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PAPER

LASIOGLOSSIN-III: ANTIMICROBIAL CHARACTERIZATION AND FEASIBILITY STUDY FOR IMMOBILIZATION APPLICATIONS

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Prevalent bacterial colonization and subsequent biofilm formation in biomedical implants demand for improved antimicrobial properties of these devices. To address this problem, immobilizing antimicrobial peptides (AMPs) on implants is a promising solution because of their biocompatibility and lesser
10 likelihood to incur pathogen to resistance. This study presents a systematic approach towards evaluating the feasibility of Lasioglossin-III (Lasio-III) (a new bee venom AMP, found in *Lasioglossum laticeps*) to be tethered onto biodevices. Antimicrobial characterization of Lasio-III in solution confirms the peptide's membranolytic mode of action and its salt-resistant, broad antimicrobial spectrum activity and anti-biofilm properties against Gram negative and Gram positive bacteria. Lasio-III was covalently
15 immobilized on silicon surfaces using APTES and PEG spacers of varying lengths. Surface characterization of the AMP-immobilized silicon was done using water contact angle measurements, XPS analysis and ellipsometry. Even at modest surface peptide concentrations of $\sim 180 \text{ ng cm}^{-2}$, Lasio-III showed antibacterial activities which were further enhanced with increasing PEG spacer lengths, as determined by live CFU counting and ATP leakage experiments. This proof-of-concept study
20 demonstrates the potential of Lasio-III as an antimicrobial coating candidate.

Introduction

Biofilm mediated microbial infection is a common problem associated with the use of implantable biodevices.¹ Biofilms can colonize almost any material including metals, ceramics and
25 polymers,² resulting in rising infections and mortality rates. While there is an increased demand for implants,³ the rapid rise in antibiotic resistant strains is predicted to further increase infection risks in patients equipped with these implants.⁴ Antimicrobial peptide (AMP)-based coatings are a promising solution to confer
30 antimicrobial functionalities to biodevices whilst simultaneously reducing the emergence of resistant pathogens.^{1, 4, 5} AMPs form an important part of the innate immune system in plants, animals and insects,^{6, 7} and are reported to be effective even against several antibiotic resistant strains due to differing modes of
35 pathogen killing from those of conventional antibiotics.⁴ However, coating bioimplants with AMPs is often challenged by the possibility of reduced AMP activity following immobilization, due to steric hindrance or ionic effects.^{8, 9} To address this challenge, choosing AMP candidates that retains

45 broad antimicrobial spectrum under physiological conditions post-immobilization is critical. Lasioglossin-III (dubbed as Lasio-III here onwards) is a new AMP recently discovered in the venom of *Lasioglossum laticeps*,¹⁰ and is reported to possess characteristically broad antimicrobial spectrum and low toxicity
50 in solution.¹¹ These attributes render Lasio-III an attractive candidate for immobilization studies. To establish the suitability of Lasio-III as a coating agent, the peptide's mode of action must first be studied, which forms the basis of this study. Earlier studies have shown that a tethered peptide's efficacy is dependent
55 on the peptide's mechanism of action. For example, some AMPs such as BUF2 that confers its antimicrobial action *via* intracellular targets, are reported to lose their antimicrobial activity post immobilization,⁹ presumably due to the loss of ability to diffuse to the corresponding binding site(s) to initiate
60 bacterial killing. Membrane active peptides like Tet-213, on the other hand, retain activity in the tethered state.¹² This study investigates the interaction of Lasio-III with bacteria and membrane-mimicking models with the aim to elucidate the peptide's mode of antimicrobial action. A platform for covalent
65 immobilization of Lasio-III *via* polyethylene glycol (PEG) spacer arms was developed to evaluate the tethered peptide's antibacterial behavior. The findings of this proof-of-concept study point to the suitability of Lasio-III as an AMP candidate for peptide immobilization applications.

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Materials and methods

Chemicals and bacterial strains

Lasio-III which is naturally amidated in the C-terminus (VNWKILGKIIKVVK-NH₂) and its N-terminus cysteine modified variant (CLasio-III) were chemically synthesized and procured from GL Biochem, Shanghai, China. All peptides used had a purity of >90%. All other chemicals were obtained from Sigma Aldrich, unless specified otherwise. Bacterial strains used in this study were *Escherichia coli* (ATCC8739), *Pseudomonas aeruginosa* (ATCC9027) and *Staphylococcus aureus* (ATCC6538).

