Supporting Information

Encapsulated DNase Improving the Killing Efficiency of Antibiotics in Staphylococcal Biofilms

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1. Materials

N-(3-aminopropyl)-methacrylamine hydrochloride (APM), 2-methacryloyloxyethyl phosphorylcholine (MPC) and glycerol dimethacrylate (GDMA) were purchased from Heowns Co., Ltd (Tianjin, China). Ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) were purchased from Aladdin Biotech Co., Ltd (Shanghai, China). Bovine serum albumin (BSA) was purchased from Gibco, Co., Ltd. Deoxyribonuelease I (DNase), DNA sodium salt and 6× DNA loading buffer from calf thymus were purchased from Beijing Solarbio Science & Technology Co., Ltd (Beijing, China). Concanavalin A from canavalia ensiformis (Jack bean) (FITC-conA), Au crystals and size exclusion chromography (Sepharose-6B) were purchased from Sigma-Aldrich. Cy5-NHS was purchased from Beijing Oukainasi Tech Inc. Ciprofloxacin was purchased from Bailingwei Technology Co., Ltd (Beijing, China). Tryptone soy broth (TSB) was purchased from OXOID (Basingstoke, U.K.). Bacterial strains *S. aureus* ATCC12600^{GFP} and *S. aureus* ATCC 43300 were purchased from PerkinElmer Inc. (Waltham, MA, USA). Traut's Reagent, Tris (2-carboxyethyl) phosphine (TCEP) and crystal violet were purchased from J&K Company. Ethyl alcohol, dimethyl sulfoxide (DMSO), acetic acid, $CaCl_2{\cdot}2H_2O$, $MgCl_2.6H_2O$ and other chemical reagents were purchased from local chemical reagent companies.

2. Instruments

UV-Visible spectra assays were acquired with NanoDrop One^c (Thermo Scientific, USA). Dynamic light scattering (DLS) and zeta potential experiments were performed on a ZETAPALS/BI-200SM (Brookheaven Instrument, USA). Transmission electron microscopy (TEM) measurements were performed on a commercial Talos F200C electron microscope at an acceleration voltage of 120 kV. Ultraviolet absorption experiments were measured on a Spark plate reader (TECAN, Switzerland). Quartz crystal microbalance with dissipation monitoring (QCM-D) measurements were conducted using a Q-Sence E4 system (Västra Frölunda, Sweden). CLSM images were captured on TCS SP8 confocal laser scanning microscopes (Leica, Germany). Fluorescence images were captured on IVIS lumina II imaged system (Xenogen, USA). SEM images were captured on scanning electron microscope (JSM-7500F, Japan).

3. Synthesis

3.1 Synthesis of n(DNase)

The n(DNase) was synthesized using the technical method reported previously.[1, 2] Briefly, N-(3-aminopropyl) methacrylamide (APM), 2-methacryloyloxyethyl phosphorylcholine (MPC) and APS were dissolved in di-water to obtain the stock solutions (w/v, 10%), respectively. The glycerol dimethacrylate (GDMA) stock solutions (w/v, 10%) were prepared by dissolving the GDMA in anhydrous DMSO. Deoxyribonuelease I (DNase) was dissolved in phosphate buffer (10 mM, pH 7.4) to obtain 1 mg/mL stock solutions. The molar ratio of APM/MPC/GDMA/APS/DNase was 3000:3000:300:360:1, while the mass ratio of APS/TEMED was kept at 1:2. Polymerization was initiated by the addition of TEMED and APS and kept at 4 °C for 1.5 h. After reaction, the n(DNase) solutions were purified to remove the unreacted monomer by dialyzed against phosphate buffer (10 mM, pH 7.4) with a dialysis tube (MWCO = 20 kDa, Sigma-Aldrich). Then, the n(DNase) was further purified to remove the DNase by size exclusion chromography (Sepharose-6B) and stored at 4 °C for further use.

