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Synthetic cationic amphiphilic α-helical peptides as antimicrobial agents

Nikken Wiradharma [a], Ulung Khoe [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] Yong Loo Lin School of Medicine, National University of Singapore, 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 (AMPs) secreted by the innate immune system are prevalent as the effective first-line of defense to overcome recurring microbial invasions. They have been widely accepted as the blueprints for the development of new antimicrobial agents for the treatment of drug resistant infections. However, there is also a growing concern that AMPs with a sequence that is too close to the host organism's AMP may inevitably compromise its own natural defense. In this study, we design a series of synthetic (non-natural) short α-helical AMPs to expand the arsenal of the AMP families and to gain further insights on their antimicrobial activities. These cationic and amphiphilic peptides have a general sequence of $(XXYY)_n$ (X: hydrophobic residue, Y: cationic residue, and n: the number of repeat units), and are designed to mimic the folding behavior of the naturally-occurring α-helical AMPs. The synthetic α-helical AMPs with 3 repeat units, $(FFRR)_3$, $(LLRR)_3$, and $(LLKK)_3$, are found to be more selective towards microbial cells than rat red blood cells, with minimum inhibitory concentration (MIC) values that are more than 10 times lower than their 50% hemolytic concentrations (HC_{50}). They are effective against Gram-positive *B. subtilis* and yeast *C. albicans*; and the studies using scanning electron microscopy (SEM) have elucidated that these peptides possess membrane-lytic activities against microbial cells. Furthermore, non-specific immune stimulation assays of a typical peptide shows negligible IFN-α, IFN-γ, and TNF-α inductions in human peripheral blood mononuclear cells, which implies additional safety aspects of the peptide for both systemic and topical use. Therefore, the peptides designed in this study can be promising antimicrobial agents against the frequently-encountered Gram-positive bacteria- or yeast-induced infections.

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1. Introduction

Antimicrobial peptides (AMPs) secreted by innate immune system of various organisms have been reported since three decades ago as the first form of natural defense against environmental parasitic infections [1]. To date, the primary structure of these peptides is so diverse that more than 1000 AMP sequences have been reported and documented in the AMP database [2]. Most of these peptides were derived from larger precursors [1], often empirically "optimized" by means of chemical modifications, such as glycosylation [3], fluorination [4], cyclization [5], or introduction of point amino acids mutations [6,7]. In addition, some peptides were also derived from a larger protein sequence through a proteolysis, such as lactoferricin α-helical AMP from lactoferrin [8]. These derived AMPs often adopt different conformational structures in aqueous or membrane-like environments; however, they exhibit a certain degree of generalities, such as net cationic charge, amphiphilicity, primarily targeting the microbial cell membrane, and possessing antimicrobial activities through disruption of the microbial cell membrane [9–13]. Since most of these peptides targeted to disrupt bacterial cell membranes in exerting their antimicrobial actions, it suggests that AMPs can potentially escape the mechanisms involved in multidrug resistance, which is an increasingly difficult phenomenon faced as a result of repeated treatments with small molecular antibiotics [14]. Indeed, more recent works of AMPs have shown that these antimicrobial peptides are less susceptible to microbial resistance as compared to small molecular antimicrobial agents [15]. In particular, A. Mor et al., reported that repeated exposure of Gram-positive bacteria towards different AMPs at sub-minimum inhibitory concentrations (MIC) did not alter the MIC value significantly for up to more than 10–15 passes of the bacterial passage [16]. With such great

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

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potential in overcoming microbial resistance, the topical applications of these peptides have gained rising attentions as there are less toxic implications in comparison to systemic applications [17,18].

Even though natural AMPs have shown to be superior in overcoming multidrug resistance, the development of natural AMPs has been lacking of design principles and not systematic. It mostly begins with a known natural peptide/short protein sequence, followed by modification and/or "optimization" to obtain improved antimicrobial effects, while reducing the undesirable cytotoxic effects towards mammalian cells. As such, this process becomes "random" involving "black box trial-and-error" procedures. Moreover, there is an increasing argument recently that clinical use of AMPs with sequences that are too close to those of human AMPs would inevitably compromise own natural defenses, possibly posing threat to public health [19]. In this view, non-natural (or synthetic) peptides were developed to provide wider arsenals of AMPs. One of the first attempts to design a synthetic class of AMP was demonstrated by G. Stephanopoulos et al., by introducing rules analogous to "grammatical rules" in linguistics to describe certain commonalities observed from the reported AMP database. These rules were used as algorithms to produce permutations of different non-natural AMP primary sequences [20]. Following that, antimicrobial synthetic peptides were developed in our laboratory in the form of self-assembled core-shell nanoparticles that were effective against a wide spectrum of microbes [21], and were capable of crossing the blood–brain barrier for the treatment of brain infections in a rabbit meningitis model [22]. More recently, another self-assembled non-natural peptide was also shown to have antimicrobial activity in the form of peptide nanotubes [23].

In this study, we would like to expand the arsenals of synthetic AMPs by using material design principles to mimic the naturally-occurring α-helical AMPs. A series of cationic peptide amphiphiles were designed based on the α-helical protein folding principles, whereby the peptide carbonyl O atom and amide proton between the ith and (i + 4)th amino acid positions form a paired hydrogen bonding, resulting in a folded structure with a regular turn every 3.6 amino acids [24,25]. Even though such conformation is stabilized through paired hydrogen bonding within the backbone of the peptide molecules, theoretical framework has shown that hydrophobic interactions between the amino acid side groups contribute significantly towards the nucleation of this helical conformation [26,27]. By garnering hydrophobic interactions between the side groups of the adjacent ith and (i + 4)th amino acids, we hypothesize that α-helical folding can be enhanced if the amino acids are not selected from the helical breakers category [28]. Therefore, to maintain the α-helical periodicity, a repeat primary sequence containing 4 amino acids is adopted in designing the AMPs. Furthermore, by providing amino acids of the same charge at the (i + 2)th and (i + 3)th positions of the peptide, cationic repulsive forces would unfold the peptide molecules in solution, thereby mimicking the α-helical folding characteristics of natural α-helical AMPs, which occurs upon interaction with bacterial membranes [11]. This mimicry is ensured by keeping the cationic and hydrophobic content within the repeat units to be balanced, so that the long-range repulsive forces can overcome the short-range hydrophobic interactions. As such, the resulting primary peptide structure can be simplified as repeat of XXYY amino acid sequence (i.e. $(XXYY)_n$), whereby X is a hydrophobic amino acid, and Y is a cationic amino acid. As it was reported that the presence of zwitterionic moieties in antimicrobial agents reduced its effectiveness [29], the C-terminal of the peptides designed in this study was amidated to maintain the high net positive charges.

