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Effects of Conventional Surfactants on the Activity of Designed Antimicrobial Peptide

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ABSTRACT

In this paper, the interaction between a designed antimicrobial peptide (AMP) G(IKK)₃I-NH₂ (G₃) and four typical conventional surfactants (SDS, C₁₆TAB, C₁₂EO₂₃ and C₁₄DMAO) have been studied through surface tension measurement, circular dichroism (CD) spectroscopy. The antimicrobial activities of AMP/surfactant mixtures have also been studied with gram-negative *E. coli*, gram-positive *S. aureus*, and fungus *C. albicans*. The cytotoxicity of the AMP/surfactant mixtures has also been assessed with NIH 3T3 and HSF cells. The surface tension data showed that AMP/SDS mixture was much more surface active than SDS alone. CD results showed that G₃ conformation changed from random coil, to β -sheet, then to α -helix with increasing SDS concentration, showing a range of structural transformation driven by the different interactions with SDS. The antimicrobial activity of G₃ to gram-negative and gram-positive bacteria decreased in the presence of SDS due to the strong interaction of electrostatic attraction between peptide and surfactant. The interaction between G₃ and C₁₆TAB, C₁₂EO₂₃, C₁₄DMAO were much weaker than SDS. As a result, the surface tension of surfactants with G₃ did not change much, neither were the secondary structures of G₃. The antimicrobial activities of G₃ were little affected in presence of C₁₂EO₂₃, slightly improved by C₁₄DMAO and clearly enhanced by cationic surfactant C₁₆TAB due to its strong cationic and antimicrobial nature, consistent with their surface physical activities as binary mixtures. Although AMP G₃ didn't show activity to fungus, the mixtures of AMP/C₁₆TAB and AMP/C₁₄DMAO could kill *C. albicans* at high surfactant concentration. The mixtures had rather high

cytotoxicity to NIH 3T3 and HSF cells although G₃ is nontoxic to cells. Cationic AMPs can be formulated with nonionic, cationic and zwitterionic surfactants during product development, but care must be taken when AMPs are formulated with anionic surfactants, as the strong electrostatic interaction may undermine their antimicrobial activity.

INTRODUCTION

An important class of surfactant applications lies in sanitation, hygiene and healthcare where a biocide is used as a bioactive against microorganisms. Typical biocides include cationic surfactants such as triclosan (TCS), 1,2-benzisothiazol-3(2H)-one (BIT) and di-decyl dimethyl ammonium chloride (DDTAB), for controlling bacteria, fungi and moulds. For hygiene and personal care products such as mouthwashes, toothpastes and antiseptic creams, cationic actives such as Cetyl-pyridinium chloride (CPC) are widely used. Whilst these actives are potent against a wide range of microorganisms, they tend to be highly toxic to mammalian cells, with potentially other adverse health impact [1-7].

Antimicrobial peptides are evolutionarily conserved across a wide variety of organisms and form part of the innate immune system [8]. Synthetic derivatives have been successfully developed on the basis of natural ones [9-14]. Some of them have been explored to address the bacterial infection, such as MSI-78, M-226 and Hlf1-11 which are the analogues or derivatives of magainin-2, indolicidin and human lactoferricin, respectively [15-17]. Antimicrobial peptides are usually composed of 12~50 amino acid residues. Most of them have the common features of positive

charges and amphipathic property, allowing them to bind to the cell wall of microorganisms and adopted an α -helical secondary structure. Antimicrobial peptides have broad spectrum antimicrobial properties due to the unique membrane disruption mechanism [9, 10]. Lam *et al.* reported a class of antimicrobial agents, termed “structurally nanoengineered antimicrobial peptide polymers” (SNAPPs) which exhibit sub- μ M activity against all Gram-negative bacteria tested, including ESKAPE and colistin-resistant and MDR (CMDR) pathogens, while demonstrating low toxicity [18]. Antimicrobial peptides are potential alternatives to many biocides that are widely used in personal care, hygiene and healthcare for the merits of relative better biocompatibility and well-tunable properties. Because products utilized in hygiene and healthcare are usually formulated with surfactants, it is crucial to study how mixing of AMPs with surfactants affects their physical properties and antimicrobial activities. Rhoades *et al.* have investigated the antimicrobial activity and solution behavior of mixtures containing surfactants and antimicrobial peptides, and found that the antimicrobial peptides could be successfully used as additives in polymer coatings formulations [19]. Besides these, antimicrobial peptides also played an important role in the field of dermatology and skin care industries [20-22]. However, the relatively high synthetic costs, possible toxicity to host cells and susceptibility to proteolytic degradation that limit their potential commercial applications [23-25].

