Supporting information

Engineering polysulfoniums for enhanced antibacterial activity with extremely minimal hemolysis

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Materials

All chemical solvents were purchased from Beijing Innochem Technology Co., Ltd. and used without further purification. All other chemicals were purchased from commercial suppliers and used without further purification. Mueller–Hinton (MH) medium, Luria–Bertani (LB) medium, LB agar were purchased from Qingdao Hope Bio-Technology Co., Ltd. *Staphylococcus aureus* (*S. aureus*) (ATCC6538), *Escherichia coli* (*E. coli*) (ATCC25922), and *Pseudomonas aeruginosa* (*P. aeruginosa*) (ATCC9027) were purchased from Nanjing Lezhen Biotechnology Co., Ltd. Additionally, multi-drug resistance *S. aureus* strains (*S. aureus* USA300-R, ATCC 700699, ATCC BAA-1026) were supplied by the Academy of Military Medical Sciences, Beijing, China. Rabbit's red blood cells (RBCs) were purchased from Zhengzhou Pingrui Bio-Technology Co., Ltd. Phosphate buffered saline (PBS) were purchased from Dalian Meilun Biotechnology Co., Ltd. (Dalian, China).

Characterizations

^{1}H NMR spectrum were recorded on a Bruker Avance II 400 (400 MHz). Tandem gel permeation chromatography (GPC) experiments were carried out in tandem, employing an SSI pump linked to a Wyatt Optilab DSP. The eluent used was DMF (HPLC grade) with 0.02 M LiBr, maintained at a flow rate of 1 mL/min. The concentration of samples analyzed in GPC was around 6 mg/mL. For standard calibration procedures, Poly(methyl methacrylate) (PMMA) standards were utilized. Confocal laser scanning microscopy (CLSM) images were recorded on a Nikon AX (Nikon Corporation, Japan). The optical density (OD) reading was recorded on a microplate reader (TECAN, Switzerland).

Experiment

Synthesis of MeP-NNCA

The synthesis of MeSPG hydrochloride was performed according to a previously reported procedure.[1] MeSPG hydrochloride (20 g, 0.134 mol) was dissolved in 150 mL of deionized water. Then, tert-butyl dicarbonate (78 mL, 0.335 mol) and triethylamine (93 mL, 0.67 mol) were added and stirred at 37 °C for 48 hours. N-hexane was used for repeated extraction three times to remove the unreacted tert-butyl dicarbonate. Subsequently, the aqueous phase was adjusted to pH = 2 with 2 M HCl and repeatedly extracted three times with ethyl acetate. The combined ethyl acetate phase was washed three times with saturated salt water, dried with anhydrous magnesium sulfate, and filtered to obtain the filtrate. The filtrate was removed by rotary evaporation to obtain MeSPG acetic acid. MeSPG acetic acid (10 g, 42.0 mmol) was dissolved in 150 mL anhydrous dichloromethane under nitrogen atmosphere, and then phosphorus trichloride (4.38 mL, 50.4 mmol) was slowly added under ice bath conditions. The reaction was carried out at room temperature for 3 hours. After the reaction was completed, the solvent was removed by vacuum, and 10 mL anhydrous dichloromethane was added to dissolve the product and filtered. The solvent was removed again under vacuum to obtain a crude product of a pale yellow oily liquid. Then, 10 mL of anhydrous tetrahydrofuran was added under a nitrogen atmosphere, and the solution was slowly poured into 100 mL of anhydrous n-hexane with stirring. This purification procedure was repeated three times, and the final product, MeP-NNCA, was obtained as a pale yellow oily liquid.

Polymerization of Allyl-NNCA and MeP-NNCA

The polymers obtained by ring-opening polymerization of Allyl-NNCA and MeP-NNCA using benzylamine ($BnNH_2$) as initiator were denoted as $PNAG_n$ and $PNSG_n$-R_1 (R_1 = Me). First, Allyl-NNCA (1.5 g, 10.629 mmol) was dissolved in anhydrous THF at the concentration of 100 mg/mL under a N_2 atmosphere, followed adding different volume of initiator solution according to the designed DP. After reacting at 55 ℃ for 48 h in a N_2 atmosphere, the mixed solution was precipitated into an excess of ice ether, and then filtered and vacuum dried to obtain a white solid. The polymerization procedure of MeP-NNCA is the same as that of Allyl-NNCA.

General procedure for the synthesis of $PNSG_n$-R_1 (R_1 = Et, n-Pr)

First, $PNAG_n$ and benzoin dimethyl ether (DMPA) was dissolved in *N, N*-dimethylformamide (DMF) with the polymer concentration at 80 mg/mL. Then, the mixture was degassed by three freeze/thaw/pump cycles, followed by addition of ethanethiol or propylmercaptan under N_2 atmosphere, and the molar ratio of [-SH]: [C=C]: [DMPA] was 70: 7: 1. The mixture was irradiated under high pressure mercury UV lamp for 6 h. After the reaction, the mixture was precipitated in ice ether for three times. After drying, the yellow solid was obtained.

Alkylation of $PNSG_n$-R_1 using epoxide

$PNSG_n$-R_1 was dissolved in acetic acid at the concentration of 40 mg/mL, and then the epoxide (5 eq per of thioether group) was added. The solution was stirred vigorously for 3 days at 37°C. The crude product was first dialyzed in 0.1 M HCl solution for 2 days and then dialyzed in deionized water for 2 days. After freeze-drying, the final product was obtained.

Antibacterial assay

The antibacterial activity of $PNSG_n$-R_1-X were measured using the minimum inhibitory concentration (MIC) assay. Bacteria in logarithmic phase of growth were diluted in MH medium to 3×10^5 colony-forming units (CFU) per milliliter as the working suspension. The polysulfonium aqueous solution in two-fold serial dilution (100 μL, MH medium) were prepared and mixed with the equal volumes of bacterial suspensions (100 μL) on a 96-well plate. Then, the 96-well plate was incubated for 18 h at 37 °C. The optical density of each well at 600 nm (OD_{600}) was recorded on a microplate reader. MH medium without bacteria was considered as the blank and that with bacteria suspension was used as the positive control. The percentage of bacterial cells survival was calculated according to the equation:

$$\%\ \text{Bacterial vitality} = \frac{OD_{600}^{\text{sample}} - OD_{600}^{\text{blank}}}{OD_{600}^{\text{positive control}} - OD_{600}^{\text{blank}}} \times 100$$

Hemolysis assay

The RBCs were washed with phosphate buffer saline (PBS) for three times, and then, RBCs were resuspended to 5% (v/v) in PBS. According to the antibacterial assay, the polysulfonium aqueous solution (32 mg/mL) in two-fold serial dilution were prepared and mixed with the equal volumes of PBS (200 μL) in microcentrifuge tubes (1.5 mL), which was incubated at 37 °C for 1 h. After incubation, all samples were centrifuged for 5 min, and the supernatant was transferred to 100 μL to 96-well plates. The OD value at 540 nm wavelength was measured under a microplate reader. PBS was used as the blank, and RBCs suspension containing Triton X-100 (0.5% in PBS) was used as the positive control. The percentage of hemolysis was calculated by the equation:

$$\%\ \text{Hemolysis} = \frac{OD_{540}^{\text{sample}} - OD_{540}^{\text{blank}}}{OD_{540}^{\text{positive control}} - OD_{540}^{\text{blank}}} \times 100$$

Stability assay

Plasma stability assay: centrifugation of fresh rabbit blood was performed at 4000 rpm for 5 minutes. Subsequently, the supernatant (plasma) was collected using a pipette gun for further use. Additionally, solutions of 2 mg/mL polysulfonium, polymyxin B, and vancomycin were prepared in fresh plasma at 37 °C for 24 h, and then the plasma was diluted for MIC test.

Long-term stability test: the polysulfonium was dissolved in a PBS solution and stored at 4 ℃ and were taken periodically for MIC testing and compared to the initial values.

Evaluation of antimicrobial resistance

S.aureus (ATCC 6538) was evaluated for antimicrobial resistance to polysulfoniums and Norfloxacin. According to the minimal inhibitory concentration (MIC) determination protocol, the MIC for cells was assessed. *S. aureus* was cultured in LB medium supplemented with the sub-MIC concentration of the preceding generation's drug at 37 °C overnight.

