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The effect of thiol functional group incorporation into cationic helical peptides on antimicrobial activities and spectra

Nikken Wiradharma [a], Majad Khan [a], Lin-Kin Yong [a], Charlotte A.E. Hauser [a], See Voon Seow [b], Shuguang Zhang [c], Yi-Yan Yang [a,*]

[a] Institute of Bioengineering and Nanotechnology, 31 Biopolis Way, Singapore 138669, Singapore
[b] National University of Singapore, Yong Loo Lin School of Medicine, Department of Pediatrics, 5 Lower Kent Ridge Rd, Singapore 119074, Singapore
[c] Massachusetts Institute of Technology, Center for Biomedical Engineering and Center for Bits & Atoms, 77 Massachusetts Avenue, Cambridge, MA 02139, USA

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ABSTRACT

Antimicrobial peptides (AMP) have been proposed as blueprints for the development of new antimicrobial agents for the treatment of drug resistant infections. A series of synthetic AMPs capable of forming α-helical structures and containing free-sulfhydryl groups are designed in this study ($(LLKK)_2C$, $C(LLKK)_2C$, $(LLKK)_3C$, $C(LLKK)_3C$). In particular, the AMP with 2 cysteine residues at the terminal ends of the peptide and 2 repeat units of LLKK, i.e., $C(LLKK)_2C$, has been demonstrated to have high selectivity towards a wide range of microbes from Gram-positive *Bacillus subtilis*, Gram-negative *Escherichia coli*, *Pseudomonas aerogenosa*, and yeast *Candida albicans* over red blood cells. At the MIC levels, this peptide does not induce significant hemolysis, and its MIC values occur at the concentration of more than 10 times of their corresponding 50% hemolysis concentrations (HC_{50}). Microscopy studies suggest that this peptide kills microbial cells by inducing pores of ~20–30 nm in size in microbial membrane on a short time scale, which further develops to grossly damaged membrane envelope on a longer time scale. Multiple treatments of microbes with this peptide at sub MIC concentration do not induce resistance, even up to passage 10. However, the same treatment with conventional antibiotics penicillin G or ciprofloxacin easily develop resistance in the treated microbes. In addition, the peptides are shown not to induce secretion of IFN-γ and TNF-α in human monocytes as compared to lipopolysaccharide, which implies additional safety aspects of the peptides to be used as both systemic and topical antimicrobial agents. Therefore, this study provides an excellent basis to develop promising antimicrobial agents that possess a broad range of antimicrobial activities with less susceptibility for development of drug resistance.

1. Introduction

Peptide-based biomaterials provide excellent avenues to a wide range of bioengineering and biomedical applications [1,2], including regenerative medicines [3,4], biomimetics materials [4,5], therapeutic delivery [6–9], and antimicrobial agents [10–12]. Secreted peptides as antimicrobial agents are abundant in nature, which provide a full-proof mechanism to fight against invasion of various microbial pathogens. Upon meeting potential harmful invasion of microorganisms, multicellular organisms secrete membrane-lytic molecules, often called antimicrobial peptides (AMP). These molecules have been reported to adopt various types of secondary conformations upon interactions with biological membranes [13–15]. On the other hand, they exist in random structures in their native forms, i.e. prior to interactions with biological membranes [15]. Owing to the robust capability to destroy microbial membranes, AMPs have been proposed as important blueprints for new generations of antibiotics in order to overcome multidrug resistant pathogenic microbes [14].

Multidrug resistant pathogens acquire their antibiotics resistance traits through a "natural selection" process in response to antibiotics exposure, which can be perceived as the "environmental pressure". The underlying mechanism of this evolution could involve any one of the following four biochemical processes [16]: (1) drug inactivations/modifications, such as in the case of production of β-lactamase or penicillinase enzyme by methicillin-

* Corresponding author. Tel.: +65 68247106; fax: +65 64789084.
E-mail address: yyyang@ibn.a-star.edu.sg (Y.-Y. Yang).

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resistant *Staphylococcus aureus* (MRSA) [17]; (2) alteration of antibiotics' inhibitory target site, such as in the case of alteration of penicillin binding protein (PBP) of drug-resistant Gram-Positive bacteria, allowing them to overcome the inhibition of peptidoglycan synthesis, which is an important layer of bacterial membrane integrity [18]; (3) reduced drug accumulation, such as in the case of low antibiotics susceptibility of *Pseudomonas aeruginosa*, and many other Gram-negative bacteria, owing to its low drug permeability of the cellular envelope and multidrug efflux pumps encoded by the drug-resistant genes [19]; and (4) alteration of metabolic pathways, such as in the case of sulfonamide-resistant bacteria, in which bacterial growth is no longer inhibited through the presence of metabolic enzyme-competing sulfonamide drugs [16]. Depending on the nature of the antibiotics, its target sites, and the bacterial species, these biochemical aspects, together with the genetic aspects responsible for the transfer of drug-resistance genes, form the basis for the emergence of multidrug resistant microbes. This, therefore, suggests that there is a great need for the development of alternative antimicrobials that can escape such microbial stress responses.

Macromolecules that attacked on microbial membranes, such as AMPs [13–15,20] or synthetic polymers mimicking AMPs [21–23] have been proposed as one of these alternative anti-infective agents. It is suggested that it may take a significantly much longer "natural selection" process for the microbial cells to totally change their cellular envelope compositions in order to overcome resistance towards AMPs' cell-lytic mechanism of actions [24,25]. Even so, the use of natural AMPs found in human's natural defense system in clinical setting in the long run has been argued to potentially increase dangers to the public's health, especially if these pathogens successfully evolve to become resistant to these natural AMPs [26]. Aware of these, Greg, first proposed a systematic method to generate series of non-natural AMPs through the use of "linguistic model" to identify the commonalities among the reported natural AMPs from various organisms [27]. In our laboratory, we recently developed nanoparticles self-assembled from an amphiphilic peptide as an alternative strategy for combating brain infections caused by Gram-positive bacteria or fungi [10,11]. Jian et al., on the other hand, adopted similar peptide self-assembly approach forming peptide nanotubes to provide alternative antimicrobials with wide antimicrobial spectra [12].