The interactions between peptides or peptide-derived molecules and surfactants have been studied due to a diverse range of morphological structures and functionalities formed and their potential use in biomaterial development. Castelletto

et al. have reported the influence of the anionic surfactant sodium dodecyl sulfonate (SDS) on the self-assembly of a peptide amphiphile C₁₆-KTTKS [26]. It was found that the addition of SDS led to a morphological transition from nanotapes to nanofibrils via the intermediate state of twisted nanoribbons. Zhang *et al.* have studied the effects of a zwitterionic surfactant C₁₂DMAO and a nonionic surfactant C₁₂E₄ on the self-assembly behavior of peptide amphiphile C₁₆-GK-3 [27, 28]. Their results revealed that different surfactant concentrations could lead to different self-assembled nanostructures from the peptide amphiphile. Jayawardane *et al.* have explored the co-adsorption of surfactant-like short peptide V₆K and conventional surfactants SDS and C₁₂TAB at solid/water interfaces [29]. The results indicated different adsorption behavior between SDS/peptide system and C₁₂TAB/peptide system, dependent on the molar ratio of the peptide and surfactant. We also demonstrated that co-assembly of SDS and a Bola type peptide KI₄K. SDS could affect the morphologies of KI₄K self-assembled nanotubes, and the morphological regulation was SDS concentration dependent [30]. Although these studies are based on surfactant-like peptides or peptide amphiphiles, they could direct the study of interactions between antimicrobial peptides and surfactants.

Formulating AMPs with conventional surfactants is a promising strategy to develop commercially affordable antimicrobial agents. In this paper, we investigated the interactions between a designed antimicrobial peptide G(IKK)₃I-NH₂ (termed as G₃) and four model conventional surfactants. G₃ was designed by our group and its antimicrobial activity has been well demonstrated [9-14]. The surfactants include SDS

(anionic), hexadecyl trimethyl ammonium bromide (C₁₆TAB, cationic), polyoxyethylene (23) lauryl ether (C₁₂EO₂₃, nonionic) and tetradecyldimethylamine oxide (C₁₄DMAO, zwitterionic). The interactions between G₃ and surfactants at the air/liquid interface and bulk solution have been studied through surface tension measurements and CD. We also examined the effects of surfactants on the antimicrobial activities of G₃ and the cytotoxicity of G₃/surfactant mixtures. The broad aim is to correlate the mode of interaction with the mixing and bioactivity against microorganisms. This work will help us understand how mixing affects their interaction with antimicrobial peptides and will improve the application of antimicrobial peptide through effective development of new products in hygiene, personal care and wound care.

Scheme 1. Chemical structure of G(IKKK)₃I-NH₂.

EXPERIMENTAL SECTION

Materials and Chemicals. Antimicrobial peptide G₃ was synthesized on a CEM Liberty microwave peptide synthesizer based on the solid phase synthesis method.

The detailed procedures of synthesis and purification have been described in our previous work [31, 32]. The molecular weight and purity of synthesized peptide were confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and reverse-phase high performance liquid chromatography (rp-HPLC) (Figure S1). The rp-HPLC result suggested the purity to be over 98%. C₁₆TAB was purchased from Sinopharm Chemical Reagent Co., Ltd, and recrystallized three times with acetone and ethanol before use [33]. SDS was purchased from Sigma-Aldrich and purified through recrystallization with ethanol and a small amount of water. C₁₂EO₂₃ was purchased from Acros Organics BVBA, and used as supplied without further purification. Tetradecyldimethylamine oxide (C₁₄DMAO) of 30 wt% aqueous solution was purchased from the China branch of Clariant Corporation. It was first lyophilized under vacuum and then recrystallized three times in acetone. All the solutions of surfactants, peptide and their mixtures were prepared with ultra-pure water from a Milli-Q ultra-pure water system (Millipore, resistivity $\geq 18.2 \text{ M}\Omega\cdot\text{cm}$).

Surface Tension. Surface tension was measured with a KRÜSS Easy Dyne tensiometer with Wilhelm plate method at $25\pm 0.1 \text{ }^{\circ}\text{C}$. The samples were kept for at least 60 min for equilibration before measurement. The measured surface tension had a deviation of $\pm 0.1 \text{ mN}\cdot\text{m}^{-1}$.

