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Surface-Conjugated Antimicrobial Peptide Leucocin A Displays High Binding to Pathogenic Gram-Positive Bacteria

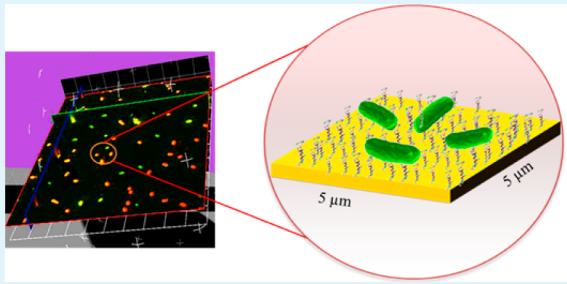
Hashem Etayash,[†,‡] Lana Norman,[‡] Thomas Thundat,[‡] Michael Stiles,[§] and Kamaljit Kaur*[,†]

[†]Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta T6G 2E1, Canada

[‡]Department of Chemical and Materials Engineering, University of Alberta, Edmonton, Alberta T6G 2V4, Canada

[§]CanBiocin, Inc., Edmonton, Alberta T5J 4P6, Canada

ABSTRACT: Leucocin A, a representative class IIa bacteriocin, is a ribosomally synthesized antimicrobial peptide (AMP) that displays potent activity against specific gram-positive bacteria. The antibacterial activity of such peptides is preceded by the binding event that can be utilized for studying specific peptide−bacteria interactions. In this study, 37-residue Leucocin A (LeuA) was synthesized using solid-phase peptide synthesis and covalently immobilized on gold substrates from either the N- or C-terminal. Both the peptide monolayers on gold substrates were incubated separately with five strains of gram-positive bacteria and displayed differential binding to different strains with highest binding to pathogenic *Listeria monocytogenes*. The C-terminally immobilized LeuA showed higher bacterial binding compared to the N-terminally attached LeuA. The full length immobilized LeuA (37-residue) was active as well as displayed higher bacterial binding (73 ± 6 bacteria/100 μm^2) compared to 24-residue inactive LeuA fragment (40 ± 8 bacteria/100 μm^2) from the C-terminal region. The high and specific bacterial binding ability of LeuA functionalized surfaces support the potential use of class IIa bacteriocins in antimicrobial peptide-based diagnostic platforms.



KEYWORDS: *antimicrobial peptide, class IIa bacteriocin, Leucocin A, surface conjugated peptide, bacterial binding, gram-positive bacteria*

1. INTRODUCTION

Bacteriocins or antimicrobial peptides (AMPs) produced by lactic acid bacteria (LAB) are ribosomally synthesized peptides that are generally active against phylogenetically related bacteria.[1−4] Bacteriocins are classified into different classes according to their genetics and biochemical characteristics. Among different classes of bacteriocins, class IIa bacteriocins are the most well-studied AMPs. Class IIa bacteriocins are unmodified single-chain peptides varying in length between 37 and 49 amino acids that display potent antibacterial activity (active in the nanomolar range). These AMPs exhibit significant activity against gram-positive *Listeria* strains; therefore, these are also known as antilisterial peptides. However, their inhibitory spectrum includes several other genera of gram-positive bacteria such as *Enterococcus*, *Carnobacterium*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Clostridium* and are inactive toward gram-negative bacteria.[1,5−8] Structurally, these peptides consist of two distinct domains: N-terminal domain and C-terminal domain. While the N-terminal domain has a well-maintained disulfide bond and a YGNGV motif, the C-terminal domain consists of an amphipathic α-helical structure, ending with a loop or hairpin-like structure at the C-terminal tail.[5]

The exact mechanism of action of class IIa bacteriocins is not clearly understood; however, several studies suggest that a receptor-mediated mechanism is most likely behind the specificity of this class of AMPs.[8−11] It is proposed that, first, the peptide may bind the target cell surface, mediated by electrostatic interactions involving the positively charged N-terminal residues, followed by specific interactions of the C-terminal region of the peptide with a specific membrane-located protein of the mannose phosphotransferase system (man-PTS). This tight binding may lead to pore formation in the target cell membrane and eventual cell death.[12] Man-PTSs are not found in eukaryotes; therefore, these peptides are highly specific and nontoxic to humans.[8] The specific mechanism of action of class IIa bacteriocins, together with their high activity against *Listeria monocytogenes* can be exploited for utilizing these AMPs for diagnostic purposes.

