Supporting Information

A Strategy for Enhanced Antibacterial Activity against *Staphylococus aureus* by Assembly of Alamethicin with Thermo-Sensitive Polymeric Carrier

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1. General

Materials and reagents. Fluorescein isothiocyanate (FITC, 90%), SBMA (97%), and 3-(4, 5dimethylthiazol-2-yl)-2, 5-diphenyltetra-zoliumbromide (MTT, 98%) were obtained from Sigma-Aldrich (Shanghai, China). Alamethicin (95%) and Rhodamine B (95%) were obtained from *J&K* Chemical (Shanghai, China). Pepsin and trypsin were purchased from Kechuang Biotech Co. (Suzhou, China). Fetal bovine serum (FBS) and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Hyclone (Logan, Utah). AEM was synthesized according to a modified literature method (Scheme S1, Figure S1 and S2, Supporting Information).¹ 2,2'-Azoisobutyronitrile (AIBN, Sinopharm Chemical Reagent Co., Ltd., CP) was recrystallized from methanol and dried in a vacuum oven at room temperature. Human dermal fibroblasts (HDF) were obtained from the Hygia Biotech Co. (Suzhou, China). *Staphylococus aureus* ATCC 25923 was acquired from American Type Culture Collection (ATCC; Rockville, MD, USA). All other chemical agents were used as received.

Characterization methods. ¹H nuclear magnetic resonance (¹H NMR) spectra were recorded on a Varian INVOA-400 instrument working at 400 MHz. The molecular weights and polydispersities of the copolymers were determined with a Waters 1515 gel permeation chromatograph (GPC) equipped with a temperature and differential refractometer control, using PL aquagel-OH MIXED-M columns with PEO standard samples and aqueous solution (0.2 M NaNO₃, 0.1 M NaH₂PO₄, 30 % (v/v) CH₃OH) as the eluent at the flow rate of 0.5 mL/min. Fourier transform infrared (FT-IR) spectrum was recorded using KBr pellets by Varian-1000 spectrometer. The Z-average size distribution of the copolymer was measured by a Malvern Instruments Zetasizer Nano with irradiation from a 632.8 nm He-Ne laser. Field-emitting scanning electron microscopy (SEM) observations were obtained on a Hitachi S-4700 microscope operated at an accelerating voltage of 15 kV. Confocal microscopy pictures were obtained on an UltraView VoX Confocal Microscope, while nanomaterial and alamethicin were fluorescently labeled with activated rhodamine B-NHS and FITC respectively.² The circular dichroism (CD) spectra were obtained on an AVIV Model 420 circular dichroism spectrometer (Aviv Inc., USA), and scans were between 190 and 260 nm with 2 nm acquisition steps. Synergy 2 microplate reader (BioTek, USA)

read the absorbance at 590 nm in MTT. The optical density (600 nm) was recorded using UV-Vis spectrophotometer.

2. Synthesis of 2-(adenine-9-yl) ethanol methacrylate

2-(adenine-9-yl) ethanol methacrylate (AEM) was synthesized according to a modified literature method³ as shown in Scheme S1. A typical process was depicted as below: 2-bromoethanol (2.0 g, 16 mmol) was dissolved in 10 mL CH₂Cl₂ (DCM) in ice-bath. Then triethylamine (Et₃N) and methacryloyl chloride were added under nitrogen atmosphere. The reaction was allowed to proceed at room temperature for 18 h. Afterwards, the mixture was filtered to remove salt and the filtrate was washed by ice water. Then filtrate was rotary evaporated after dried by MgSO₄ and vacuum dried to get 2-bromoethanol methacrylate (Figure S6). Then adenine (1.0 g, 7.41 mmol), NaH (0.2 g, 8.33 mmol) were added into 20 mL dry dimethyl formamide (DMF) and stirred for 2 h at room temperature. Afterwards, 2-bromoethanol methacrylate (2.4 g, 12.44 mmol) was added into the system. The reaction was allowed to proceed at room temperature for 12 h. Then, the mixture was filtered to remove NaCl. The filtrate was precipitated by acetone for three times. The product was vacuum dried at room temperature. Figure S7 depicted the ¹H NMR spectra of 2-(adenine-9-yl) ethanol methacrylate.

