic implant infections. The “minimum inhibitory concentration” (MIC) was determined through optical density measurements by testing the stability of each peptide for at least 8 h after the bacteria had entered the mid log phase.

To attain the MIC of peptides, the peptide concentration was gradually increased from 10 μM to 200 μM for each bacterium. Various peptide concentrations at different time points were used for *S. mutans*, as shown in Figure 3 (a) (and also in Figure S6 (a-d), Supporting Information). The same experimental setup was utilized for the other two bacteria species (*S. epidermidis and E. coli*) where 10–25 μM and 50 μM peptide concentrations were used, respectively, as shown in Figures 3 (b) and 3 (c) (and also in Figure S7 (a-b), Figure S8 (a-b) Supporting Information).

Based on the gradually increased peptide concentrations, the MIC was found for two bifunctional peptides (TiBP1-GGG-AMP and TiBP2-GGG-AMP) to be 200 μM for *S. mutans* (Figure 3 (a)), 50 μM for *S. epidermidis* (Figure 3 (b)), and 50 μM for *E. coli* (Figure 3 (c)). To complete the analysis of all peptide sequences, the antimicrobial activity of only

TiBP1 and TiBP2 was also tested in the presence of each of the three species of bacteria as the negative control (Figure S9). As shown in Figure S9, neither of the peptides alone displayed any antimicrobial activity at any concentration from 50 to 200 μM tested.

In summary, the antimicrobial activity of the AMP in solution is the most pronounced; however, the bifunctional peptides also have significant antimicrobial activity although in slightly higher concentrations. More specifically, the AMP is effective in solution at low concentrations for *S. epidermidis* (Gram-positive) and *E. coli* (Gram-negative) (10 μM) (Figure S7 and Figure S8) and for *S. mutans* at 25 μM (Figure S6). Bifunctional peptide efficacy, on the other hand, is recognized at higher peptide concentrations, i.e., 200 μM for *S. mutans* and 50 μM for *S. epidermidis* and *E. coli*, respectively.

These results of AMP and its bifunctional molecular constructs in solution may be compared with the information in the literature regarding the models on the AMP activity based on membrane properties of the Gram-positive and the Gram-negative bacteria.[8,18,19,45,52] Although there is no consensus on the exact biological mechanism of AMPs, some basic trends are common in many systems. First of all, it is widely assumed that the cationically charged peptides are electrostatically attached to the negatively charged microbial cell membrane. Then the AMP assuming an amphipathic structure adapts to the specific conditions at the membrane-water interface which leads to an increase in the permeability of the cell membrane and loss of barrier function, resulting in the leakage of cytoplasmic components and eventual cell death.[8,20,53]

AMP activity is controlled not only by the membrane structure of the bacteria but also through a gene regulation mechanism. This has been proposed to regulate selected resistance genes, e.g. dlt-operon, mprF, and vraFG genes, when the bacteria come into contact with the AMPs[8,16,52,54] and, thereby, limit the interaction with the AMP. Gram-negative bacteria have faster permeabilization and killing kinetics compared to Gram-positive bacteria according to many studies in the literature.[44,55,56] This also confirms the conclusion of previous studies that the double membrane of the Gram-negative bacterium is a less efficient barrier than the single membrane and the thick peptidoglycan layer of the Gram-positive species.[52,55]

3.5. Antimicrobial Efficacy of the Peptide Functionalized Titanium Surfaces

The effectiveness of the antimicrobial activity of a peptide adsorbed on a solid surface may change compared to that in solution. In general, the immobilized AMPs may display an increase in their MIC value compared to the soluble peptide. In this part of the research, we evaluated the antimicrobial efficacy of each peptide on the Ti substrate using their minimum inhibitory concentrations that were determined in solution, namely, 200 μM for *S. mutans*, 50 μM for *S. epidermidis* and *E. coli* (The characteristics of substrate Ti are shown in Figure S4, Supporting Information.).