Contamination of water, food, and pharmaceutical products with pathogenic and resistant bacteria remains one of the main concerns in developed and developing nations, because of a lack of fast, inexpensive, portable, and easy-to-handle tools to detect pathogens. Currently available methods for the detection of micro-organisms utilize the old traditional techniques such as culture-based and colony-counting procedures.[13] These methods of enumerating bacteria are very slow to provide a full protection from exposure to microbial invasion. Alternatively,

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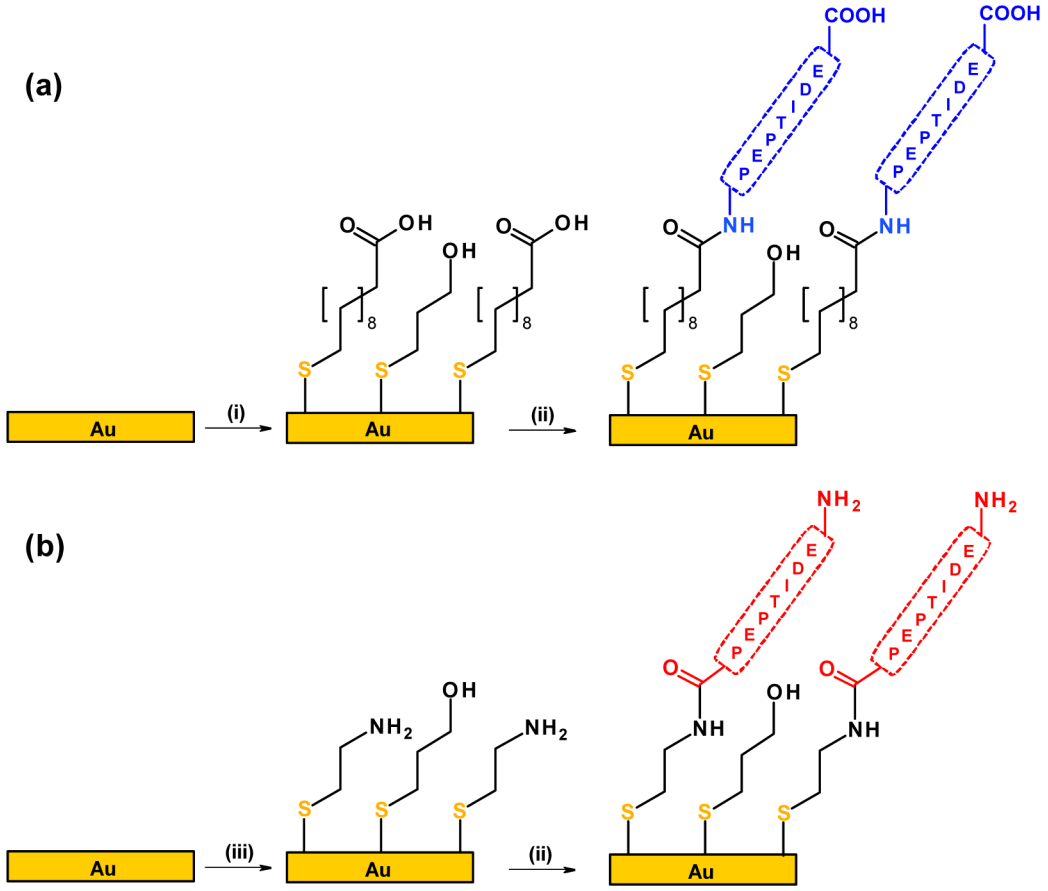

Figure 1. Schematic showing peptide immobilization on gold-coated silicon wafer substrates. Peptide leucocin A (LeuA, 37-residue) was covalently conjugated to functionalized gold substrates through the (a) N-terminus or (b) C-terminus. Reagents used were (i) 11-MUA and 3-mercapto-1-propanol in absolute ethanol, (ii) LeuA (0.8 mg/mL), EDC, and NHS in PBS, and (iii) cysteamine and 3-mercapto-1-propanol in absolute ethanol.

rapid detection methods such as polymerase chain reaction (PCR) based techniques and monoclonal antibodies-based immunoassays have proven to be fast and highly sensitive.[14,15] However, these methods suffer from numerous potential constraints, such as sample-pretreatment requirements and trained labor necessities, as well as a lack of stability under harsh environmental conditions.

Solid supports with covalently conjugated AMPs have been exploited as alternatives to traditional pathogen-detection platforms.[16−20] Our group, as well as others, have shown that AMPs can act as potential candidates for use as molecular recognition elements in biosensing platforms.[21−23] Mannoor et al. utilized AMP magainin I conjugated gold microelectrodes to show differential binding of bacterial strains, as well as detection of pathogenic strains of *E. coli* and *Salmonella* in real time.[24] We showed that 24-residue C-terminal fragment of a class IIa bacteriocin, Leucocin A (LeuA) immobilized on gold surface displayed significant binding toward gram-positive bacteria with various binding affinities for different strains and no binding to gram-negative bacteria.[21] The study highlighted the importance of the C-terminal region of class IIa bacteriocins for receptor and bacterial binding. The 24-residue LeuA fragment (24AA LeuA) showed no activity, whereas it displayed good binding to the indicator bacterial strains such as *Listeria monocytogenes*.

The full-length LeuA (37-residue) is a potent antimicrobial peptide that exhibits activity against *Listeria monocytogenes* in the nanomolar range (minimum inhibitory concentration (MIC) = 0.1 nM).[25,26] We hypothesized that full-length LeuA immobilized on a gold surface may display improved binding, compared to the LeuA fragments. Therefore, the objective of the current study was to immobilize LeuA on gold substrates seeking for improved bacterial binding and high specificity toward pathogenic gram-positive *Listeria monocytogenes*. Accordingly, full-length LeuA was synthesized and conjugated to gold substrates through the N- or C-terminal groups (Figure 1), followed by evaluation of bacterial binding using fluorescently labeled bacteria. The C-terminally immobilized LeuA showed much higher binding to *Listeria* strains compared to *S. aureus* and *E. faecalis*. The immobilized LeuA peptide displayed antibacterial activity and showed higher bacterial binding, compared to 24AA LeuA fragment immobilized on the gold surface, highlighting the potential of LeuA immobilized surfaces for developing peptide-based diagnostic platforms.