3.2 Synthesis of n(BSA)

The n(BSA) was synthesized using the technical method similar to that synthesis of n(DNase). Briefly, bovine serum albumin (BSA) was dissolved in phosphate buffer (10 mM, pH 7.4) to obtain 1 mg/mL stock solutions. The molar ratio of APM/MPC/GDMA/APS/BSA was 8000:8000:800:960:1, and the mass ratio of APS/TEMED was kept at 2:1. Polymerization was initiated by the addition of TEMED and APS and kept at 4 °C for 1.5 h. After reaction, the n(BSA) solutions were purified to remove the unreacted monomer by dialyzed against phosphate buffer (10 mM, pH 7.4) with a dialysis tube (MWCO = 20 kDa, Sigma-Aldrich). Then, the n(BSA) were further purified to remove the BSA by Sepharose-6B and stored at 4 °C for further use. As shown in **Supporting Information Figure S3**, the DLS and zeta potential results of n(BSA) were 32.1 ± 3.6 nm and +11.2 mV, respectively. These results indicated that the n(BSA) was similar to the n(DNase) in physicochemical properties.

3.3 Synthesis of Cy5-labeled n(DNase)

Cy5 was dissolved in DMSO to obtain 5 mg/mL stock solutions. Cy5 stock solutions (20 μL, 5 mg/mL) were slowly added into n(DNase) solutions (2 mL, 1 mg/mL) stirring for 4 h at 4 °C. After reaction, the Cy5-labeled n(DNase) solutions were dialyzed against phosphate buffer (10 mM,

pH 7.4) to remove the unreacted Cy5 and stored at 4 °C for further use. The DNase was labeled with Cy5 using the same method.

4. Characterization of n(DNase)

4.1 Zeta potential and DLS measurements

Zeta potential measurements were performed using ZETA PALS/BI-200SM. The DLS measurements were performed on a laser-light-scattering spectrometer (BI-200SM) equipped with a digital correlator (BI-9000AT) at 635 nm under 90° scattering angle. The n(DNase) was diluted with phosphate buffer (10 mM, pH 5.0) to 0.2 mg/mL.

4.2 TEM measurements

The morphology of DNase and n(DNase) was analyzed by transmission electron microscope (TEM) (Talos F200C, Czech Republic, USA). The TEM samples were prepared by dropping samples solutions (2 μL, 1 mg/mL) onto a copper grid coated with carbon for 2 min, blotting with filter paper to remove excess liquid, staining with 1% sodium phosphotungstate (10 μL) for 2 min and drying in vacuum before TEM observation.

4.3 EDS measurements

The n(DNase) was dissolved in di-water at the concentration of 1 mg/mL, and dropped on the copper grid coated with carbon. The procedure of sample preparation was similar to that for TEM measurements. The images were captured by TEM equipped with energy dispersive spectrometer (EDS, JEM-2800, Japan).

5. Activity assays of n(DNase)

5.1 Ultraviolet measurements

The enzymatic activity of n(DNase) was evaluated by monitoring the elevated absorbance at 260 nm within 1 min at 37 °C. Briefly, the calf thymus DNA was dissolved in Tris-HCl (10 mM, pH 7.4) for overnight under static condition. The n(DNase) was hydrolyzed in acetate buffer (10 mM, pH 5.0) at 37 °C for different time. 25 mM $MgCl_2$ and 5 mM $CaCl_2$ were simultaneous dissolved in acetate buffer (10 mM, pH 5.0) to obtain the acetate buffer working solutions. After hydrolysis, the n(DNase) was diluted with acetate buffer working solutions to 0.01 mg/mL. The elevated absorbance at 260 nm within 1 min of the mixture solutions containing n(DNase) (400 μL, 0.01 mg/mL) and DNA (40 μL, 0.25 mg/mL) was monitored at 37 °C by Nanodrop One^c (Thermo Scientific, USA). The enzymatic activity of DNase was assessed under the same condition (denoted

as 100%). Briefly, DNase was dissolved in the acetate buffer working solutions at the concentration of 0.01 mg/mL, then DNase (400 μL, 0.01 mg/mL) and DNA (40 μL, 0.25 mg/mL) was monitored at 37 °C by Nanodrop One^{c} (Thermo Scientific, USA). The nuclease activity of n(DNase) was calculated by the formula:

$$\text{Nuclease activity of n(DNase)} = \frac{A_{260}[\text{n(DNase)}]_{1\text{min}} - A_{260}[\text{n(DNase)}]_{0}}{A_{260}(\text{DNase})_{1\text{min}} - A_{260}(\text{DNase})_{0}} \times 100$$

$A_{260}[\text{n(DNase)}]_{1\text{min}}$ and $A_{260}(\text{DNase})_{1\text{min}}$ represent the absorbance of n(DNase) and DNase at 260 nm at the end time, respectively. While $A_{260}[\text{n(DNase)}]_{0}$ and $A_{260}(\text{DNase})_{0}$ represent the absorbance of n(DNase) and DNase at 260nm at the initial time, respectively.

