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COMMUNICATION

Antimicrobial Peptide with the Aggregation-Induced Emission (AIE) Luminogen for Studying Bacterial Membrane Interactions and Antibacterial Actions†

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A fluorescence technique to investigate the interactions between bacterial membranes and an aggregation-induced emission (AIE) luminogen-decorated AMP (TPE-AMP) was reported. Our simple and fast method consists of mixing TPE-AMP and bacterial suspensions and recording the fluorescence signals by flow cytometry and confocal microscopy in a "non-washing" manner.

Antimicrobial peptides (AMPs), which form a key component of innate immune systems in animals, plants and many other organisms,[1-3] offer immediate and effective defenses against infections. They have been proposed and are being used as novel antimicrobial agents to treat infectious pathogens in the past decades, due to their broad-spectrum activities and infrequent bacterial resistance.[4,5] The largest group of AMPs are cationic (cationic AMPs), and are usually composed of 10-50 amino acids with at least two excess lysine and arginine residues and up to 50% hydrophobic amino acids.[6,7] Cationic AMPs always interact with anionic bacterial surfaces as part of their mechanisms of action, resulting in either disruption of membrane integrity (e.g., by membrane destabilization, pore formation, etc.) or translocation across the membrane to attack negatively-charged targets such as RNA.[8-10] Thus, the affinity of cationic AMPs for the bacterial membranes is integral to mechanisms of action of these peptides.

A variety of techniques have been employed to investigate the interactions between AMPs and bacterial membranes from biochemical, biophysical and structural aspects.[10,11] Lipid membranes/vesicles as model bacterial membranes have also been used to assess the interactions with AMPs.[12] The model predictions indicate AMPs capable of high bacterial membrane coverages could be effective antimicrobial agents, and the antibacterial activities of AMPs depend on their ability to bind to the bacterial membranes.[13,14] Therefore, it is important to obtain the information on interfacial enrichments of AMPs on the bacterial membranes. Despite previous efforts, there remains a strong demand for simple techniques for studying membrane interactions and antibacterial activities of AMPs.

Aggregation-induced emission (AIE) is an anomalous photophysical phenomenon, offering a new platform to monitor the light-emitting processes from the aggregation of non-emissive or weakly emissive luminogens, such as tetraphenylethene (TPE).[15-19] The innovative work of Tang *et al.* reported in 2001[20] have opened a new avenue for applications in sensors, chemotherapy, bioimaging and optoelectronic devices.[21-31] AIE phenomenon has shown great potential in bacterial research, and AIE luminogens-based materials have been used for bacterial detection, imaging and elimination.[32-36] Since the interactions between AMPs and bacterial membranes result in the surface enrichment of these peptides, the light-up characteristics of AIE luminogens upon aggregation inspire us to explore their applicability for studying the interactions between TPE-containing AMPs and bacterial membranes. As far as we know, the combination of AIE technique and AMPs to study the bacterial membrane interactions and antibacterial activities of AMPs has yet to be reported.

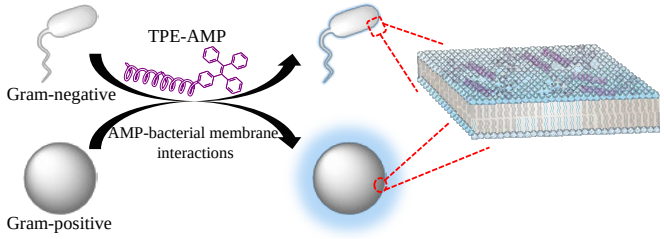

Fig. 1 Schematic illustration for the study of AMP interactions with bacterial membranes via the AIE technique.

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† Electronic Supplementary Information (ESI) available: Experimental section, MIC values, ^{1}H NMR, MALDI-TOF, fluorescence spectra of bacteria and MFI of bacteria. See DOI: 10.1039/x0xx00000x

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In this work, the TPE-containing AMP (TPE-AMP) was synthesized via thiol-ene conjugation between cysteine-terminated AMP (CysHHC10, Sequence: Cys-Lys-Arg-Trp-Trp-Lys-Trp-Ile-Arg-Trp-NH_2) and 4-(1,2,2-triphenylethenyl)benzenemethyl methacrylate (TPEMA, see Scheme S1 in ESI). The successful preparation of TPE-AMP was verified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS, Fig. S3, ESI) and HPLC. TPE-AMP is soluble in deionized water and phosphate-buffered saline (PBS, pH = 7.4). The UV-visible absorption spectrum of TEP-AMP in PBS solution (Fig. 2a) exhibits two absorption peaks at 289 and 280 nm. Owing to its good solubility, TPE-AMP emits weak fluorescence in PBS, with a quantum yield of 1.5% using quinine sulfate as the standard. TPE-AMP aggregates in water/ethanol mixture (1:4, v/v), showing an enhanced quantum yield of 4.7%.