The peptides were first characterized for their secondary conformation in solution and simulated membrane environment. The hemolytic properties of the peptides were then characterized and the effectiveness of the designed peptides in killing microbial cells was tested against the model microbes such as *B. subtilis* (Gram-positive) and *C. albicans* (Yeast). The mechanism of actions was examined against *B. subtilis* bacterial membrane using SEM technique. Meanwhile, kinetics of antibacterial effect was also observed. In addition, non-specific immune stimulation *via* cytokines pathways was examined by measuring the *in vitro* secretion level of IFN-α, IFN-γ, and TNF-α by treated human peripheral blood mononuclear cells (PBMCs).

2. Materials and methods

2.1. Materials

Peptides were obtained from GL Biochem (Shanghai, China), and their fidelity 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 being re-crystallized. Sodium dodecyl sulphate (SDS) micelle solution (10% w/v in DI water) was obtained from 1st Base (Malaysia) and used upon dilution to the desirable concentration range. Tryptic soy broth (TSB) powder and yeast mould broth (YMB) powder were purchased from BD Diagnostics (Singapore) and used to prepare the microbial broths according to the manufacturer's instructions. Phosphate-buffered saline solution at 10× concentration was purchased from 1st Base (Malaysia) and used after dilution to the desired concentration. Ethanol (analytical grade, 99%) and glutaraldehyde (synthetic grade, 50% in H_2O) were purchased from Sigma–Aldrich (Singapore) and used as received. *B. subtilis* (ATCC No. 23857) and *C. albicans* (ATCC No. 10231) were obtained from ATCC (U.S.A) and re-constituted according to the suggested protocols. Red blood cells used in the experiments were obtained from rats maintained at the Animal Handling Units of the Biomedical Research Centers (Singapore). Human 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. Granulocyte macrophage-colony stimulating factor (GM-CSF) and lipopolysaccharide were purchased from Sigma–Aldrich and used as received. Enzyme-linked immuno-sorbent assay (ELISA) kit for human interferons alpha (IFN-α, Cat No. 41100-1) and gamma (IFN-γ, Cat No. 41500-1) detection were purchased from PBL Interferon Source (U.S.A.), while that for the detection of human tumor necrosis factor alpha (TNF-α, Cat No. BMS223INST) was purchased from Bender MedSystem (Austria). These kits were used according to the manufacturer's protocols.

2.2. Peptide characterization

The peptides designed in this study were synthesized by the standard Fmoc-solid phase peptide synthesis protocol at GL Biochem (Shanghai, P. R. China). The fidelity of the synthesis was confirmed *via* MALDI-TOF MS and RP-HPLC techniques. An equal volume of peptide aqueous solution (0.5 mg/mL) and α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution (saturated in acetonitrile/water mixture at 1:1 volume ratio) were pre-mixed and spotted onto the MALDI ground-steel target plate to measure the molecular weight of the peptide. The same peptide solution was also run through an RP-HPLC (with C-18 as the stationary phase, and the mixture of acetonitrile and water as the mobile phase with a gradient being varied from 5% to 20% acetonitrile from 0 to 20 min). From the area of the chromatograms obtained, the purity of the peptides was estimated to be more than 95%.

2.3. Circular dichroism (CD) spectroscopy

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

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

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 amino acids), c is peptide concentration [mg/mL], and l is the path length [cm].

2.4. Minimum inhibitory concentration (MIC) measurements

Microbial cells were firstly re-constituted from its lyophilized form according to the manufacturer's protocol. Bacterial cells were cultured in TSB at 37 °C, and yeast cells were cultured in YMB at room temperature under constant shaking at 100 rpm. MIC was measured using the broth microdilution method as reported previously [21,22]. Briefly, broth containing an antimicrobial peptide was prepared at various concentrations (0.98, 1.95, 3.90, 7.81, 15.63, 31.25, 62.5, 125, 250, 500 mg/L) and transferred into a 96-well tissue culture plate (100 μL/well). Microorganism was first inoculated overnight to enter its log growth-phase. An equal volume of microorganism solution (100 μL) was added into each well of the 96-well plate containing the antimicrobial peptide. Prior to this mixing, the concentration of microorganism solution was adjusted to give an initial optical density (O.D.) reading of approximately 0.1–0.2 at 600 nm wavelength. The growth of the microorganism was observed upon introducing the antimicrobial peptide by measuring the O.D. readings at 2 h intervals for up to 8 h. The MIC was taken as the concentration of the antimicrobial peptide at which no microbial growth was observed with unaided eye and microplate reader (TECAN, Switzerland) at the end of 8 h incubation. Broth containing microbial cells was used as the negative control, and each test was carried out in 6 replicates. The standard deviations of the 6 readings were reported as the error bars.

2.5. Hemolytic activity test

Hemolytic activity tests were performed according to the previously reported protocol [21,22]. Briefly, fresh red blood cells were washed with PBS for three times, and were subjected to 25 times dilution with PBS to reach a concentration of approximately 4% (in volume) of the blood cells. The red blood cell suspension (100 μL) was placed into a 96-well cell culture plate and mixed with 100 μL of the antimicrobial peptide solution in PBS at concentrations of 0.98, 1.95, 3.90, 7.81, 15.63, 31.25, 62.5, 125, 250, 500 mg/L. The mixture was then incubated at 37 °C for 1 h to allow for the hemolysis process to take place. At the end of incubation time, the non-hemolysed red blood cells were separated by centrifugation at 13000 g for 5 min. Aliquots (100 μL) of the supernatant were transferred into a new 96-well plate, and the hemoglobin release was measured by checking the UV–absorbance of the samples at 576 nm using a microplate reader (TECAN, Switzerland). Two controls were provided in this assay: an untreated red blood cell suspension in PBS was used as the negative control, and a solution containing red blood cells lysed with 0.1% Triton-X was used as the positive control. Each assay was performed in 6 replicates, and the data were expressed as means and standard deviations of the 6 replicates. Percentage of hemolysis was calculated using the following formula:

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

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

Bacterial cells were cultured in tryptic soy broth at 37 °C under constant shaking at 100 rpm. Similar treatments as the broth microdilution method were performed for a shorter period of incubation to prepare the treated bacterial sample. Briefly, broth containing an antimicrobial peptide (100 μL) was prepared at 50 mg/L (a concentration above the MIC of the peptide) and pipetted into a 96-well cell culture plate. Microorganism was first inoculated to enter its log growth-phase. An equal volume of microorganism solution (100 μL) was added into each well of the 96-well plate containing the antimicrobial peptide. The concentration of microorganism solution was adjusted to give an initial optical density (O.D.) reading of 0.1–0.2 at 600 nm wavelength. Peptide treatment of the bacterial cells was at 37 °C for 2 h with 8 replicates, after which all the replicates were mixed into a microfuge tube and pelleted down at 5000 g for 5 min. For kinetic study of the antimicrobial actions, the incubation time with the antimicrobial agent was varied from 10, 20, 30, 40, 60, to 120 min. Following the incubation, bacterial cells were then washed with PBS for three times and fixed with 2.5% glutaraldehyde for 15 min, followed by washing with PBS twice. A final wash was performed with DI water, before the bacterial cells were pelleted down and dehydrated with a series of graded ethanol solution (30%, 50%, 70%, 95%, and 100%). Upon dehydration, the cells were then dried using a critical point dryer. Dried bacterial cells were then mounted on carbon tape, sputtered with platinum coating, and imaged under a field emission scanning electron microscope (JEOL JSM-7400F, Japan).

Table 1
Peptide designs and their molecular weights.

Number of repeat units (n)	Repeat units	Peptide sequence	Theoretical M_w	Measured M_w[a]
2	FFRR	FFRRFFRR-NH_2	1230.49	1231.37
	LLRR	LLRRLLRR-NH_2	1094.42	1097.27
	LLKK	LLKKLLKK-NH_2	982.37	984.14
3	AARR	AARRAARRAARR-NH_2	1380.63	1381.99
	FFRR	FFRRFFRRFFRR-NH_2	1837.22	1839.55
	LLRR	LLRRLLRRLLRR-NH_2	1633.11	1634.74
	LLKK	LLKKLLKKLLKK-NH_2	1465.03	1467.53
4	FFRR	FFRRFFRRFFRRFFRR-NH_2	2443.94	2443.60
	LLRR	LLRRLLRRLLRRLLRR-NH_2	2171.81	2174.35
	LLKK	LLKKLLKKLLKKLLKK-NH_2	1947.70	1951.07

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

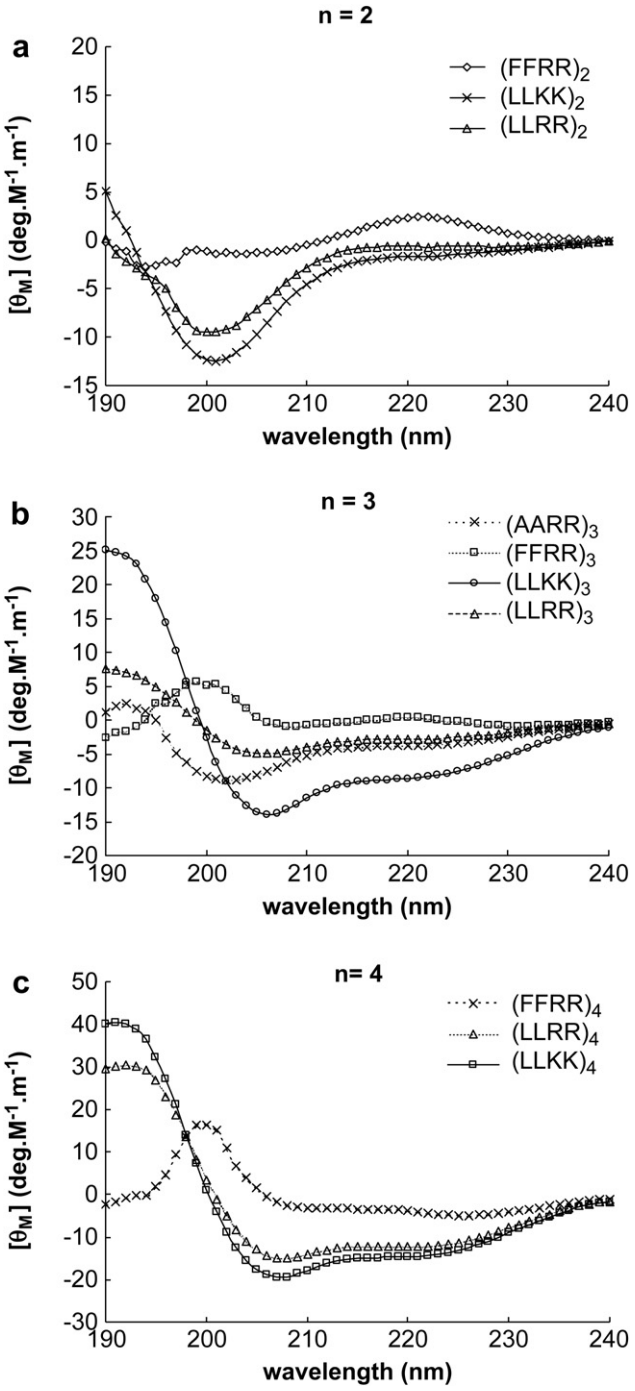


Fig. 1. CD spectra of designed antimicrobial peptides with number of repeat units, n = 2 (a), 3 (b), and 4 (c), measured in 25 mM SDS micelles solution (simulating microbial membrane environment). α-Helical signals are detected for peptides having n = 3 and 4, and the strength of the α-helical signal is a function of the composition of amino acids as well as repeat unit n.

2.7. In vitro immunogenicity test

Human PBMCs were used to test on the effect of the peptides to induce non-specific immune response by monitoring the secretion of IFN-α, IFN-γ, and TNF-α, three major cytokines produced by the innate immune system in its response to fight against infections/intrusions of foreign materials. These three cytokines were selected out of many cytokines as IFN-α has been widely accepted as the specific marker for bacterial and/or viral infections, whereas IFN-γ is known to be a more general marker resulting from immune responses towards foreign materials [30]. TNF-α also plays a major role in response to perceived "invader" molecules in the body, which activates neutrophils and macrophages to clear the foreign "invaders".