Peptides were immobilized on the Ti substrate by allowing self-adsorption for an incubation time of 4 h at 37 °C with agitation. Upon incubation, the substrate surface was washed twice to remove any excess amount of nonspecifically adsorbed peptide. Meanwhile, each bacterium was grown until their mid log phase and 10^8 cells/mL were exposed to the peptide

modified titanium surface and incubated for a period of 2 h. After this procedure, the cells are fixed and labeled with Syto 9 and visualized under fluoresce microscopy (FM) which allowed correlation to be formed between bacterial binding and killing efficacy on the AMP-coated surfaces. The FM results demonstrate clearly that the amounts of adhered bacteria on the bare titanium surface among the three species are significantly different from each other (Figure 4, first row). In the presence of *S. mutans*, the difference in adhered bacteria on the peptide-functionalized surface vs the bare surface is significant. Specifically, there is an ~45-fold difference between the TiBP1-GGG-AMP biofunctionalized titanium surface versus the positive control (bare titanium). This is compared with the case where there is an ~20× difference with the negative control (only AMP) (Figure 4 left column and Figure 5 (a)). The surface antimicrobial activity of TiBP2-GGG-AMP was not as high as TiBP1-GGG-AMP although it is higher than the negative control. The difference in the ratio of surface coverage between the TiBP2-GGG-AMP modified surface and the negative control is ~2-fold, while the difference is ~4-fold with the positive control (Figure 5 (a)). In the presence of *S. epidermidis*, a decrease similar to that in *S. mutans* was observed in bacterial adhesion between the positive control (bare titanium) and the negative control (AMP). However, antimicrobial behaviors of TiBP1-GGG-AMP and TiBP2-GGG-AMP were very similar in the case of *S. epidermidis*. The efficiency of TiBP2-GGG-AMP is higher against *S. epidermidis* than that in *S. mutans* (Figure 4 middle column and Figure 5 (b)). In the case of *E. coli*, AMP was highly efficient to prevent bacterial adhesion on the titanium surface. The difference in the ratio of surface coverage between AMP and TiBP1-GGG-AMP is very low. Although TiBP2-GGG-AMP had a significant decrease on cell adhesion compared to the positive control, the difference relies on the surface coverage ratio not being higher than AMP (negative control) (Figure 4 right column and Figure 5 (c)).

The surfaces modified with bifunctional peptides were found to significantly reduce bacterial adhesion against all three bacteria when compared to adhesion on bare titanium and the AMP functionalized Ti surface.

The substrates modified with TiBP2-GGG-AMP were found to have less bacterial adhesion on average than those modified with AMP when incubated with *S. mutans* and *S. epidermidis*; however; interestingly, they had more bacterial adhesion on average than substrates modified with AMP when incubated with *E. coli*. The solid surfaces modified with TiBP1-GGG-AMP were found to reduce bacterial adhesion of *S. mutans* and *E. coli* better than surfaces modified with TiBP2-GGG-AMP.

The antimicrobial activity of the bifunctional peptides was found to be more profound toward Gram-positive bacteria, i.e. *S. mutans and S. epidermidis*. This may be due to α-helical conformation of the cationic peptides having two unique features: a net positive charge and an amphipathic character that can effect not only the antimicrobial activity but also the selectivity between Gram-negative and Gram-positive bacteria.[43,44,52]

The surface functionalization domain in the case of TiBP1 has a positively charged peptide and has a tendency to be an α-helical structure as shown in Figure 2 (c) compared to TiBP2 as may be observed from the respective Ramachandran plots (Figure S5). Our bifunctional peptide TiBP1-GGG-AMP resulted in an increase in the charge value (+9) compared to

TiBP2-GGG-AMP (+6) and AMP (+6) alone. Their molecular models reveal that TiBP1-GGG-AMP has a tendency to have an open structure potentially due to the repulsion between charged residues TiBP1 and AMP domains. These repulsions may let bacteria reach the AMP domain easier or display the AMP domain better to damage bacterial membrane. The molecular model shows that it has turns that are similar to an α-helical structure of AMP. Although TiBP2-GGG-AMP has the same charge value, i.e. +6, with AMP it has a more compact and closed structure compared to AMP.