Most recently, we have proposed using protein folding theory as the first principle to design a series of non-natural α-helical AMPs that possess a general primary structure of $(XXYY)_n$, whereby X is a hydrophobic amino acid, Y is a cationic amino acid, and *n* is the number of repeat units varying from 2 to 4. These AMPs are effective in inhibiting microbes belonging to both Gram-positive and yeast families, with AMP having $(LLKK)_3$ sequence that exhibits the highest selectivity towards microbes over mammalian cells [20]. In this study, we attempt to broaden the antimicrobial spectrum of these synthetic α-helical peptides to combat Gram-negative bacteria by providing systematic modification on their primary structure. As highlighted in several earlier studies, the presence of free-sulfhydryl (thiol) group(s) in natural AMPs [28] or in Bismuth-derived organometallic antimicrobials [29] was suggested to significantly increase the potency of the antimicrobials. We hypothesize that by modifying the end-terminal(s) of the peptides with L-cysteine residue, which carries free-thiol functionality on its side group, the antimicrobial spectrum of the previously reported α-helical peptides can be broadened. From the previous series of α-helical peptides, the most optimal amino acid compositions forming α-helical signatures with two and three repeat units, i.e., $(LLKK)_n$, where $n = 2,3$, are used to incorporate thiol groups in this study, owing to their antimicrobial efficacy yet low hemolytic properties. Based on this principle, a new series of peptides are designed: $(LLKK)_2C$, $C(LLKK)_2C$, $(LLKK)_3C$, $C(LLKK)_3C$ (Table 1). To justify the importance of the free-sulfhydryl functionality in this series of peptides, two control peptides with two L-methionine residues (substituting the L-cysteine residues) on the terminal ends of the peptide, $M(LLKK)_2M$ and $M(LLKK)_3M$, are also provided in this study. The antimicrobial properties of these new peptides were studied by bacteriostatic MIC measurement against wider selections of clinically threatening microbes from Gram-positive bacteria: *Bacillus subtilis*, Gram-negative bacteria: *Escheria coli*, *P. aerogenosa*, and yeast: *Candida albicans*. Confocal and scanning electron microscopy techniques were employed to investigate pore formation and membrane destruction mechanism of the peptides. Potential cytotoxic effect of peptides against mammalian cells was characterized by measuring their hemolytic effect on rat's red blood cells (rRBCs). Undesirable immmunogenicity of peptides was also tested *in vitro* by measuring the secretion level of TNF-α and IFN-γ cytokines in human monocytes treated with the peptides. Finally, the capability of the peptide to overcome bacterial resistance was evaluated by repeated treatment of the bacteria with the peptide, in comparison to the conventional β-lactam (penicillin G) and fluoroquinolone (ciprofloxacin) antibiotics.

2. Materials and methods

2.1. Materials

Peptides were purchased from GL Biochem (Shanghai, China), and their molecular weights was further confirmed *via* matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS, Model Autoflex II, Bruker Daltonics Inc., U.S.A.), using α-cyano-4-hydroxycinnamic acid as matrix. The purity of the peptides was also tested to be more than 95% with analytical reverse phase (RP)-HPLC. α-Cyano-4-hydroxycinnamic acid (HCCA) was purchased from Sigma–Aldrich (Singapore) and used in saturated acetonitrile/water (1:1 volume ratio) after re-crystallization. Ethanol (analytical grade, 99%) and dimethylsulfoxide (DMSO, synthesis grade, 99%) were purchased from Tee Hai (Singapore). Tryptic soy broth (TSB) powder and yeast mould broth (YMB) powder were purchased from BD Diagnostics (Singapore) and used to prepare the microbial growth media according to the manufacturer's instructions. RPMI growth medium, penicillin-streptomycin solution, and low-endotoxin fetal bovine serum were supplied by Invitrogen and used as received. Sodium dodecyl sulfate (SDS) micelle solution (10% w/v in DI water) was obtained from 1st Base (Malaysia) and used upon dilution to the desirable concentration range. Phosphate-buffered saline solution at 10 × concentration was purchased from 1st Base (Malaysia) and used after dilution to the desired concentration. 100 kDa dextran, 500 kDa dextran, fluorescein isothiocyanate (FITC), and glutaraldehyde (synthetic grade, 50% in H_2O), ciprofloxacin, penicillin G, and sodium tert-butoxide were purchased from Sigma–Aldrich (Singapore) and used as received. *B. subtilis* (ATCC No. 23857), *C. albicans* (ATCC No. 10231), *E. coli* (ATCC No. 25922), and *P. aerogenosa* (ATCC No. 9027), were obtained from ATCC (U.S.A) and reconstituted according to the suggested protocols. Red blood cells (RBCs) used in the experiments were obtained from rats maintained at the Animal Handling Units of

Table 1
α-helical peptide designs with sulfhydryl modification strategy and their molecular weights.

Number of Repeat Units (n)	Notation	Cysteine group	Peptide Sequence	Theoretical M_w	Measured M_w[a]
2	$M(LLKK)_2M$ (negative control 1)	0	MLLKKLLKKM-NH_2	1244.76	1246.39
	$(LLKK)_2C$	1	LLKKLLKKC-NH_2	1085.51	1087.23
	$C(LLKK)_2C$	2	CLLKKLLKKC-NH_2	1188.66	1190.11
3	$M(LLKK)_3M$ (negative control 2)	0	MLLKKLLKKLLK KM-NH_2	1727.43	1729.16
	$(LLKK)_3C$	1	LLKKLLKKLLKKC-NH_2	1568.18	1569.25
	$C(LLKK)_3C$	2	CLLKKLLKKLLKKC-NH_2	1671.32	1672.71

[a] Measured by MALDI-TOF, apparent $M_w = [M_w + H]^+$.

the Biomedical Research Centers (AHU, BRC, Singapore). Human peripheral blood mononuclear cells (PBMCs) were extracted from healthy blood donors and maintained with RPMI medium supplemented with 10% low-endotoxin fetal bovine serum (FBS) and 1% penicillin-streptomycin. Enzyme-linked immuno-sorbent assay (ELISA) kit for human interferon gamma (IFN-γ, Cat No. 41500-1) detection was purchased from PBL Interferon Source (U.S.A.), while that for the detection of human tumor necrosis factor alfa (TNF-α, Cat No. BMS223INST) was purchased from Bender MedSystem (Austria). These kits were used based on the manufacturer's recommended protocols.

2.2. Peptide characterization

Peptides designed and used in this study were synthesized *via* Fmoc-solid phase protocol at GL Biochem (Shanghai, China), and their characteristics were further confirmed with MALDI-TOF MS and HPLC. To measure the molecular weight, equal volume of peptide solution (0.5 mg/mL in deionised water) and HCCA solution (saturated in acetonitrile/water mixture at 1:1 volume ratio) were spotted onto MALDI ground-steel target plate after mixing. To estimate the purity of the peptide products, the same peptide solutions were run through an RP-HPLC (with C-18 column as the stationery phase and equal-volume of acetonitrile/water mixture as the mobile phase).

2.3. Circular dichroism (CD) spectroscopy

Peptide solutions were prepared at 0.5 mg/mL peptide, which contained 25 mM SDS surfactant. The CD experiments were recorded with a CD spectropolarimeter (JASCO, J-810) at room temperature, using a quartz cell having 1.0 mm path length. CD spectra of the peptide solutions were acquired with solvent subtraction from 190 to 240 nm wavelength at 10 nm/min scanning speed, and were averaged from 3 runs for each peptide sample. The acquired CD signal spectra were then converted to mean residue ellipticity by using the following equation:

$$\theta_M = \frac{\theta_{obs}}{10} \cdot \frac{M_{RW}}{c \cdot l}$$

where θ_M is residue ellipticity [deg. M^{-1}. m^{-1}], θ_{obs} is the observed ellipticity corrected for the buffer at a given wavelength [mdeg], M_{RW} is residue molecular weight (M_w/number of amino acids), c is peptide concentration [mg/mL], and l is the path length [cm].