The human PBMCs used for this immune stimulation assay were extracted from freshly donated whole blood (from healthy donors from the National University Hospital, Singapore), *via* a standard Ficoll-Hypaque density centrifugation technique. Upon extraction from the human blood, PBMCs were re-constituted and maintained in RPMI growth medium (supplemented with 10% FBS and 1% penicillin-streptomycin) at 37 °C in the presence of 5% CO_2. To test the stimulation of IFN-α secretion by the human PBMCs after the peptide treatment, PBMCs were seeded at 3×10^5 cells/well in a 96-well plate (100 μL/well), and the cells were maintained with RPMI supplemented with granulocyte macrophage-colony stimulating factor (GM-CSF) (100 ng/mL) for 48 h prior to treatment, according to the reported procedures [31]. Priming of monocytes with GM-CSF was earlier reported to be an absolute requirement for IFN-α secretion stimulated by many immunogenic materials such as lipopolysaccharide [31,32]. Upon 48 h of priming, PBMCs were treated with the designed antimicrobial peptide for 12 h (100 μL at concentration of 250 mg/L), after which the growth medium was taken for ELISA test to detect the presence of any IFN-α protein in the growth medium. Similarly, in order to test on the possibility of the antimicrobial peptide to stimulate IFN-γ and TNF-α secretion, which may result in an undesirable non-specific immunogenic response *in vivo*, PBMCs were seeded at 3×10^5 cells/well in a 96-well plate (100 μL/well). Unlike immune response test for IFN-α, there is no requirement for PBMCs priming for IFN-γ and TNF-α stimulation [31]. The seeded PBMCs were then treated with the antimicrobial peptide for 2 days (100 μL at concentration of 250 mg/L), after which the growth medium was taken for ELISA test to detect the presence of any IFN-γ or TNF-α protein in the growth medium. These tests were performed in triplicates, and were repeated with a blood sample from a different donor (National University Hospital, Singapore), to ensure the reproducibility from different blood samples. In these experiments, two controls were used: treatment with growth medium only (as negative control), and treatment with lipopolysaccharide (100 ng/μL) (as positive control).

3. Results and discussion

3.1. Peptide characterization

The structure and molecular weight of the peptides were verified by MALDI-TOF MS. Table 1 summarizes the theoretically calculated molecular weights of the peptides and their measured molecular weights *via* MALDI-TOF MS. It can be seen that each peptide has a measured molecular weight value that is in a very close agreement with its theoretical value. This suggests that the peptides were successfully synthesized.

3.2. Circular dichroism (CD) spectroscopic studies

Most of the previously reported natural amphiphilic antimicrobial peptides adopt flexible random structure in aqueous solutions and assemble into a more rigid α-helical structure in the presence of a simulated membrane environment. Previous studies on peptide-lipid and protein-lipid interactions have well established that SDS micelles could be used as a model lipid system [11,33,34], because a similar detergent like SDS molecules had been reported to form micellar rings around the transmembrane helical region of a membrane protein, which were similar to the structure of the natural bilayer membrane [35,36]. In order to investigate the

Table 2
Minimum inhibitory concentrations (MIC) and 50% hemolytic concentrations (HC_{50}) of the synthetic antimicrobial peptides.

Antimicrobial peptides	Peptide sequence	MIC (mg/L)		HC_{50} (mg/L)
		B. subtilis	*C. albicans*	
$(FFRR)_2$	FFRRFFRR-NH_2	125	>500	>500
(LLRR)2	LLRRLLRR-NH_2	62.5	125	>500
(LLKK)2	LLKKLLKK-NH_2	500	250	>500
$(FFRR)_3$	FFRRFFRRFFRR-NH_2	31.3	125	>500
$(AARR)_3$	AARRAARRAARR-NH_2	>500	>500	>500
$(LLRR)_3$	LLRRLLRRLLRR-NH_2	15.6	500	>500
$(LLKK)_3$	LLKKLLKKLLKK-NH_2	31.3	125	>500
$(FFRR)_4$	FFRRFFRRFFRRFFRR-NH_2	20	>50	~26
$(LLRR)_4$	LLRRLLRRLLRRLLRR-NH_2	10	>50	~12
$(LLKK)_4$	LLKKLLKKLLKKLLKK-NH_2	10	>50	~24