3. Synthesis of poly(AEM-co-SBMA)

A typical method is described below¹: the solution comprising AEM (0.35 mmol), SBMA (0.35 mmol), AIBN (0.0055 g) and DMSO (4.0 mL) was prepared and placed in an ampoule. The contents were purged with argon for 20 min to eliminate the dissolved oxygen, and then the ampoules were flame-sealed and placed in oil bath thermostat at 70 °C for three days. Each ampoule was cold with ice water and opened, and the mixture was diluted with DMSO (10 mL) and then precipitated in acetone (100 mL) for three times. The obtained polymer was dried in vacuum at 40 °C until a constant weight was obtained gravimetrically. The copolymer was characterized by ¹H NMR and FT-IR spectra.

Three samples of copolymers with different molar ratio of SBMA and AEM were synthesized by changing the amount of AEM (0.35, 0.175 and 0.117 mmol).

4. Assembly of poly(AEM-co-SBMA) and alamethicin

Poly(AEM-co-SBMA) (1.0 mg) was dissolved in hot water (10 mL), and then dialyzed against

distilled water with a membrane (MWCO = 3,500 Da) for 24 h to obtain the copolymer aggregation.

The assembly complex was obtained as follows: poly(AEM-*co*-SBMA) (1.0 mg) was dissolved in distilled water (10 mL) when heating, and 3 mL of the solution was mixed with alamethicin in ethanol (1 mg/mL, 150 μ L) at room temperature. The mixture solution dialyzed against distilled water overnight with a membrane (MWCO = 1,000 Da). Then it was placed into another dialysis bag (MWCO = 3,500 Da) to remove unpackaged alamethicin. In order to ensure that free alamethicin had been removed completely by dialysis bag (MWCO = 3,500 Da), the model experiment with flurorescently labeled alamethicin was carried out. The assembly complex was investigated by using SEM, size distribution, CD and confocal microscopy spectra.

Three samples of alamethicin/poly(AEM-*co*-SBMA) were obtained by changing the concentrations of alamethicin in ethanol (0.03, 0.06, 0.15 mg/mL; 1.0 mL). The entrapment efficiency was estimated according to the related model experiment.

5. Model experiment of alamethicin entrapment

It is difficult to measure concentration of alamethicin in solution due to there is no characteristic spectrum of alamethicin. Therefore, in order to evaluate the entrapment efficiency of alamethicin by poly(AEM-*co*-SBMA), a model experiment was carried out with flurorescently labeled alamethicin⁴ as follows: alamethicin (5.0 mg) was dissolved in ethanol (1.0 mL), and solution pH value was adjusted to pH = 9. Then, FITC (5.0 mg) was added and the solution was stirred for 4 h at room temperature. Afterwards, the solution dialyzed against ethanol with a dialysis bag (MWCO = 1,000 Da) to remove free FITC. Therefore, FITC-labeled alamethicin was obtained.

FITC-labeled alamethicin (5 mg/mL, 1.0 mL) was slowly added into poly(AEM-*co*-SBMA) aqueous solution (2 mg/mL, 5 mL) under strong stirring. The mixture solution dialyzed against distilled water overnight with a membrane (MWCO = 1,000 Da). Then the suspension was placed into another dialysis bag (MWCO = 3,500 Da) to remove unpackaged alamethicin. In the process, absorbance of the suspension was recorded by UV-Vis spectrophotometer at 490 nm. The result was shown in Figure S8, which manifested that free alamethicin could be removed completely after dialyzing for 24 h.

On the other hand, the concentration of FITC-labeled alamethicin in ethanol was determined

according to the standard curve (Figure S9) by UV-Vis spectrophotometer at 490 nm. On the basis of the calculation of absorbance of alamethicin in assembly complex (A_1) and alamethicin in ethanol (A_0), alamethicin entrapment efficiency (EE) was estimated as EE = $A_1/A_0 \times 100\%$.