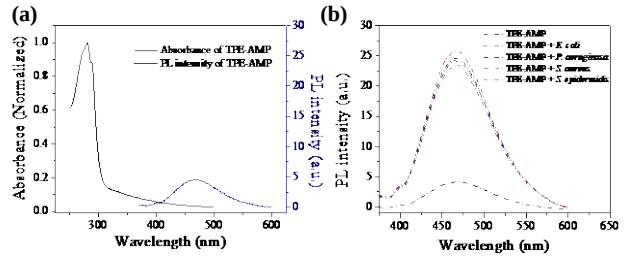

Fig. 2 (a) UV-visible absorption and PL spectra of TPE-AMP in PBS (pH = 7.4, 0.1 mg/mL) with an excitation wavelength of 350 nm; (b) PL spectra of TPE-AMP in the absence and presence of *E. coli*, *P. aeruginosa*, *S. aureus* and *S. epidermidis* in PBS (pH = 7.4) with an excitation wavelength of 350 nm.

CysHHC10 is a synthetic AMP, and exhibit strong antimicrobial properties against both gram-positive and gram-negative bacteria.[4,7,37] Upon conjugation with TPEMA, the resulting TPE-AMP also exhibits good antimicrobial properties. As shown in Table S1 (ESI), the minimum inhibitory concentration (MIC) values of TPE-AMP against Gram-negative *E. coli* and *P. aeruginosa*, as well as Gram-positive *S. aureus* and *S. epidermidis*, are 15.8, 31.8, 15.8 and 7.9 μM, respectively, as compared to the corresponding MIC values of 10.1, 20.2, 2.5 and 1.3 μM for CysHHC10. The increased hydrophobility by conjugation with TPEMA may alter the insertion of CysHHC10 into bacterial membranes, resulting in higher MIC values. After storing at 4 °C for more than 1 month, the PBS stock solution of TPE-AMP shows the same MIC values against these bacteria, indicating good stability of TPE-AMP in PBS. In general, TPE-AMP has a higher antibacterial efficacy against gram-positive bacteria than gram-negative bacteria. Since the affinity of AMP for the bacterial membranes is an critical factor in the antibacterial actions,[38] it is plausible to state that TPE-AMP has a higher affinity for the gram-positive bacterial membranes. This phenomenon is probably associated with the presence of arginine and tryptophan residues in TPE-AMP, favoring its insertion into gram-positive bacterial membranes.[39] The spread-plate method was also utilized to investigate the antibacterial efficacy of TPE-AMP. After mixing TPE-AMP with the bacterial suspensions, aliquots of samples were immediately spread onto the agar plates. The mobility of bacteria and TPE-AMP becomes restricted, once placed on the agar plates. The antibacterial action of TPE-AMP is thus mainly due to its initial uptake by the bacterial membranes. Fig. S4 (ESI) shows the photograph of agar plates spread with TPE-AMP-treated *E. coli*, *P. aeruginosa*, *S. aureus* and *S. epidermidis*. The agar plates are eventually covered by significantly larger amounts of gram-negative bacteria than gram-negative bacteria. The larger number of colonies of Gram-negative bacteria translates into poorer antibacterial efficacy of TPE-AMP against Gram-negative bacteria. These is a simple correlation between the spread plate assays and the MIC values, indicating these methods can be used to evaluate the antibacterial property of TPE-AMP. However, the spread plate and MIC assays are tedious and time-consuming to obtain the formation of bacterial colony or the growth of bacteria. The potential light-up characteristics of TPE-AMP in the presence of