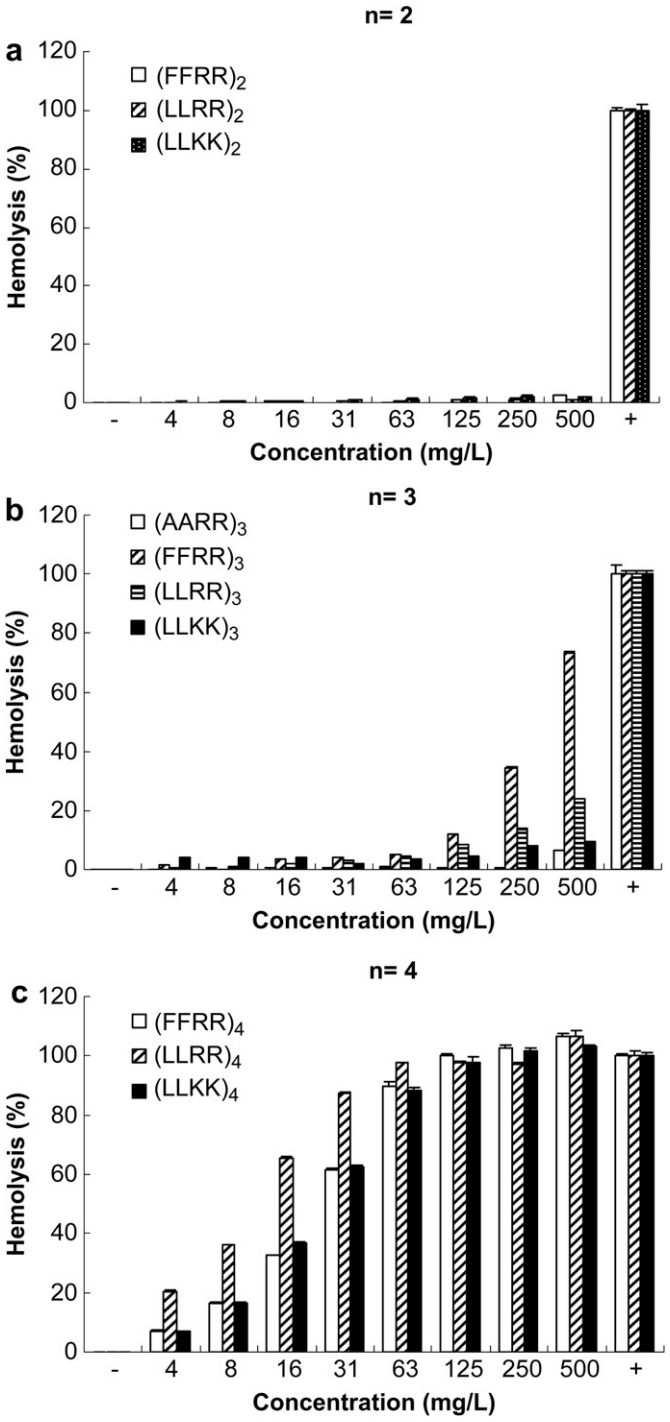
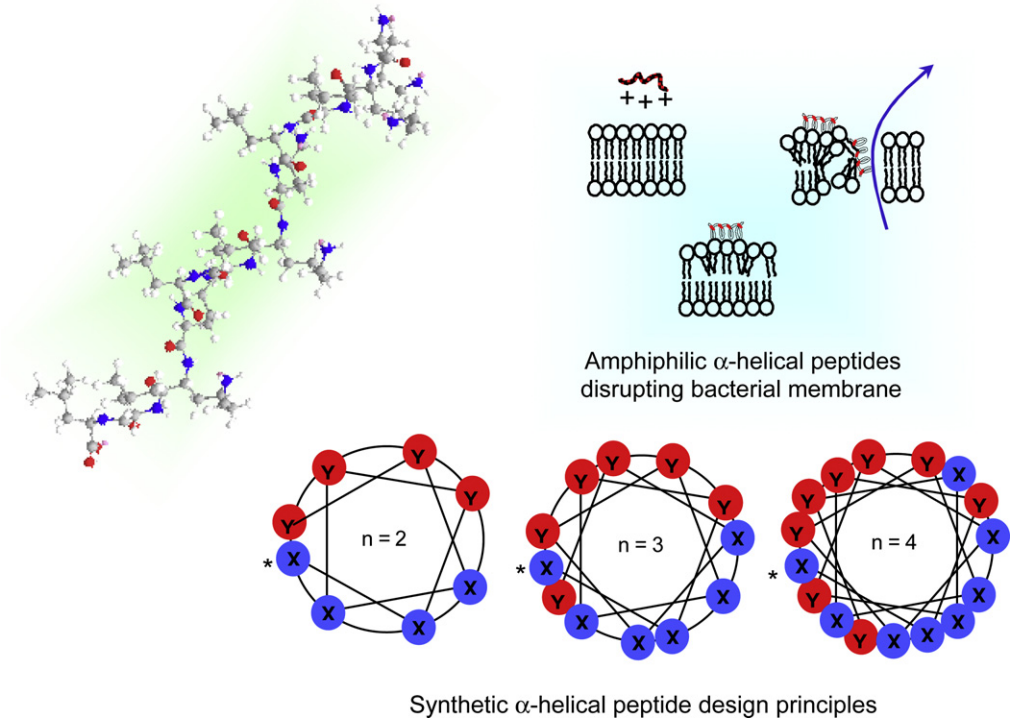


Fig. 2. Hemolytic properties of the synthetic antimicrobial peptides (n = 6 replicates) having various number of repeat units (n): (a) n = 2, (b) n = 3, (c) n = 4; with PBS treatment as negative control, and 0.1% Tripton-X treatment as positive control. Peptides with n = 2 and 3 provides minimal hemolytic properties at concentrations that are effective in inhibiting the growth of microbial cells.

proclivity of the conformational structures of the peptides in both water and membrane-like environments, they were first tested with CD spectroscopy technique at room temperature, by using both water and aqueous SDS micelle solution as the solvent. When water was used as the solvating medium, all the peptides did not adopt any α-helical conformations (data not shown), while in the membrane-mimicking environment (i.e. in water containing SDS micelles), most of the peptides tested assembled into α-helical conformation (Fig. 1). This finding is in direct agreement to the solution behavior of the α-helical natural antimicrobial peptides reported earlier [10–13,33,34]. It is generally believed that the cationic residues of these peptides could have initiated the association of the peptides onto the surface of SDS micelle through electrostatic interaction, which allows the folding of the peptides to occur [12,13]. From Fig. 1, it can also be seen that both the composition and the length of the peptides affected the helicity of the peptide conformation. Peptides having phenylalanine as the hydrophobic residues did not show strong signal of α-helical secondary structure in solution, which was characterized by the presence of double minima at 220 nm and 208 nm UV–vis wavelengths [6]. In contrast, peptides having leucine and lysine amino acid residues were found to exhibit strong α-helical signals. In addition, peptides having 2 repeat units (n = 2) were mostly found to form random coils regardless of the composition of the amino acids (Fig. 1 (a)). By comparing the molecular residual ellipticity $[-\theta_M]$ values of the peptides at these wavelengths, the propensity of the peptides in forming α-helical conformation could be arranged according to the order of $(FFRR)_2$, $(LLRR)_2$, $(LLKK)_2$, $(FFRR)_3$, $(LLRR)_3$, $(AARR)_3$, $(FFRR)_4$, $(LLKK)_3$, $(LLRR)_4$, and $(LLKK)_4$, from the weakest to the strongest inclinations. This observation is in agreement with the widely accepted knowledge of protein folding inclination that alanine, lysine and leucine residues have a stronger propensity of α-helical formation, while both phenylalanine and arginine residues are rather indifferent for the formation of such conformation [26,37]. As such, peptides containing amino acids having weaker propensities to form helical structure required more repeat units to give typical α-helical CD spectra. In addition, peptides with more repeat units were also observed to form the α-helical structures more readily when compared to the shorter peptides, as implied by lower minimum value of the molecular elipiticy, $[-\theta_M]$, at 222 nm wavelength, which has been referenced as a measure of helical fraction of proteins in solution [28,38].