2. MATERIAL AND METHODS

Antimicrobial Peptide Leucocin A. Leucocin A (LeuA) consisting of 37 amino acid residues (NH_2-KYYGNGVHC-TKSGCSVNWGEAFSAGVHRLANGGNGFW-COOH) was chemically synthesized using standard Fmoc solid-phase peptide synthesis (Fmoc-SPPS). The synthesis was carried out on Fmoc−Trp(Boc)−Wang resin (0.2 mmol scale, 0.27 mmol/g) using an automated peptide synthesizer (Tribute, Protein Technology, Inc., USA) as described previously.[26] Briefly, the resin was loaded to the peptide synthesizer reaction vessel and Fmoc−amino acids were loaded into the amino acid

vials (4 equiv, 0.8 mM). The synthesis protocol was set up in order for washing, Fmoc deprotection, washing, and coupling, respectively, for each cycle. HCTU (0.8 mM) was used as an activating agent, while NMM/DMF (1M/L) acted as a base. Freshly prepared 20% piperidine in DMF was used for Fmoc-deprotection. The completed peptide sequence (37 residues) was ultimately released from the resin with concomitant removal of the acid-labile side-chain protecting groups by adding a mixture of 90% TFA, 9% DCM, and 1% triisopropylsilane (~10 mL) for 90 min at room temperature with automatic shaking. The filtrate from the cleavage reaction was collected in a collection tube attached to the synthesizer and was concentrated under vacuum and precipitated by adding cold diethyl ether (~15 mL). After triturating for 2 min, the peptide was collected by centrifugation and decantation of ether. A lyophilized crude peptide was then obtained by freeze-drying the collected product.

The purification and characterization of the peptide was carried out on a reversed-phase (RP) HPLC (Varian Prostar 210, USA) using Vydac semipreparative C18 (1 cm × 25 cm, 5 μm) and analytical C8 (0.46 cm × 25 cm, 5 μm) columns. Initially, the crude peptide was reconstituted in a 1:1 acetonitrile/water solution and introduced to the HPLC. The peptide was monitored at a 220 nm wavelength, using a linear gradient of acetonitrile/water (0.05% TFA, v/v) mixture. Peak (fraction) showing the correct mass $[M + H]^+$ was collected and evaporated on a rotary evaporator followed by lyophilization in a freeze dryer to obtain the pure peptide. Next, the peptide was oxidized to form disulfide bond between Cys9 and Cys14 by dissolving in Tris buffer (pH 8.4) at a concentration of 1 mg mL^{-1}. DMSO (10%) was added to the buffer to increase peptide solubility and expedite oxidation. Peptide solution was stirred overnight and the oxidized product was subjected to RP-HPLC to obtain pure LeuA. The identity and purity of LeuA was confirmed by MALDI-TOF spectrometry (calcd. [M+H]+ 3928.7, found [M+H]+ 3929.2) and semi-preparative C18 RP-HPLC (elution time 29 min using a gradient of 15%−55% ACN/water (0.1% TFA) in 55 min with a flow rate of 1 mL/min).

Bacterial Cells. The bacterial strains used in this study were *Listeria monocytogenes* ATCC 43256, *L. monocytogenes* ATCC 19116, *Carnobacterium divergens* LV13, *Staphylococcus aureus* ATCC 13565, and *Enterococcus faecalis* ATCC 19433. All bacterial stocks were obtained from CanBiocin, Inc. (Edmonton, AB, Canada). The experiments with the bacterial subculture, maintenance, and treatments were conducted in a level II biosafety cabinet.

Surface Functionalization. Gold-coated silicon substrates (0.5 cm × 1 cm, 50 mm thickness, Ultrasil, Hayward, CA) were cleaned by immersion in a freshly prepared piranha solution (30% H_2O_2/H_2SO_4, 1:3, v/v) prior to use. Highly reactive piranha solution was handled with extreme caution. The clean substrates were rinsed with copious amount of Milli-Q water followed by washing with 95% ethanol. The substrates were dried under a stream of nitrogen. The peptide (LeuA) was covalently immobilized on gold surface either from the N- or C-terminal.

For the N-terminal immobilization of the peptide, gold surface was first treated with 11-mercaptoundecenoic acid (11-MUA).[27] The substrates were incubated overnight with 0.01 M (75/25) of 11-mercaptoundecenoic acid (7.5 mM) and 3-mercapto-1-propanol (2.5 mM) in absolute ethanol at room temperature. After washing with ethanol, the substrates were rinsed with Milli-Q water in order to remove any unbound 11-MUA. The SAM of 11-MUA was activated for about 2 h using a mixture of EDC (0.2 M) and NHS (0.05 M). Thereafter, peptide immobilization was carried out by depositing LeuA peptide (200 μL, 0.8 mg/mL) in PBS on the 11-MUA monolayer and kept overnight at room temperature for the amide bond formation between amine group of the peptide and the activated carboxylic terminal of 11-MUA monolayer (Figure 1a). The surfaces were then vigorously rinsed in PBS with agitation and finally dried under nitrogen.