Study on bacterial killing kinetics

S.aureus USA300-R in logarithmic phase of growth were washed three times with PBS, and diluted to 3×10^5 CFU/mL as the working suspension. Bacteria were treated with polysulfoniums at concentrations of 1 × MIC, 2 × MIC, and 4 × MIC, respectively. At various time points (0 min, 2 min, 5 min, 10 min, and 20 min), the bacterial suspension was diluted to an appropriate concentration and evenly spread on LB agar plates. Subsequently, the plates were incubated in a biochemical incubator at 37 ℃ for 15 to 18 hours for colony counting and activity assessment.

Inhibition of methicillin-resistant *Staphylococcus aureus* (MRSA) biofilm formation

S.aureus USA300-R in the logarithmic growth phase were diluted to a concentration of 3×10^5 CFU/mL in MH medium supplemented with 1% glucose as the working suspension. The polysulfonium solution was subsequently diluted to the desired concentration in MH medium containing 1% glucose by two-fold serial dilution in a 96-well plate. Following this, 50 μL of the bacterial suspension was added to each well and mixed thoroughly. The plate was then incubated at 37 °C for 24 hours. After incubation, the original medium was carefully removed, and 100 μL of an MTT solution (0.5 mg/mL) in PBS was added to each well, followed by an additional incubation at 37 °C for 4 hours. Subsequently, the MTT solution was aspirated, and 150 μL of DMSO was added to each well. After a shaking period of 30 minutes, the OD value was recorded at 570 nm on a microplate reader. In this experiment, MH medium containing the bacterial suspension served as the positive control, while MH medium lacking the bacterial suspension

served as the blank control. The bacterial cell viability was calculated according to the equation, which was used for evaluating the ability of the sample in inhibiting MRSA biofilm formation:

$$\%\ \text{Bacterial vitality} = \frac{OD_{570}^{\text{sample}} - OD_{570}^{\text{blank}}}{OD_{570}^{\text{positive control}} - OD_{570}^{\text{blank}}} \times 100$$

Activity against mature MRSA biofilm

MRSA in the logarithmic growth phase were diluted to a concentration of 3×10^5 CFU/mL in MH medium containing 1% glucose as the working suspension. Bacterial suspension (100 μL) was added to each well of a 96-well plate and incubated at 37 °C for 24 hours. Subsequently, the original medium was carefully aspirated, and polysulfonium solution at the desired concentrations was added into each well. Following a second incubation at 37 ℃ for 24 hours, the medium was again aspirated, and 100 μL of MTT solution (PBS, 0.25 mg/mL) was added to each well and incubated at 37 °C for 4 hours. After the incubation, the MTT solution was aspirated, and 150 μL of DMSO was added to each well. The plates were shaken for 30 minutes, and the OD value was recorded at 570 nm on a microplate reader. In this experiment, MH medium containing the bacterial suspension served as the positive control, while MH medium lacking the bacterial suspension served as the blank control. The bacterial cell viability was calculated according to the equation, which was used for evaluating the ability of the polysulfoniums in eradicating mature biofilm formation:

$$\%\ \text{Bacterial vitality} = \frac{OD_{570}^{\text{sample}} - OD_{570}^{\text{blank}}}{OD_{570}^{\text{positive control}} - OD_{570}^{\text{blank}}} \times 100$$

Confocal laser scanning microscopy study

For CLSM images of the inhibiting and eradicating of MRSA biofilm formation, we used the confocal dish instead of 96-well plate. Prior to imaging, the MRSA biofilm was stained with STYO 9 and propidium iodide (PI). After 10 min, the biofilms were imaged by CLSM at different wavelengths.

Confocal microscopy imaging of bacteria

The fluorescein isothiocyanate (FITC)-labeled $PNSG_{58}$-Me-Cl was synthesized following the previously reported method.[2] Bacteria in the logarithmic growth phase were harvested, washed three times with PBS, and resuspended in PBS to a concentration of 3×10^8 CFU/mL. An aliquot of FITC-labeled $PNSG_{58}$-Me-Cl was then added to 1 mL of the bacterial suspension, along with 20 μL of Nile Red (5 mg/mL), achieving a final concentration of 8 × MIC. The bacterial suspension was subsequently incubated in the dark at 37 °C for 5 minutes MRSA. Images of the bacteria were captured using confocal microscopy.

Cytoplasmic membrane permeabilization assay

MRSA, cultured overnight, was harvested and subjected to two washes with HEPES buffer (5 mM HEPES, 20 mM glucose, pH 7.4). The resultant cell concentration was adjusted to 3×10^8 CFU/mL, yielding a bacterial suspension prepared in HEPES buffer. Subsequently, propidium iodide (PI) was introduced to the suspension at a final concentration of 10 μM. This mixture was then dispensed into a 96-well black plate. After allowing the system to stabilize until the fluorescence intensity remained relatively constant, the antibacterial agent at a final concentration of 32 μg/mL was introduced (time = 0 s). The fluorescence intensity of PI was continuously monitored using a multifunctional microplate reader, with an excitation wavelength set at 535 nm and an emission wavelength at 617 nm. An enhancement of PI fluorescence was observed within 15 minutes, serving as an indicator of cell membrane permeability and facilitating the assessment of PI uptake.

Cytoplasmic leakage assay

MRSA incubated overnight was collected and washed twice with PBS buffer, and then diluted in PBS buffer to a cell concentration of 1×10^9 CFU/mL as a bacterial suspension to be used. The polysulfoniums were introduced into the bacterial suspension at concentrations corresponding to 1 × MIC, 2 × MIC, and 4 × MIC. An untreated bacterial suspension served as the control. Following the addition of the polysulfoniums, the mixture was incubated at 37 °C for 2 hours. Subsequently, the sample was filtered through a 0.22 μm filter to remove cellular debris, and the supernatant was obtained for further analysis. The optical density at 260 nm was measured using a microplate reader to record.

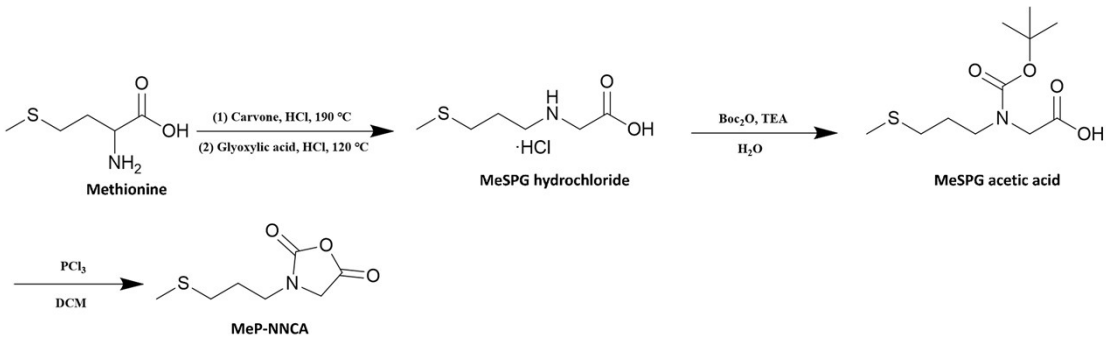
Bacterial Morphology Assays

Regulus 8100 scanning electron microscope (HITACHI, Japan) was used to observe morphological changes in bacterial cells before and after treatment with polysulfonium. Briefly, an overnight culture of MRSA was centrifuged

to collect bacterial cells, which were then washed twice with PBS. The bacterial was resuspended in PBS and adjusted to a concentration of 3×10^8 CFU/mL. A 500 μL bacterial suspension was mixed with 500 μL polysulfonium solution and incubated on a shaker at 37 °C for 2 h. A control group, consisting of bacterial suspension without treatment, was also prepared. Following incubation, the mixture was centrifuged at 5000 rpm for 10 minutes, and the supernatant was carefully removed. The bacterial was washed once more with PBS and fixed using electron microscope fixative (2.5% glutaraldehyde in PBS), followed by overnight incubation at 4 °C. The next day, the sample was centrifuged again, the supernatant discarded, and the bacterial pellet was washed with PBS. Dehydration was performed using a gradient of 30%, 50%, 70%, 80%, 90%, and 100% ethanol. Finally, the samples were dried and observed using scanning electron microscopy.