2.4. Minimum inhibitory concentration (MIC) measurements

Microbes were re-constituted from their dried pellet form obtained from ATCC. Bacteria (*B. subtilis*, *E. coli*, and *P. aerogenosa*) were cultured in TSB under constant shaking at 100 rpm at 37 °C, while yeast (*C. albicans*) was cultured in YMB under constant shaking at 100 rpm at room temperature. MIC of each peptide against different microbes was measured using broth microdilution method as described previously [10,11,20]. Briefly, antimicrobial solutions were prepared by dissolving peptides in the respective broth medium from 0 to 1000 mg/mL concentration range by performing serial dilutions. Microbial cells were inoculated overnight prior to MIC experiments, and adjusted spectrophotometrically to give an absorbance reading at 600 nm wavelength equivalent to that of the standard McFarland 1. Under this condition, the O.D. reading was approximately 0.07, and it has been established that the bacteria counts can be approximated as 3×10^8 CFU/mL. Equal volumes of the adjusted bacterial culture (100 μL) were then mixed with the antimicrobial solutions (100 μL) in a 96-well microplate; and the growth of the microbes was monitored visually and spectrophotometrically by measuring the O.D. readings (TECAN, Switzerland) at 2 hourly intervals for up to 8 h. The MIC was reported as the minimum concentration of the peptides required to inhibit the growth of microbial cells at the 8th hours of incubation. In order to ensure aseptic handling environment, a negative control of pure broth without microbes was accompanied for each MIC test, and each MIC test was reproduced 3 times, using 6 numbers of replicates for each experiment.

2.5. Hemolytic activity test

The undesired biological activity of the peptides designed in this study against mammalian cells was tested using freshly drawn rRBCs obtained from AHU, BRC, Singapore. Briefly, rRBCs were washed with PBS thrice and subjected to 25× volumetric dilutions in PBS to achieve 4% blood content (by volume) as reported previously [10,11,20]. Antimicrobial solutions were prepared by dissolving peptides in PBS at concentrations ranging from 0 to 5000 mg/L (for peptides with 2 repeat units), and 0–1000 mg/L (for peptides with 3 repeat units). Equal volumes of antimicrobial solutions (100 μL) were then mixed with the diluted blood suspension (100 μL). The mixtures were then incubated at 37 °C for 1 h to allow for the interactions between rRBC and the AMPs to take place. Following the incubation, the mixture was subjected to centrifugation (3000 g for 5 min), after which, the supernatant (100 mL) was transferred into a 96-well microplate. The hemoglobin release was measured spectrophotometrically by measuring the absorbance of the samples at 576 nm using a microplate reader (TECAN, Switzerland) [10,11,20]. Two control groups were provided for this assay: untreated rRBC suspension (as negative control), and rRBC suspension treated with 0.1% Triton-X (as positive control). Each assay was performed in 4 replicates and repeated 3 times to ensure reproducibility of the experiments. Percentage of hemolysis was as follows:

Hemolysis (%) = [(O.D.$_{576\ nm}$ of the treated sample − O.D.$_{576\ nm}$ of the negative control)/(O.D.$_{576\ nm}$ of positive control − O.D.$_{576\ nm}$ of negative control)] × 100%.

2.6. Antibacterial mechanism study

The mechanism of the antimicrobial actions of the peptides was elucidated by direct observation of the bacterial membrane structure with the field emission scanning electron microscopy, as well as indirect probing of pore-formation of the bacterial membrane using confocal microscopy. Prior to sample preparation for microscopy imaging, bacterial cells were cultured in TSB at 37 °C under constant shaking at 100 rpm.

2.6.1. Field emission-scanning electron microscopy (FE-SEM) analysis of bacterial cells

For FE-SEM sample preparation, similar bacterial treatment with peptides as the broth microdilution assay was performed for a shorter incubation time (2 h). As previously shown that the cell-lytic peptides at concentration >MIC could induce membrane corrugation on bacterial cell walls [20], in this study, *E. coli* were treated with the effective peptides $C(LLKK)_2C$ and $(LLKK)_3C$) as representatives. Briefly, *E. coli* suspension at ~3×10^8 CFU/mL (100 μL) were mixed with peptide ($C(LLKK)_2C$ and $(LLKK)_3C$) solutions (100 μL) at their respective MIC concentration, and incubated in a 96-well microplate for 2 h. 8 replicates of the same conditions were used to increase the number of cells for the imaging experiments. After the incubation, the 8 replicates were mixed into a microfuge tube and centrifuged at 5000 g for 5 min. Bacterial pellets were then washed 3 times with PBS, and subjected to similar centrifugation after each wash. Following that, fixation of bacterial cells was performed with 2.5% glutaraldehyde for 15 min, followed by washing with PBS twice. Final wash was performed with deionised water before bacterial cells were dehydrated using graded ethanol solution as described previously [10,20]. Upon dehydration, the cells were dried with critical point dryer, mounted on carbon tape, sputtered with platinum coating (20 mA, 20 s), and imaged with an FE-SEM setup (JEOL JSM-7400F, Japan).

2.6.2. Confocal microscopy study

Unlike observation with FE-SEM, in order to estimate the porosity of the bacterial membrane using confocal microscopy, a fluorescent probe is needed. In this study, dextrans having molecular weights of 100 kDa and 500 kDa, labeled with FITC, were used as the probe molecules. FITC-labeled dextrans were first synthesized according to the following protocol, which is a modified version of reaction between isothiocyanate and hydroxyl functional group as reported before [30]. Dextran (0.5 g) was dissolved in 50 mL DMSO in a round bottom flask under nitrogen purging. The mixture was stirred at 80 °C until the polymer was completely dissolved. Next, 0.1 g (0.0010 M) of sodium tert-butoxide was added to the stirring mixture, the contents of the flask were left to stir at 80 °C for a further 15 min. Then, 0.20 g (0.00051 M) of FITC was added to the stirred polymer solution. The reaction was carried out in the dark for a further 16 h under nitrogen. After 16 h, the contents of the flask were cooled down to 30 °C and the crude product was precipitated into excess tetrahydrofuran (THF) and the precipitate was then dissolved into a small amount of alkaline water (pH ≈ 8.0). Under darkness this mixture was then dialyzed against water for 1 day with a continuous flow by a membrane dialysis method using dialysis tubing with a molecular weight cut-off (MWCO) of 3.5 kDa (Spectrum Laboratories, U.S.A.). Dextran conjugated with FITC was harvested by freeze-drying.