The peptide was similarly immobilized on the gold surface via the C-terminal except that the gold substrates were first treated with 0.01 M (75/25) of cysteamine hydrochloride (7.5 mM) and 3-mercapto-1-propanol (2.5 mM) in absolute ethanol.[27] The reaction mixture was kept overnight at room temperature followed by washing and rinsing with ethanol and Milli-Q water. The cysteamine functionalized-substrates were dispersed in a phosphate buffer solution (pH 7.4) containing LeuA (0.8 mg/mL), followed by addition of EDC (0.2 M) and NHS (0.05 M) for activation of the peptide COOH group. The activated carboxyl group reacts with the NH_2 group of the cysteamine leading to formation of amide bond (Figure 1b). Prior to surface analysis, the substrates were removed from the solution and rinsed with copious amounts of PBS to remove any physically adsorbed materials. The samples were then dried with nitrogen and characterized immediately using FTIR-RAS and ellipsometric spectroscopy. Here we assumed that both N- and C-terminal conjugations give comparable peptide density as both methods used similar steps. In addition, LeuA has two lysines with side chain NH_2 group (1K and 11K) and one glutamic acid with side chain COOH (20E). Both lysines are in the N-terminal region close to the N-terminal free amino group, and COOH of 20E is most likely sterically hindered giving less chance for immobilization (amide bond formation) from different sites.

Fourier Transform Infrared Reflection−Absorption Spectroscopy (FTIR-RAS). FTIR-RAS of the surface immobilized peptide was acquired using a Nexus 670 FTIR (Thermo Nicolet, Madison, WI) equipped with a surface-grazing angle attachment (SAGA, Thermo Spectra-Tech, Shelton, CT) and a liquid-nitrogen-cooled MCT detector. The grazing angle was 75°, and all spectra were averaged over 1064 scans at a resolution of 2 cm^{-1}. Peak information was obtained using OMNIC provided by Thermo Nicolet.

Ellipsometric Spectroscopy. A Sopra GES5 variable angle spectroscopic ellipsometer (Sopra Inc., Palo Alto, CA) was used to determine the film thickness of the SAMs on the gold surfaces. The measurements were acquired under ambient conditions on the gold-coated substrates before and after incubation with the peptide solution in order to determine the thickness of the chemisorbed peptide monolayer. The ellipsometer equipped with a He−Ne Laser (λ = 632.8 nm) with fixed incidence angle of 70° and using an analyzer at 45°. The thickness of the peptide monolayer was calculated using the regression method with Sopra WinElli (version 4.07) by setting the n and k values for the peptide immobilized monolayers as 1.5 and 0.0, respectively. The data presented are the averages of at least five measurements.

Biological Activity. Leucocin A activity was measured against five bacterial strains: *Listeria monocytogenes* ATCC 43256 (grown in TSBYE, 37 °C), *Listeria monocytogenes* ATCC 19116 (TSBYE, 37 °C), *Enterococcus faecalis* ATCC 19433 (APT broth, 37 °C), *Staphylococcus aureus* ATCC 13565 (APT

broth, 37 °C), and *Carnobacterium divergens* UAL9 (APT broth, 25 °C). Antimicrobial activity assay, followed by determination of minimum inhibitory concentrations (MICs), were done using the liquid growth inhibition assay in microtiter plates, as described previously.[28,29] Briefly, peptide stock solution (25 μM) was prepared in methanol/water (1:3) and serial dilutions were made as required. Peptide concentrations of the stock solutions was determined by measuring UV absorbance at 280 nm, following the literature procedure.[30] The final volume of each well of the microtiter plate was made 200 μL with the culture medium containing LeuA fractions at 2-fold dilutions and an indicator strain at an OD (610 nm) of 0.01. The microtiter plate culture was incubated overnight, after which the growth of the indicator strain was measured spectrophotometrically at 600 nm using a microtiter plate reader (TECAN, Männedorf, Switzerland). MICs were defined as the peptide concentration that inhibited the growth of the indicator strain by 50%.

Confocal Microscopy for Monitoring Bacterial Adhesion. Samples of bacteria (*C. divergens*) with varying concentrations (1×10, 10^2, 10^3, 10^4 cfu mL^{-1}) were incubated independently with the N- and C-terminally immobilized peptide substrates for 30 min at room temperature. Previously, we found that longer incubation times (40−90 min) did not increase binding between the immobilized peptide and bacteria.[21] After incubation, the surfaces were washed with PBS buffer several times. Stock solution of nucleic acid stain propidium iodide (PI) (Molecular Probes) was made by dissolving PI in deionized water at 1 mg/mL (1.5 mM) and was stored at 4 °C protected from light. PI solution (3 μM), made by diluting 1 mg/mL stock solution 1:500 in buffer, was deposited on top of the substrates and the substrates were incubated for 3 h in darkness at 37 °C. Thereafter, the substrates were rinsed with fresh PBS (1×) and dried under nitrogen followed by analysis using confocal microscopy.