The compact molecular structure may have a drastic effect on its antimicrobial efficiency. In addition to the molecular structure of the peptides, antimicrobial behavior of bifunctional peptides may be further examined in terms of their hydropathy index. First of all, the relative hydrophobicity of amino acid residues in a given protein is defined by the values of the hydrophobicity scales. The domains of amino acids, which are more positive, are located on the more hydrophobic location along a protein sequence. These domains, for example, are commonly used to predict the transmembrane α-helices along a given protein sequence. Therefore, the hydropathy values of a sequence of a given domain can be an indicator of a specific hydrophobic region of a protein inside a lipid bilayer. Since the AMPs have such hydrophobic characteristics, the question is whether they retain their antimicrobial function when conjugated to solid-binding sequences. The fluorescence microscopy results of the chimeric peptides clearly demonstrate (given in Figure 4) the behavior of the overall peptide constructs displaying antimicrobial activity when bound to the Ti surface (given in Figure 4).

To understand basic correlation in between peptide function and hydrophobic character, the hydropathy index of the peptides was determined, as listed as GRAVY in Table 1. Normally, the AMP sequence when examined alone has a hydropathy value of 0.5, i.e., a high potential for disrupting the cell membranes. On the contrary, the solid-binding peptides have highly negative hydropathy numbers, so they do not have the tendency to interact with the negatively charged cell membranes. Since both functionalities are presented in the chimeric peptides, it can be concluded that the AMP and the solid binding domain at each of the two terminals separated by a flexible GGG linker in the middle retain their biofunctionality, i.e., the hydrophobic AMP displaying its hydrophobic chemistry while the solid-binding sequences preferring to be bound to the Ti surface. The structurally chimeric peptides, therefore, maintain their bifunctionality, just as designed, regardless of the fact that the overall peptide sequences are still highly hydropathic (−0.89 and −0.45 for TiBP1 and TiBP2, respectively). Maintaining its activity, and while the chimera is still bound to the solid substrate via the solid binding domain, the AMP domain is probably an attack on the bacterial membrane.

Equally significant to the AMP activity of chimera is the fact that they retain bound to the solid surface without disrupting the bioactivity of the functional domain at the opposite terminal. Among the two chimera, the bifunctional activity is probably more prominent with TiBP1 than with TiBP2 as evidenced from the results in Figures 4, 5, and 6 that show the effectiveness of the peptide constructs in deflecting and/or killing the surface-bound bacteria. It appears that the TiBP1 bound surface is more hydrophilic than TiBP2 as the former has a net charge of 3, while the latter is neutral. The difference in behavior of the Ti-binding peptides probably is also originating from their conformational behavior on Ti when

exposed to the solid. Based on the hydrophobicity values, therefore, the TiBP1 construct should work more effectively than TiBP2, confirmed by the observations of its antimicrobial activity as shown in Figures 4, 5, and 6.

Overall biological functionality of the chimeric peptide is a result of its molecular folding characteristics. In the present case, there is an interplay between the solid binding property and antimicrobial activity inherently preserved through the molecular folding that is adapted upon adsorption to the solid surface. This appears to be more effective in the case of TiBP1 compared to TiBP2. In both peptides, however, the introduction of the "GGG" linker turned out to be crucial to keep these two separate activity domains from interfering with each other to retain their specific functions. Ideally, when bound to the surface the construct should make contact with the solid substrate at multiple positions for a better grab of the substrate while faithfully displaying the AMP domain extending fully into the solution, effectively incorporating into the membrane of the bacteria. Interestingly, extensive molecular dynamics studies showed that, probably as a result of kinks across the linker position, the AMP domain bends into the solution, away from the solid surface.

As demonstrated by the molecular dynamic modeling studies (Figure 2 (c) and (d)), in both constructs, the GGG linker, at the carboxy-terminus, is connected to the AMP with a GL sequence, and hence a kink appears in both cases to display the AMP domain into the solution. Coincidentally, the GL amino acid sequence also appears in TiBP1 at the amine-terminus of the GGG linker; therefore, there is an additional kink, possibly affecting the display of the AMP domain more freely into the solution, a possible explanation of its high effectiveness. This can be seen comparatively in the fluorescence images demonstrating antimicrobial activity of the two constructs in Figure 4 third vs fourth rows.