Intracellular ROS assay

The bacterial suspension of MRSA was diluted with PBS buffer (pH = 7.4) to achieve a concentration of 3×10^8 CFU/mL. Subsequently, the reactive oxygen species probe, DCFH-DA, was introduced to the bacterial suspension, resulting in a final concentration of 20 μM within the system. In a black 96-well plate, 180 μL of the bacterial suspension was aliquoted into each well, after which 20 μL of the antibacterial polymer was added. Consequently, the concentration of the polysulfoniums in each well was established at 4 × MIC μg/mL. The fluorescence intensity of DCFH was continuously monitored using a multifunctional microplate reader, with excitation and emission wavelengths set at 488 nm and 530 nm, respectively.



Scheme S1. Synthetic routes of MeP-NNCA.

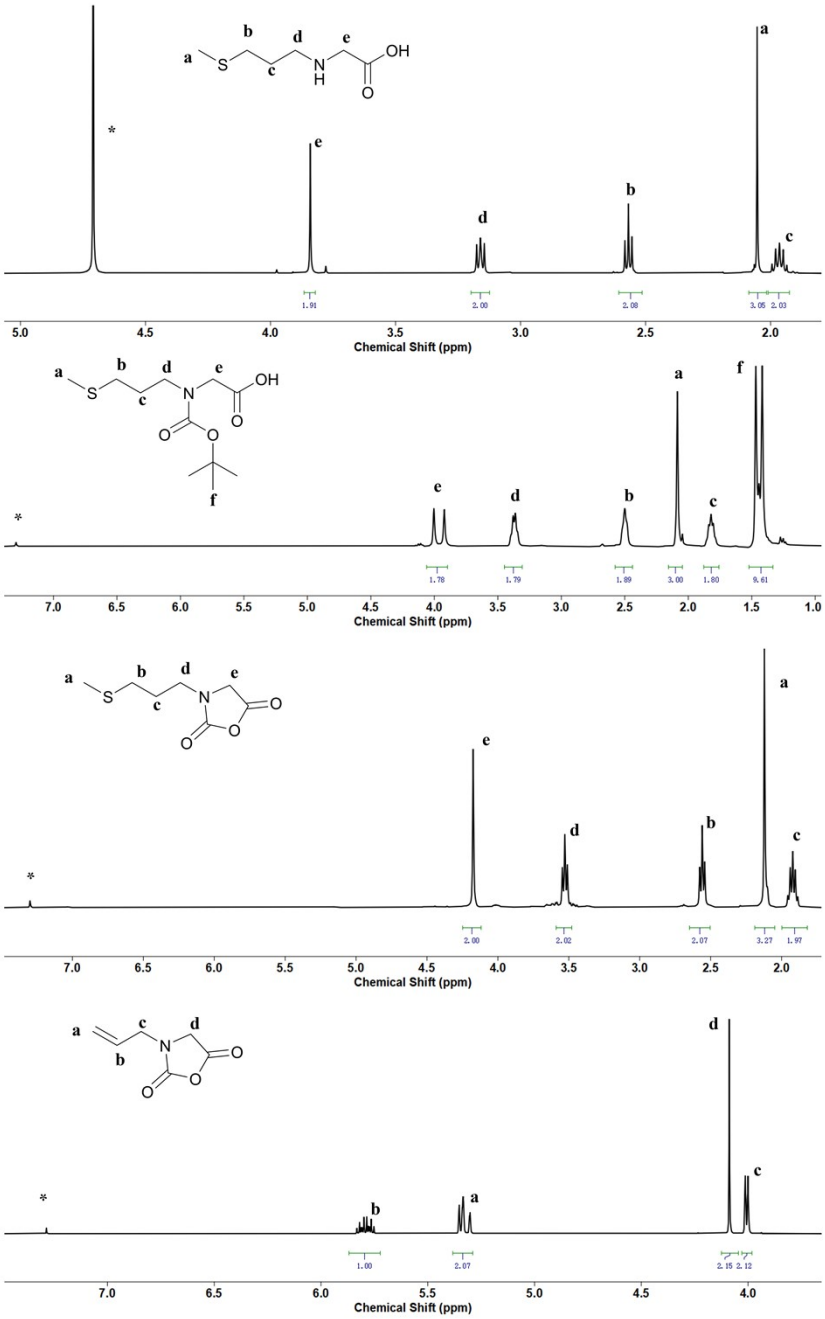


Figure S1. ^{1}H NMR spectra of MeSPG hydrochloride in H_2O, MeSPG acetic acid in $CDCl_3$, MeP-NNCA in $CDCl_3$ and Allyl-NNCA in $CDCl_3$.

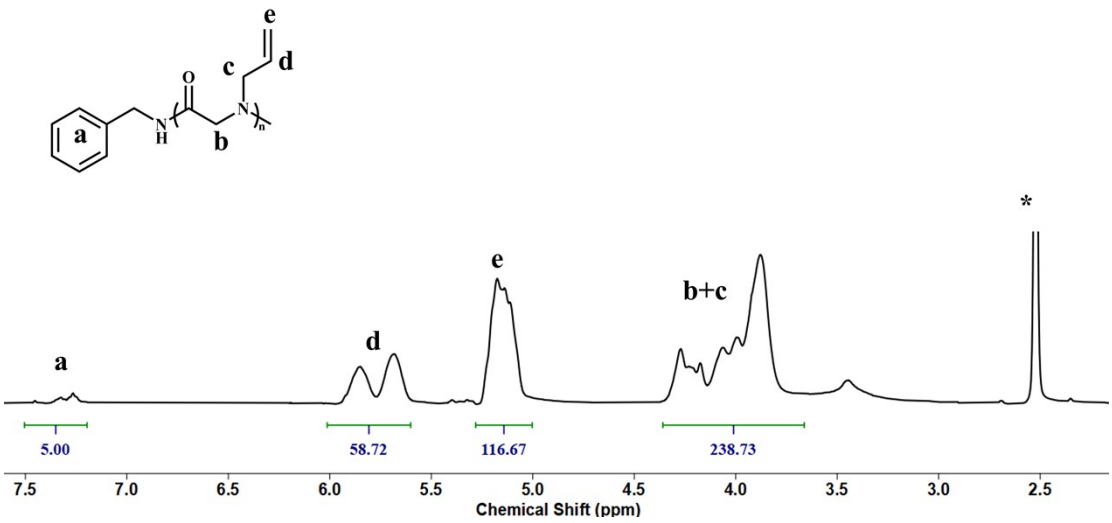


Figure S2. ^{1}H NMR spectrum of $PNAG_{58}$ in DMSO-d_6.