In order to prepare the bacterial sample, *E. coli* were treated with the $C(LLKK)_2C$ peptide (with the highest selectivity towards microbes) at its MIC concentration for 15 min, in the presence of FITC-labeled dextran having different molecular weights as probes. Treatment for a short time-scale will induce pore-formation, and the presence of the pores on the membrane will allow the probe molecules to diffuse into the bacterial cells, only if the pore is larger than the size of the probe molecules. By this way, one can also estimate the size of the pores being induced by the antimicrobials, as shown in the similar experiments before [31]. Following this treatment, the bacterial cells were pelleted by centrifugation at 5000 g for 5 min, and washed thoroughly three times with PBS to remove the presence of free probe molecules in the solution. Next, bacterial cells were fixated with 2.5% glutaraldehyde solution, and washed with PBS twice. The bacterial cells were then re-suspended in PBS, dripped onto a poly-L-lysine coated glass slide, and aired in the dark for a few hours to allow adsorption of the cells onto the glass slide. Next, the non-adhering bacterial cells were washed with PBS thoroughly, and the slides were air-dried and sealed with a coverslip for imaging under the confocal microscope.

2.7. Drug resistant stimulation study

Drug resistance was induced by treating bacterial cells repeatedly with antimicrobial agents. In this study, we attempt to induce drug resistance in Gram-positive *B. subtilis* and Gram-negative *E. coli* bacteria as the model microorganisms from different families. With the same broth microdilution method, the MIC of

C(LLKK)$_2$C peptide against these microorganisms was tested for up to 10 passages of bacterial growth. For each passage's MIC measurement, the bacterial cells exposed to the sub-MIC concentration (1/8 of MIC at that particular passage) were re-grown to a logarithmic growth phase, and re-used for the subsequent passage's MIC measurement for the same antimicrobial agents. By recording the changes in the MIC, i.e., MIC at passage n normalized to that at the first passage, drug resistant behavior of the bacteria could be shown. C(LLKK)$_2$C peptide having the highest selectivity towards microbial cells was used in this study, in comparison to two other conventional antibiotics known to have different growth inhibitory mechanisms: penicillin G (β-lactams antibiotics), and ciprofloxacin (fluoroquinolone antibiotics).

2.8. In vitro immunogenicity test

The *in vitro* immunogenicity of the peptides was tested using human PBMCs obtained from healthy donors. The PBMCs were extracted from the donated whole human blood by the standard Ficoll-Hypaque density centrifugation technique. Upon extraction, the PBMCs were maintained in RPMI growth medium, supplemented with 10% low-endotoxin FBS and 1% penicillin-streptomycin, in a 37 °C incubator supplied with 5% CO_2. Secretion of IFN-γ and TNF-α was monitored upon treatment of PBMCs with the peptides designed in this study at concentration of 250 mg/L, in comparison to the untreated negative control group and lipopolysaccharide (LPS, 100 ng/μL)-treated positive control group. Briefly, 100 μL of peptide (or LPS) in growth medium is mixed with 100 μL of 3×10^6 cells/mL, and were seeded onto a 96-well plate. The cells were allowed to incubate with the potentially immunogenic substance for 2 days, after which the secretion of the cytokines in the growth medium was measured with ELISA kit.

3. Results and discussion

3.1. Peptide design and characterization

The sequences of the peptides in this study were designed based on the commonalities shown in the naturally-occurring host defense AMPs, with the following two criteria: containing both cationic and hydrophobic amino acid residues in their primary structures, as well as folding into α-helical structures [14,15]. We have recently reported that synthetic α-helical peptides containing L-lysine and L-leucine residues arranged regularly in a periodic manner according to $(LLKK)_n$ rule can fold into α-helical structure and induce high selectivity towards Gram-positive bacteria and yeast (particularly when $n = 3$) [20]. Based on this study, 4 different sequences of new peptides were designed to contain sulfhydryl groups at the end-terminal(s) of the peptides, while the helical folding behavior is expected to be retained. The structure and molecular weight of the peptides were verified by comparing the theoretical and measured molecular weight using MALDI-TOF-MS. As shown in Table 1, these two values are in close agreement. In addition, the purity of the peptides was further confirmed with RP-HPLC to be above 95%. Furthermore, the presence of free thiol groups was tested with the standard Elmann's test for the detection of thiol (data not shown).

3.2. Circular dichroism (CD) spectroscopic studies

Both composition and length of the peptides have been shown to affect the α-helical folding behavior of peptides in solution. Generally, α-helical peptides containing lysine and leucine residues possess strong α-helical signature as confirmed with our recent report [20]. In order to investigate if the incorporation of thiol group on the terminal ends of the peptide affects the α-helical folding propensity, CD experiments were conducted using 25 mM SDS solution as the model biological bilayer component. Fig. 1(a) and (b) summarize the CD spectra of peptides with $n = 2$ and 3, respectively. The presence of additional amino acids (cysteine or methionine) increases the propensity for α-helical folding, as characterized by the presence of the double minima in the figure, plausibly due to the increased size of the peptide molecule, which enhances the hydrogen bonding between the carbonyl O atom of ith amino acid position and amide proton of the $(i + 4)$th amino acid position in the peptide backbone. This is highly probable since both cysteine and methionine does not belong to the helical breaker amino acids [32]. Similarly, an increased number of repeat units (n) also increases the α-helical folding by the same virtue, which is characterized by the extent of the negative values of the molecular elipticity (θ_M) at the minima points.

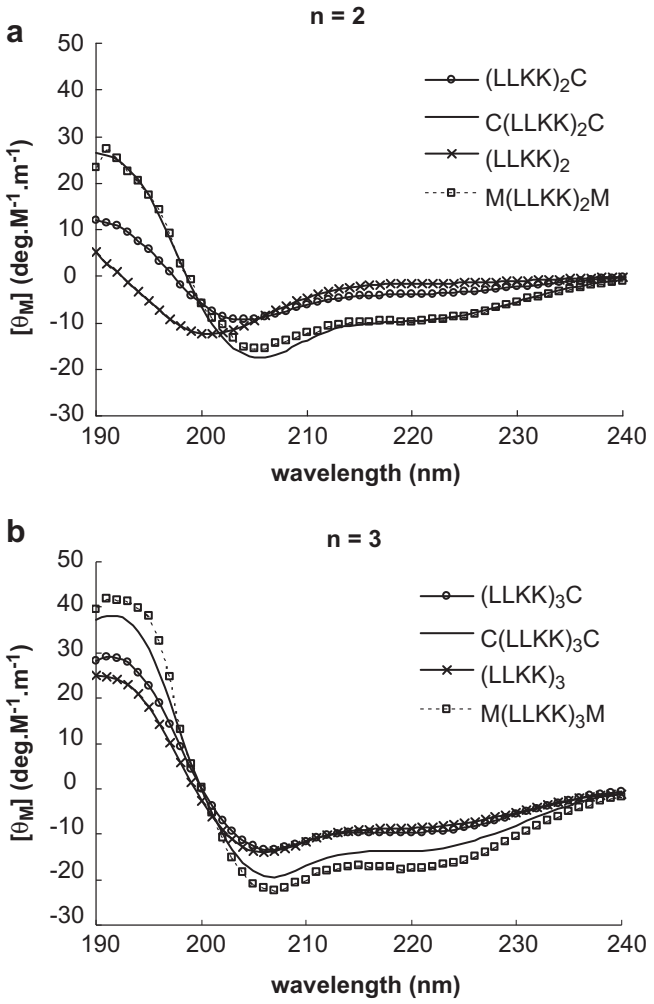

Fig. 1. CD Spectra of cysteine-modified α-helical peptides with (a) two repeat units ($n = 2$), and (b) three repeat units ($n = 3$).