3.6. Bacterial Adhesion Control on the Patterned Titanium Surface

Simple single step peptide based functionalization of surfaces might also provide control of bacterial adhesion over different regions; this approach could be useful for creating specific surface regions to analyze different bacterial species. We next investigated if the bacterial adhesion could be controlled under certain patterns using simple chimeric peptide motifs. We examined the difference in bacterial adhesion behavior on bare titanium as well as the titanium surface functionalized with TiBP1-GGG-AMP. The peptide was stamped on the surface using a soft lithography technique, i.e. PDMS stamp, and antibacterial efficacy of the TiBP1-GGG-AMP was tested against *S. mutans* and *E. coli* on the patterned areas (Figure 6). The observed bacterial pattern in Figure 6 demonstrates the ease of applying the peptide on the surface for any type of pattern to decorate surfaces for a variety of applications, analysis of bacterial growth, susceptibility, or protecting certain high risk regions.

Building upon experimental findings, bifunctional peptides have demonstrated improved antimicrobial activity on the surfaces in the presence of three different sets of bacteria using a simple single step surface functionalization procedure. TiBP1-GGG-AMP demonstrated the best antimicrobial activity between the two bifunctional peptides on the titanium implant materials being more efficient against *S. mutans*.

Here we demonstrate the applicability of chimeric peptides as antimicrobial implant coatings; however, their effects on osteoblast-like cells need to be further evaluated. M. Kazemzadeh-Narbat et al. previously showed that loading one of the antimicrobial peptides (HHC-36) onto octacalcium phosphate (OCP) coatings in self-organized TiO_2 nanotube arrays did not negatively affect MG-63 osteoblast-like cells. They also reported incorporation of antimicrobial peptides (HHC-36) into OCP coatings could even moderately enhance bone growth in vivo.[31,57] In another study, Zhang et al. demonstrated the differentiation of circulating monocytes treated with an antimicrobial peptide called LL-37 resulted in novel bone forming cells. Monocytes are often recruited to sites of injury and may differentiate into various cell types including osteoclasts, and the peptide interaction may contribute to bone repair.[58] Similarly Kraus et al. reported a positive role of Human β-Defensins, which is a class of antimicrobial peptides, in proliferation, differentiation, and mineralization of MG-63 osteoblast-like cells.[59] In different studies, human MSCs were shown to produce LL-37 having a role in the prevention of bacterial infection.[60,61] It is quiet interesting to note that so far studies have been showing the preferred action of antimicrobial peptides to be on the prokaryotic membranes rather than eukaryotic membranes. AMPs and functionalizing surfaces using bioenabled approaches with AMPs may provide an interesting avenue to explore combating bacterial infection in a bimimetic way.

4. CONCLUSIONS

Herein, we offer a simple way toward tailoring biomaterial surfaces capable of preventing bacterial infection by using engineered chimeric peptide based surface biofunctionalization. We designed two chimeric peptides comprised of titanium binding and antimicrobial peptide (AMP) motifs; these bifunctional peptides rely on the titanium-binding property that allows the molecular construct to preferentially bind to the biomaterial while freely exposing the AMP motif to combat invading bacteria. The efficacy of our bifunctional peptides, TiBP1-GGG-AMP and TiBP2-GGG-AMP, was evaluated both in-solution and on the solid surface of titanium substrate against *Streptococcus mutans, Staphylococcus epidermidis,* and *Escherichia coli*. Surfaces modified with both of the chimeric peptides were found to significantly reduce bacterial adhesion against all three bacteria compared to the bare titanium. The results of the presented work indicate that surface modification with engineered biomolecules consisting of antimicrobial and titanium-binding peptide domains is a promising approach to prevent bacterial infection on implant surfaces. Based on the chimeric approach that incorporates modular units various peptides can be designed against a broad range of bacteria by utilizing sequence, structure, and charge relations of AMPs and solid-binding peptides. The controlled binding to and release of biomolecules on implant surfaces are critical in the development of smart implant materials. More detailed studies may be necessary to test the underlying mechanism of antimicrobial activity in such bioconjugated peptides using a variety of modular sequences and to determine the stability of adsorbed peptides on the implant surface in a variety of biological conditions, including change in pH, buffers, and body fluids.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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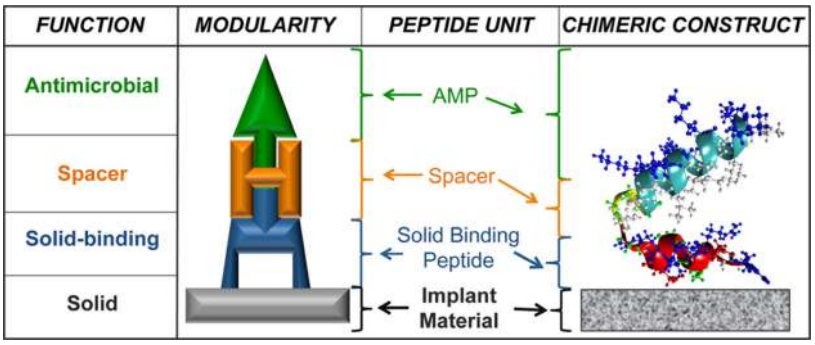