Circular Dichroism (CD) Spectroscopy. CD spectra were collected on a MOS-450 spectrometer (Biologic, France) at room temperature. Wavelength scan was recorded from 190 to 270 nm with an interval of 0.5 nm. Quartz cuvettes with different path

length (10, 5, 1, 0.5 and 0.1 mm) were used according to the sample concentration. The spectra were the average of at least 5 measurements after the solvent background was subtracted. The signal was expressed in the unit of $[\theta]$ ($10^3 \text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$).

Antimicrobial Activity. Gram-negative *E. coli DH5 α* and gram-positive *S. aureus* were cultivated in LB and TSB media respectively with continuous shaking at 37 °C for 24 h. Then the bacterial cells were centrifuged and washed with PBS twice, and suspended in the media again. A volume of 100 μL bacterial suspension with the concentration of 2×10^6 CFU/mL was added into 100 μL G₃/surfactant solutions (surfactant was SDS, C₁₆TAB, C₁₄DMAO or C₁₂EO₂₃) in a 96-well plate. The plate was incubated at 37 °C for 18 h and then the absorbance at 600 nm (A_{600}) was measured using a microplate autoreader (Molecular Devices, M2e). For fungus, *C. albicans* was cultivated in Sabouraud medium with continuous shaking at 37 °C for 48 h. When mixed with G₃/surfactant, it was further incubated for 24 h before measuring A_{600} . The microbial survival ratio was calculated with Equation 1.

$$\text{survival ratio} = \frac{A - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \quad (1)$$

The absorbance value of wells without peptides was used as control and the wells without cells as blank in the measurements. The survival ratio of bacteria was measured with A_{600} .

MTT Assay. MTT assay was analyzed the cytotoxicity of surfactants, G₃, and G₃/surfactant mixtures with HSF and NIH 3T3 cells. In brief, cells were firstly seeded and incubated overnight in a 96 well plate at 37 °C. Then, the cells were treated with certain concentration of surfactant or G₃ for 24 h. Subsequently, a volume of 20 μL (5

mg/mL) MTT was added to each well and the plate was incubated for another 4 h. Finally, the supernatant was removed and the precipitated formazan was dissolved with 150 μ L of dimethyl sulfoxide (DMSO), and the absorbance at 490 nm (A_{490}) was measured. The cell survival ratio was also calculated with Equation 1 with A_{490} value.

RESULTS AND DISCUSSION

Surface Tension of G_3 , SDS and their binary mixtures. The surface tension of AMP G_3 , surfactants and their mixtures of different ratios were measured at 25 ± 0.1 °C. The mixing of positively charged G_3 and anionic surfactant SDS might result in precipitation within a certain concentration ratio range due to the charge neutralization. Precipitation of G_3 /SDS mixture occurred over the SDS concentration range of 0.35~3.5 mM and the G_3 concentration range of 0.025~0.050 mM. The solution was cloudy when SDS/ G_3 ratio was between 5 to 10, especially around 7, which also confirmed the electrostatic interaction between SDS and G_3 . Over all other concentration ratios we studied, the G_3 /SDS mixed solutions were transparent without precipitates.

Figure 1. Plot of surface tension against $\lg(c)$ for G_3 in pure water at 25 °C.

The surface tension (γ) versus the concentration ($\lg c$) for individual G_3 and SDS were plotted in Figure 1 and Figure 2A, respectively. The cmc (critical micellar concentration) and γ_{cmc} (surface tension at the cmc) of G_3 were 1.07 mM and 56.3 $\text{mN}\cdot\text{m}^{-1}$, respectively. Figure 2A showed that cmc and γ_{cmc} of SDS were consistent with the values reported in literature under the solution conditions [34]. The surface tension changes from the G_3 /SDS mixtures were subsequently measured and the profiles showed significant deviations from those of G_3 and SDS alone.