5.2 Agarose gel electrophoresis

The n(DNase) was hydrolyzed in acetate buffer (10 mM, pH 5.0) for 4 h at 37 °C, and then diluted with acetate buffer working solutions to 1 μg/mL. Next, calf thymus DNA (20 μL, 0.25 mg/mL) and n(DNase) (20 μL, 1 μg/mL) were mixed for 30 min at 37 °C under static conditions. The DNA was treated with DNase and n(BSA) at the same condition, respectively. The electrophoresis was performed using 1% agarose gel. The images were taken directly after running the gels under UV light using a digital camera. The agarose gel electrophoresis image was shown in **Supporting Information Figure S1.**

6. Stability evaluation of n(DNase)

The n(DNase) were incubated at 37 ºC over different time spans (i.e. 0 h, 24 h, 48 h, 72 h, 96 h and 120 h) to assess the size of n(DNase) via DLS. The procedures of DLS measurement was same to those in Section 4.1.

The enzyme activity of n(DNase) was also measured after lyophilization. 10 mL of n(DNase) suspension (1 mg/mL) were lyophilized for 24 h using a vacuum pump accompanied by a vapor condenser (- 72 ºC). After the lyophilization, 10 ml of PB buffer (pH 5.0, 10 mM) was added to the n(DNase) powder to resuspend the n(DNase) in a vial. The vial was then incubated at 37 ºC for 4 hours to degrade the polymer coating of n(DNase) and recover the DNA cleavage activity. The enzyme activity of n(DNase) was determined following the same procedure as described in Section 5.1.

To evaluate the thermal stability, n(DNase) (1 mg/mL) was incubated at 65 ºC for 1 hour. After the incubation, the heat was removed and the n(DNase) solution was allowed to restore to room

temperature for activity measurement. The enzyme activity of n(DNase) was determined following the same procedure as described in Section 5.1.

7. Culturing and harvesting of bacterial strains

In this study, *S. aureus* ATCC12600^{GFP} and *S. aureus* ATCC 43300 (PerkinElmer Inc., Waltham, MA, USA) were employed. The two strains were cultured and harvested according to the previous protocol and suspended in PBS to the concentrations required in the respective experiments.[3] The concentration of bacterial suspensions was determined by OD_{600} via Nanodrop One^{c} (Thermo Scientific, USA).

8. The formation of biofilm

The bacterial suspensions (1 mL, OD_{600} = 0.1) were adhered on sterile cell culture dishes for 2 h at 37 °C under static condition. After washing three times with PBS, the adherent bacteria was grown in TSB medium (2 mL) for 24 h at 37 °C under static. The TSB medium was replaced with fresh TSB (2 mL) and incubated for another 24 h. Finally, 48 h old biofilms were obtained through washing three times with PBS. 100 μL and 500 μL of bacterial suspensions (OD_{600} = 0.1) were adhered on 96-well plates and 48-well plates, respectively. The following incubation procedures were completely similar to the above described method. Biofilm in the text represents 48 h old biofilm without otherwise noted.

9. Assess accumulation of n(DNase) in biofilm

9.1 CLSM measurements

To observe the distribution of n(DNase) in biofilm, FITC-conA labeled *S. aureus* ATCC43300 biofilms were exposed to the Cy5-labeled n(DNase) solutions. Briefly, biofilms were formed in cell culture dishes by the method as previously description. Next, the biofilms were fixed with glutaraldehy (v/v, 4% in PBS) for 4 h at 4 °C, and subsequently stained with FITC-conA solutions (2 mL, 0.01 mg/mL) for 20 min at 4 °C in darkness. The FITC-conA was dissolved in PBS. The FITC-conA labeled biofilms were exposed to Cy5-labeded n(DNase) solutions (2 mL, 0.2 mg/mL) for 30 min in darkness at 37 °C, followed by washing three times with PBS to completely remove n(DNase) that did not penetrate and retain in the biofilms. CLSM (Leica, German) was used to study the distribution of n(DNase) into the biofilm. FITC-conA and Cy5 were excited with 488 nm argon ion laser and 633 nm red HeNe laser, respectively. All data were acquired and analyzed using Leica software.