To examine binding affinity of the immobilized peptide to five different strains, each bacterial strain (10^3 cfu/mL) was incubated separately with surfaces immobilized LeuA for 30 min at room temperature. The bound cells were treated as described above prior to the microscopic analysis.

The viability of bacterial cells attached to the substrates was evaluated using a live/dead Bacterial Viability Kit (Life Technologies Inc., Burlington, ON, Canada). Live/dead bacterial viability stains, CyQUANT green and propidium iodide (PI), respectively, were prepared separately by dilution with sterile water (1:10) and were mixed together in equivalent ratio. The mixed live/dead solution (100 μL) was deposited on the surface of the samples, and the samples were incubated for 10 min in darkness at room temperature prior to analysis using confocal microscopy.

Peptide-bacterial interaction and bacterial viability were examined with a confocal inverted microscope, Quorum WaveFX spinning disk confocal system (Quorum Technologies, Inc., Guelph, Canada). Images were acquired with an oil immersion lens at a magnification of 60×/1.4. Multiple fluorescence signals were acquired sequentially to avoid cross talk between image channels. Fluorophores were excited with the 488 nm line of an argon laser (for CyQUANT) and the 543 nm line of a HeNe laser (for PI). The emitted fluorescence was detected through spectral detection channels between 500−530 nm and 555−655 nm for green and red fluorescence, respectively.

3. RESULTS AND DISCUSSION

Peptide Leucocin A. The synthesis of peptide Leucocin A (LeuA) was attempted by stepwise solid phase peptide synthesis using standard Fmoc-chemistry. Preloaded Wang resin with Fmoc-protected tryptophan amino acid (Fmoc−Trp(Boc)−Wang resin) was used for automated peptide synthesis on a peptide synthesizer. The crude 37-residue peptide obtained was purified using semipreparative RP-HPLC. Oxidative folding was carried out in a buffer solution overnight to form the native disulfide bond between Cys9 and Cys14, followed by purification using RP-HPLC. Class IIa bacteriocins contain a conserved disulfide bond in the N-terminal region. LeuA was obtained with an overall yield of 18% and purity of ≥95%. The pure peptide was characterized using analytical HPLC and MALDI-TOF mass spectrometry.[26]

Peptide Immobilization. The peptide was covalently conjugated to a gold surface using gold−thiol chemistry. A thiol linker was used to anchor the peptide to the gold surface (Figure 1). For immobilizing peptide through the N-terminal amine, a monolayer of 11-MUA was first created on the gold surface. Peptide was conjugated to the carboxylic acid group of 11-MUA using EDC and NHS as coupling reagents. Peptide was conjugated from the C-terminal using cysteamine as the linker. A monolayer of cysteamine on a gold surface was created followed by peptide conjugation to the free amine of cysteamine. In order to obtain low density of functionalization, the monolayers on Au surface (11-MUA or cysteamine) were prepared in the presence of 3-mercaptopropanol (25%).

Surface Characterization. FTIR-RA spectroscopy was used to estimate the orientation and conformation of the surface-conjugated peptide.[21] Figure 2a shows the spectra for the peptide immobilized through the N-terminal and with free C-terminal domain. The observed absorbance bands at 1663 and 1535 cm^{-1} correspond to the amide I and amide II bands, respectively, and are characteristic of a helical conformation of the surface-conjugated peptide.[31−34] A shoulder peak at 1722 nm next to the amide bands is likely due to the C-terminal free carboxylate (C═O stretch). The intense bands recorded at 2922 and 2850 cm^{-1} are assigned for CH_2 chains, which suggest rather good crystallinity of the 11-MUA layer as also observed previously.[35] The weak absorbance band at 1251 cm^{-1} is likely attributed to the OH twist of the mercaptopropanol. Similarly, the peptide immobilized through the C-terminal and with free N-terminal region shows characteristic peaks for the peptide (1663 and 1535 cm^{-1}) as well as OH of mercaptopropanol (Figure 2b). The signal for the cysteamine CH_2 was weak in this case; therefore, clear bands between 2950 and 2850 cm^{-1} for the C−H stretch were not observed. Furthermore, the orientation of the helical peptide on the gold substrate, with respect to the surface normal, was deciphered from the difference in the intensity ratio between the amide I and amide II peaks. The calculated ratio for both the N- and C-terminally conjugated LeuA was on the order of 1.4 suggesting that the peptide lies predominantly parallel to the gold surface. This is similar to our previous observation where a 24-residue fragment of LeuA was found to position itself parallel to the gold surface in a random orientation.[21] The film thickness for both the N- and C-terminally immobilized LeuA was found to be 1.4 (±0.5) nm, suggesting a flat lying configuration for the peptide layers. This is in close agreement with the peptide width of 1.6 nm found in the three-dimensional (3-D) solution structure of LeuA (based on NMR spectroscopy).[5]