Characterization of the antimicrobial properties of Lasio-III in solution from

Peptide activity and selectivity determination

Minimal inhibitory concentration (MIC) of Lasio-III was determined by the micro-broth dilution method.¹³ In brief, overnight cultures of bacteria in Mueller–Hinton (MH) broth were sub-cultured to mid-log phase. The bacteria were then diluted to 10⁶ CFU/ml, 50 µl of which was added to 50 µl of serially diluted peptide solutions (28–0.4 µM). The plates were incubated at 37°C for 18 h. Wells without peptide served as the positive control while uninoculated media served as the negative control. MIC was determined as the lowest peptide concentration that inhibited complete growth of the bacteria. Experiments were conducted in triplicates and the mean values were reported. For the assessment of the effects of salt ions on peptide activity, media containing NaCl and MgCl₂ at final concentrations of 150 mM and 2 mM, respectively, was used. Minimal haemolysis concentration (MHC) of Lasio-III was measured using freshly collected human Red Blood Cells (hRBC) according to an established method.¹⁴ Fresh hRBCs were washed 3 times with PBS and re-suspended in the same to obtain a 4% (v/v) suspension. 100 µl of this solution was then added to 100 µl of serially diluted peptide solutions in PBS. Samples were then incubated for 1 h at 37°C and centrifuged at 1000 × g for 5 min. The release of hemoglobin was monitored by measuring the absorbance of the supernatant at 405 nm by a microplate reader (Biorad, USA). Absorbance measurements of hRBCs in PBS (A_{blank}) and 0.1% Triton X-100 (A_{triton}) were recorded as negative and positive controls respectively. The percentage of hemolysis was calculated according to the equation:

$$\% \text{ hemolysis} = 100 \times [(A_{\text{sample}} - A_{\text{blank}}) / (A_{\text{triton}} - A_{\text{blank}})].$$

The MHC was taken to be the minimal peptide concentration that produces 50% hemolysis. The MHC and MIC values were used to determine the cell selectivity index (CI) of the peptide.^{15, 16} A similar methodology was used to perform the hemolysis assay for the peptide coated surfaces.

Field emission scanning electron microscopy (FESEM) analysis

FESEM analysis of Lasio-III-treated and untreated bacteria cells were performed using a field electron microscope (Jsm-6700F, JEOL, Japan) based on a reported protocol,¹⁷ to study cell morphology changes with peptide incubation. Briefly, *E. coli*, *S. aureus* and *P. aeruginosa* cells were incubated with Lasio-III at 1X MIC for 15 min, after which the bacteria cells were fixed on glass slides with 4% glutaraldehyde in 0.15 M sodium phosphate buffer (pH 7.4). The slides were rinsed with 0.15 M sodium phosphate buffer and dehydrated through a graded ethanol series

(30–100%). The dehydrated slides were dried at 60°C for 5 min and then coated with platinum metal for 80 s at 20 mA.

Mechanistic Investigations

Outer membrane permeabilization assay based on NPN dye uptake

The outer membrane permeabilization activity of Lasio-III was studied by measuring the extent of 1-*N*-phenylnaphthylamine (NPN) dye uptake. *E. coli* cells grown to mid-log phase were centrifuged at 3000 × g and resuspended in 10 mM phosphate buffer (pH 7.2) to OD₆₀₀ of 0.6 (6 × 10⁸ CFU/ml). A stock solution of NPN dissolved in acetone was added to 500 µl *E. coli* cells containing varying peptide concentration (1–15 µM), to a final concentration of 10 µM. The NPN dye was excited at 350 nm and the increase in fluorescence intensity from emission maxima at 410 nm was recorded using a fluorescence spectrophotometer (LS5, Perkin Elmer, U.S.A.). The basal fluorescence value (i.e. fluorescence of NPN in 500 µl *E. coli* cells) was subtracted from fluorescent readings of all peptide-containing samples. The maximum value of NPN uptake was determined by treating the cells with 10 µl polymyxin B sulfate (from 0.64 µg/ml stock solution), which is an efficient outer membrane permeabilizing agent.¹⁸ The outer membrane permeabilization activity of Lasio-III was assessed by comparing fluorescence readings of peptide-treated cells relative to polymyxin B-treated cells.

Inner membrane permeabilization assay

Overnight grown *E. coli*, *S. aureus* and *P. aeruginosa* cells were subcultured in fresh MH media to OD₆₀₀ ~ 0.35. Cells were then harvested by centrifugation (10000 × g, 5 min) and washed with PBS thrice. The washed bacteria cells were resuspended in PBS to final a concentration of 10⁸ CFU/ml. 50 µl of the cell suspension was incubated with 50 µl of peptide in PBS at concentrations of 8X, 4X, 2X and 1X MIC for 60 min at 37°C. 5 µl of Propidium iodide (PI) (1 µg/ml) was then added to the cell suspensions and fluorescence was measured using a multi-mode microplate reader (Synergy HT, BioTek Instruments, U.S.A.) at excitation and emission wavelengths of 520 nm and 620 nm, respectively. Cells in buffer with and without peptide served as the positive and negative controls, respectively. The mean value for three triplicates subtracted from those of corresponding negative controls was reported.