9.2 IVIS lumina measurements

To assess the accumulation of n(DNase) in biofilm, *S. aureus* ATCC43300 biofilms were formed in 48-well plates. The biofilms were exposed to Cy5-labeled n(DNase) solutions (1 mL, 0.2 mg/mL) for 30 min at 37 °C in darkness and then washed different times with PBS, respectively. Fluorescence images were captured by IVIS lumina II imaged system (Xenogen, USA).

10. Assessment of binding affinity between EPS and n(DNase)

To evaluate the binding affinity between EPS and n(DNase), quartz crystal microbalance with dissipation monitoring (QCM-D) measurements were performed. QCM-D measurements were conducted using a Q-Sence E4 system (Västra Frölunda, Sweden). EPS solutions (10 mg/mL) were obtained as following steps. Biofilms were scraped and dissolved in PBS, centrifuged at 10000 rpm for 60 min and lyophilized overnight to obtain solid power of EPS. Then, the EPS was dissolved in PBS (10 mM, pH 5.0) to obtain 10 mg/mL EPS solutions. The n(DNase) was coated onto the surface of Au crystals by two steps. Firstly, (DNase) was activated using Traut's Reagent and TCEP for 2 h. The mole ratio of n(DNase) : Traut : TCEP was 1 : 5 : 10. Next, the Au crystals were immersed into the solutions of activated n(DNase) for 48 h at room temperature. The n(DNase)-coated Au crystals were rinsed with di-water, dried under nitrogen gas and equipped into the standard flow module before measurements. The n(DNase)-coated Au crystals were washed with PBS (10 mM, pH 7.4) for 1 h at a flow rate of 30 μL/min and then equilibrated at 10 μL/min until the baseline was stable. Then, EPS solutions (10 mg/mL, pH 5.0) were injected for 30 min at 10 μL/min and followed by fluid of PBS buffer for 30 min. The binding affinity between EPS and DNase was measured by the same method.

11. Biofilm disintegration assay

11.1 CLSM measurements

S. aureus ATCC43300 biofilms were exposed to the n(DNase) solutions (2 mL, 0.2 mg/mL) for 30 min at 37 °C and subsequently washed three times with PBS. Then, the biofilms were incubated with acetate buffer working solutions for 2, 4 and 6 h at 37 °C. After incubation, biofilms were washed three times with PBS and stained with FITC-conA solutions (2 mL, 0.01 mg/mL) for 20 min at 4 °C in darkness. The CLSM images were captured by CLSM (Leica, Germany) and FITC-conA was excited with argon ion laser at 488 nm. Biofilms treated with PBS, DNase and n(BSA) were measured at the same condition.

11.2 Crystal violet staining measurements

S. aureus ATCC43300 biofilms formed in 96-well plates were exposed to the n(DNase) solutions (200 μL, 0.2 mg/mL, pH 7.4) for 30 min at 37 °C and washed three times with PBS. Biofilms were exposed to acetate buffer working solutions (200 μL) for 2, 4 and 6 h at 37 °C. Followed by washing three times with PBS to remove the disintegrated biofilm and thoroughly dried, the biofilms were fixed with absolute methanol (150 μL) for 15 min. After discarding the absolute methanol, crystal violet solutions (150 μL, w/w 0.5% in PBS) were added to each well for 20 min to stain the biofilms. After crystal violet staining, the biofilms were washed three times with PBS to remove the excess crystal violet. The stained biofilms were thoroughly dissolved by adding acetic acid (200 μL, v/v, 33% in PBS) and measured at 595 nm on a microplates reader. Biofilms treated with PBS, DNase and n(BSA) were measured at the same condition.