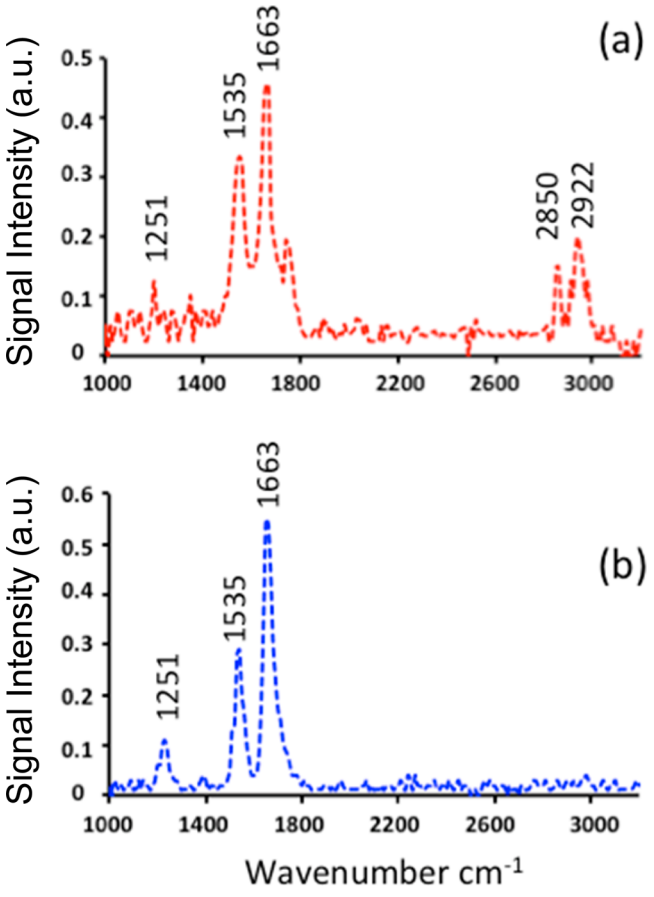

Figure 2. FTIR-RA spectra of the peptide monolayers on gold substrates. The peptide (LeuA) was covalently immobilized on functionalized gold substrates through the (a) N-terminus or (b) C-terminus.

Bacterial Interaction with N- or C-Terminally Immobilized Peptide. Confocal microscopy was used to detect peptide−bacteria interactions, as well as to examine the viability of the surface-adhered bacteria. Full-length LeuA was conjugated to Au surface through the N- or C-terminal amino acids. This was done to evaluate whether peptide with free C-terminal domain or free N-terminal domain will lead to enhanced bacterial binding. LeuA conjugated to the Au surface was incubated with different concentrations (10^1−10^4 cfu mL^{-1}) of gram-positive *C. divergens*. A peptide-free gold surface was used as a control. At low concentrations (10−10^2 cfu mL^{-1}), there was no significant difference between the bacterial binding to a peptide-free or peptide-conjugated gold surface (Figure 3a). However, at higher concentrations (10^3 and 10^4 cfu mL^{-1}), the bacterial binding on both the N- and C-terminally immobilized peptide surfaces was much higher, compared to the peptide-free surface. Furthermore, it was found that peptide immobilized from the C-terminus (81 ± 7 bacteria/100 μm^2) showed higher bacterial binding, compared to the N-terminally immobilized peptide (73 ± 6 bacteria/100 μm^2). This suggests that the free N-terminal region in the C-terminally immobilized peptide provides a better interaction interface between the immobilized peptide and bacteria, and the increase in the binding activity could be due to the exposure of the target bacteria to the highly charged N-terminus region of LeuA. This observation supports the hypothesis that the initial interaction of helical AMPs with the membranes of the target bacteria occurs via electrostatic attraction between the positively charged amino acids of the AMP and the negatively charged phospholipids of the bacterial membrane.[36,37] This is likely followed by specific interaction between the C-terminal amphipathic helical region of the peptide and the extracellular domain of the target cell receptor protein (man-PTS).

Peptide Specificity for Different Bacterial Strains. LeuA immobilized from the C-terminus was used to study the interaction with different bacterial strains. The binding affinity of the surface-immobilized peptide was tested against five bacterial strains: *L. monocytogenes* 43256, *L. monocytogenes* 191116, *C. divergens* LV13, *S. aureus*, and *E. faecalis* (Figure 3b). The binding was compared between pathogenic *L. monocytogenes* and two other gram-positive pathogens, *S. aureus*, and *E. faecalis*. The results showed highest binding affinity toward *L. monocytogenes* 43256 and *L. monocytogenes* 19116, where an