6. Cell toxicity of assembly complex

The MTT assay is an effective method to determine cellular viability in vitro.⁵ The HDF cells were maintained in DMEM supplemented with 10% FBS at 37 °C. And then, the cells were cultured into 96-well pates at a density of 5×10^3 cells and were incubated overnight under a humid, 5% CO₂ atmosphere for MTT assay. The media was removed from the wells and replaced with 200 µL media containing alamethicin (2-30 µM) or assembly complex equivalent. Concentrations of assembly complex were determined based on alamethicin concentrations. Empty polymeric carrier (equal to assembly complex) was used to account for any effect of copolymers on cell viability. Untreated cells containing media were used as control.

After incubation for 24 h, the wells were washed once with PBS and incubated with MTT dye (0.5 mg/mL of MTT dye in DMEM) in the dark for 4 h at 37 °C and then DMSO (150 μ L) was added to each well. The absorbance at 590 nm was recorded by a spectrophotometer. Subtract blank absorbance values (wells containing media, MTT dye and DMSO) from each value and express cellular viability as a percentage.

7. Enzymatic stability assessment of assembly complex

The enzymatic stability assessment of assembly complex was performed according to the literature⁶: aliquots of alamethicin and assembly complex solutions in PBS (pH = 7.4) were processed with different enzymes (pepsin and trypsin) at 37 °C for 30 min, respectively. Afterwards, treated solutions were added to *Staphylococus aureus* bacterial media at 37 °C for 24 h. The reaction systems untreated with alamethicin and assembly complex were performed in parallel as negative control. The more stable alamethicin would lead to the better inhibitory ratio of bacteria. The bacterial suspension concentration is proportional to optical density (OD, λ = 600 nm) within a certain range in UV-Vis measurement. Therefore, enzymatic stability was assessed according to residual alamethicin activity using the formula below:

Inhibition ratio (%) = $(A_1-A)/A_1 * 100\%$

where A and A_1 are the absorbance at $OD_{600 nm}$ after treatment with enzyme and the negative control, respectively.

8. Antibacterial activity of assembly complex

Investigation of the minimum inhibitory concentrations (MICs) and the minimum bactericidal concentrations (MBCs). Broth dilution method was used to determine the MICs and MBCs.⁷⁻⁸ Briefly, the reference strain *Staphylococus aureus* was exposed to different dilutions of free alamethicin and assembly complex with a starting alamethicin concentration of 256 µM at 37 °C. Besides, *Staphylococus aureus* was exposed to empty polymeric carrier, and the simple mixture of alamethicin and polymer carrier (equal to assembly complex) in parallel, respectively. Broth medium and untreated bacterial cultures were used as negative and positive controls, respectively. MIC is defined as the lowest drug concentration required for preventing the appearance of a visible growth after overnight incubation, while the MBC is the lowest drug concentration required for killing 99.9 % of selected microorganisms.⁹

Bacterial growth curve of assembly complex. The bacterial growth curve is divided into four parts: slow period, logarithmic period, plateau period and decline period. *Staphylococus aureus* in logarithmic phase was diluted into 10^6 CFU/mL using broth medium, and then assembly complex was added to make final concentration of solutions as 1, 2 and 3 times the MIC. Untreated *Staphylococus aureus* and broth medium were used as control. *Staphylococus aureus* was cultured in a thermostat at 37 °C with an oscillation 150 r/min for 24 h.¹⁰ The bacterial growth was indicated by recording the optical density (OD_{600 nm}) on a UV-Vis spectrophotometer every 2 h.

9. Release of FITC-labeled alamethicin from the assembly complex

In order to investigate the release of alamethicin from assembly complex, flurorescently labeled alamethicin was also used in the model experiments. The release of FITC-labeled alamethicin from the assembly complex was determined by UV-Vis spectrophotometer after 0, 0.5, 1, 2, 3, 4, 5, 5.5 and 6 h incubation (Figure S9). Assembly complex (EE: 28.81 ± 4.09 %) was placed into a dialysis bag (MWCO = 3,500 Da). The dialysis bag was then immersed in 200 mL of PBS buffer (pH = 7.4) at 25 °C and 37 °C with a stirrer agitating continuously, respectively. The absorbance of assembly complex was

measured by UV-Vis spectrophotometer at 490 nm, and the alamethicin retention was calculated on the assumption that all released alamethicin went into PBS buffer.