Following previous studies of interactions between surfactants and proteins [35], we have studied the effect of mixing on surface tension by measuring how surface tension changes as a function of SDS concentration at different fixed G_3 concentrations. Although G_3 is a small molecule relative to proteins, it carries 7 positive charges with 3 repeats of IIKK (peptide segment of Ile-Ile-Lys-Lys), it is treated as a model of polypeptide or polymer in this case. The surface tension was subsequently measured with the G_3 concentrations fixed at 0.005, 0.01, 0.015, 0.025 and 0.05 mM. It can be seen from Figure 2 that even at the lowest G_3 concentration of 0.005 mM and over the lowest SDS concentration range the surface tension was decreased significantly (Figure 2A, Region i). In this region, the surface tension of both G_3 and SDS alone were above 70 $\text{mN}\cdot\text{m}^{-1}$, while the surface tension of the G_3 /SDS mixture was much lower ($\sim 38 - 47 \text{ mN}\cdot\text{m}^{-1}$) than that of each component alone. Because of the opposite charges, SDS molecules might bind to G_3 to form complexes, with the exact structure and composition of the complexes varying with SDS concentration. With each SDS molecule binding, G_3 loses a net charge but gains

an SDS carrying a dodecyl chain, resulting in enhanced hydrophobicity. Thus, a characteristic feature in this region is the steady decline of surface tension with rising SDS concentration, consistent with the expectation of increasing hydrophobicity of the complexes with more SDS binding. But all complexes formed in this region clearly have better surface activities than their individual components as evident from surface tension changes.

As the SDS concentration further increased above 0.014 mM (Figure 2A, Region ii), a break point occurred above which increase in SDS concentration did not cause any measurable change in surface tension. This feature is similar to that of polymer/surfactant and protein/surfactant systems with opposite charges. In spite of a lack of change in surface tension, the surface composition must change due to the increasing SDS concentration. In fact, both the compositions of the complexes at the surface and those in the bulk solution must vary with the increase of SDS concentration due to the combined actions of charge neutralization and hydrophobicity effect as indicated earlier [35].

Figure 2. Surface tension changes from G_3 /surfactant mixtures against $\lg c_{\text{surf}}$. The concentrations of G_3 were fixed at 0.005, 0.01, 0.015, 0.025, 0.05 and 0.10 mM, respectively. (A) G_3 /SDS mixtures, (B) G_3 / $C_{16}\text{TAB}$ mixtures, (C) G_3 / $C_{12}\text{EO}_{23}$ mixtures, and (D) G_3 / $C_{14}\text{DMAO}$ mixtures.

As SDS concentration reaches the boundary between Region ii and Region iii, the surface tension suddenly starts to rise at the G_3 concentration of 0.005 mM, with the peak occurring near the SDS concentration of 0.35 mM (Figure 2A, Region iii). There are two possible explanations here concerning the rising surface tension, the changing composition and structure of the G_3 /SDS complexes, or with the rising SDS concentration, SDS monomers gradually replace the G_3 /SDS complexes saturated with SDS binding. Above the peak position, the surface tension subsequently becomes declined with further increasing SDS concentration and eventually approached the

value of SDS alone, indicating that the surface G_3 /SDS complexes were gradually replaced by the SDS monomers.

For the binary mixtures with G_3 concentration greater than 0.005 mM, the surface tension changes follow the same pattern with increasing SDS concentration, except that the peak in Region iii is far less pronounced. Instead the surface tension in this region rises slightly, then decreases to a lower value than that of SDS with 0.005 mM of G_3 . The surface tension drop clearly indicated the association of G_3 with SDS again in the form of the G_3 /SDS complexes at the surface. With the increased concentration of G_3 , more G_3 /SDS complexes adsorbed at the surface and they did not get entirely removed even if the SDS concentration reached its cmc value.

The cmc of the G_3 /SDS mixture with 0.005 mM G_3 was around 2.5 mM, while the cmc of SDS alone was 8 mM in the ultrapure water. The surface tension above the cmc of G_3 /SDS was almost the same with that of SDS (Figure 2A, Region iv), except for the SDS solution with 0.05 mM G_3 . When the SDS concentration was high enough, it would adsorb on the surface and finally replaced the G_3 /SDS complexes. When the concentration of G_3 was high enough (0.050 mM), parts of the G_3 /SDS complexes could adsorb on the surface even under high SDS concentration. Thus, its surface tension was lower than that of SDS in Region iv. When the SDS concentration was above its cmc, the surface activity of the mixtures was mainly dominated by SDS, so the surface tension curves were similar in Region iv. For high G_3 concentrations of 0.025 and 0.05 mM, the G_3 /SDS complex might also contribute to the surface activity, and their surface tension was a bit lower than other curves. All the results indicated

that there was a strong interaction between G_3 and SDS molecules to form complexes that have a varying range of surface activity.