Figure 1.
Chimeric peptide design to incorporate solid binding and antimicrobial peptide for bifunctionality.

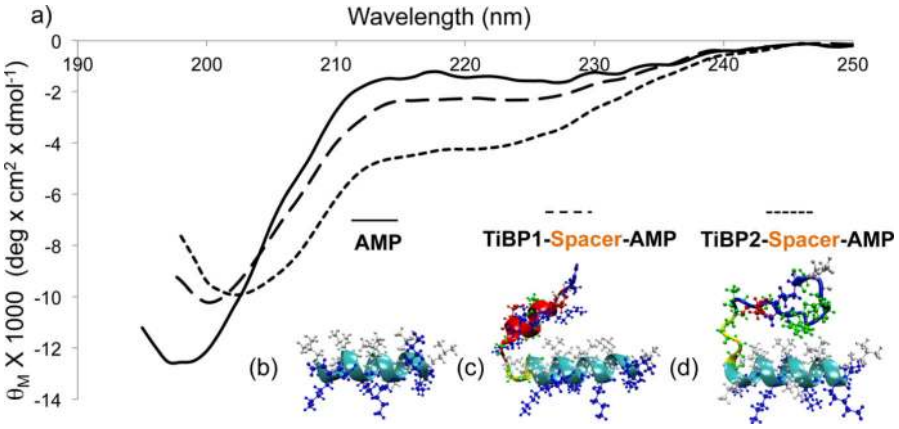

Figure 2.

(a) CD spectra of peptides (50 μM peptide in PBS) and (b-d) molecular dynamics models of the peptides. The colors of the amino acids shown in the CPK representation indicate the following residues: white, nonpolar; green, polar; red, acidic; and blue, basic.

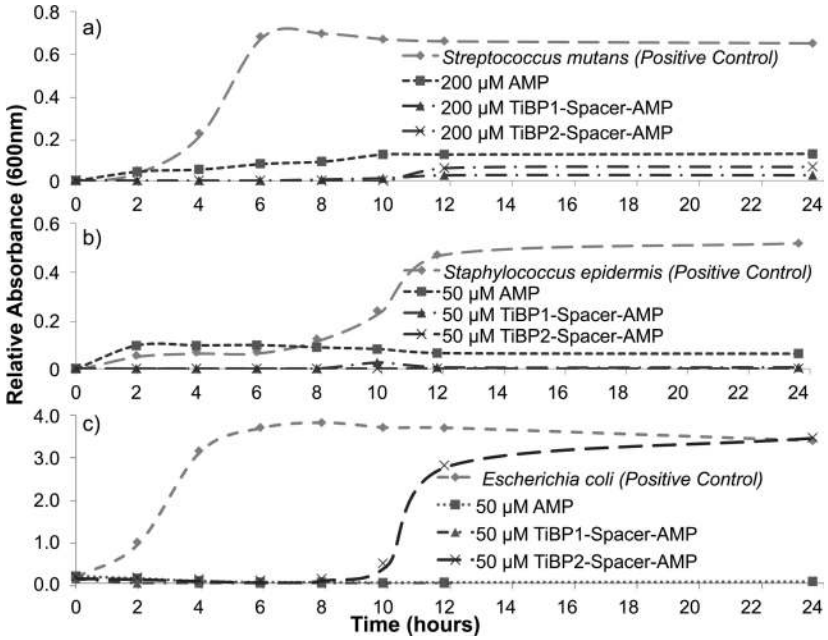

Figure 3.

Minimum inhibitory concentration of peptides (AMP and bifunctional counterparts) against *Streptococcus mutans* (a), *Staphylococcus epidermidis* (b), and *Escherichia coli* (c).