10. Data analysis

The presented data are as mean \pm S.E.M. of three independent experiments. Comparisons between individual groups were made by paired Student's *t*-test, and *p* < 0.01 was considered as the level of significance.



Figure S1. FT-IR spectra of $poly(AEM_n-co-SBMA_m)$: (a) $poly(AEM_{0.32}-co-SBMA_{0.68})$; (b) $poly(AEM_{0.23}-co-SBMA_{0.77})$; (c) $poly(AEM_{0.18}-co-SBMA_{0.82})$. (The characteristic peaks at 3448 and 1729 cm⁻¹ were corresponding to $-NH_2$ and -C=N for AEM, and 1484, 1181 and 1039 cm⁻¹ for $-N(CH_3)_2^-$, $-SO_3^-$ and -S-O stretching vibrations of SBMA, respectively).



Figure S2. SEM images (scale bar: 3 µm) and particle size distributions of poly(AEM-*co*-SBMA) in distilled water (~ 25 °C): (A, D) poly(AEM_{0.32}-*co*-SBMA_{0.68}); (B, E) poly(AEM_{0.23}-*co*-SBMA_{0.77}); (C, F) poly(AEM_{0.18}-*co*-SBMA_{0.82}). (Elliptic morphologies were presented, and the size of copolymer aggregations increased from 395 nm to 869 nm with increasing hydrophilic PSBMA molar fraction in copolymer chain (Figure S4, Supporting Information), which may be related with the ability of adenine groups to form self-complementarity hydrogen-bonding and propensity for aromatic π - π stacking.¹¹)



Figure S3. Confocal microscopy pictures of alamethicin (A, labeled with FITC), copolymer poly(AEM_{0.32}-*co*-SBMA_{0.68}) (B, labeled with rhodamine B-NHS), and their coexistence (C).



Figure S4. CD spectra of alamethicin/poly(AEM_{0.32}-*co*-SBMA_{0.68}) solutions: (a) alamethicin/ poly(AEM_{0.32}-*co*-SBMA_{0.68}) (w/w) = 1/10; (b) alamethicin/poly(AEM_{0.32}-*co*-SBMA_{0.68}) (w/w) = 1/5; (c) alamethicin/poly(AEM_{0.32}-*co*-SBMA_{0.68}) (w/w) = 1/2. (In general, alamethicin remains helical conformation in liposome assemblies while displaying a random conformation in aqueous solutions.¹²⁻¹³ In this study, the data illustrated that alamethicin in assembly complex displayed a distinct doublenegative-peak at 208 and 222 nm, deriving from that blank CD spectrum of empty polymeric carrier was excluded from the matching spectra of assembly complex.¹⁴⁻¹⁵ The minima at 208 nm and 222 nm increased their intensities with increasing amounts of alamethicin, suggesting that more alamethicin were assembled into poly(AEM-*co*-SBMA) copolymers, and exhibited alpha helical structure.¹⁶)



Figure S5. Release of alamethicin from polymeric carrier $poly(AEM_{0.32}$ -*co*-SBMA_{0.68}) in PBS at 37 °C (a) and 25 °C (b) (mean \pm SD, n = 3).



Scheme S1. Synthesis route of 2-(adenine-9-yl) ethanol methacrylate.



Figure S6. ¹H NMR spectra (400MHz, CDCl₃) of 2-bromoethyl methacrylate.



Figure S7. ¹H NMR spectra (D₂O, 400 MHz) of 2-(adenine-9-yl) ethanol methacrylate.



Figure S8. The absorbance of assembly suspension in dialysis bag (MWCO = 3,500 Da) in the process of removing unpackaged alamethicin.



Figure S9. (A) The absorbance and (B) the standard curve of alamethic in ethanol ($\lambda_{max} = 490$ nm).

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