3.3. Hemolytic analysis of peptides

Development of anti-infective agents has to be accompanied with rational design such that it does not cause undesired cytotoxic effects. Generally, depending on the target applications of the biomaterials, cytotoxic effects need to be characterized in different manners. Cytotoxicity of synthetic antimicrobial materials is often analyzed in terms of its activity to lyse mammalian red blood cells, which is implied by its hemolysis properties. Table 2 summarizes the 50% hemolysis concentration of the peptides (HC_{50}), a concentration estimated based on the hemolysis test as shown in Fig. 2. As indicated in both Table 2 and Fig. 2, the value of the HC_{50} is a strong function of the number of both repeat units and cysteine residues. For example, the peptides with $n = 2$ induce very low hemolysis (i.e. less than 5%) up to 500 mg/L, with significant cytotoxicity difference between peptides with and without free sulfhydril group(s) being observed only above 1000 mg/L concentration. On

Table 2
Minimum inhibitory concentration (MIC) and 50% hemolysis concentration (HC_{50}) of the synthetic antimicrobial peptides with the incorporation of sulfhydryl group.

Modification	Peptide Sequence	MIC (mg/L)				HC_{50} (mg/L)
		Gram +	Gram -		Yeast	
		B. subtilis	*E. coli*	*P. aerogenosa*	*C. albicans*	
2 Met, $n = 2$ (negative control 1)	MLLKKLLKKM-NH2	125	>500	500	500	>2500
1 Cys, $n = 2$	LLKKLLKKC-NH_2	125	>500	63	250	>2500
2 Cys, $n = 2$	CLLKKLLKKC-NH_2	125	225	125	250	>2500
2 Met, $n = 3$ (negative control 2)	MLLKKLLKKLLKKM-NH_2	31	250	31	125	~158
1 Cys, $n = 3$	LLKKLLKKLLKKC-NH_2	16	150	16	63	~402
2 Cys, $n = 3$	CLLKKLLKKLLKKC-NH_2	31	125	125	125	~82

the contrary, peptides with $n = 3$ have more significant hemolysis, presumably due to higher helical folding tendency of these peptides, which results in increased molecular rigidity upon folding, thereby inducing higher hemolysis. Furthermore, within the series of peptides with $n = 3$, the effect of free sulfhydril group becomes more apparent. At 500 mg/L, <10% hemolysis is induced by the peptide without cysteine ($(LLKK)_3$) [20], while ~60% hemolysis is observed for the peptide with 1 cysteine ($(LLKK)_3C$), and ~94% for the peptide with 2 cysteine ($C(LLKK)_3C$), respectively. These phenomena can be understood from the literature that the presence of free-thiol group in some bacteria's pore-forming toxins has the capability to induce interactions with cholesterol present in the mammalian erythrocytes' membranes. Thiol-activated cytolysin, for example, has been reported to interact with cholesterol in mammalian cell membrane as their binding receptor to subsequently induce pore formation [33]. Even though the thiol decoration on the terminal end(s) of the α-helical peptides can undesirably increase the hemolytic activity, the benefit of this functional group in broadening the antimicrobial activity of the peptides outweigh such adverse observation, especially for peptides with $n = 2$, which will be further discussed in more details in the following section.

3.4. Antimicrobial activities of the peptides

The antimicrobial activities of the peptides were tested in terms of their corresponding bacteriostatic inhibitory concentrations, indicated by the MIC, as summarized in Table 2 and Figs. S1–S6 in the supplementary information. The peptides designed in this study were able to inhibit a wide range of microbial cells at different concentrations, depending on the number of thiol and number of repeat units per peptide molecule. The MICs of the peptides were tested against Gram-positive bacteria *B. subtilis,* Gram-negative bacteria *E. coli, P. aerogenosa*, and yeast *C. albicans*. Generally, all the peptides are effective to inhibit the growth of Gram-positive bacteria and yeast, at a concentration that is predominantly lower than (if not the same as) the previous series of α-helical peptides without thiol group [20]. From comparison of the MIC and HC_{50} values of the peptides with $n = 2$ and $n = 3$, an interestingly different antimicrobial effect resulting from the thiol incorporation is observed between peptides with $n = 2$ and $n = 3$. For peptides with $n = 2$, which were only marginally potent towards microbial cells before thiol incorporation [20], the presence of thiol enhances antimicrobial potency towards Gram-positive bacteria and yeast, and broadens its activities towards

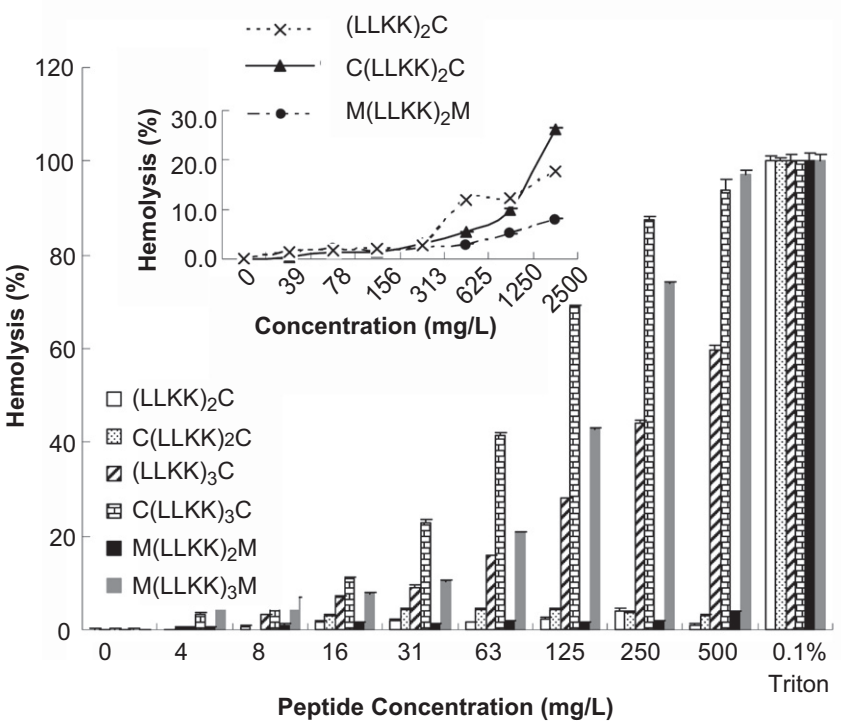

Fig. 2. Hemolytic activity of the cysteine-modified α-helical antimicrobial peptides.