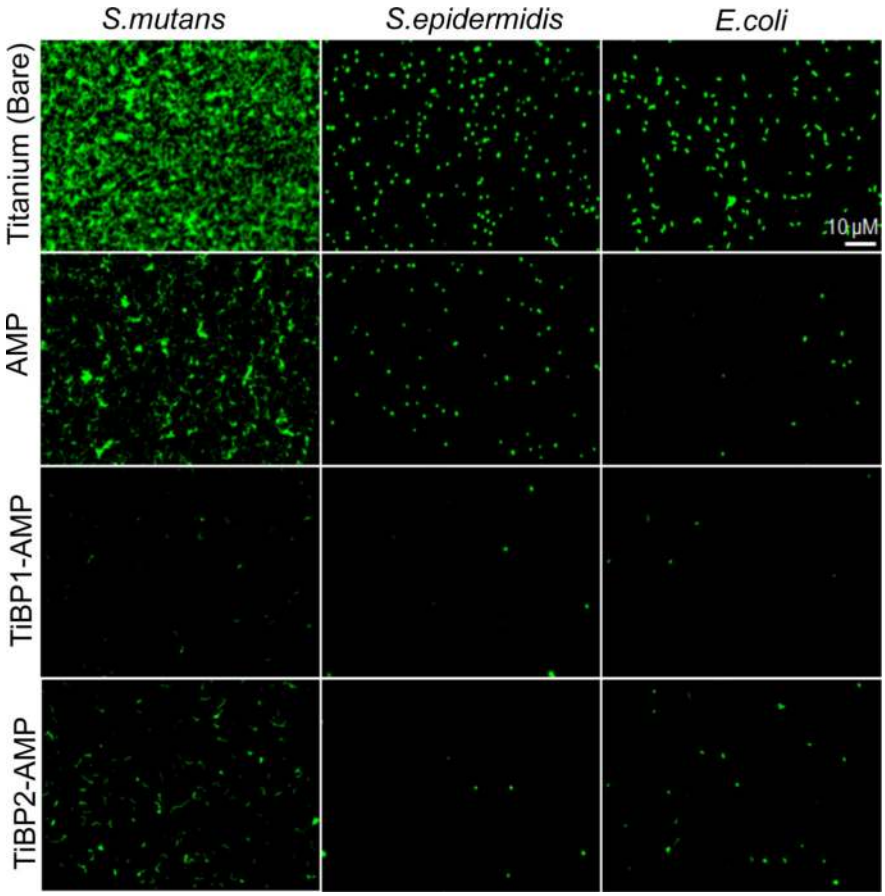

Figure 4.

Bacterial adhesion on AMP, TiBP1-Spacer-AMP, and TiBP2-Spacer-AMP peptide modified titanium surfaces against *Streptococcus mutans* (left column), *Staphylococcus epidermidis* (middle column), and *Escherichia coli* (right column).

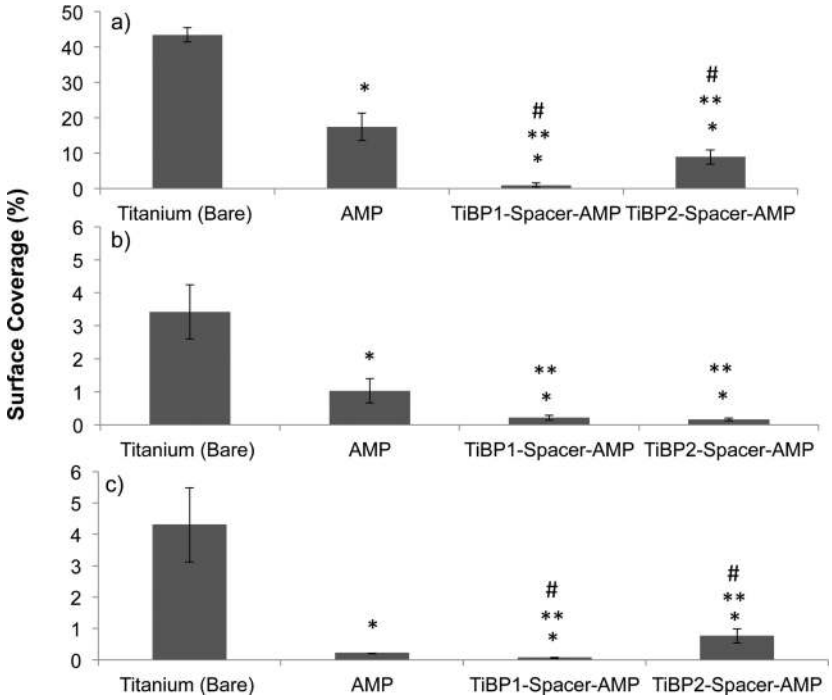

Figure 5.