Surface Tension of Binary mixtures of $G_3/C_{16}TAB$, $G_3/C_{12}EO_{23}$ and $G_3/C_{14}DMAO$. The surface tension curves of the $G_3/C_{16}TAB$ mixtures are shown in Figure 2B. The cmc and γ_{cmc} of mixtures were almost the same with $C_{16}TAB$ (0.96 mM). When $C_{16}TAB$ concentration was below cmc, the surface tension of $G_3/C_{16}TAB$ mixtures was lower than that of $C_{16}TAB$ alone, especially when G_3 amount was high enough. The surface tension of $G_3/C_{16}TAB$ mixtures was the same with $C_{16}TAB$ above cmc. It is deduced that G_3 could adsorb on the surface at low $C_{16}TAB$ concentrations, and could be gradually replaced at high $C_{16}TAB$ concentrations due to the high surface activity of $C_{16}TAB$. As G_3 and $C_{16}TAB$ bear the same charge type, the interaction is based on their relative surface activity.

The surface tension curves of $G_3/C_{12}EO_{23}$ and $G_3/C_{14}DMAO$ mixtures are shown in Figures 2C and 2D. They are similar to those of $G_3/C_{16}TAB$. The γ_{cmc} values of $G_3/C_{12}EO_{23}$ and $G_3/C_{14}DMAO$ were a bit lower than the surfactants because G_3 was also surface active. And their cmc values were the same with $C_{12}EO_{23}$ (0.06 mM) and $C_{14}DMAO$ (0.06 mM). It was indicated that there were only weak interactions between peptide and surfactants, because $C_{12}EO_{23}$ is neutral and $C_{14}DMAO$ is weakly positively charged (The pH of mixture solution was 5.5, and isoelectric point of $C_{14}DMAO$ is 8.8 (Figure S2)). So the interaction between G_3 and $C_{12}EO_{23}$ or $C_{14}DMAO$ was also very weak. As shown in Figure 1, G_3 also possessed weak surface activity. As there were no strong interactions between G_3 and $C_{16}TAB$, $C_{12}EO_{23}$,

C₁₄DMAO, both G₃ and surfactants contributed to the surface activities of the mixtures, so their surface tensions below the cmc values were lower than the surfactants alone.

Circular Dichroism (CD). CD spectroscopy was employed to investigate the interactions of AMP G₃ with surfactants. Figure 3A are CD spectra of 0.050 mM G₃ under different concentrations of SDS. The secondary structure of G₃ in the mixtures gradually changed from random coil to α -helix with increasing SDS concentration. The G₃ conformation in the G₃/SDS mixtures related with the surface tensions. The conformation of AMP G₃ alone was random coil, but transformed to β -sheet when SDS concentration was below the cmc, and then to α -helix when SDS concentration was close to or above the cmc. Combining with the surface tension data, we can see that only when G₃ adopts random coil or β -sheet structure, the mixed G₃/SDS system shows a higher surface activity than single components alone. It was indicated the G₃/SDS complexes were more surface active than SDS alone. While G₃ molecules were wrapped in the SDS micelles, they moved to the bulk solution with the micelles and no longer contributed to the surface activities. G₃/SDS mixtures at other G₃ concentrations show similar CD spectra, thus also supporting the proposed interaction mechanism (Figure S3).

Figure 3. CD spectra of 0.05 mM G_3 mixed with different surfactants: (A) SDS, (B) C_{16} TAB, (C) C_{12} EO₂₃ and (D) C_{14} DMAO.

CD spectra of 0.050 mM G_3 mixed with C_{16} TAB, C_{12} EO₂₃ and C_{14} DMAO are shown in Figures 3B, 3C and 3D, respectively. G_3 adopted random coil conformation with these three surfactants over all the concentrations studied. And the conformation of G_3 did not changing even at higher surfactant concentrations. The conformation also remained random coil even when G_3 concentration was as high as 0.1 mM (Figures S4, S5 and S6). The results thus reveal that cationic surfactant C_{16} TAB, nonionic C_{12} EO₂₃ and zwitterionic surfactant C_{14} DMAO have no effect on the secondary structure of G_3 and that the interactions between these surfactants and AMP G_3 are very weak under these conditions. The antimicrobial activity of AMPs should not be affected if they are formulated with these surfactants.