Circular dichroism (CD) spectroscopy

The secondary structure of Lasio-III in the presence and absence of SDS (sodium dodecyl sulphate) and DPC (dodecylphosphocholine) lipid micelles were determined using the Chirascan™ Circular Dichroism Spectrometer (Applied Photophysics Limited, U.K.). Peptide concentrations for the CD studies were fixed at 100 µg/ml in 10 mM phosphate buffer (pH 7.2). The final concentration of the lipid micelles was 10 mM. The spectra of peptide solutions contained in a 0.01 cm path length quartz cuvette (Hellma) were recorded at 25°C from 190 to 240 nm using a bandwidth of 1 nm and averaged over three scans. Data was collected over a step size of 0.5 and time constant of 0.5 s. Baseline scans were obtained using the same parameters for samples containing buffer, micelles and vesicles only, and were subtracted from the respective data scans with peptide.

Isothermal titration calorimetry (ITC)

All ITC experiments were performed on a VP-ITC calorimeter (Micro Cal Inc.) at 30°C with DPC lipid micelles. For titration with DPC micelles, Lasio-III and lipid were both dissolved in 10 mM phosphate buffer (pH 7.2). Twenty injections of 5 µl aliquots of the DPC micelles (100 mM) were added to the sample cell containing Lasio-III at 5 µM, while the reference cell contained 10 mM phosphate buffer. Each titration was performed at an interval of 300 s, stirring speed of 300 rpm and the heat exchange was recorded. In all cases, the heats of dilution of the micelle alone into the buffer were subtracted from the titration data. The resulting data were integrated with Micro Cal Origin 5.0, fitted with models provided in the software and analyzed to determine the association constant, K_a , and the enthalpy change, ΔH . The Gibb's free energy change, ΔG , and entropy change, ΔS , were calculated from the fundamental thermodynamic equations: $\Delta G = -RT \ln K_a$ and $\Delta S = (\Delta H - \Delta G)/T$, respectively.

Fluorescence and paramagnetic probe quenching

The interaction of Lasio-III with negatively charged SDS micelles were analyzed by step wise titration of the peptide (5 µM) in 10 mM phosphate buffer (pH 7.2) with addition of SDS from 2 to 20 mM. The lone tryptophan of the peptide was excited at a wavelength of 280 nm and the intrinsic fluorescence was monitored between 300-460 nm using a fluorescence spectrophotometer (LS5, Perkin Elmer, Inc.) with dual monochromators. Measurements were recorded using a 0.1 cm path length cuvette and a slit width of 3nm.

The fluorescence quenching of the micelle bound peptide was performed with two paramagnetic probe quenchers, i.e. 5-doxyl stearic acid (5-DSA) and 16-doxyl stearic acid (16-DSA). The stock solutions of the paramagnetic probes were prepared by dissolving 10 mg of each 5- and 16-DSA in methanol. 1 mM solutions of the probes were then prepared in phosphate buffer for the quenching experiments. Small aliquots of the paramagnetic probe (5-20µM) were serially added into the buffer solution containing 5 µM of the peptide bound to 15 mM SDS micelles. The corresponding tryptophan fluorescence intensity quenching was monitored between 300-400 nm.

Bacteria inhibition assays

Time dependent killing kinetics

Bacteria strains were grown overnight at 37°C in MH broth with ambient air supply. Aliquots of exponentially growing bacteria were resuspended in fresh MH broth in a 96-well plate at approximately 10^5 CFU/ml and treated with Lasio-III at 2X, 1X, X/2 and X/4 MIC. The cells were incubated at 37°C and the OD₆₀₀ was measured at 15 min intervals. All experiments were done in triplicates and the mean $\pm$ standard deviations (SD) of the OD readings were reported.

Anti-biofilm assay

Biofilm production was performed on flat-bottomed polystyrene plates (Corning Inc. #3799) according to the protocol described previously¹⁹ with slight modifications. Briefly, *E. coli*, *S. aureus* and *P. aeruginosa* cells grown to logarithmic phase were diluted to 10^5 CFU in 200 µl of LB medium and were further incubated with Lasio-III at 2X, 1X, X/2 and X/4 MIC. Each peptide concentration of the test was replicated for six wells (n = 6). Plates were incubated for 24 h at 37°C and analysed for biofilm formation by crystal violet staining²⁰ and OD₆₀₀ measurement for bacterial count. Bacteria cells in media without any peptide

served as the positive control, while sterile media served as the negative control.

Statistical analysis

The antimicrobial assays were performed in triplicates and the average values were reported, while the anti-biofilm assays were done with n = 6. Each figure was represented with standard deviation for the mean. For all other assays, p values were determined using paired student's t-test for analysis of significance, P-values less than 0.05 were considered to be significant.

Peptide immobilization

Substrate preparation

Silicon wafers (diameter = 2.54 cm) were obtained from Latech, Singapore. The wafers were first washed by boiling with 0.05% SDS solution for 30 min followed by washing with copious amounts of DI water. The washed surfaces were then heated to 70°C in basic piranha solution comprising ammonium hydroxide, hydrogen peroxide and water (1:1:5 v/v). The oxidised silicon surfaces were thoroughly washed with DI water and dried in a stream of nitrogen. The surfaces were then amino-silanized with 2% v/v amino propyl triethoxy silane (APTES) solution in anhydrous toluene for 1 h. The slides were cured at 100°C for 1 h and washed with dry dimethyl formamide (DMF) before further treatment for peptide immobilization. Since, the DMF wash leaches out the adsorbed superficial layers of APTES that are held together by weak interactions, only a single covalently immobilized layer is leftover.