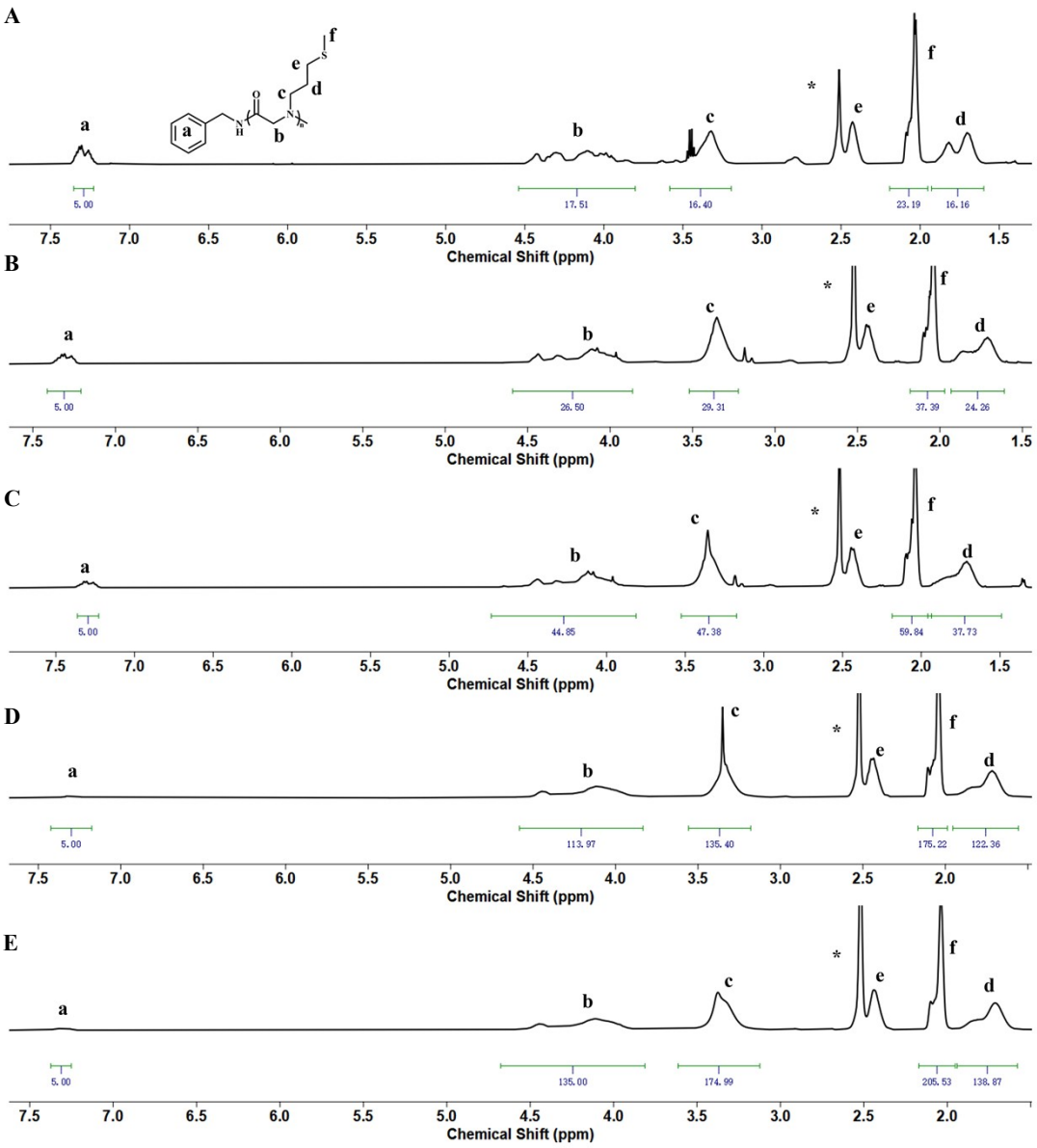


Figure S3. ^{1}H NMR spectra of (A) $PNSG_{8}$-Me, (B) $PNSG_{12}$-Me, (C) $PNSG_{20}$-Me, (D) $PNSG_{58}$-Me and (E) $PNSG_{69}$-Me in DMSO-d_6.

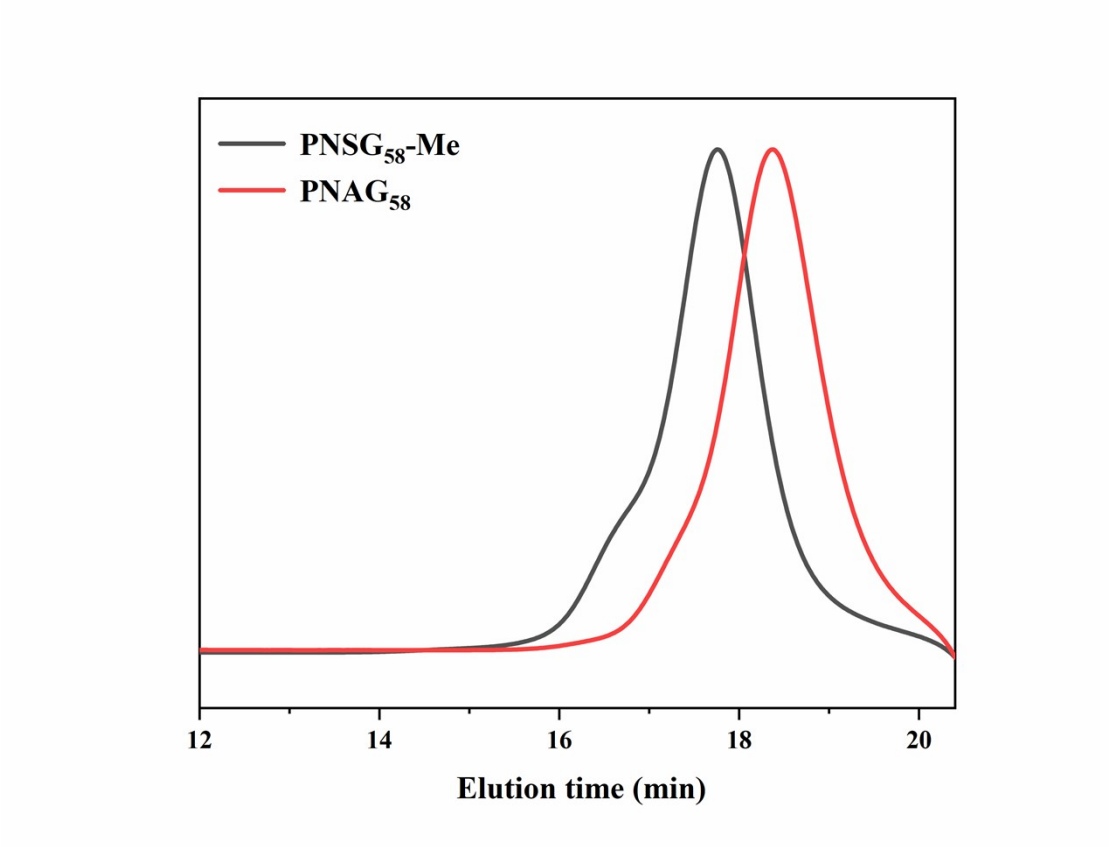


Figure S4. GPC traces of $PNAG_{58}$ and $PNSG_{58}$-Me using DMF as the mobile phase at a flow rate of 1 mL/min.

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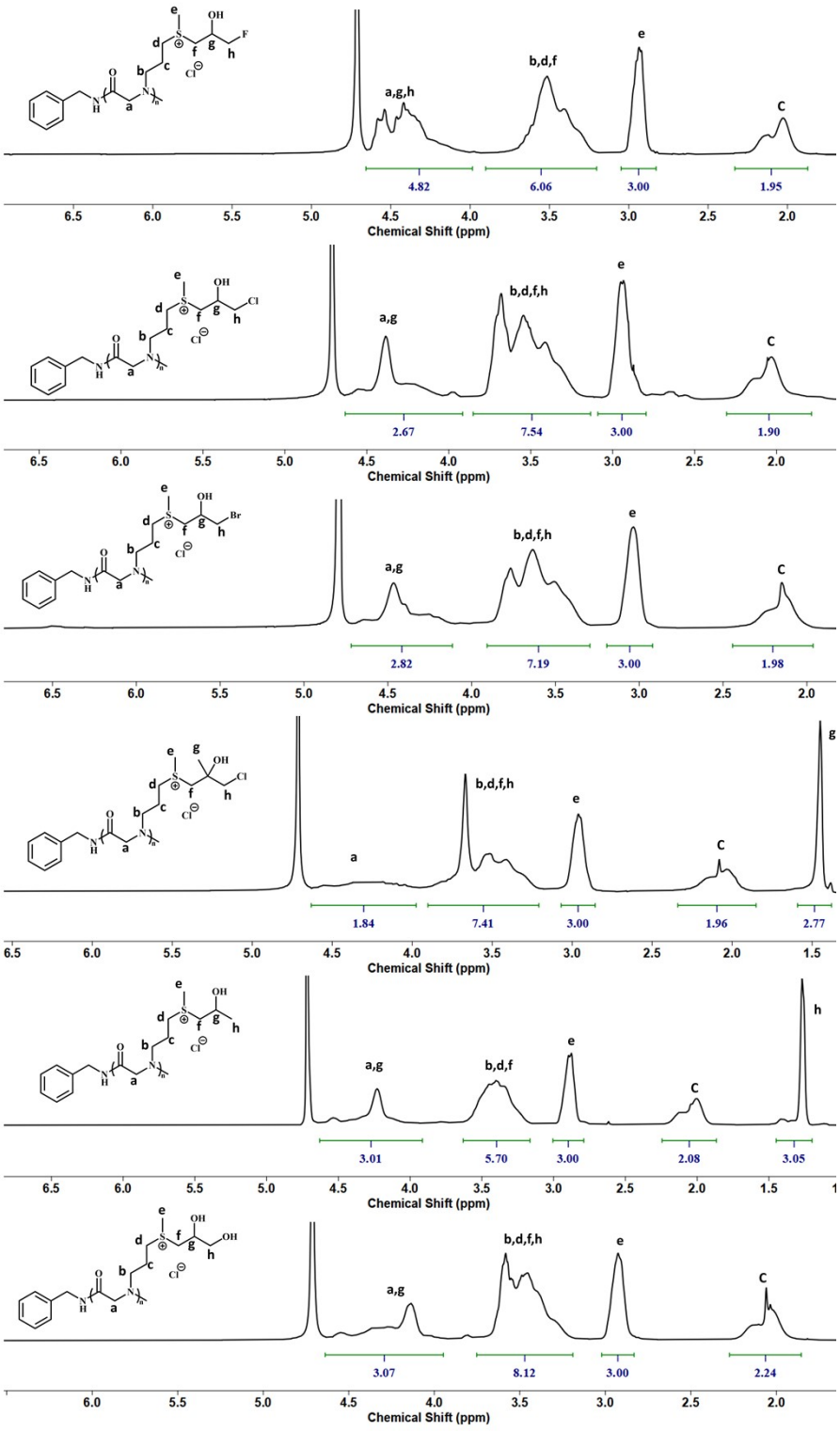