11.3 SEM measurements

Glass slides were sterilized with anhydrous ethanol and then thoroughly washed with sterile water and air dried. Next, *S. aureus* ATCC43300 biofilms were formed on the glass slides placed in the 48-well plates and treated with n(DNase) for 6 h at 37 °C. Then, the biofilms were fixed similar to that for CLSM measurements of biofilm disintegration assay. Followed by dehydrating with ethanol solutions (1 mL) at a series of concentration (v/v, 30%, 50%, 70%, 90%, 95%, and 100% in PBS) for 5 min, respectively, and dried in vacuum for 2 h. The biofilms were observed and imaged using SEM (JSM-7500F, Japan). Biofilms treated with PBS, DNase, n(BSA) were measured at the same condition.

12. Bacterial killing of ciprofloxacin combined with n(DNase)

12.1 CLSM measurements

S. aureus ATCC12600^{GFP} biofilms were exposed to the n(DNase) solutions (2 mL, 0.2 mg/mL) for 30 min at 37 °C, and subsequently washed three times with PBS. The biofilms were then incubated with acetate buffer working solutions for another 6 h at 37 °C. Next, the biofilms were treated with ciprofloxacin solutions (2 mL, 1.56 μg/mL) for 3 h at 37 °C. After discarding the ciprofloxacin solutions, the biofilms were fixed with glutaraldehy (v/v, 4% in PBS) for 4 h at 4 °C. The dead bacteria was stained with ethidium bromide (EB) solutions (2 mL, 0.01 mg/mL) for 20 min at 4 °C in darkness. The EB was dissolved in DMSO to obtain 1 mg/mL stock solutions and then diluted with PBS to 0.01 mg/mL. The green fluorescent protein of *S. aureus* ATCC12600^{GFP}

and EB were excited with 488 nm and 514 nm, respectively. Biofilms treated with PBS, DNase and n(BSA) were measured at the same condition.

12.2 Flat colony counting measurements

S. aureus ATCC12600^{GFP} biofilms were exposed to the n(DNase) solutions (200 μL, 0.2 mg/mL) for 30 min at 37 °C, and subsequently washed three times with PBS. The biofilms were then incubated with acetate buffer working solutions for another 6 h at 37 °C. After washing three times with PBS, the biofilms were treated with ciprofloxacin solutions (200 μL) at different concentration (1.56, 3.12, 6.24, 12.5, 25, 50 and 100 μg/mL) for 3 h at 37 °C, respectively. The biofilms were sonicated for 5 min (three times) and diluted in 10-folds steps from 1:10 to 1:10^6 in PBS. The diluted bacterial suspensions (100 μL) were inoculated onto the TSB agars plates, respectively, and then incubated for 24 h at 37 °C. After incubation, the number of visible colonies was counted. Survival rate of the bacteria treated with n(DNase) was calculated according to the formula:

$$\text{Survival rate} = \frac{\text{N[n(DNase)]}}{\text{N[PBS]}} \times 100\%$$

N[n(DNase)] and N[PBS] represent the number of colonies pretreated with n(DNase) and PBS in the TSB agar plates, respectively. Biofilms treated with DNase and n(BSA) were assessed at the same condition.

13. Statistics

All statistical analyses were performed using the Prism software package (PRISM 8, GraphPad Software, USA).

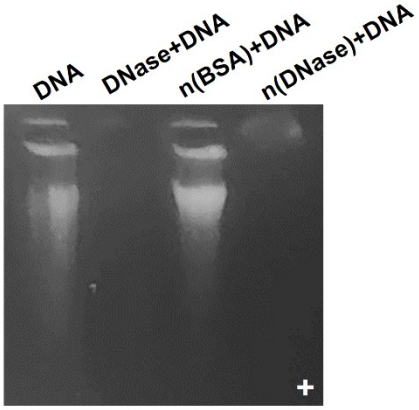

Fig. S1 Agarose gel electrophoresis results of DNA incubating with PBS, DNase, n(BSA) and

n(DNase) for 30 min at 37 °C, respectively. The "+" represents the positive direction of the electrophoresis.