Spacer mediated immobilization of CLasio-III on silanized surface

The amino- silanized surfaces prepared above were first PEGylated using Succinimidyl-([N-maleimidopropionamido]-polyethyleneglycol ester (NHS-PEG-Mal) (Thermo Scientific Inc.). The NHS-PEG-Mal molecule consists of two functional groups viz. NHS (N-hydroxy succinimide) and maleimide that are separated by a PEG spacer arm. In this study, two different PEG spacer arms consisting of 12 and 24 ethylene oxide moieties were used and designated as NHS-PEG(12)-Mal and NHS-PEG(24)-Mal, respectively, throughout this study. 0.2 mg/ml NHS-PEG-Mal solutions were prepared in dry DMF and allowed to react with two sets of amino-silanized surfaces for 4 h. The resulting surfaces were washed thoroughly with DMF followed by water and PBS. The PEGylated silicon surfaces produced were treated with N-terminus cysteine-modified Lasio-III (CLasio-III) in PBS at a concentration of 0.2 mg/ml. Details of the reaction scheme is presented as Fig. 1.

Surface characterization

Contact angle measurement

Static water contact angle measurements of the coated and the uncoated silicon wafers were carried out using a dynamic contact angle analyser (Fta200, FTA, U.K.). Images of 5 µl water droplets on sample surfaces were taken using a digital camera provided with the instrument and analysed using the Fta32 software. At least three different spots were tested for each sample and the average value $\pm$ SD (standard deviation) were reported.

X-ray photoelectron spectroscopy (XPS)

Fig. 1 Schematic representation of the reaction steps involved in the covalent immobilization of CLasio-III on a silicon surface. The bifunctional PEG spacers were attached to the amino groups provided by APTES on one side followed by a specific cysteine-maleimide reaction for peptide coupling on the other side.

the silicon surface was performed using an X-ray photoelectron spectrometer (Axis-ULTRA, Kratos) equipped with Al K α source operating at a power of 10 mA, 15 kV. Elements were identified from the survey spectra. High resolution spectra of Carbon, Nitrogen and Oxygen were recorded individually for elemental ratio comparisons.

Measurement of coated film thickness using ellipsometry

Coated film thickness was measured using a variable angle spectroscopic ellipsometer (VASE, UVI-SEL, Horiba Jobin Yvon) fitted with a 75 W xenon arc lamp as the light source. All measurements were performed using an incident angle of 70° with the incident photon energy ranging from 1.5 – 4.7 eV with a step size of 0.1 eV. Film thickness calculations were performed by the DP2 software using the standard slab model based on the isotropy of the optical properties of the polymer and the peptide films²¹; the selected parameters in the models were optimised to best fit the spectral data. All calculations were done using the Levenberg-Marquardt optimization algorithm, where Goodness of Fit (GOF) was evaluated using χ^2 values. All fitting results provided χ^2 values less than 1, indicating high quality of fit. The APTES layer was modelled using the Aminosilane dispersion formula, while the PEG and the bound peptide layers were modelled using the Cauchy approximation provided within the DP2 software. The refractive index of the APTES layer was fixed as 1.422, while that of the PEG and peptide layer was set at 1.460.^{12, 22}

Determination of peptide concentration on coated silicon surface

Film thickness obtained from ellipsometry measurements was used to measure CLasio-III concentration on the surface, using the same method reported in previous studies.^{22, 23} For a pure substance coated on a surface, the mass per unit area (σ) of the adsorbed layer is estimated using Equation 1

$$\sigma = d \cdot \rho^0 \quad (1)$$

where d is the thickness of the adsorbed layer (in nm) and ρ^0 is the density (mass per unit volume) of the desired substance.²³ For a pure substance, ρ^0 is given by

$$\rho^0 = M_w \cdot N \quad (2)$$

where M_w is the molecular weight and N the number of moles of the substance. Using the Lorentz-Lorenz equation, the value of N can be determined from the optical properties of the substance²⁴ and thus Equation 2 can be re-written as

$$\rho^0 = M_w \cdot N = \frac{M_w}{A} \frac{n^2 - 1}{n^2 + 2} \quad (3)$$

where A is the molar refractivity of the adsorbed substance and n is its refractive index. Hence for a pure substance, σ can be determined from Eq 4

$$\sigma = d \cdot \rho^0 = \frac{0.1 M_w \cdot d}{A} \frac{n^2 - 1}{n^2 + 2} \quad (4)$$

where, σ is expressed in $\mu\text{g}/\text{cm}^2$. The M_w/A value for adsorbed Lasio-III is calculated to be 4.254 based on previous findings.²⁵