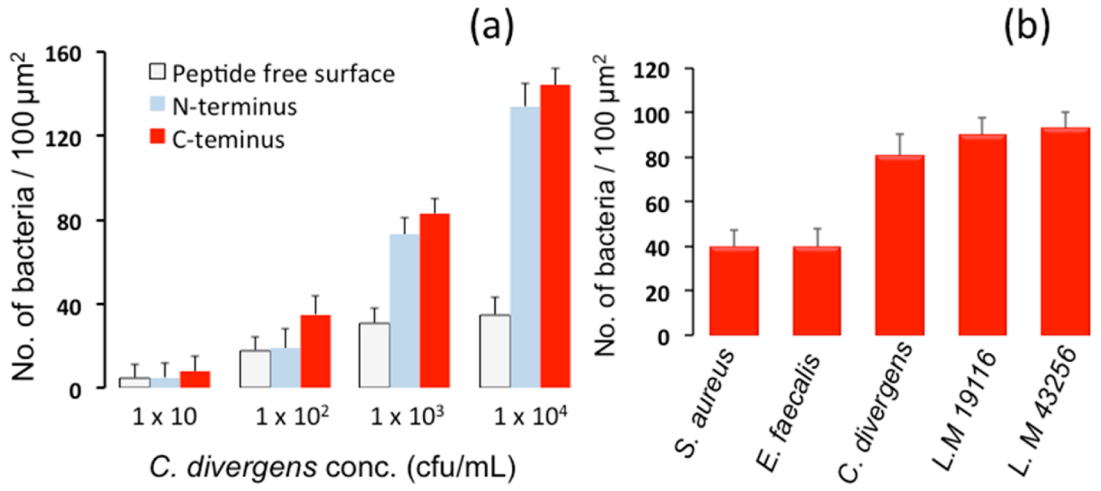

Figure 3. Bacterial interactions of surface-immobilized antimicrobial peptide LeuA using confocal microscopy: (a) average number of gram-positive bacteria (*C. divergens*) bound to a gold surface with no peptide (peptide-free), N- or C-terminally immobilized peptide as a function of bacterial concentration (10^1−10^4 cfu mL^{-1}); (b) average number of bacteria bound to gold surface with C-terminally immobilized peptide when incubated with five different bacterial strains at 10^3 cfu mL^{-1}. The average number of bacteria was counted from confocal microscopy images using ImageJ 1.46. Error bars are based on three individual samples prepared under the same conditions. ("L.M." stands for *Listeria monocytogenes*.)

average surface concentration of 101 ± 11 and 97 ± 12 bacteria/100 μm^2, respectively, was observed. *E. faecalis* and *S. aureus* displayed much less binding, with an average surface concentration of 39 ± 5 and 38 ± 5 bacteria/100 μm^2, respectively. In comparison, the nonpathogenic *C. divergens* also showed good bacterial binding (81 ± 7 bacteria/100 μm^2). The binding results can be correlated to the bactericidal activity of LeuA toward these strains. All three strains that showed higher binding are very sensitive to LeuA with a MIC value of ~39 nM (Table 1). While LeuA displays MIC values in the μM range for *S. aureus* and *E. faecalis*. In addition, the variations in the peptide-bacteria interactions between strains can be correlated with the differential expression levels of the man-PTS receptors from one bacterial strain to another.[2,8,11] Many recent studies have shown that the number of receptors on the cellular membrane of bacteria play a crucial role in the peptide–bacteria interactions.[9,10]

Table 1. Antibacterial Activity of LeuA against Gram-Positive Bacterial Strains

strain[a]	medium	MIC[b] (nM)
L. monocytogenes ATCC 43256	TSBYE	37 ± 0.5
L. monocytogenes ATCC 19116	TSBYE	39 ± 0.4
C. divergens LV13	APT broth	42 ± 0.6
E. faecalis ATCC 19433	APT broth	10 000 ± 300
S. aureus ATCC 13565	APT broth	32 000 ± 500

[a]All strains were obtained from the Strain collection of CanBiocin, Inc., Edmonton, Canada (ATCC: American Type Culture Collection). [b]MIC values represent peptide concentration that inhibited the growth by 50%. Data are represented as means ± standard errors of the means. The values are results of at least three independent measurements.

Bacterial Interaction with the Full Length LeuA or 24-Residue LeuA Fragment. Previously, we observed that the 24AA LeuA fragment immobilized (from the N-terminal) on gold surface displayed significant binding toward gram-positive bacteria with various binding affinities from one strain to another.[21] Here, we compare the binding affinities of the 24AA LeuA with the full length LeuA when conjugated to the gold surface through the N-terminal. We found that full length LeuA bound more bacteria (73 ± 6 bacteria/100 μm^2) compared to the 24AA fragment (40 ± 8 bacteria/100 μm^2) (Figure 4a). Class IIa bacteriocins like LeuA are characterized by a conserved and positively charged N-terminal region involved in the AMP activity and the C-terminal domain with the amphiphilic α-helix responsible for antimicrobial specificity. The C-terminal peptide, 24AA LeuA, which spans most of the C-terminal region, shows less binding to bacteria compared to the full-length peptide, suggesting that the full-length peptide is required not only for antibacterial activity but also for enhancing the binding affinity toward the target bacteria.

Next, we used a live/dead viability kit to stain the bacterial cells bound to 24AA LeuA and full-length LeuA. *C. divergens* (10^3 cfu mL^{-1}) were incubated with the peptide-functionalized slides for 30 min at room temperature, followed by incubation with a solution of a live/dead stain from the viability kit for 10 min at 37 °C. The live/dead stain contains two fluorescent dyes, CyQUANT, which stains all live bacteria green, and propidium iodide (PI), which gives red stain to the dead cells as it can only crossover damaged cells membranes. Figure 4b shows the bacterial cells bound to 24AA LeuA after staining. More than 73.5% of the cells attached to the 24AA LeuA functionalized surface appear green, indicating that the majority are still alive and have bound to the immobilized peptide without being harmed. On the other hand, more than 72.5% bacteria attached to the LeuA functionalized surface carry the red color (red-stained cells) and few cells (<27.5%) appear with the green color (Figure 4c). Similar viability results were obtained for the C-terminally immobilized LeuA (not shown here). This suggests that the majority of the cells that bound to the LeuA coated surface have been killed by coming in contact with the AMP, and the full-length peptide maintains a high portion of its activity when immobilized on surface. Furthermore, the full-length peptide (LeuA-functionalized surface) shows higher binding to bacterial cells than its shorter C-terminal fragment (24AA LeuA-functionalized surface). The LeuA-functionalized surface exhibits nearly 2-fold bacterial binding, compared to the 24 AA LeuA functionalized gold surface (Figure 4a).