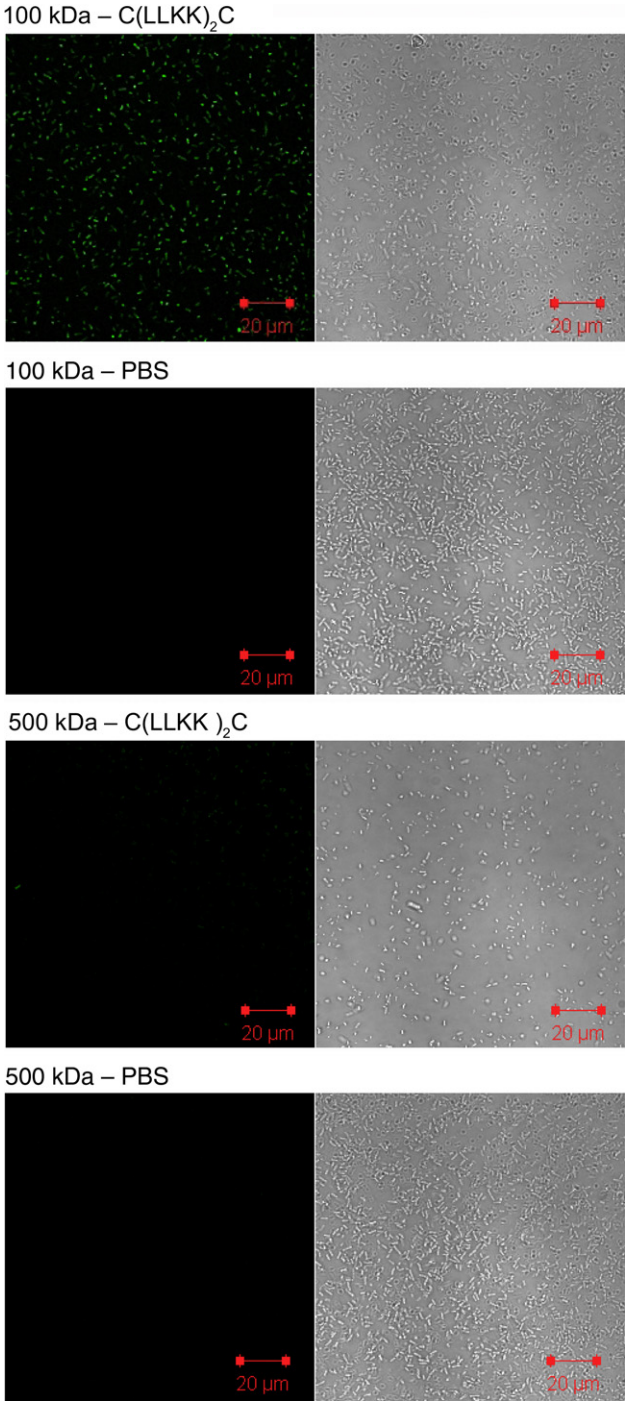

Fig. 3. Confocal images of *E. coli* after treatment with either PBS or the $C(LLKK)_2C$ peptide at its corresponding MIC in the presence of FITC-labeled dextran having different molecular weights (100 kDa FITC-dextran, Z-average: ~32 nm as measured by DLS; 500 kDa FITC-dextran, Z-average: ~89 nm as measured by DLS). This serves to estimate the pore size formed on the bacterial membrane after treatment with the $C(LLKK)_2C$ peptide for ~15 min. Left column shows FITC filtered channel and right column shows bright field channel.

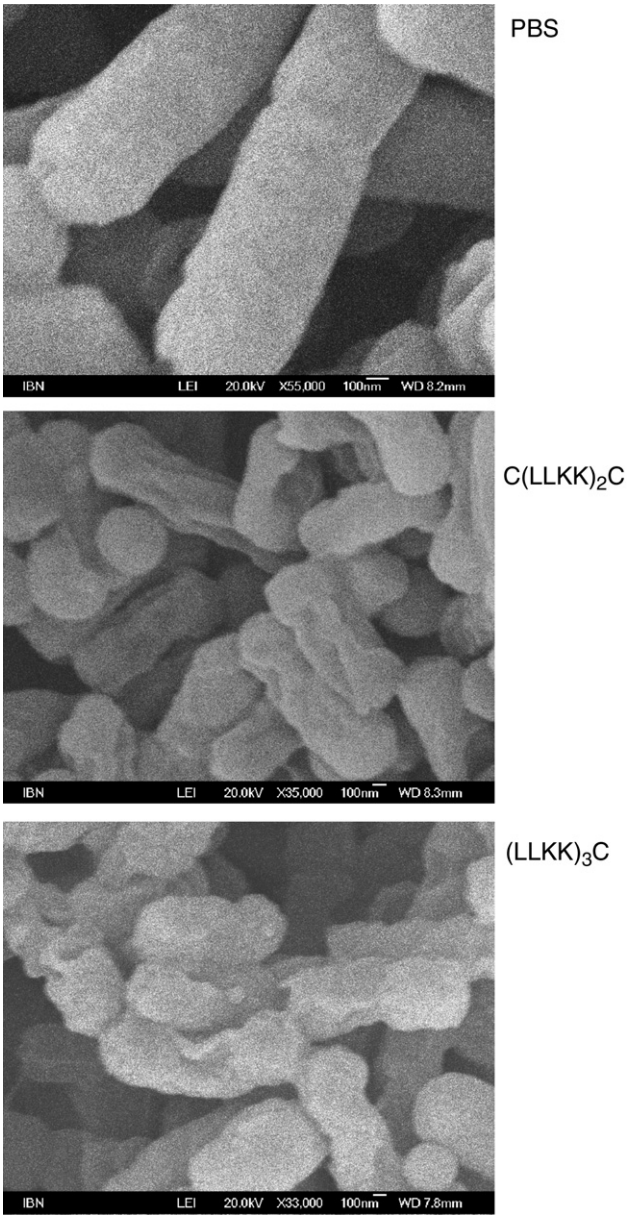

Fig. 4. FESEM images of *E. coli* showing the difference on the membrane morphology between treated and untreated bacteria. Bacterial membrane damages were seen upon treatment with $C(LLKK)_2C$ and $(LLKK)_3C$ peptides for 2 h, in comparison to the untreated control (top).