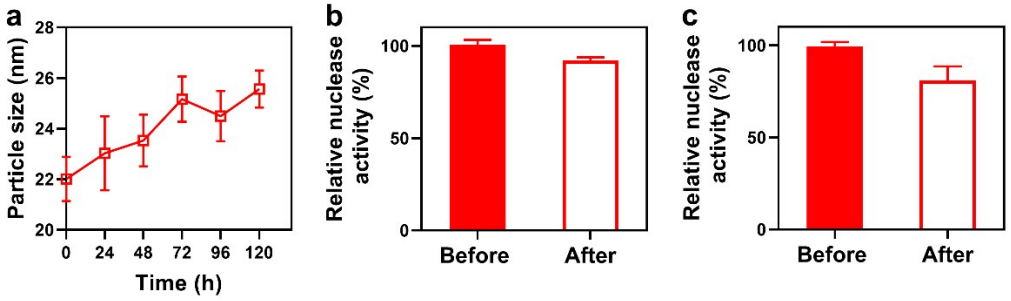

Fig. S2 (a) Summary of size changes of n(DNase) at 37 °C in phosphate buffer saline (PBS) over 120 hours. (b) The nuclease activities of n(DNase) at 65 °C for 1 hour. The nuclease activity of n(DNase) without heat treatment was set to 100%. (c) The nuclease activity of froze- dried n(DNase). the nuclease activity of n(DNase) without freeze-drying was set to 100%. Data are presented as mean ± standard error of the mean ± s.e.m. with n independent experiments (n = 3).

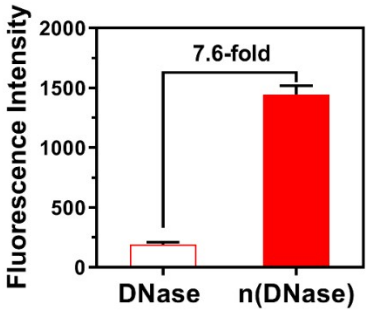

Fig. S3 Quantitative analysis of total fluorescence intensity of the DNase (white) and n(DNase) (red) in the biofilm.

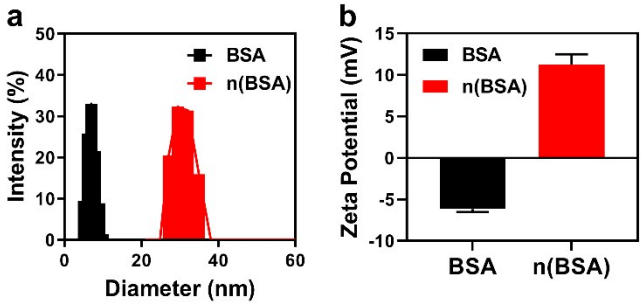

Fig. S4 (a) The hydrodynamic diameter distribution of BSA and n(BSA) measured by dynamic light scattering and in phosphate buffer (10 mM, pH 5.0). (b) Zeta potentials of BSA and n(BSA) in phosphate buffer (10 mM, pH 5.0).

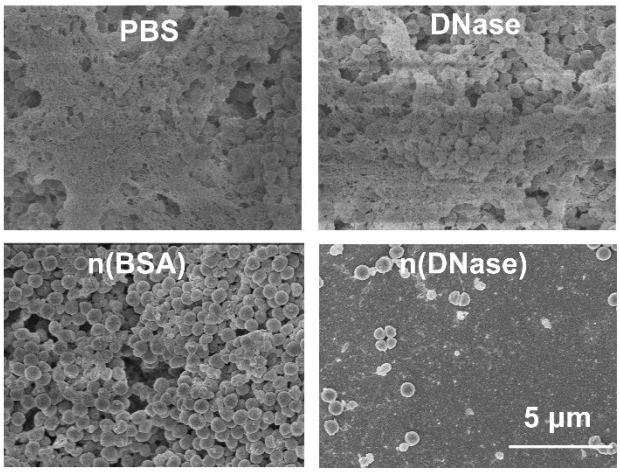

Fig. S5 SEM images of *S.aureus* ATCC 43300 biofilms treated with PBS, DNase, n(BSA) and n(DNase) for 6 hours at 37 °C, respectively. Scar bar, 5 μm.

References

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