Figure S5. ^{1}H NMR spectra of $PNSG_{58}$-Me-F, $PNSG_{58}$-Me-Cl, $PNSG_{58}$-Me-Br, $PNSG_{58}$-Me-Cl-Me, $PNSG_{58}$-Me-H, $PNSG_{58}$-Me-OH in D_2O.

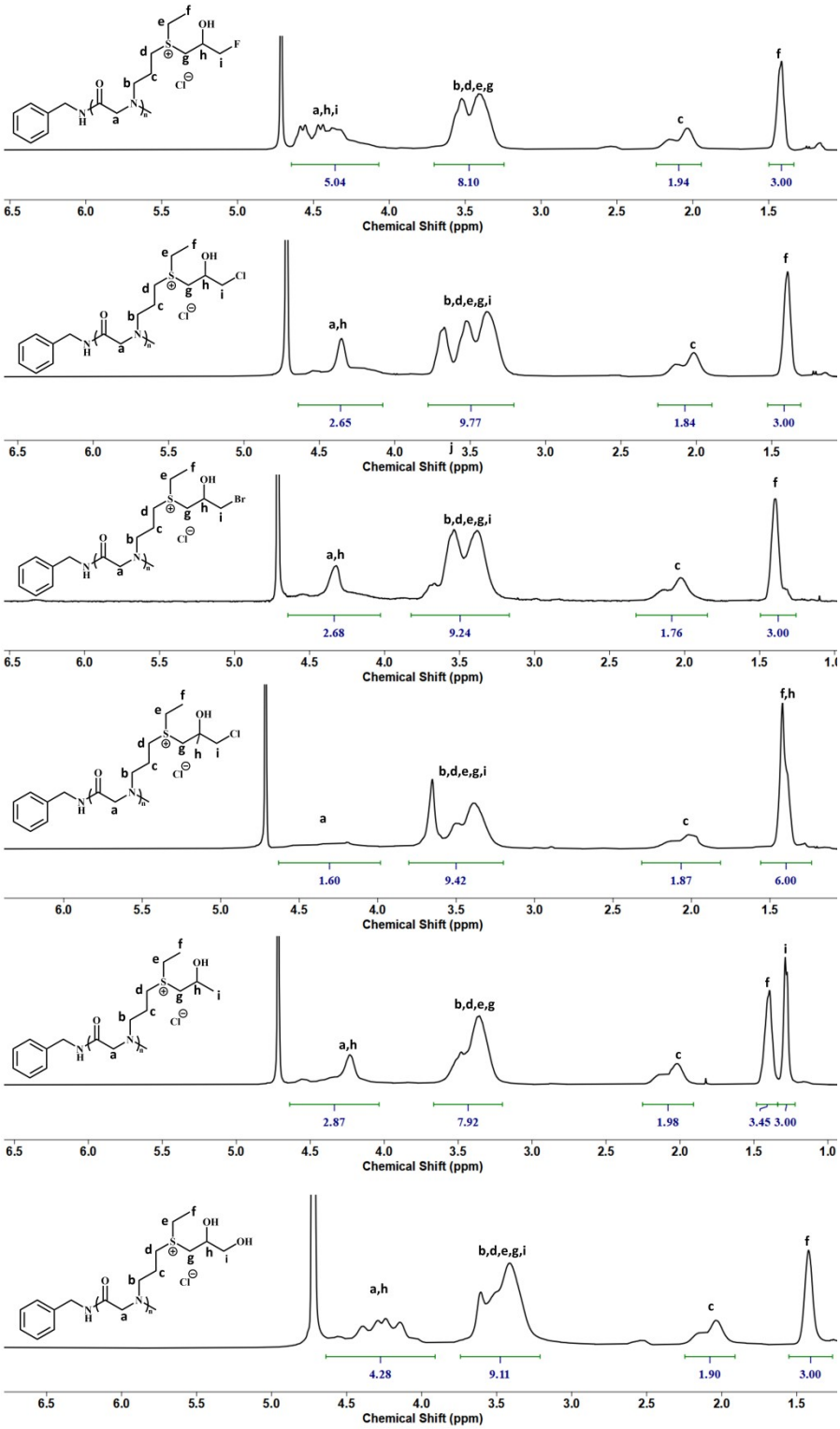

Figure S6. ^{1}H NMR spectra of $PNSG_{58}$-Et-F, $PNSG_{58}$-Et-Cl, $PNSG_{58}$-Et-Br, $PNSG_{58}$-Et-Cl-Me, $PNSG_{58}$-Et-H, $PNSG_{58}$-Et-OH in D_2O.