Surface coverage analysis of titanium surfaces functionalized by the peptides, i.e. AMP, TiBP1-Spacer-AMP, and TiBP2-Spacer-AMP against *Streptococcus mutans* (a), *Staphylococcus epidermidis* (b), and *Escherichia coli* (c), $*p < 0.01$ compared to titanium bare, $**p < 0.01$ compared to AMP, $^{\#}p < 0.01$ comparison in between TiBP1-Spacer-AMP and TiBP2-Spacer-AMP.

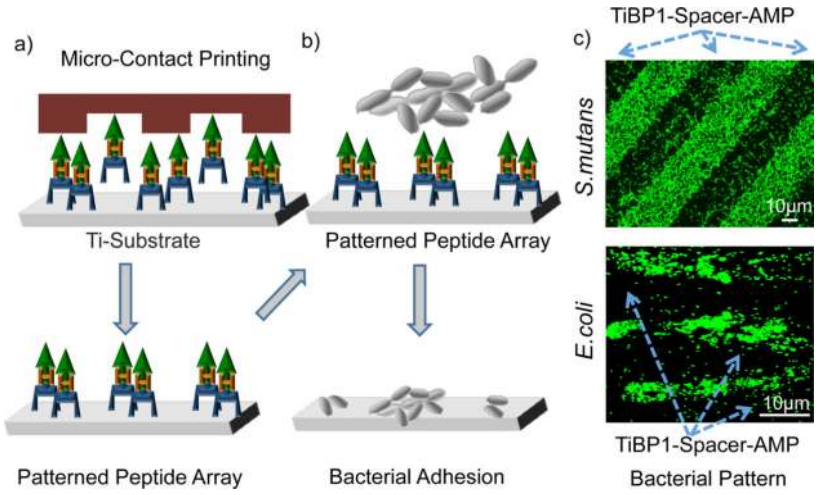

Figure 6.

Stamping chimeric peptide on a titanium substrate provides control of the bacterial adhesion on desired patterns. a) Schematics of PDMS patterning of the engineered chimeric peptide on the Ti substrate; b) Adhesion of *S. mutans* and *E. coli* (10^8 cell/mL) on the patterned peptide array; c) FM images of Syto9 labeled *S. mutans* and *E. coli* on the patterned peptide array.

Table 1

Molecular Characteristics of the Engineered Peptides Used in This Work

Peptide	Sequence*	MW#	pI#	GRAVY**	Chr#
AMP	LKLLKKLLKLLKKL	1692.34	10.70	0.5	+6
TiBP1-Spacer-AMP	RPRENRGRERGL GGGLKLLKKLLKLLKKL	3342.14	11.85	-0.89 (-2.63)	+9/+3
TiBP2-Spacer-AMP	SRPNGYGGSESSGGG LKLLKKLLKLLKKL	3042.66	10.39	-0.45 (-1.57)	+6/0

* Color Scheme for the AAs: R: Polar +; E: Polar –; S: Polar 0; A: Non-Polar Aliphatic; Y: Non-Polar Aromatic; P and G; C; .
# MW: Molecular Weight; pI: ; Chr: Net Charge (expassy.org)
**GRAVY: Grant Average Hydropathy Index (http://www.gravy-calculator.de/)

Table 2

Binding Affinity of the Peptides on Titanium Determined by Using QCM Measurement[a]

Peptide Name	K_D (μM)	ΔG (kcal/mol)
AMP	0.40 ± 0.04	-8.78 ± 0.22
TiBP1-Spacer-AMP	0.14 ± 0.06	-9.00 ± 0.35
TiBP2-Spacer-AMP	0.26 ± 0.06	-9.01 ± 0.11

[a] K_D is the dissociation constant, and ΔG is the free energy of adsorption.