Anti-bacterial assay of CLasio-III-immobilized silicon surfaces

The antibacterial activity of the CLasio-III immobilized silicon surfaces were done as per the ISO 22196 protocol²⁶ with some modifications. *E. coli* cells in the mid-log phase were diluted to 10^5 CFU/ml in LB medium. 20 μl of the cell suspension was added into the wells of a six-well culture plate, and covered with

the coated silicon wafers to allow the inoculums to spread over

Fig. 2 FESEM images of Lasio-III induced membrane disruption of the bacteria cells. Lasio-III (1X MIC) incubated with *E. coli*, *P. aeruginosa* and *S. aureus* (down panel) and non-peptide treated controls (up panel).

the entire peptide-tethered surface. The plates were incubated at 37°C for 2 h, followed by washing with 2 ml of fresh LB broth. The viable cells in solution were then serially diluted and 20 µl aliquots were spread on LB agar plates for CFU determination after 16 h incubation. Silicon wafers coated with NHS-PEG-Mal moieties only were used as the control surfaces.

ATP leakage assay

The effect of immobilization on the activity of immobilized CLasio-III was studied by determining ATP leakage of bacteria cells. CLasio-III immobilized silicon slides were treated with 300 µl of 10⁵ CFU/ml *E. coli* cells in PBS buffer. The cells were incubated for 30 min at 37°C following which ATP leakage was quantified using the BacTiter-Glo kit (Promega) by measuring sample luminescence using a luminometer (Modulus Fluorometer 9200-000, Turner BioSystems, U.S.A.). PEG coated silicon wafers were used as the negative control while ATP leakage from bacteria treated with 1X MIC of Lasio-III in solution was considered as the positive control.

Results

The antimicrobial activity of Lasio-III was tested against Gram negative bacteria (*E. coli* and *P. aeruginosa*) and Gram positive bacteria (*S. aureus*) (Table 1). The antimicrobial potency of Lasio-III against the three bacteria strains tested is shown by the low MIC values. The MHC value of Lasio-III was found to be 135 µM. A high CI value for all the strains tested indicate that Lasio-III has good selectivity towards bacteria cells compared to the host cells, demonstrating good biocompatibility. The efficacy of many AMPs is hindered by ions present in urine and serum. Lasio-III, however, exhibits salt tolerant properties, where the peptide retains its antimicrobial activity under physiological salt concentrations. FESEM analysis shows that Lasio-III treated bacteria cells had significantly compromised outer membrane integrity (Fig. 2). Obvious differences in the membrane morphology of peptide-treated cells and untreated cells are apparent, where the former exhibit significant membrane wrinkling, blister formation and rupture.

Following the observation that Lasio-III can induce outer

membrane disruption from FESEM analysis, we set out to perform detailed studies to ascertain whether Lasio-III can induce both outer and inner membrane permeabilization of bacteria cells. The ability of Lasio-III to permeabilise the outer membrane was assessed through the NPN dye uptake assay. NPN molecules which are hydrophobic, do not fluoresce in hydrophilic aqueous environment but generate a strong fluorescence when the molecules enter a hydrophobic environment like the lipid bilayer of a bacteria outer membrane.²⁷ Fig. 3A shows that significant fluorescence relative to polymyxin B-treated cells is obtained when *E. coli* cells were incubated with Lasio-III, which confirms the occurrence of outer membrane perturbation, leading to incorporation of the dye into the hydrophobic milieu. A bacteria cell that is intact does not absorb the dye.

Inner membrane permeabilization of Lasio-III was tested based on its capability to induce PI uptake by the bacteria cells. PI fluoresces strongly upon intercalating with DNA inside the cell, which indicates a compromised inner membrane integrity.²⁸ Fig. 3B shows the PI fluorescence (after subtraction of negative control fluorescence readings) upon incubation of bacteria and Lasio-III. The increasing fluorescence readings with increasing peptide concentrations suggest dose-dependent inner membrane permeabilization behaviour within the range of peptide concentration tested. The increased fluorescence of *P. aeruginosa* compared to that of *E. coli* and *S. aureus* is attributed to the larger genome size of *P. aeruginosa* (>1.3 times).^{29, 30}

Table 1 Antimicrobial and cell selectivity index of Lasio-III against different bacteria.

Bacteria	MIC (µM)			CI ^a
	Normal (MH) Broth	150 mM NaCl	2 mM MgCl ₂	
<i>E. coli</i>	1.75	1.75	1.75	154
<i>S. aureus</i>	1.75	6.2	1.75	154
<i>P. aeruginosa</i>	3.5	3.5	7.0	77

^a The cell selectivity index (CI) was calculated using the formula $CI = 2 \times \text{MHC} / \text{MIC}$, where the MHC value of Lasio-III was 135 µM.

Fig. 3 *In vitro* characterization of the mechanism of action of soluble Lasio-III. Permeabilization of the outer membrane is shown by the uptake of the NPN dye at 410 nm. Membrane permeabilizing activity of Lasio-III is expressed as percentage membrane permeabilization relative to polymyxin B (A). Inner membrane permeabilization study by DNA intercalation with PI dye (B). The induction of alpha helicity upon the interaction of Lasio-III with negatively charged SDS and zwitterionic DPC is elucidated by CD spectroscopy (C). ITC with DPC micelles for thermodynamic characterization of membrane peptide interaction is provided in (D). Fluorescence based quenching of 5- (left) and 16-DSA (right) paramagnetic probes with SDS bound peptide micelles is presented in (E).