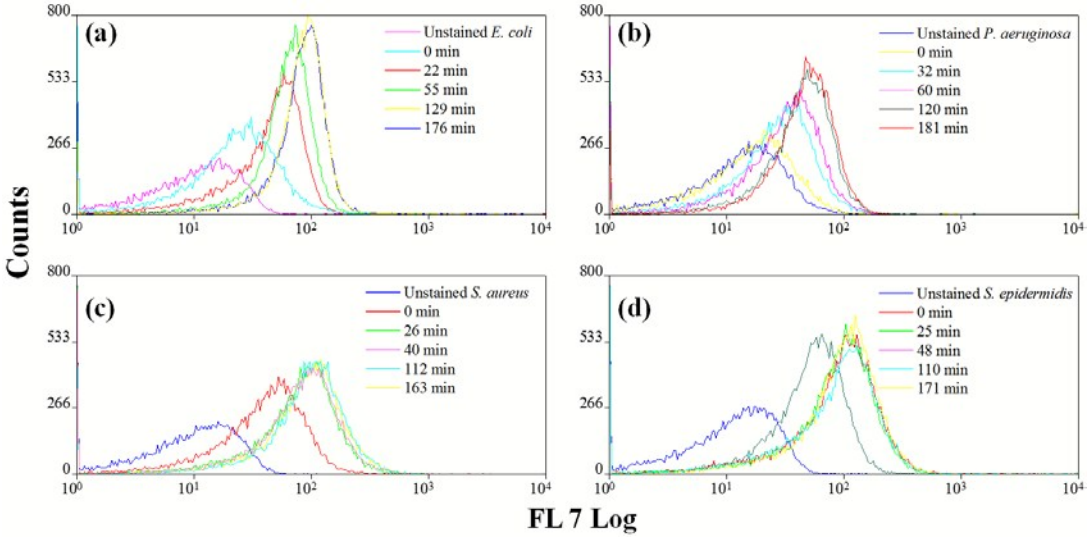

Fig. 3 Time-dependent flow cytometry analyses of (a) *E. coli*, (b) *P. aeruginosa*, (c) *S. aureus* and (d) *S. epidermidis* after incubation with TPE-AMP.

bacteria may provide an alternative approach to investigate its interactions with the bacterial membrane and its antibacterial activities via fluorescence techniques.

Fig. 2b shows the fluorescence responses of TPE-AMP towards *E. coli*, *P. aeruginosa*, *S. aureus* and *S. epidermidis*. The introduction of bacteria results in the enhanced blue fluorescence centered at about 467 nm. The bacteria show no fluorescence in PBS solution via excitation at 350 nm (Fig. S5, ESI). After passing through 0.22 μm syringe filter, the TPE-AMP and bacteria mixtures also exhibit negligible fluorescence (Fig. S6, ESI). These results suggest that the increase in fluorescence intensities of TPE-AMP are mainly due to its uptake by bacterial membranes and its AIE phenomenon on the membrane surfaces. Unfortunately, no correlation between fluorescence response at macroscopic level and MIC values (or affinity for the bacterial membrane) is observed. The secretion of extracellular polysaccharides by the bacteria in the presence of AMP[40] may cause the aggregation of TPE-AMP in PBS and interfere the fluorescence response at macroscopic level. Microscopic characterizations of the interactions between TPE-AMP and bacteria via flow cytometry and confocal microscopy were carried out.

To gain insight into the interactions at microscopic level, the bacteria treated with TPE-AMP were analyzed by flow cytometry (Fig. 3). After incubation with TPE-AMP, all of the fluorescence (Pacific Blue) peaks shift to the right compared to peaks for the bacteria alone. Fig. S7 (ESI) shows the evolution of the mean fluorescence intensity (MFI) in the flow cytometry histograms. The unstained bacteria show negligible MFIs, while they exhibit increased MFIs with the addition of TPE-AMP. The MFIs show an overall tendency to increase with the increase in incubation time, reach maximums and then decrease gradually. Gram-positive bacteria exhibit higher MFIs and faster membrane interactions than gram-negative bacteria. This phenomenon is correlated with the MIC values of TPE-AMP against these bacteria. The results suggest that flow cytometry together with the light-up characteristics of TPE-AMP can be used to evaluate TPE-AMP's affinity for the bacterial membranes and antimicrobial properties.

Confocal microscopy was then used to investigate the interactions between TPE-AMP and bacterial membranes. After incubation with TPE-AMP, the bacterial suspensions were immediately transferred to glass cover-slips without washing. Fig. 4 shows the confocal laser scanning microscope (CLSM) images of bacteria treated with TPE-AMP. In comparison to the bright-field images, blue fluorescence can be observed from the fluorescence images of Gram-positive bacteria, while weak blue emission is visible on the fluorescence images of Gram-negative bacteria. The weakly fluorescent Gram-negative bacteria imply that the affinity of TPE-AMP for Gram-negative bacterial membranes is weaker than that for Gram-positive bacterial membranes. This finding is consistent with that observed from flow cytometry, and correlates with MIC and spread plate assays. Thus, the flow cytometry and confocal microscopy can provide alternative approaches for the study of bacterial membranes interactions and antibacterial actions of TPE-AMP.

Although several AMPs have demonstrated efficacy in phase II/III clinical trials,[2] *in vivo* toxicity remains a primary concern for the clinical applications of AMPs.[41] The *in vivo* toxicity of CysHHC10 and TPE-AMP was assessed using five-week-old ICR mice (n = 5) after single-dose intravenous administration through the tail vein at a dose of 5 mg kg^{-1}. The mice show their normal behavior, and no mouse becomes moribund or died after 5 days of examination. The mice were then sacrificed. Heart, liver, femoral muscle, lung and kidney were examined histopathologically. Fig. 5 show the representative histological sections of tissues stained with haematoxylin and eosin (H&E). In comparison to the control, CysHHC10 and TPE-AMP show no or negligible toxic side effects on the regular anatomical structures of the organs, reflecting their safe use as a potential therapeutic.