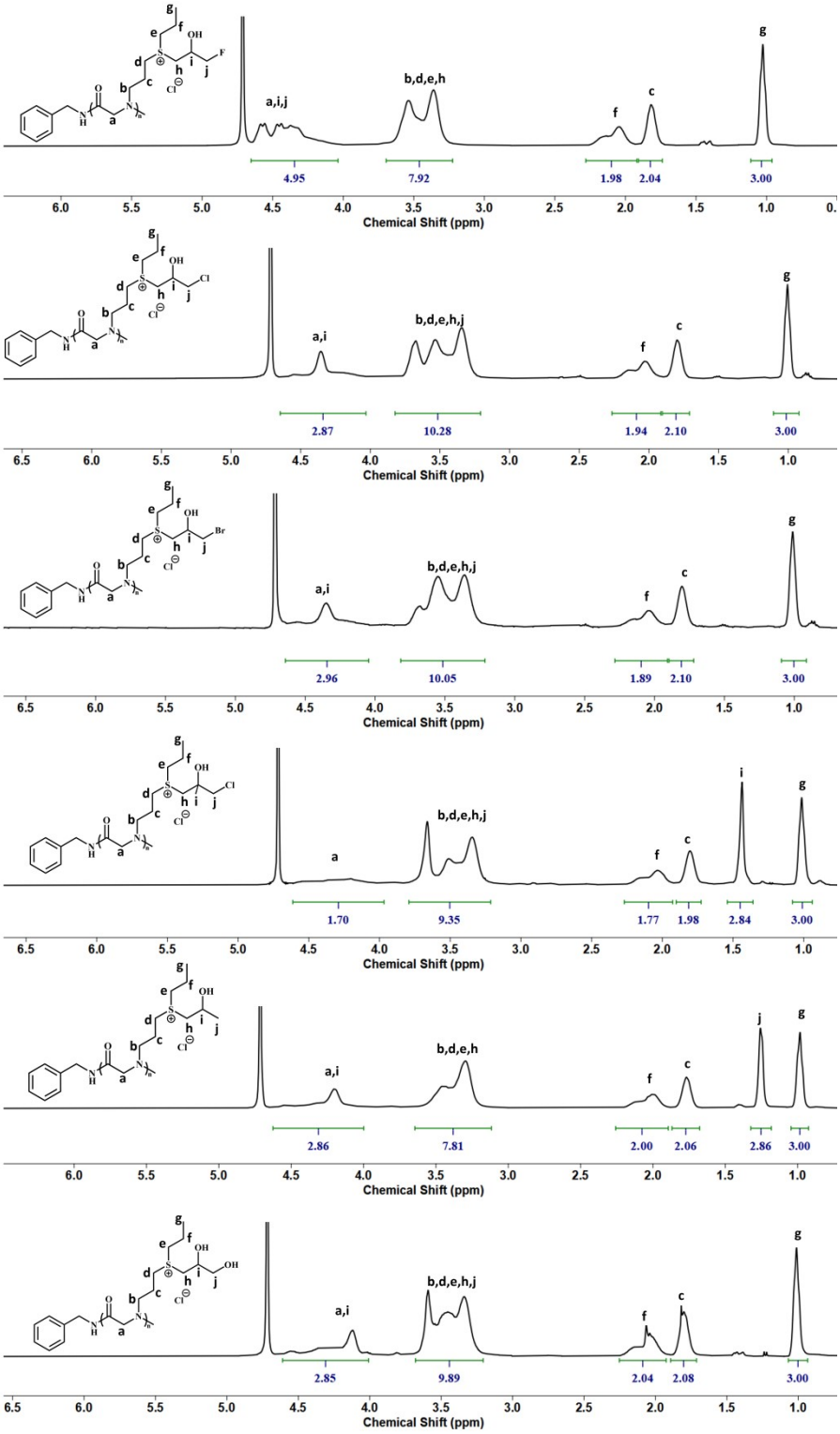

Figure S7. ^{1}H NMR spectra of $PNSG_{58}$-Pr-F, $PNSG_{58}$-Pr-Cl, $PNSG_{58}$-Pr-Br, $PNSG_{58}$-Pr-Cl-Me, $PNSG_{58}$-Pr-H, $PNSG_{58}$-Pr-OH in D_2O.

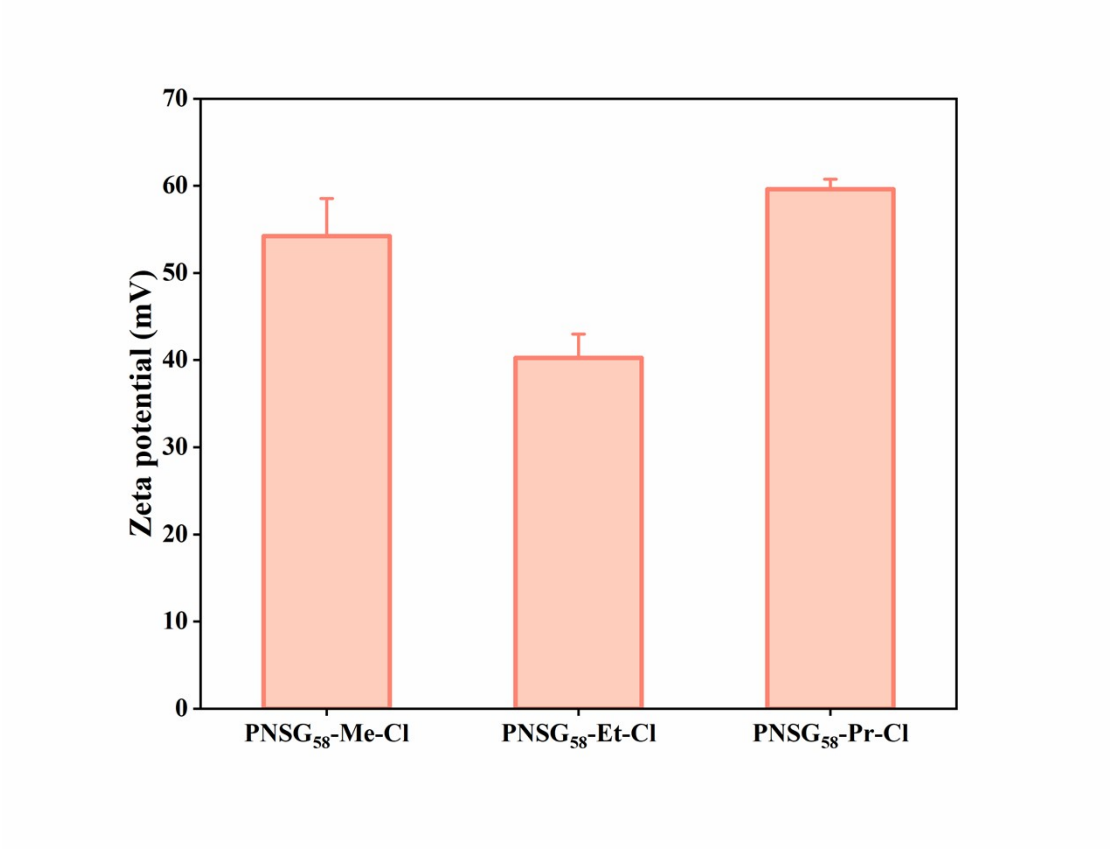

Figure S8. Zeta potential plots of $PNSG_{58}$-Me-Cl, $PNSG_{58}$-Et-Cl and $PNSG_{58}$-Pr-Cl at the concentration of 1 mg/mL.

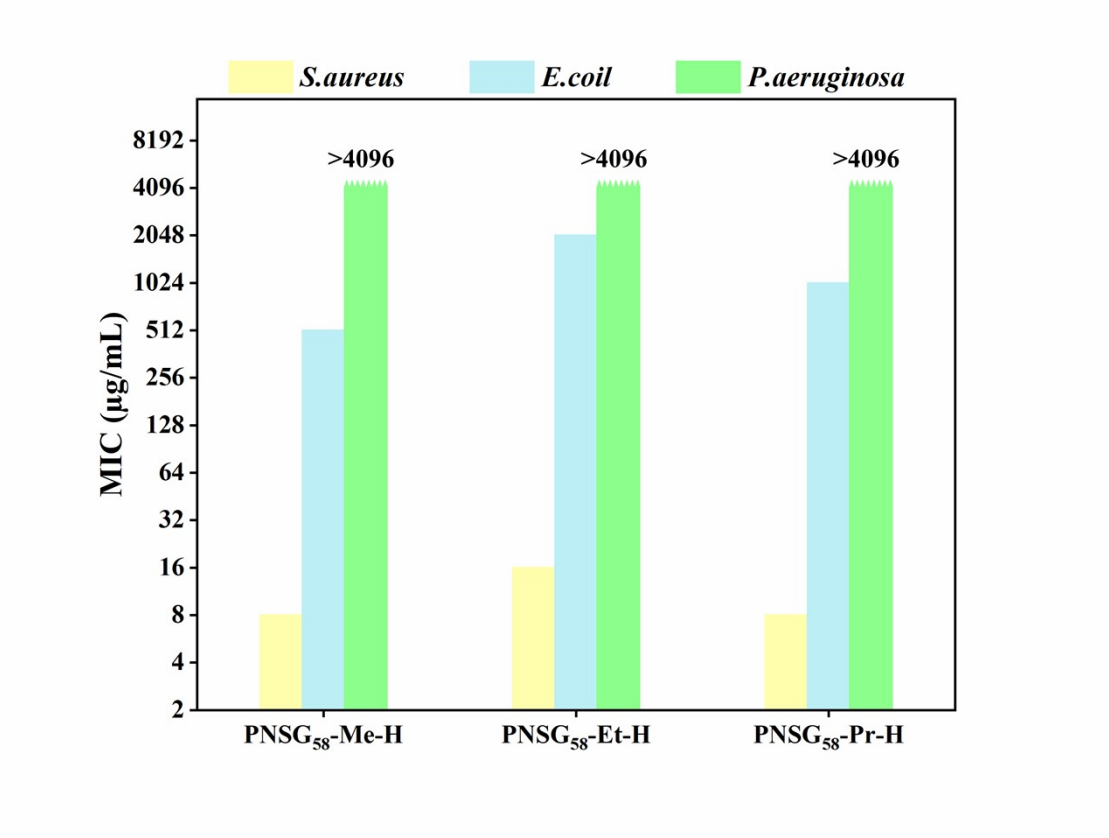

Figure S9. MICs of $PNSG_{58}$-Me-H, $PNSG_{58}$-Et-H and $PNSG_{58}$-Pr-H.