To investigate the effect of membrane-peptide interactions on peptide secondary structure, the CD spectra of Lasio-III was measured in the presence of negatively charged SDS and zwitterionic DPC micelles, which are commonly used anionic and zwitterionic membrane-mimicking models.³¹ Fig. 3C shows that interaction of Lasio-III with both lipid micelles resulted in a dip in the CD spectrum at 208 and 222 nm, which indicates helicity development. Fig. 3D shows the binding profile of DPC micelles with Lasio-III under iso-thermal conditions. The thermodynamic profile of the DPC injections is shown in the upper panel, while the corresponding fitted curve is shown in the panel below. The data indicate that the binding results in an exothermic reaction with a binding constant, K_a , of $13.8 \times 10^7 \text{ M}^{-1}$ and enthalpy change (ΔH) in the order of -10.8 kcal/mol which are comparable to those of other bee venom peptides such as melittin.³²

To further understand the membrane disturbing and antimicrobial property of Lasio-III, we utilized the intrinsic tryptophan fluorescence emission of its lone tryptophan residue in presence of the negatively charged SDS micelles. Membrane-peptide interaction was monitored by the shift in fluorescence emission maximum upon treatment of the peptide with SDS micelles. The emission maximum of the peptide in the buffer was observed at 360 nm. Upon addition of saturating concentration of the SDS micelles at 15 mM, which was previously determined

through fluorescence binding assay (data not shown), the emission maximum of Lasio-III showed a blue shift to 330 nm. This significant blue shift of $\sim 20 \text{ nm}$ indicates the peptide's high affinity for negatively charged lipid membrane (Fig. 3E). The orientation of the peptide in the SDS micelles was analyzed by addition of either 5-DSA or 16-DSA paramagnetic probes to the micelle bound peptide.³³ Since 5-DSA (doxyl moiety at the 5th position) is known to perturb the residues closer to the micelle surface, whilst 16-DSA (doxyl moiety at the 16th position) perturbs residues inserted in the lipid micelle (interfacial hydrophobic),^{32, 34} these probes were used to locate the position of the Lasio-III tryptophan residue within the SDS micelle. A drastic quenching in the fluorescence intensity, observed only with addition of increasing concentration of the 16-DSA compared to that in 5-DSA was observed (Fig. 3E), indicating that the N-terminal fragment of the peptide is inserted within the micelle environment. Addition of the paramagnetic probes beyond $20 \mu\text{M}$ did not cause any further quenching in fluorescence intensity.

Further, killing kinetics test was performed to assess the efficiency of Lasio-III in inhibiting bacteria cells. Fig. 4A-C show fast killing rates of Lasio-III against *E. coli*, *S. aureus* and *P. aeruginosa*, where complete inhibition was observed for three bacteria strains at $\geq 1 \times \text{MIC}$ within 15-30 min. Biofilm formation by the three bacteria strains was tested in the presence of Lasio-

Fig. 4 Effectiveness of Lasio-III on real time growth and antibiofilm activity. The growth kinetics of *E. coli* (A), *P. aeruginosa* (B) and *S. aureus* (C) in the absence and presence of peptide at different concentrations. The biofilm inhibitory ability against *E. coli* (D), *P. aeruginosa* (E) and *S. aureus* (F) with varying peptide concentrations normalized against non-peptide treated controls (taken as 100%). The dark grey bars represent the absorbance of crystal violet at 570 nm while the light grey bars denote the absorbance at 600 nm. The asterisk (*) indicates statistical difference from the positive control ($p < 0.05$).

III at different concentrations. The percentage of bacteria growth and the corresponding biofilm obtained from Lasio-III treated samples relative to those for non-peptide treated control samples (considered as 100%) are shown in Fig. 4D-F. Significant reduction in biofilm formation was observed for all the bacteria incubated at $\geq 1 \times \text{MIC}$ of Lasio-III. Amongst the three organisms, *P. aeruginosa* showed the highest inhibition in biofilm formation at sub-MIC levels of Lasio-III. Based on these findings, it is reasonable to conclude that Lasio-III is a broad spectrum AMP that remains active even at physiological salt concentrations. The peptide's fast killing kinetics as well as anti-biofilm properties render it a suitable candidate for immobilization studies. A platform for covalent immobilization of Lasio-III on silicon surfaces was developed and the characterization studies of the peptide-immobilized surfaces were subsequently performed. Terminal cysteine-based attachments of peptides is favourable, where it allows a fixed peptide oriented coupling mediated by covalent bonding and eliminates the problem of position independent attachment of peptides that can result in loss of activity.³⁵ Cysteine modification at both the N- and C-terminus of Lasio-III was tested, but the N-terminal modification was found to be more active (data not shown) and hence was used for the successive immobilization studies. The immobilization

procedures are performed by treating the silicon surfaces according to the reaction scheme shown in Fig. 1.