Gram-negative bacteria (Table 2). For example, MIC of $(LLKK)_2$ was reported to be 500 mg/L against *B. subtilis* [20], and it is lowered to 125 mg/L for both $(LLKK)_2C$ and $C(LLKK)_2C$. Similar observations were also reported by Cunha B.A. et al., where the bacteriostatic concentration of Bismuth compound was significantly lowered with complexation of lipophilic thiol chelator [29], and by Yamashita T. et al., where acetylation of sulfhydryl group on the cysteine residues in the AMP reduced antimicrobial activity [28]. In addition, the increase in its antimicrobial activity for the $n = 2$ peptides due to thiol incorporation is observed at concentrations well-below their corresponding HC_{50} of >2500 mg/L. In another example, both $(LLKK)_2$ [20] and the control $M(LLKK)_2M$ peptides are ineffective against *E. coli*, but $C(LLKK)_2C$ peptide becomes active in inhibiting the growth of *E. coli* at 225 mg/L. In addition, the presence of thiol also enhances antimicrobial activity against *P. aerogenosa*. Even though the underlying mechanism of such observation is still unclear, the study of the effect of lipopolysaccharide (LPS) from Gram-negative bacteria on the level of thiol in the blood platelets performed by Gowacki et al. may serve as a premise and provide some insights to this observation. In that particular study, it was concluded that there might be some interactions between thiol and LPS taking places in the form of

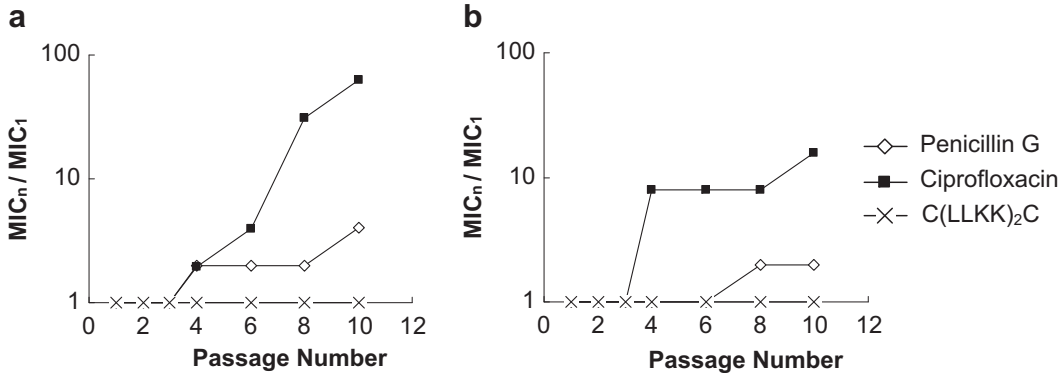

Fig. 5. Changes in MIC's of antimicrobial substances upon multiple sub-lethal dose exposures, signifying drug resistant development as a result of evolutionary survival of (a) Gram-positive *B. subtilis* and (b) Gram-negative *E. coli*.

complexes [34]. In this view, it is possible that a similar type of interactions between the sulfhydryl group(s) on the terminal end(s) of the AMPs and the LPS on the outer membrane of the Gram-negative bacteria provides an avenue to draw the AMP molecules closer towards the outer membranes of the bacteria for the helical folding to occur, which eventually leads to bacterial lysis.

Further analysis of the effect of thiol group on antimicrobial effect of peptides with $n = 3$ series, which were already a potent inhibitor against Gram-positive bacteria and Yeast [20], reveals a different trend. Generally, incorporation of one thiol group into $(LLKK)_3$, i.e., $(LLKK)_3C$, also enhances the antimicrobial activity and broadens its spectrum of activity towards Gram-negative bacteria. However, this increase of antimicrobial activity is also accompanied by the increase in hemolytic activity as the HC_{50} of $(LLKK)_3$ originally at >500 mg/L [20] is lowered to ~402 mg/L, reducing its bacterial selectivity. In addition, incorporation of another thiol group into $C(LLKK)_3C$ reduces the antimicrobial activity and selectivity towards microbial cells even more. This is severely marked by the increase in hemolytic activity at HC_{50} to ~82 mg/L, a concentration at which it is still ineffective to inhibit *E. coli* and *C. albicans*. Comparison of $C(LLKK)_3C$ with the second control peptide $M(LLKK)_3M$ also shows that incorporation of 2 thiol groups (from cysteine) or 2 thioether groups (from methionine) does not provide significant difference in its antimicrobial activity, which is presumably because the non-modified $(LLKK)_3$ peptide was already a strong antimicrobial agent itself [20], hence dampening the advantage of having the thiol groups. It is also possible that $C(LLKK)_3C$ and $M(LLKK)_3M$ are sparingly soluble at higher concentration as shown from the high solution turbidity (O.D. reading >0.5) at 0 h (Fig. S4 and Fig. S6). Based on these observations, $C(LLKK)_2C$ is the best candidate among the peptides tested. Therefore, the antimicrobial actions of $C(LLKK)_2C$ will be further analyzed in the following sections because of its broad antimicrobial activity accompanied by excellent selectivity towards microbial cells.

3.5. Antimicrobial mechanism

AMPs have been reported to act on the bacterial membrane by forming pores and eventually destroying the integrity of the bacterial membrane. In order to further elucidate the functional mechanism of this series of AMPs, we first performed a short-term treatment of *E. coli* with $C(LLKK)_2C$ peptide for ~15 min, in the presence of FITC-labeled dextran (100 kDa or 500 kDa). The presence of FITC-dextran serves as a probe to show the capacity of the

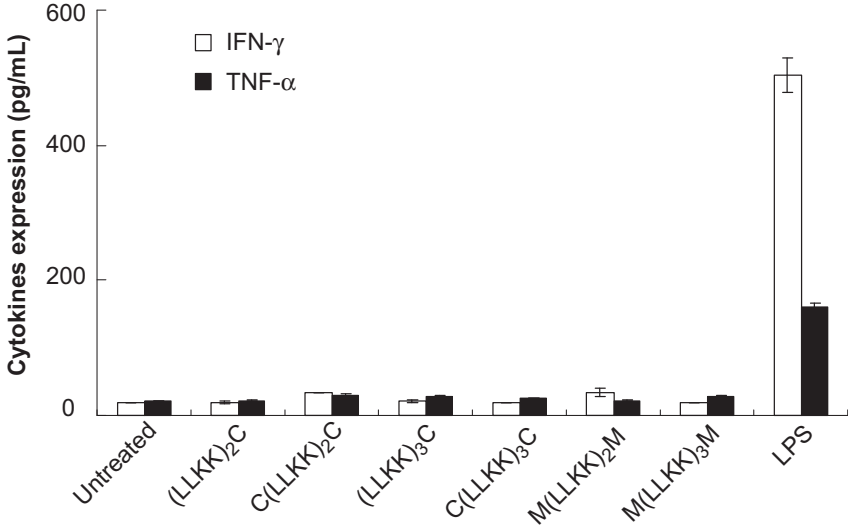

Fig. 6. Secretion of IFN-γ and TNF-α by PBMCs treated with various antimicrobial peptides. Untreated and lipopolysaccharide (LPS)-treated PBMCs were provided as negative and positive control groups, respectively.