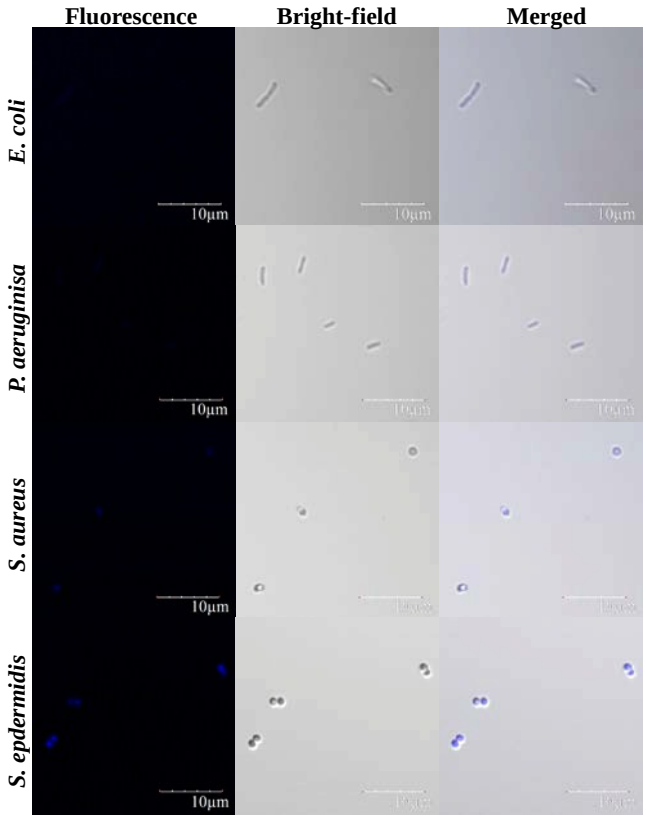

Fig. 4 CLSM images of bacteria (*E. coli*, *P. aeruginosa*, *S. aureus* and *S. epidermidis*) treated with TPE-AMP.

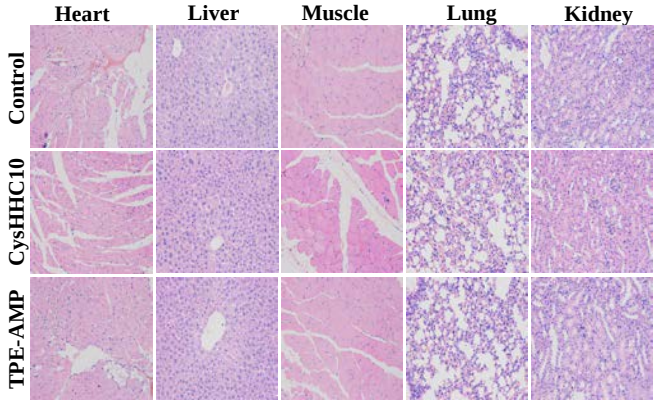

Fig. 5 Histological examination of organs of mice treated with CysHHC10 and TEP-AMP (5 mg/kg).

In summary, a simple strategy has been presented to study the bacterial membrane interactions and antibacterial actions of AMPs. TPE-AMP emits weak fluorescence in aqueous media. Upon the addition of bacteria, its light-up characteristic could be utilized to visualize the affinity of TPE-AMP for the bacterial membranes at microscopic level. Stronger fluorescence signals are detectable in Gram-positive bacteria by flow cytometry and confocal microscopy, which are correlated with the higher antibacterial activities of TPE-AMP against Gram-positive bacteria. This study provides an important approach to the prediction of bacterial membrane interactions and antibacterial activities of AMP via the fluorescence techniques. In comparison to traditional MIC and spread plate assays, the AIE-aided fluorescence investigation at microscopic level provides a simple and quick approach to the study of AMPs' affinity for the bacterial membranes and their concomitant antibacterial actions. Also, *in vivo* toxicity tests show TPE-AMP exhibits no or negligible toxicity. TPE-AMP could be further developed into a fluorescent probe to visualize the *in vivo* localization of AMPs in the infected tissues or normal organs of an animal infection model. Since the excitation wavelengths of TPE-AMP are in the UV range and have poor tissue penetrations, further development of near-infrared emissive AIE luminogens-containing AMPs will benefit the study of *in vivo* localization of AMPs.

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