Static water contact angle measurement was used to assess the success of each surface reaction. The water contact angle trends for the respective surfaces are shown in Table 2. The first reaction step involves surface oxidation using basic piranha, which is expected to increase surface hydrophilicity; this was confirmed by the reduction in water contact angle from 53° for the untreated silicon wafer to 15° . In the second step, after APTES reaction, the surface hydrophilicity is expected to decrease due to the presence of propyl groups of the APTES molecule that increased the contact angle to 35.6° . Following further treatment with NHS-PEG-Mal, the water contact angle remains within the range of $55^\circ - 65^\circ$, and this is attributed to the amphipathicity of PEG. Following peptide immobilization, our results confirm that the wettability of the peptide-immobilized silicon surface increased, which led to a reduction in the contact angle ($33^\circ - 38^\circ$).³⁶ XPS analysis provided in Fig. S1 (Supporting information) presents high resolution X-ray photoelectron spectra of individual elements i.e. Carbon 1s, Nitrogen 1s and Oxygen 1s for the Silicon surfaces following different stages of reaction. A good correlation of the measured change in atomic percentage of C/N and C/O (Table 2) with each surface modification step was obtained. In brief, an increased

C/N ratio is expected after the reaction of APTES with PEG molecules, since the latter does not contain nitrogen. After peptide immobilization, the C/N ratio decreased as expected, due to the presence of peptide-derived nitrogen.

Table 2 presents the thickness of the different coated layers on the silicon surfaces as determined by spectroscopic ellipsometry. Incubation of APTES on silicon surfaces can lead to its aggregation mainly through hydrogen bonding.³⁷ Therefore, curing the surfaces at elevated temperatures (following treatment with APTES) is necessary to ensure covalent interaction with the silicon surface. Following washing of excess APTES molecules during subsequent DMF washing steps, a monolayer of approximately 10 Å in thickness is expected.³⁸ The monolayer thickness of CLasio-III which comprises 15 amino acid residues was measured to be ~10 – 15 Å, as determined by ellipsometry measurements, and correspond to that expected for a given

Table 2 Water contact angle measurement, dry-thickness of individual layers coated on silicon wafers determined by ellipsometry, and elemental surface composition analysis determined by XPS.

Sl. No.	Layer	Water contact angle (°)	Thickness (Å)	Surface composition from XPS analysis	
				C/N	C/O
1.	APTES	N.A.	94.73 ± 5.65	N.A.	N.A.
2.	APTES**	35.6 ± 1.8	11.20 ± 3.14	5.0	1.63
3.	PEG(12) Mal [^]	61.4 ± 5.0	42.51 ± 10.4	6.59	1.81
4.	PEG(24) Mal [^]	53.7 ± 3.9	34.01 ± 1.25	8.19	2.48
5.	PEG(12) Lasio [#]	37.8 ± 2.5	14.92 ± 1.12	6.18	1.52
6.	PEG(24) Lasio [#]	33.5 ± 0.7	15.47 ± 3.10	5.13	1.76

**Thickness measured post-curing of APTES layer and DMF washing

[^]Thickness of the corresponding PEG layer

[#]Thickness of the bound Lasio-III layer on the respective PEG surface
N.A.: Not analysed

peptide monolayer on the PEG coated silicon surfaces. Using Equation 4 to estimate peptide concentration on the surface, the amount of CLasio-III present on the PEG(12) and PEG(24) surfaces were found to be comparable, i.e. 172 ± 10 ng/cm² and 180 ± 15 ng/cm², respectively.

Bioactivity assays on immobilized CLasio-III show that the peptides retain anti-bacterial activity post immobilization. Immobilized CLasio-III were also non-hemolytic, where less than 1% hemolysis was observed (data not shown). The percentage bacteria inhibition relative to control surfaces containing PEG(12) and PEG(24) without peptides was found to be slightly higher for CLasio-III immobilized on PEG(24) surface (70% ± 10) than the PEG(12) surface (54% ± 8) (Fig. 5A). The membrane-permeabilizing capability of immobilized CLasio-III was confirmed by the ATP leakage assay results on the respective peptide-immobilized surfaces after incubation with bacteria. Fig. 5B shows the net luminescence of the samples after subtraction of the blank sample luminescence which consists of the ATP reaction mixture only. It is clear that higher luminescence is observed for both peptide-immobilized surfaces compared to the non-peptide treated surfaces, which indicates that the immobilized peptides can still induce membrane permeabilization

in the bacteria cells.

Fig. 5 Characterization of the bactericidal activity of immobilized CLasio-III. Antimicrobial activity of the CLasio-III immobilized silicon surfaces against *E. coli* (determined by CFU counts), expressed as percentage killing relative to that of PEG-treated surfaces without peptide (A). ATP leakage of *E. coli* cells upon the interaction with immobilized CLasio-III (B).