AMP in inducing pore formation in the outer membrane of the bacteria. When the membrane becomes porous because of the treatment with the AMP, small fluorescent-labeled molecules can passively diffuse into the bacterial cells. This permits visual inspection of the bacteria using confocal microscopy. At the same time, different size of FITC-labeled dextran allows one to estimate the size of the pores induced by means of size exclusion as demonstrated elsewhere [31]. Fig. 3 shows the confocal images of *E. coli* populations treated with the AMP in the presence of different size of FITC-dextran. In comparison to the control group of *E. coli* incubated with FITC-dextran only, it can be shown clearly that the treatment with the AMP indeed leads to significant uptake of FITC-dextran especially with 100 kDa. The uptake of FITC-dextran was not as obvious for 500 kDa dextran, most plausibly due to small pores in the membrane, which excluded the uptake of larger molecular weight dextran molecules. The solution behavior of FITC-dextran molecules was analyzed with DLS, and the size distribution of the FITC-dextran with 100 kDa and 500 kDa is shown in Fig. S7 in the supplementary information. As 100 kDa FITC-dextran spread from 7 nm, 39 nm–253 nm, while the 500 kDa FITC-dextran measured around 29 nm, 239 nm and 5371 nm; and due to the fact that the uptake of the 500 kDa dextran molecules by the treated *E. coli* was prevented, it is apparent that the size of the pores that were induced by the AMP on the surface of the bacteria might fall within the range of 7–29 nm. This is in agreement to the findings observed from a similar type of experiments performed by Ghosh J.K. et al., where the size of pores induced by serial analogs of melititin AMPs was around 23 nm [31].

In the treatment for a longer time scale ($\sim$1 h), the damage on the bacterial membrane can be studied with FE-SEM microscopy that allows observation of the surface morphology of the bacteria. Fig. 4 shows that the integrity of *E. coli* membrane upon the treatment with $C(LLKK)_2C$ and $(LLKK)_3C$ was grossly affected. Comparison with the untreated control group clearly shows that the AMP molecules "chew" up the membrane of *E. coli*, changing the overall morphology of the bacterial cells. In our recent report with Gram-positive bacteria *B. subtilis*, similar observation was also noticed that by varying the treatment duration of the bacteria, more and more destructive effect was observed on the overall morphology of the bacteria, presumably due to the more severe loss of membrane integrity upon prolonged treatment of the bacteria with AMP [20].

3.6. Induction of drug resistance with antimicrobial agents

Multiple exposures of microbial cells towards certain drugs at doses below their lethal concentrations have been understood to induce drug resistance in microbial pathogenesis [16]. This phenomenon is simulated as shown in Fig. 5, where MIC of *B. subtilis* (Gram-positive) and *E. coli* (Gram-negative), re-grown from their sub MIC concentration, is monitored over the course of repeated treatments with penicillin G, ciprofloxacin, and the $C(LLKK)_2C$ peptide. From the figure, it can be seen that bacteria develop resistance towards different antibiotics at varying passages. This indicates that different antimicrobial mechanism of actions present different levels of difficulty for the bacteria to "alter" their metabolic pathway to overcome the environmental stress factor induced by the presence of the antimicrobials. For example, the multiple treatments with ciprofloxacin gave rise to onset of resistance to as early as passage 4, for both *B. subtilis* and *E. coli* (Fig. S9 in the Supplementary information). In addition, the sensitivity of *B. subtilis* with ciprofloxacin was greatly abolished as soon as the onset of resistance occurred as the MIC at the subsequent passages continued to shoot up reaching nearly as high as 2 orders of magnitudes by passage 10. This effect was not that severe in Gram-negative *E. coli*. On the other hand, insensitivity towards penicillin occurred less severely, as after passage 10, the MIC of both *B. subtilis* and *E. coli* did not increase beyond an order of magnitude (Fig. S8). This signified that the different mechanisms of antimicrobial action presented different levels of difficulty for bacteria to overcome. Furthermore, it is also apparent that certain mechanism of action is easier to be overcome by different families of bacteria. More importantly, by looking at the evolution of MIC values for the multiple treatments with the $C(LLKK)_2C$ peptide, we can infer that the membrane lysis mechanism of antimicrobial actions with the macromolecular peptide offer advantages over the small molecular antibiotics as the multiple treatments up to passage 10 do not show any onset of antibacterial insensitivity for both Gram-positive and Gram-negative bacteria (Fig. S10). This offers an attractive solution in preventing the evolution of "super bugs", thereby providing new paradigm shifts in antibiotics treatment.

3.7. In vitro immunogenicity test

Another undesired property of biomaterials came from its immunogenic aspect upon contact with tissues [35]. Most of these immunogenic properties arise from the non-specific immune response of the host organism to react to the foreign body objects, which are often perceived as "invaders", even though biomaterials are generally used in contact with the tissues for regenerative or healing purposes. Secretion of cyokines initiates the upstream pathways of immunogenic response in multicellular organisms; therefore in this study we measure the elevation of pro-inflammatory cytokines IFN-γ and TNF-α secretions by PBMC upon contact with the AMPs, which are generally secreted upon detection of "foreign molecules". In Fig. 6, the effect of these secretions was summarized for treatment with all the 6 peptides designed in this study. From this figure, it is apparent that since the peptides induced a very low level of IFN-γ and TNF-α as compared to the positive control bacterial LPS, they show negligible immunogenic response in comparison to that given by the control LPS. This finding is important as it opens up the possibility for further development of the peptides as anti-infective agents both systemically and topically. In addition, this preliminary *in vitro* immunogenicity test of the peptides also serves to measure their safety in terms of non-specific immune response reactions, which is an important point of consideration as immunogenicity of therapeutics has the potential to induce unwanted adverse systemic inflammation.

4. Conclusion

In this study, we have highlighted that simple thiol decoration of our previously designed AMPs *via* the incorporation of cysteine(s) at the terminal end(s) of the AMPs significantly broadens their antimicrobial activity to Gram-positive and Gram-negative bacteria as well as yeast. Among the series of peptides studied, peptide with 2 cysteines and 2 repeat units (i.e. $C(LLKK)_2C$) provides the highest selectivity towards a wide range of microbes as opposed to mammalian cells. The antimicrobial mechanism of this peptide is shown *via* pore-formation and membrane damage. The $C(LLKK)_2C$ peptide does not induce significant secretion of IFN-γ and TNF-α. More importantly, the microbes do not develop into resistance after multiple treatments with the peptide. The findings reported in this study provide a strong premise to further develop these AMPs as active anti-infective agents for treating drug resistant infections caused by various families of microbes.

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2011.08.020.

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