Antimicrobial Activities and Cytotoxicity. The effect of SDS on the antimicrobial activity of G_3 has been assessed with *E. coli DH5 α* , *S. aureus* and *C. albicans*. The results of *E. coli DH5 α* are shown in Figure 4A. The antimicrobial activity of G_3 /SDS mixtures was dependent on SDS concentration. The MIC (minimal inhibitory concentration) value of G_3 was about 0.005 mM according to our previous study [9], and the *E. coli DH5 α* survival ratio with 0.0015 mM G_3 was about 80%. The antimicrobial activity of G_3 /SDS at low SDS concentration was slightly stronger than that of SDS alone, as SDS also has weak antimicrobial activity at this concentration range. However, the bacteria could be completely killed when SDS concentration was up to 1.7 mM. On the other hand, when the concentration of SDS was high enough, the antimicrobial activity of the G_3 /SDS mixture was similar to that of SDS alone, indicating that G_3 lost its own antimicrobial activity due to the strong interaction with SDS. When G_3 was at 0.005 mM, the antimicrobial activity of the G_3 /SDS mixture decreased first and then increased with the increase of SDS concentration. When the SDS concentration was below 0.035 mM, SDS molecules were bound to G_3 molecules through electrostatic attraction. Thus, the antimicrobial activity decreased with the increasing concentration of SDS. When the concentration of SDS was above 0.035 mM, G_3 molecules were completely bound by SDS and loss its activity almost entirely. Under these conditions, the antimicrobial activity was mainly contributed by SDS and increased with SDS concentration. The antimicrobial activity of 0.010 mM G_3 with SDS was similar to that of 0.005 mM G_3 /SDS mixture. In short, although both the surface tension and the G_3 conformation depended on the ratio of SDS to G_3 ,

there were no direct relationships between the surface tension and the antimicrobial activity. The G_3 antimicrobial activities were basically reduced by the electrostatic interactions with SDS. When G_3 interacts with the microbes, it usually adopts the α -helix conformation and disrupts the cell membranes to kill the microbes. But the G_3 molecules bounded tightly in SDS micelles would not have antibacterial activities although it also adopted the α -helix conformation.

Figure 4. Antimicrobial activity of G_3 /surfactant mixtures against *E. coli DH5a*. (A) G_3 /SDS, (B) G_3 /C₁₆TAB, (C) G_3 /C₁₂EO₂₃, (D) G_3 /C₁₄DMAO. The concentrations of G_3 were fixed at 0.0015, 0.005 and 0.01 mM for G_3 /SDS, G_3 /C₁₂EO₂₃ and G_3 /C₁₄DMAO mixtures, respectively. The concentration of G_3 for G_3 /C₁₆TAB mixture was fixed at 0.002 mM.

The antimicrobial activity of the G_3 /C₁₆TAB mixture is shown in Figure 4B. It is well-known that most of cationic surfactants can be used as antimicrobial agents at a

certain concentration. We have determined the MIC value of C₁₆TAB that was around 0.01 mM. The *E. coli DH5α* survival ratio was about 0.8 when the concentration of C₁₆TAB was 0.005 mM. However, the bacteria could be killed completely when it was mixed with 0.002 mM G₃. Thus, the G₃/C₁₆TAB mixture showed well synergistic antimicrobial activity at this condition.

The antimicrobial activity of the G₃/C₁₂EO₂₃ mixture is shown in Figure 4C. Nonionic surfactant C₁₂EO₂₃ did not show obvious antimicrobial activity even up to the concentration of 0.2 mM. The antimicrobial activity of C₁₂EO₂₃ with 0.0015 mM G₃ was similar to that of pure C₁₂EO₂₃, because G₃ concentration was much lower than its MIC. The survival ratio of C₁₂EO₂₃ with 0.0015 mM G₃ was even higher than C₁₂EO₂₃ alone at low concentration. It seemed abnormal. However, we have found that the EO chains could induced the aggregation of peptide molecules in our previous study [36]. In the C₁₂EO₂₃/G₃, G₃ might also induced the surfactants to form metastable premicelles below its CMC, and increased the apparent A₆₀₀ values through scattering. For C₁₂EO₂₃ with 0.005 mM G₃, when the C₁₂EO₂₃ concentration was lower than its cmc (0.057 mM), the antimicrobial activity of G₃/C₁₂EO₂₃ was the same as G₃. When the concentration of C₁₂EO₂₃ was above its cmc, however, the antimicrobial activity of the G₃/C₁₂EO₂₃ mixture decreased obviously because some G₃ molecules must be wrapped or dissolved in the C₁₂EO₂₃ micelles. For C₁₂EO₂₃ with 0.010 mM G₃, the effective antimicrobial activity concentration of G₃ was still greater than 0.005 mM, even if some of the molecules were deactivated by the C₁₂EO₂₃ micelles. Thus, the G₃/C₁₂EO₂₃ mixture still shows a high antimicrobial

activity.