Discussion

In this study, the antimicrobial profile, biocompatibility and bacteria-killing mechanisms of Lasio-III were investigated, with the aim to systematically assess the suitability of Lasio-III as a potential AMP-based coating agent. Lasio-III was chosen based on its broad antimicrobial spectrum and low toxicity.^{10, 11} We found Lasio-III to be highly active against *E. coli*, *S. aureus* and *P. aeruginosa* (with MIC in the micromolar range) and is resistant to salt and ions at physiological concentrations (Table 1). These attributes render Lasio-III superior to many conventional AMPs which lose antimicrobial activity when in contact with salt and ions.⁸ However, better understanding of the peptide's mechanism of action both in the soluble and immobilized form is critical to assess its suitability as an antimicrobial coating agent.³⁹ A set of studies to investigate the mode of killing of Lasio-III in the soluble form indicate its ability to bind both the outer and inner membranes of the three bacteria strains studied (Fig. 3A-E). CD and ITC studies to elucidate peptide interaction and binding behaviour with membrane mimicking lipid models point towards its membrane targeting mechanisms. The tryptophan quenching assay with 16-DSA further confirmed the membrane perturbing potential of Lasio-III. Based on these results, we conclude that the mechanism of action of Lasio-III certainly involves membrane-interaction. Studies on the rate of Lasio-III-induced pathogen killing and biofilm

inhibition (Fig. 4A-C) clearly show that Lasio-III possesses a fast killing ability toward both Gram positive and negative bacteria compared to many other active AMPs⁴⁰⁻⁴² and could also inhibit

biofilm formation for all the tested bacteria (Fig. 4D-F). Considering the promising antimicrobial characteristics of Lasio-III, further studies to evaluate the activity of Lasio-III post immobilization was performed. We proceeded to develop a platform for Lasio-III immobilization. Considering that a large fraction of implantable biodevices are made of silicone based polymeric materials,⁴³ we chose to develop the peptide immobilization platform on a silicon surface. In this study, N-terminal cysteine modified Lasio-III immobilization on the silicon surface was performed using suitable spacers. Direct surface immobilization strategies were not considered because of the high probability for loss of peptide activity due to steric hindrance, as reported in earlier studies.⁹ PEG spacers were chosen for this work due to its anti-bacteria adhesion properties,⁴⁴ Silicon surfaces were first amino-silanized using APTES to form a layer that will provide free -NH₂ groups for easy coupling with the N-hydroxy-succinimide moiety of NHS-PEG-Mal.³⁵ Two PEG moieties which had 12 and 24 ethylene oxide moieties, respectively, were used, i.e., (i) NHS-PEG(12)-Mal (spacer length ~ 53.4 Å⁰) and (ii) NHS-PEG(24)-Mal (spacer length ~ 95.2 Å⁰). The free maleimide groups on the PEG-immobilized silicon surface allow thio-ether linkage formation with the free N-terminal cysteine residues of CLasio-III, to yield a covalently linked monolayer of the peptide⁴⁵. Surface characterization of the silicon wafers including static water contact angles, XPS and ellipsometry (Table 2) at different reaction stages confirmed the success of the reaction scheme presented in Fig. 1. A surface peptide concentration of 170-180 ng/cm² was obtained, as determined by ellipsometry, which lies within the expected concentration range for a peptide monolayer. Growth inhibition for *E. coli* was observed even at this modest surface concentration (Fig. 5A). As the surface peptide concentration was comparable with the use of both spacer lengths, the use of PEG(24) spacer gives a higher bacteria inhibition compared to PEG(12). Our findings agree well with earlier studies which also reported that the antibacterial action of immobilized AMPs improved with increasing spacer lengths for penetrating-based mechanisms.^{9,46} Furthermore, the ATP leakage test on the immobilized CLasio-III (Fig. 5B) and the fluorescence quenching assays (Fig. 3E), suggest that the peptide activity is mediated through a membrane interactive mode of kill. However, some short peptides (~9-10 residues) which are tethered without spacers, have also been reported to retain their antimicrobial activities. This could be the result of possible electrostatic interferences leading to alteration of the cytoplasmic membrane Donnan potential. Therefore, other plausible modes of action related to ionic imbalance or autolytic cell death cannot be ruled out for immobilised AMPs.¹ The antimicrobial behaviour of tethered AMPs will vary with coupling chemistry, immobilization surfaces, spacers and individual AMP characteristics.

Conclusions

Antimicrobial activity characterization of a new broad spectrum and salt-resistant AMP, Lasio-III, in both the soluble and immobilized form is reported. An immobilization platform to

covalently impregnate and assemble Lasioglossin-III on silicon wafers was successfully developed, leading to the first proof-of-concept study of antimicrobial characterization of immobilized Lasioglossin-III. The results of this study point to the potential of Lasio-III as an AMP candidate for a variety of immobilization applications.

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Supplementary information

† Electronic Supplementary Information (ESI) available. See DOI: 10.1039/b000000x/

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