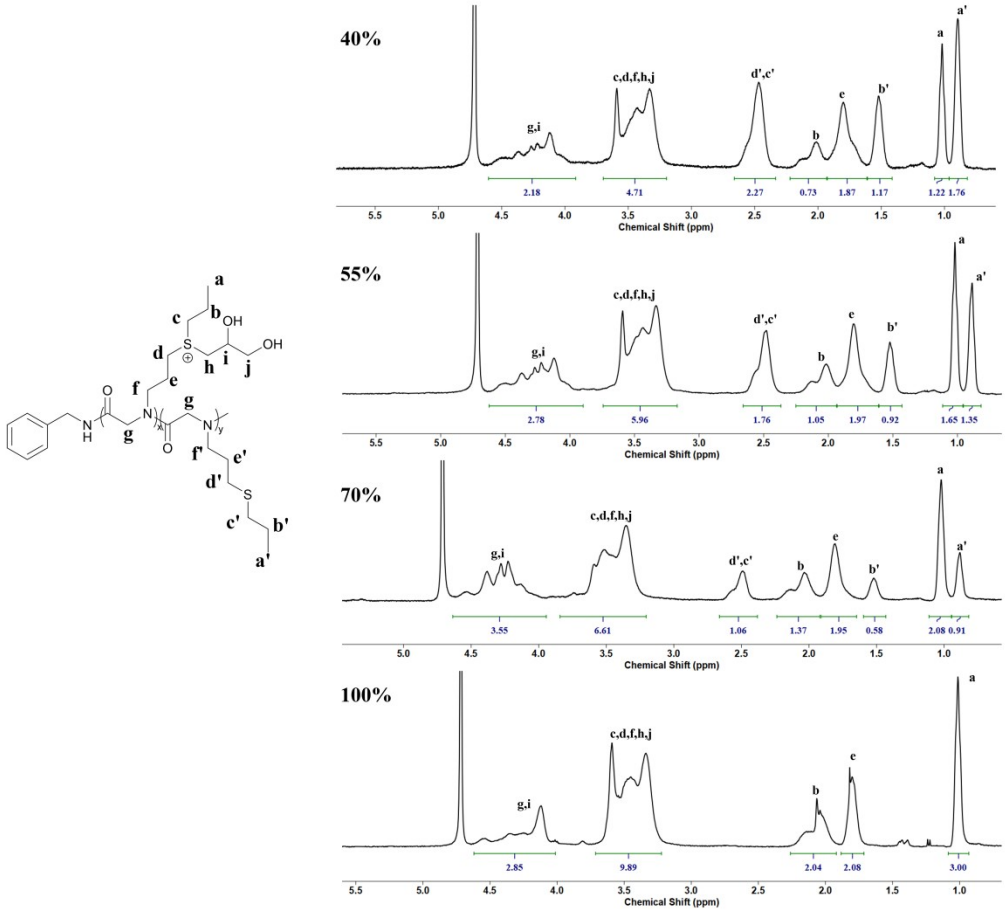

Figure S10. ^{1}H NMR spectra of PNSG$_{58}$-Pr-OH with different degree of functionalization in D_2O.

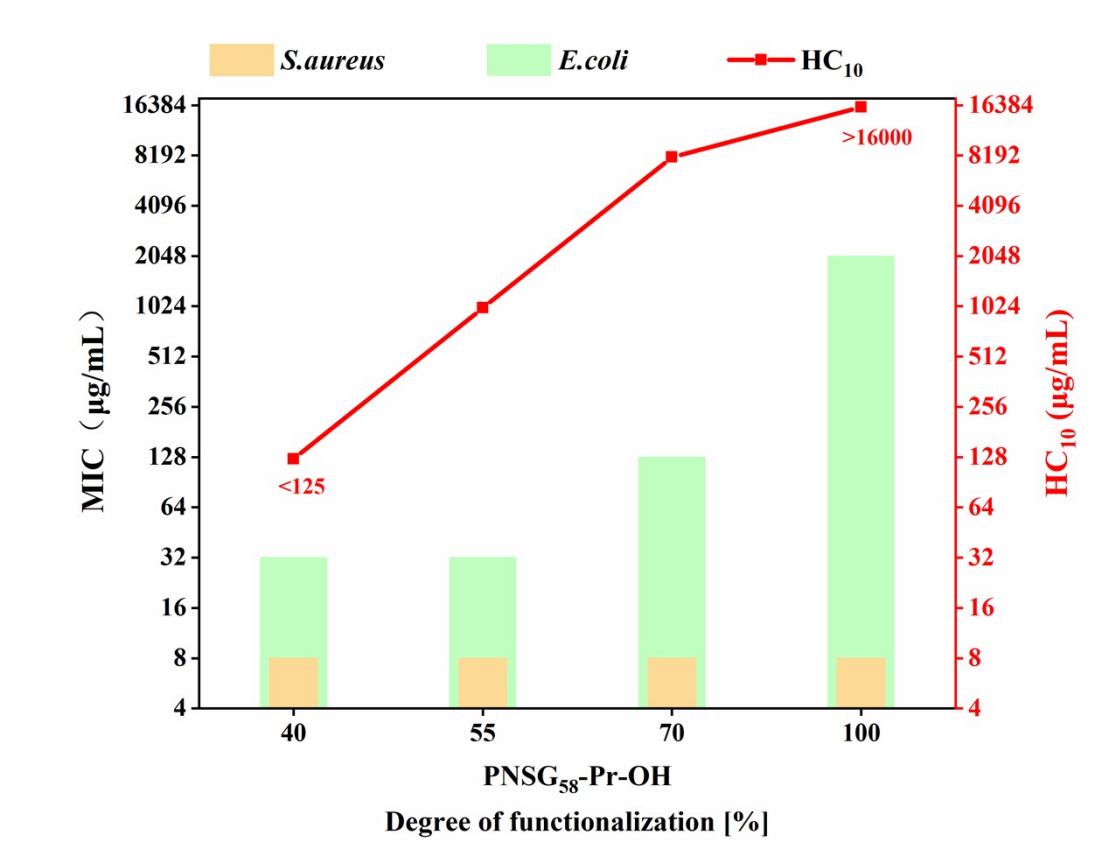

Figure S11. MICs and hemolytic activity of $PNSG_{58}$-Pr-OH with different degree of functionalization.

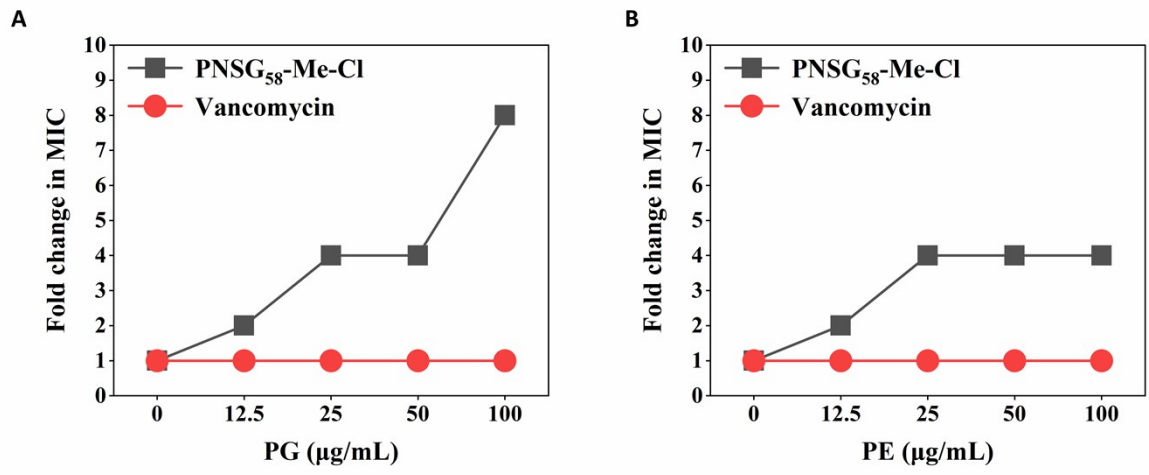

Figure S12. Antibacterial activity of $PNSG_{58}$-Me-Cl in the presence of phosphatidylglycerol (A) and phosphatidylethanolamine (B), using vancomycin for comparison.