3.3. Hemolytic activity of peptides

As the peptides designed in this study were aimed for the purpose of biomedical applications in fighting against infectious diseases, it is important to evaluate the extent of undesirable cytotoxic effect of the peptides. In the context of antimicrobial agents, cytotoxic effect is generally tested by analyzing the hemolytic properties of the antibiotics against mammalian red blood cells. Table 2 summarizes the concentrations of the peptides that resulted in 50% lysis of red blood cells (HC_{50}), which were estimated from the hemolysis tests of the peptides across a range of peptide concentrations as plotted in Fig. 2. It is desirable that the HC_{50} value of the peptide is as high as possible in comparison to the effective antimicrobial concentration (listed in the same table). This effect will be discussed in greater details in the following section. By comparing the HC_{50} values of the different peptides, it is apparent that the hemolytic property of the peptide is affected by both the number of repeat units (length of peptides), and the nature of hydrophobic/cationic residues of the peptides. In general, peptides having 2 and 3 repeat units were not hemolytic up to 500 mg/L (the highest concentrations tested in this study), while the peptides having 4 repeat units were highly hemolytic (>60% hemolytic) even at low concentration of 31 mg/L (Fig. 2). In this view, more repeat units seemed to possess greater ability of penetrating and/or disrupting cell membrane owing to the increased hydrophobicity of the longer peptide molecule, hence exhibiting an increased hemolytic activity. This trend was also observed earlier in the



Scheme 1.

natural antimicrobial peptide systems [6] or synthetic antimicrobial polymer systems [39].

3.4. Antimicrobial activities of peptides

All the peptides were tested for their MIC values against Gram-positive bacteria *B. subtilis* and yeast *C. albicans* with the standard broth microdilution assay. Most of the peptides efficiently suppressed these microbial cells at different effective concentration levels. Table 2 summarizes the MIC values of all the peptides designed in this study. Recalling the HC_{50} values listed in the same table, most of the peptides with 3 repeat units have a MIC value lower than the HC_{50} value, implying a wider therapeutic window, hence their potential usefulness as systemic antimicrobial agents. For example, $(FFRR)_3$, $(LLRR)_3$, and $(LLKK)_3$ peptides had MIC values of 31.3, 15.6, and 31.3 mg/L against *B. subtilis* (Table 2), respectively, and at these concentrations, less than 5% hemolysis was observed (i.e. less than 5% mammalian red blood cells were lysed) (Fig. 2). On the other hand, for the yeast *C. albicans*, which are generally more difficult to kill [21], the MIC values were significantly increased as compared to those for *B. subtilis*, i.e., 125, 500, and 125 mg/L, respectively. At the MIC levels for *C. albicans*, these peptides induced 12–24% hemolysis, except for $(LLKK)_3$ which induced only about 4.2% hemolysis (Fig. 2). This suggests a good selectivity of these peptides towards the anionic constituent of microbial cell membranes, over the zwitterionic mammalian cell membranes. However, $(AARR)_3$ peptide was particularly ineffective against the microbial cells due to weak lipophilicity of the alanine residue, resulting in poor hydrophilicity/lipophilicity balance of the amphiphiles. In contrast to the peptides with 3 repeat units, peptides designed with 2 repeat units exhibited much weaker antimicrobial activities as it was only effective to inhibit the growth of *B. subtilis* and *C. albicans* at much higher concentrations (Table 2). Similarly, the peptides with 4 repeat units also exhibited much poorer selectivity towards bacterial cells over mammalian red blood cells, even though they were generally more effective to

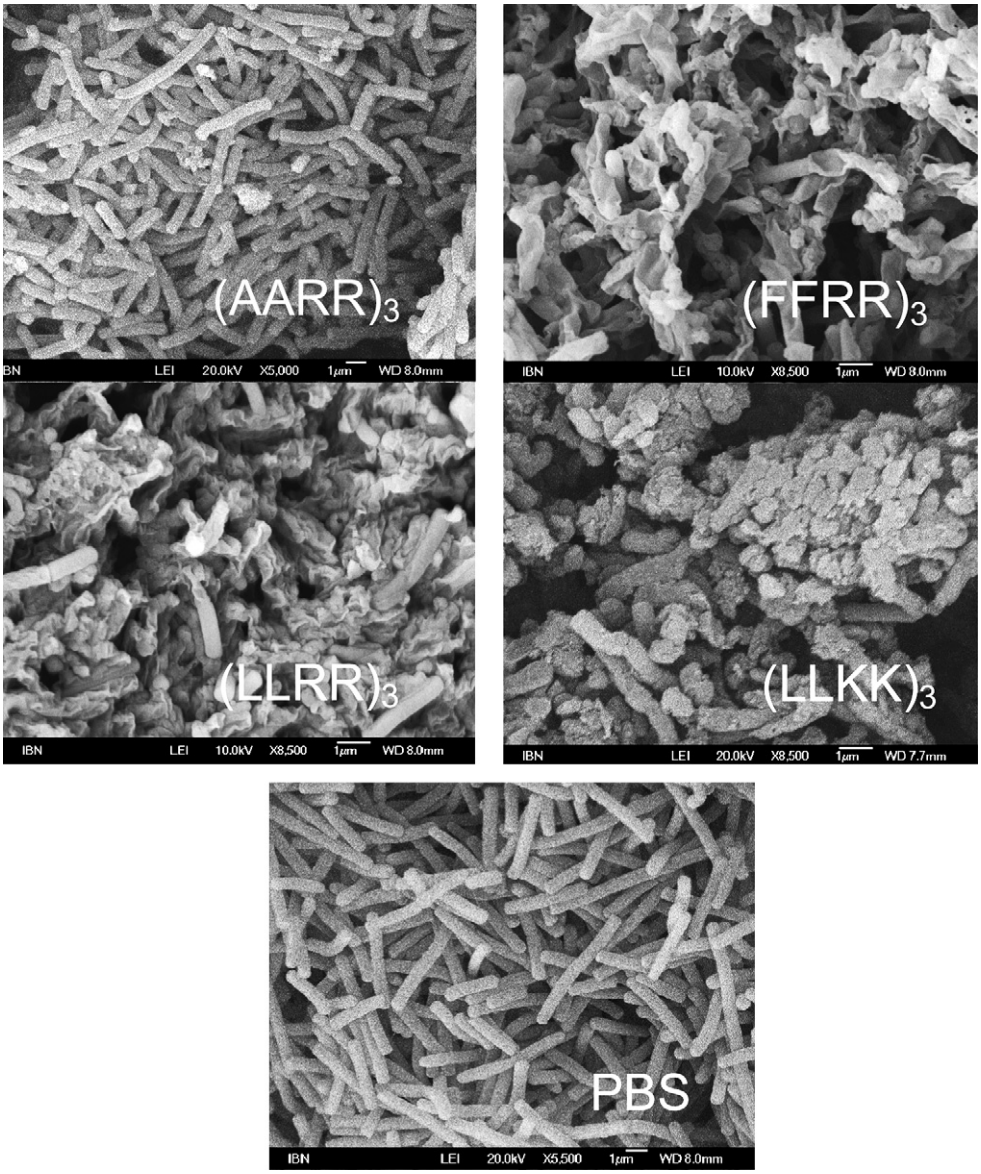


Fig. 3. SEM images of *B. subtilis* after incubation with the peptides of three repeat units at 50 mg/L (above the MICs) for 2 h. Bacterial membrane destabilization is observed after incubation with these peptides, in comparison to the control treatment with PBS buffer. For peptides having effective antimicrobial properties, i.e., $(FFRR)_3$, $(LLKK)_3$, and $(LLRR)_3$, membrane disruption is observed for the majority of the bacterial population.