The antimicrobial activity of the G_3/C_{14} DMAO mixture is shown in Figure 4D where the data indicate synergistic effect. The results were similar to that of G_3/C_{16} TAB mixture. In G_3/C_{14} DMAO mixtures, C_{14} DMAO was positively charged (similar to C_{16} TAB). Therefore, both G_3 and C_{14} DMAO could kill the bacteria and the mixture also showed good antimicrobial activity.

Figure 5. Antimicrobial activity of G_3 /surfactant mixtures against *S. aureus*. (A) G_3 /SDS, (B) G_3/C_{16} TAB, (C) G_3/C_{12} EO₂₃, (D) G_3/C_{14} DMAO. The concentrations of G_3 were fixed at 0.0015, 0.005 and 0.01 mM.

The antimicrobial activity of G_3 /surfactant mixtures against *S. aureus*, a gram positive microbe, was shown in Figure 5. The MIC value of G_3 against *S. aureus* was about 0.005 mM (Table S1). Although C_{12} EO₂₃ showed no antimicrobial activity and

SDS showed no antimicrobial activity at low concentration, the G_3 /SDS and G_3 / $C_{12}EO_{23}$ mixtures could kill *S. aureus* with the addition of G_3 . For G_3 /SDS, the activities against *S. aureus* were similar with those against *E. coli*. For G_3 / $C_{12}EO_{23}$, the G_3 activities hadn't been affected by the surfactant. And there were obvious synergistic effects in G_3 / $C_{16}TAB$ and G_3 / $C_{14}DMAO$ mixed systems.

Figure 6. Antimicrobial activity of G_3 /surfactant mixtures against *C. albicans*. (A) G_3 /SDS, (B) G_3 / $C_{16}TAB$, (C) G_3 / $C_{12}EO_{23}$, (D) G_3 / $C_{14}DMAO$. The concentrations of G_3 were fixed at 0.0015, 0.005 and 0.01 mM.

The antifungal activity of the G_3 /surfactant mixtures is shown in Figure 6. Although AMP G_3 has no activity to *C. albicans* (Figure S7) because of the different cell membrane structures of bacteria and fungi, mixtures of G_3 / $C_{16}TAB$ and G_3 / $C_{14}DMAO$ did show antifungal activity for the positive charge bearing property of $C_{16}TAB$ and

C_{14} DMAO. As the activity of G_3/C_{16} TAB mixture was higher than individual C_{16} TAB, synergistic effect might exist in G_3/C_{16} TAB mixed system (Figure 6B). For G_3/C_{14} DMAO, the antifungal activity of mixtures was consistent with that of individual C_{14} DMAO (Figure 6D). G_3/SDS and $G_3/C_{12}EO_{23}$ mixtures could not kill *C. albicans* (Figure 6A, 6C). The antifungal activity of G_3 /surfactant mixtures mainly derived from the cationic surfactants.

Figure 7. Cytotoxicity of G_3 /surfactant mixtures against NIH 3T3 cells. (A) G_3/SDS , (B) G_3/C_{16} TAB, (C) $G_3/C_{12}EO_{23}$, (D) G_3/C_{14} DMAO. The concentrations of G_3 were fixed at 0.0015, 0.005 and 0.01 mM.

The cytotoxicity of G_3 /surfactant mixtures has been assessed with NIH 3T3 and HSF cells and the results are shown in Figures 7 and 8. The IC_{50} values (concentrations to induce 50 % cell growth inhibition) of G_3 to NIH 3T3 and HSF

cells were more than 0.05 mM and 0.1 mM. In our previous work, we also showed that G₃ had inconsiderable impact to normal cells at the concentration below 0.05 mM [11]. Although G₃ had also no cytotoxicity (Figure S7) to these cells at the concentration as high as 0.01 mM, all four mixtures were cytotoxic to NIH 3T3 cells because surfactants themselves have certain cytotoxicity to cells. When G₃ was mixed with surfactants, the cytotoxicity to normal cells was enhanced unexpectedly. The surfactants might change the cell membrane structures through hydrophobic or electrostatic interactions, which made that the G₃ molecules interacted with the cells more easily.

Figure 8. Cytotoxicity of G₃/SDS mixture against HSF cells.