While the C-terminal region has the ability to bind cells without any harmful effect, the full-length peptide has the ability to capture and kill the cells at the same time, supporting the conjecture that binding is a precursor to the biological activity. Several studies have characterized the relationship

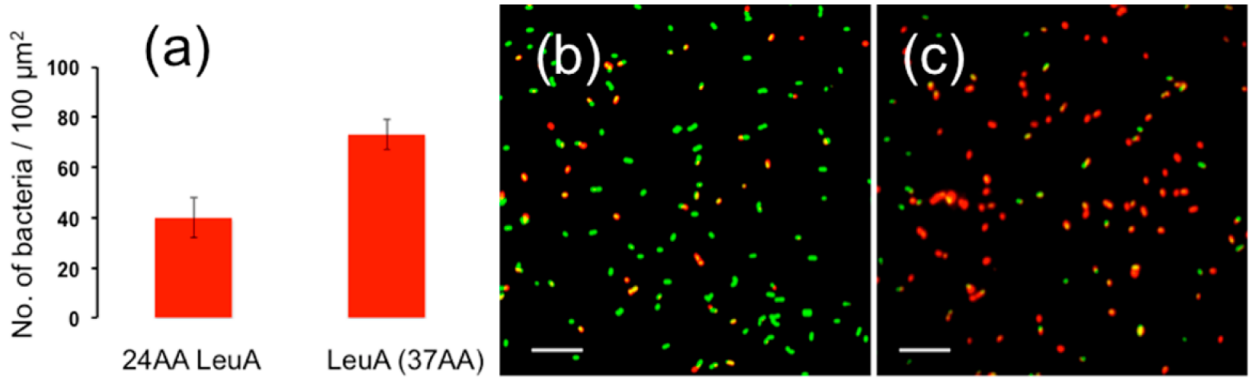

Figure 4. Binding and viability of bacteria (*C. divergens*) attached to peptide coated surfaces. (a) Average number of bacteria bound to 24AA LeuA or full-length LeuA functionalized gold surfaces. The bound bacteria were counted from confocal microscopy images based on five individual studies performed under the same conditions. A gold surface coated with (b) 24 AA LeuA (24-mer) or (c) full-length LeuA (37-mer) was incubated with *C. divergens* (10^3 cfu/mL) for 30 min and a live/dead viability kit was used to stain live cells green and dead cells red. Cells were imaged using a confocal microscope (scale bar = 10 μm).

between surface binding of the AMPs and its subsequent ability to produce biological activity.[38−40] Such peptide-based platforms are promising for developing AMP based diagnostics as well as surface-coating materials with bactericidal properties. Other studies have shown the ability of peptides to bind to bacterial cells in which no activity was observed, suggesting that antimicrobial peptides exhibit a broader range of binding than antibacterial activity.[41−43] Inactive peptides that bind bacteria specifically may prove to be better molecular recognition elements in diagnostic platforms as active peptides damage cells, which may lead to a loss of binding between the peptide and the dead bacteria.

To summarize, the antimicrobial peptide LeuA from class IIa bacteriocins was chemically synthesized and immobilized on a gold substrate. Ellipsometric spectroscopy and FTIR confirmed the proper surface functionalization with peptide monolayer. The immobilized peptide was then evaluated for its binding properties toward various gram-positive bacterial strains. Results of the bacterial screening displayed significantly higher binding of the immobilized LeuA to *L. monocytogenes* species, compared to *S. aureus* and *E. faecalis*, following the same trend as that observed for the bactericidal activity of LeuA toward these strains (Table 1). Moreover, the full-length active LeuA (37-residue) turned out to be a much better interacting peptide with bacteria than the shorter inactive fragment (24 AA LeuA).

4. CONCLUSIONS

The binding of the surface-immobilized LeuA to five strains of gram-positive bacteria has been examined. The peptide immobilized through the C-terminus shows higher binding to bacteria compared to the N-terminally attached peptide, suggesting that the free N-terminal positively charged region of the peptide allows better interaction with bacteria. Moreover, the full-length peptide (37-residue) binds to a higher number of bacteria, compared to the 24AA LeuA fragment. Most bacteria bound to the full-length LeuA immobilized on gold surface were found dead. The activity and better binding of the LeuA functionalized surface emphasizes the importance of the full-length peptide for application in AMP-based bacterial diagnostic platforms.

AUTHOR INFORMATION

Corresponding Author

*Tel.: 780-492-8917. Fax: 780-492-1217. E-mail: kkaur@ualberta.ca.

Notes

The authors declare no competing financial interest.

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