inhibit the growth of microbial cells at lower concentrations than those peptides with 3 repeat units. For example, $(FFRR)_4$, $(LLRR)_4$, and $(LLKK)_4$ peptides had MIC values of 20, 10, and 10 mg/L against *B. subtilis* (Table 2), respectively. However, at these concentrations, around 50%, 50%, and 30% of mammalian red blood cells were lysed (Fig. 2). Presumably, such poorer selectivity was encountered due to the increased hydrophobicity and poorer facial amphiphilicity of these peptides. Scheme 1 shows the helical wheel schematic representation of the antimicrobial peptides designed in this study for the 2, 3, and 4 repeat units' peptides. It can be seen that the peptides with 3 repeat units resulted in the most "optimum" facially amphiphilic conformation, upon rearrangement into the corresponding α-helical secondary structures. This finding signifies the postulated factors that would affect the selectivity of antimicrobial peptides earlier [12]. In summary, among all the peptides designed in this study, excellent selectivity over both *B. subtilis* and *C. albicans* was particularly demonstrated by $(LLKK)_3$ peptide, which could be due to a combination of proper hydrophobic-cationic balance, stronger inclination to form α-helical conformation in a membrane-like environment, as well as optimum facially amphiphilic helical conformation.

3.5. Antimicrobial mechanism

Based on the MIC and HC_{50} values of the peptides, the potential use of the peptides with 3 repeat units are more apparent than the others. Hence, these peptides were further studied in its antimicrobial actions in killing microbial cells by observing the integrity of bacterial cell membrane upon treatment with these peptides. Fig. 3 shows SEM images of *B. subtilis*, which were treated with different

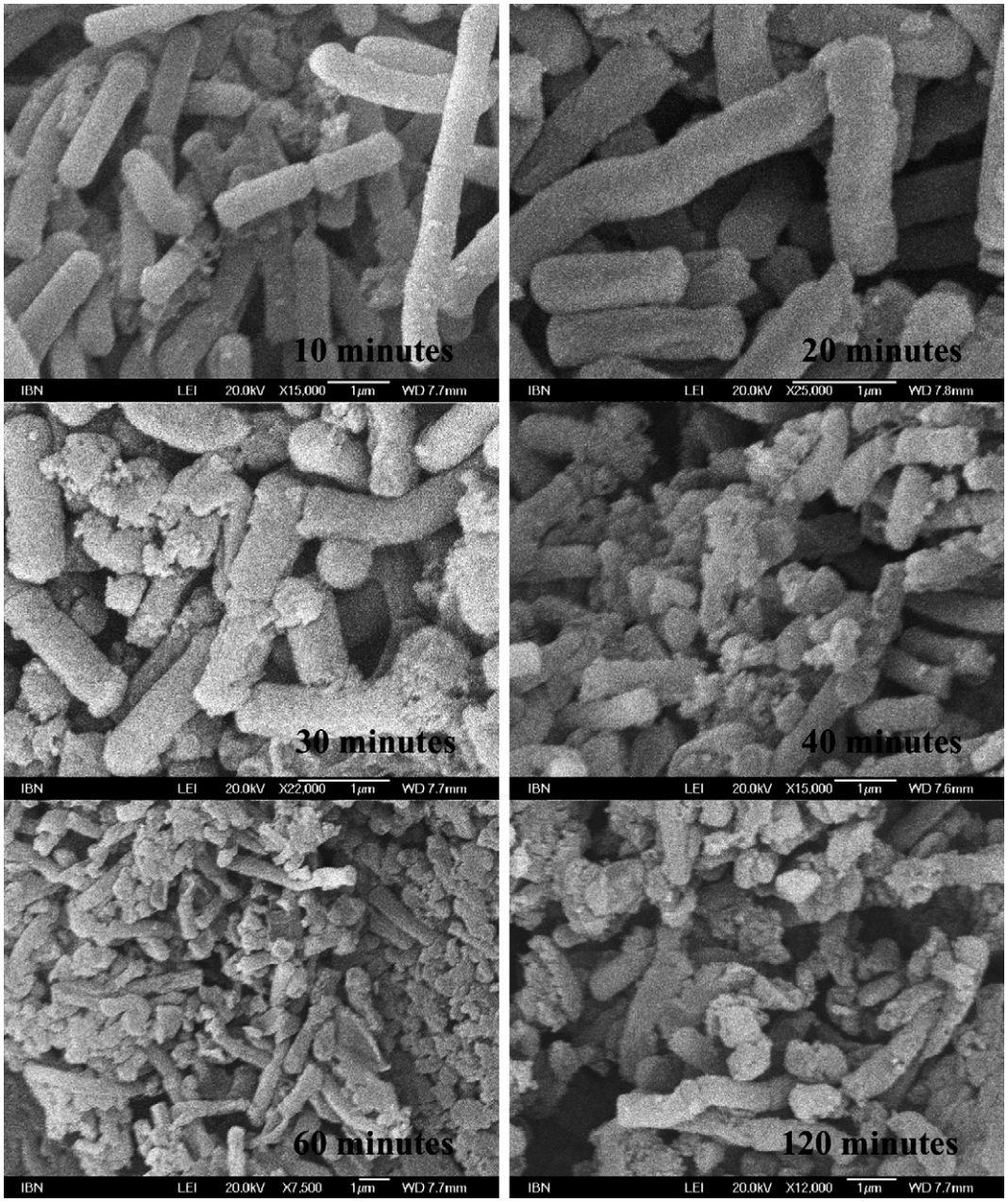


Fig. 4. SEM images of *B. subtilis* after incubation with $(LLKK)_3$ peptide at 50 mg/L (above the MIC) for various periods of incubation time varying from 10 to 120 min. Membrane damage is observed as soon as 10 min post incubation. Extensive membrane damage and significant amount of bacterial debris are seen at 30 min or beyond. This observation implies the membrane-lytic mechanism of action of the designed antimicrobial peptides.

peptides of 3 repeat units at a concentration of 50 mg/L (above their corresponding MIC, except for $(AARR)_3$ peptide) for 2 h, and observed under FE-SEM after drying. As seen from these images, the treatment with most of the peptides induced a significant membrane damaging effect, in comparison to that with PBS as the negative control. In addition, the treatment with $(AARR)_3$, which were not effective to inhibit the growth of *B. subtilis*, was observed to result in minimal membrane damage in minor population of the bacteria.