The G₃/SDS mixture was also cytotoxic to HSF cells when the concentration of SDS at a low level. And when the concentration of SDS was low (<0.52 mM), the cytotoxicity of G₃/SDS increased with the concentration of G₃ increased. It was indicated that the G₃/SDS complex was more toxic to cells than SDS itself. Because the SDS could change the cell membrane structures through hydrophobic interaction,

which made that the G₃ molecules interacted with the cells more easily. And thus, the cytotoxicity of G₃/SDS increased. The results also implied that it should be more careful in the sanitizer formulation development with antibacterial peptides, especially the surfactant concentration.

CONCLUSIONS

The interactions between G₃ and four typical surfactants have been investigated through the combination of surface tension measurement and CD spectroscopy. The effects of surfactants on G₃ antimicrobial activity and cytotoxicity of G₃/surfactant mixtures have also been assessed. Only a small amount of G₃ could cause a drastic decrease of surface tension of SDS solution, indicating strong cooperative interaction between them. The cmc values of G₃/SDS were lower than those of SDS, and the γ_{cmc} values were much lower as well, over the low SDS concentration range. The CD results showed that the secondary structure of G₃ changed from random coil, to β sheet, then to α -helix along with increasing SDS concentration, indicating a substantial transformation of structural conformations associated with different interactions. The surface activities of C₁₂EO₂₃, C₁₆TAB, and C₁₄DMAO did not change obviously after the addition of G₃, and the secondary structure of G₃ did not alter by these three surfactants either. Taken together, the strong interaction between G₃ and SDS system is driven by electrostatic attraction. The charge neutralization reduced the antimicrobial activities of G₃. The G₃ molecules bounded tightly in SDS micelles would not have antimicrobial activities although it also adopted the α -helix conformation. In contrast, the antimicrobial activities of G₃ were almost unaffected by

nonionic surfactant C₁₂EO₂₃ due to weak physical interaction between them. As for C₁₄DMAO, the antimicrobial activity of G₃ was increased by the zwitterionic surfactant because C₁₄DMAO displayed a weak cationic nature under the pH studied. And the synergistic antimicrobial effect was also found between G₃ and C₁₆TAB due to C₁₆TAB performed strong cationic and antimicrobial nature. Although G₃ was nontoxic to cells, all the G₃/surfactant mixtures showed cytotoxicity because the surfactants could interact with the cell membrane and change its structures through hydrophobic or electrostatic interactions, which made that the G₃ molecules interacted with the cells more easily. Understanding how antimicrobial AMP interacts with different types of surfactants is a crucial step towards developing hygiene and healthcare products where surfactants are readily used. Cationic antimicrobial peptide G₃ could be formulated with nonionic, zwitterionic and cationic surfactants, but careful attention must be paid when using with anionic surfactants.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Content: HPLC and MS spectra of G₃; isoelectric point of C₁₄DMAO; CD spectra of G₃ with SDS, CTAB, C₁₂EO₂₃, and C₁₄DMAO; Antimicrobial activity and cytotoxicity of G₃.

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Notes

There are no conflicts to declare.

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Supporting Information:

Effects of Conventional Surfactants on the Activity of Designed Antimicrobial Peptide

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Figures: 7

Table: 1

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Figure S1. (A) HPLC profiles and (B) mass spectra of the designed antimicrobial peptide $G_3(IKK)_3I-NH_2$.

Figure S2. Plot of isoelectric point of C₁₄DMAO.

Figure S3. CD spectra of different concentrations of G₃ with SDS. (A) 0.005 mM, (B) 0.01 mM, (C) 0.015 mM and (D) 0.025 mM.

Figure S4. CD spectra of different concentrations of G_3 with CTAB. (A) 0.005 mM, (B) 0.01 mM, (C) 0.025 mM and (D) 0.1 mM.

Figure S5. CD spectra of different concentrations of G_3 with $C_{12}EO_{23}$. (A) 0.005 mM, (B) 0.01 mM, (C) 0.025 mM and (D) 0.1 mM.

Figure S6. CD spectra of different concentrations of G₃ with C₁₄DMAO. (A) 0.005 mM, (B) 0.01 mM, (C) 0.025 mM and (D) 0.1 mM.

Figure S7. Antimicrobial activity (bacteria: *E. coli* DH5α, *S. aureus*, *C. albicans*) and cytotoxicity (cells: NIH 3T3 and HSF) of G₃.

Table S1 The MIC and IC₅₀ values of G₃ to microbes and cells

Microbe or Cells	<i>E. coli</i>	<i>S. aureus</i>	<i>C. albicans</i>	NIH 3T3	HSF
MIC or IC ₅₀ (mM)	0.005	0.005	/	> 0.05	> 0.1