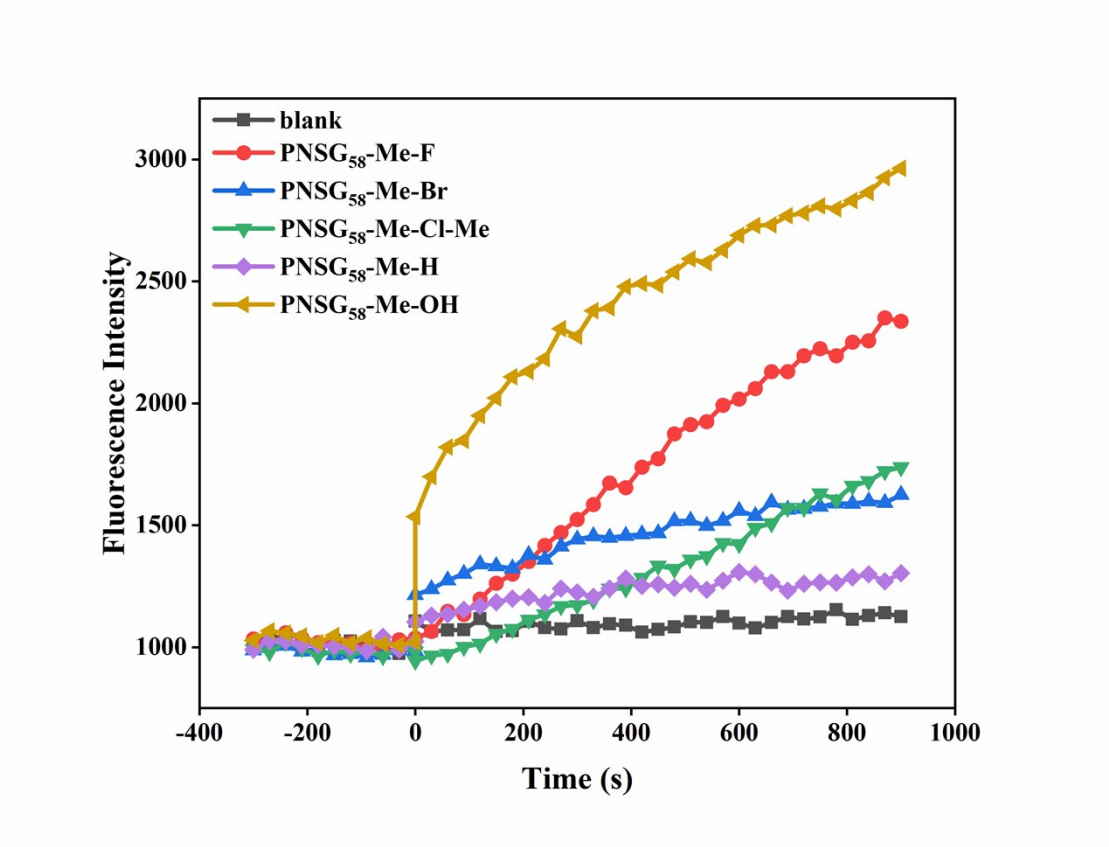

Figure S13. Cytoplasmic membrane permeabilization of MRSA following treatment with $PNSG_{58}$-Me-F, $PNSG_{58}$-Me-Br, $PNSG_{58}$-Me-Cl-Me, $PNSG_{58}$-Me-H, $PNSG_{58}$-Me-OH and PBS buffer as blank.

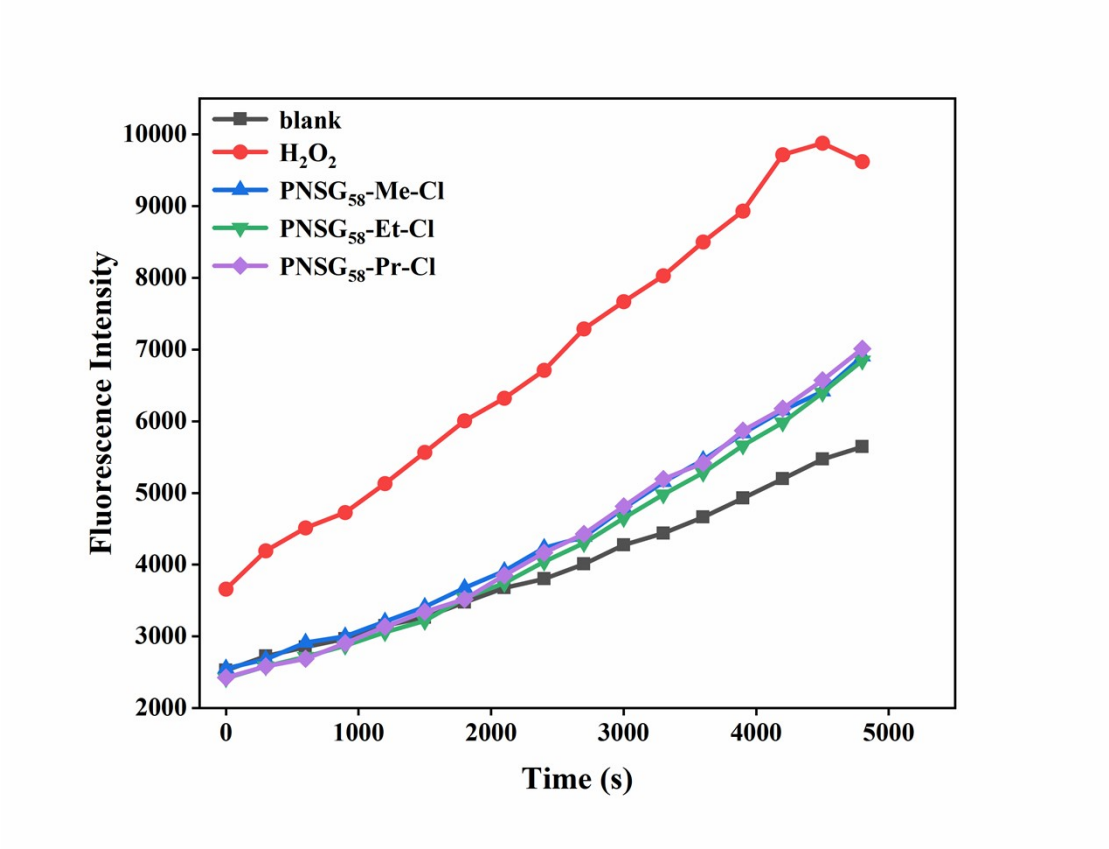

Figure S14. Fluorescent intensities of DCFH-DA probe by MRSA treated with $PNSG_{58}$-Me-Cl, $PNSG_{58}$-Et-Cl, $PNSG_{58}$-Pr-Cl at 32 μg/mL, 40 mM H_2O_2 as positive control and PBS buffer as blank.

References

1. Y. Deng, H. Chen, X. Tao, F. Cao, S. Trépout, J. Ling and M.-H. Li, *Biomacromolecules*, 2019, **20**, 3435-3444.
2. Z. Wang, M. Lin, C. Bonduelle, R. Li, Z. Shi, C. Zhu, S. Lecommandoux, Z. Li and J. Sun, *Biomacromolecules*, 2020, **21**, 3411-3419.