The kinetics of the antimicrobial effect of the most selective and effective peptide in this study, $(LLKK)_3$, was further studied qualitatively by observing the membrane structure of *B. subtilis* treated with the peptide for various time length. As shown in Fig. 4, even for the short treatment of 10 min with the antimicrobial peptide, a significant extent of membrane damage on some of the bacterial cells were observed. This observation is in agreement with the more recent study conducted by AM Belcher et al, in which a special setup of high-speed atomic force microscopy technique was used to study the kinetics of the antimicrobial activities of peptides [40]. In their study, it was observed that in some bacterial cells, membrane disruption occurred as early as 65 s after treatment with antimicrobial peptides of different origins. It was also found that the effect of membrane corrugation of the bacterial cell remains a variable among different bacterial cells, as there were bacterial cells without any signs of membrane disruption even after 16 min of exposure to the same peptide.

3.6. In vitro immunogenicity test

Use of synthetic biomaterials often inevitably leads to the interactions between the biomaterials and the host. One important aspect of biomaterial development other than its functionality is its biocompatibility, which entails a long list of material properties as a reaction upon contact with biological tissues. Some of these properties include both cytotoxic as well as immunogenicity aspects [41]. Even though some studies arguably reported that it might be useful to have an antimicrobial peptide that could induce immunogenic response by the host organism *via* the Toll-like Receptor (TLR) pathways, in view of providing synergy for its microbial invasion clearance [9], uncontrolled non-specific immunogenic response can become detrimental towards the host organism. This, under a more severe condition, may result in a medical condition termed as *hypercytokinemia* or *cytokine storm*, causing further severe inflammations. For example, the experimental immunomodulator drug, TGN1412, which belong to a humanized recombinant protein drug class originating from mouse, caused 100% *hypercytokinemia* response within all the 6 Phase I clinical trial patients [42].

In this study, we attempted to gauge the probability of the $(LLKK)_3$ peptide, which is the most effective and selective in killing microbial cells, in inducing non-specific immunogenic response *in vitro* through the cytokine pathways, by measuring the level of IFN-α, IFN-γ, and TNF-α (Fig. 5) secretion by human PBMCs upon stimulation with the peptide. These cytokines form the major upstream signaling molecules that are involved in the innate immune system upon recognizing a perceived "invader" in the human body. As summarized in Fig. 5, the secretion of these cytokines was relatively low upon stimulation with the peptide, which practically occurred at the basal-secretion level of the non-stimulated PBMCs. Furthermore, to ensure the validity of the test, the capability of the PBMCs to secrete these three cytokines was also demonstrated by a positive control group, at which PBMCs were stimulated with a highly immunogenic lipopolysaccharide. Prolonged cytokine secretion activates macrophages, which often leads to necrosis and surrounding tissue fibrosis/scarring. Therefore, these findings suggest that the use of the designed $(LLKK)_3$ peptide as an anti-infective agent (either topically or systemically) could potentially avoid undesired tissue fibrosis.

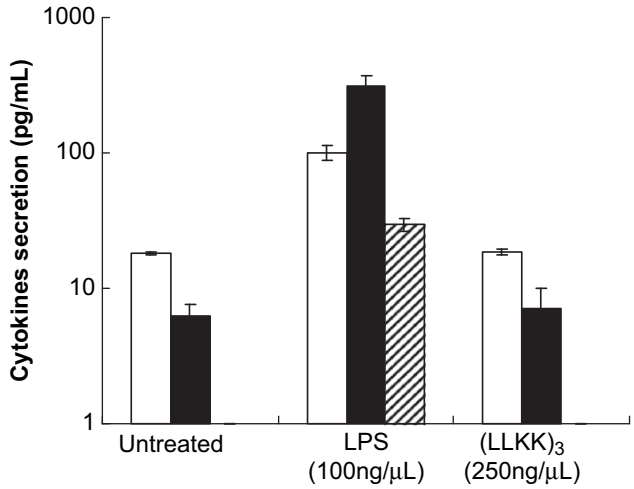


Fig. 5. Secretion of Cytokines [(□) IFN-α, (■) IFN-γ, and (▨) TNF-α] by human peripheral blood mononuclear cells stimulated by $(LLKK)_3$ and lipopolysaccharide (LPS). Stimulation with $(LLKK)_3$ peptide does not show signification expression of all IFN-α, IFN-γ, and TNF-α cytokines, in comparison to the untreated negative control and the stimulation with LPS positive control. This ensures the nonimmunogenic aspects of the $(LLKK)_3$ peptide to be used as antimicrobial agents.

4. Conclusion

In this paper, we have proposed a systematic approach to design antimicrobial peptides based on the principles of α-helical peptide/protein folding theory. A series of synthetic antimicrobial peptides have been synthesized and evaluated for their efficacy against Gram-positive bacteria and yeast. These peptides are unfolded in a random structure in aqueous solution, but folded into α-helical structure upon interacting with a membrane environment. Peptides with more repeat units formed the α-helical structure more readily than the shorter peptides. An increased number of repeat units led to stronger antimicrobial activities and a higher degree of hemolysis. However, the peptides with 3 repeat units such as $(FFRR)_3$, $(LLRR)_3$, and $(LLKK)_3$ had low MIC values, yet induced insignificant hemolysis. In particular, $(LLKK)_3$ peptide had MIC values of 31.3 and 125 mg/L against *B. subtilis* and *C. albicans* respectively. At these concentrations, it caused less than 5% hemolysis. At a concentration slightly above its MIC, the peptide was shown to damage the membrane of *B. subtilis* as soon as 10 min post incubation. This antimicrobial peptide did not induce non-specific immunogenicity *in vitro via* cytokines pathway involving TNF-α, IFN-α and IFN-γ. All these findings demonstrated that this peptide could be a promising antimicrobial agent against Gram-positive bacteria and yeast.

Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.biomaterials.2010.11.054

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