Support information

Influence of Cationic Groups on Antibacterial Behavior of Cationic Nano-sized Hyperbranched Polymers for Enhancing Bacteria-infected Wound Healing

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Contents

1 Experimental Section

1.1 Materials

Diethylenetriamine, methyl acrylate (MA), methanol, methyl tert-butyl ether, glycidyl trimethyl ammonium chloride(GTA), NaBr, methyl iodide, 1-iododododecane, epichlorohydrin, methyl tert-butyl ether, N,N-Dimethyldodecylamine, disodium phosphate, HEPES (0.5M, pH 7.4), N-phenyl-1-naphthylamine (NPN), potassium dihydrogen phosphate, luria-bertani (LB) broth, LB agar, minimum essential medium (MEM) culture, sterile saline was purchased from Aladdin(Shanghai, China), Macklin (Shanghai, China), Sinopharm (Shanghai, China), Qingdao Hope Bio-Technology Co., Ltd (Qingdao, China), Cienry Biotechnology Co., Ltd (Huzhou, China), or Shanghaiyuanye Bio-Technology Co., Ltd (Shanghai, China), respectively. Besides, fetal bovine serum (FBS), DNA (Herring sperm), ethidium bromide (EB) and ortho-nitrophenyl-β-galactoside (ONPG) and 3,3'-dipropylthiadicarbocyanine iodide ($DiSC_3(5)$) were supplied by Sigma Aldrich (New Jersey, USA) and TCI (Shanghai, China), respectively. Phosphate buffered saline (PBS, 0.03 mol/L, pH 7.4) and ultrapure water (>18 MΩ cm−1) were used throughout this study.

1.2 Characterization and Instruments.

Chemical compositions and surface structures of all functionalized CNHBP polymers were characterized by both X-ray photoelectron spectroscopy (XPS) and nuclear magnetic resonance (NMR). XPS measurements were performed using a Thermo Scientific K-alpha XPS system with a microfocused monochromated Al Kα X-ray source and a 180° hemispherical analyzer with a 128-channel detector using an analyzer pass energy of 50 eV. XPS data were analyzed using Casa XPS software32 and were energy-referenced to the C 1s peak at 284.8 eV binding energy. Samples for XPS were prepared by drop-casting 20 μL of a dilute sample onto doped silicon wafers. ^{1}H NMR measurements were performed using a Bruker Avance-500 spectrometer with a TCI-F cryoprobe. FTIR spectra were recorded by Fourier transform infrared spectroscopy (Nicolet iS50 FT-IR, Thermo Fisher Scientific Corp., USA). The size and

size distribution of CNHBP series were determined by dynamic light scattering system (DLS), using a Brookhaven ZetaPALS particle size and ζ potential analyzer (Brookhaven Instruments Corp., USA). All measurements were repeated three times for each sample. The morphology of CNHBP series were examined by transmission electron microscope (TEM, JEM-2100, JEOL, Japan). The CNHBPs at the concentration of 10 mg/mL was dripped on the copper grids and then dried at room temperature. After drying, copper grids were used for TEM characterization. The critical aggregate concentrations (CACs) of CNHBPs were determined by a conductivity meter (CM, DDS, 11A, China). The measurements were repeated at least twice. Mn (Daltons) was examined via Gel Permeation Chromatography (GPC) method (waters1525 & Agilent PL-GPC220).

1.3 Preparation of Cationic Nano-sized Hyperbranched Polymer (CNHBP) with Different Cationic Groups.

1.3.1 Synthesis of Primary Amine-functionalized CNHBP (P-CNHBP).

P-CNHBP was synthesized by one-pot method. Briefly, Diethylenetriamine (71.91 g) was added into a round-bottomed flask under N_2 atmosphere. MA (60.00 g) dissolved in methanol (30.00 g) was added slowly and dropwise, and then reacted under room temperature for 4 h. After, the mixture was step heated to 100 °C and 120 °C, reacted for 2 h and 4 h under vacuum, respectively. Finally, the resultant solution was precipitated by methyl tert-butyl ether twice, and then dried in vacuum. The product yield was 98.65%.

1.3.2 Determination of Total Ammonia Value for P-CNHBP.

The content of terminal primary amino groups and total amino groups in the product were determined by acid-base titration. The total ammonia value of P-CNHBP can be obtained by above equal. Then continue to titrate when the pH jumps for the second time, the content of primary and secondary amino groups can be further calculated by eq. (1):

$$M_{primary\ amine\ values} = C_{HCl} \times V_{NaOH} - C_{HCl} \times V_{NaOH}\ mmol \quad (1)$$

where, C_{HCl} was the concentration of HCl standard solution, mol/L; V_{HCl} was the volume of added hydrochloric acid standard solution, ml; C_{NaOH} was the concentration of NaOH standard solution, mol/L; V_{NaOH} was the volume of NaOH standard solution added when the first pH jump occurred, mL.

In this experiment, the concentration of HCl standard solution and NaOH standard solution was 0.50 mol/L, respectively. Briefly, 0.11 g P-CNHBP was dissolved in water to get the test solution (20.01 g/mL). And then excess of HCl standard solution was added to make all amino groups convert into salts. Thereafter, the salt solution was titrated with NaOH standard solution to get the titration curve (Figure S1). According to eq. (1) and Figure S1, the total amine value of P-CNHBP was 0.0092 mol/g.

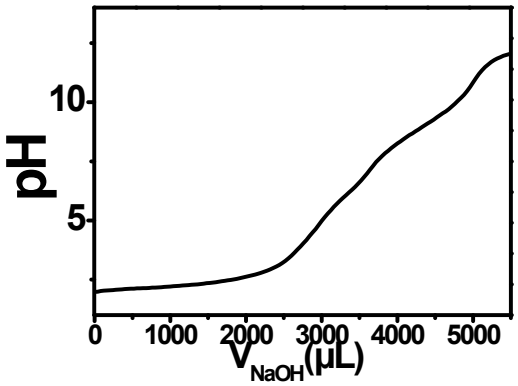


Figure S1. The titration curve of P-CNHBP.

1.3.3 Synthesis of Secondary Amine-functionalized CNHBP (S-CNHBP).

Firstly, P-CNHBP (2.00 g) was dissolved in methanol and methyl iodide (with respect to the molar amount of primary amines of P-CNHBP) was added. The mixture was reacted at 60 °C for 6 h. After, the product was washed with methyl tert-butyl ether three times, and then dried in vacuum. The yield of S-CNHBP was 98.43%.

1.3.4 Synthesis of Quaternary Ammonium-functionalized CNHBP (Q-CNHBP).

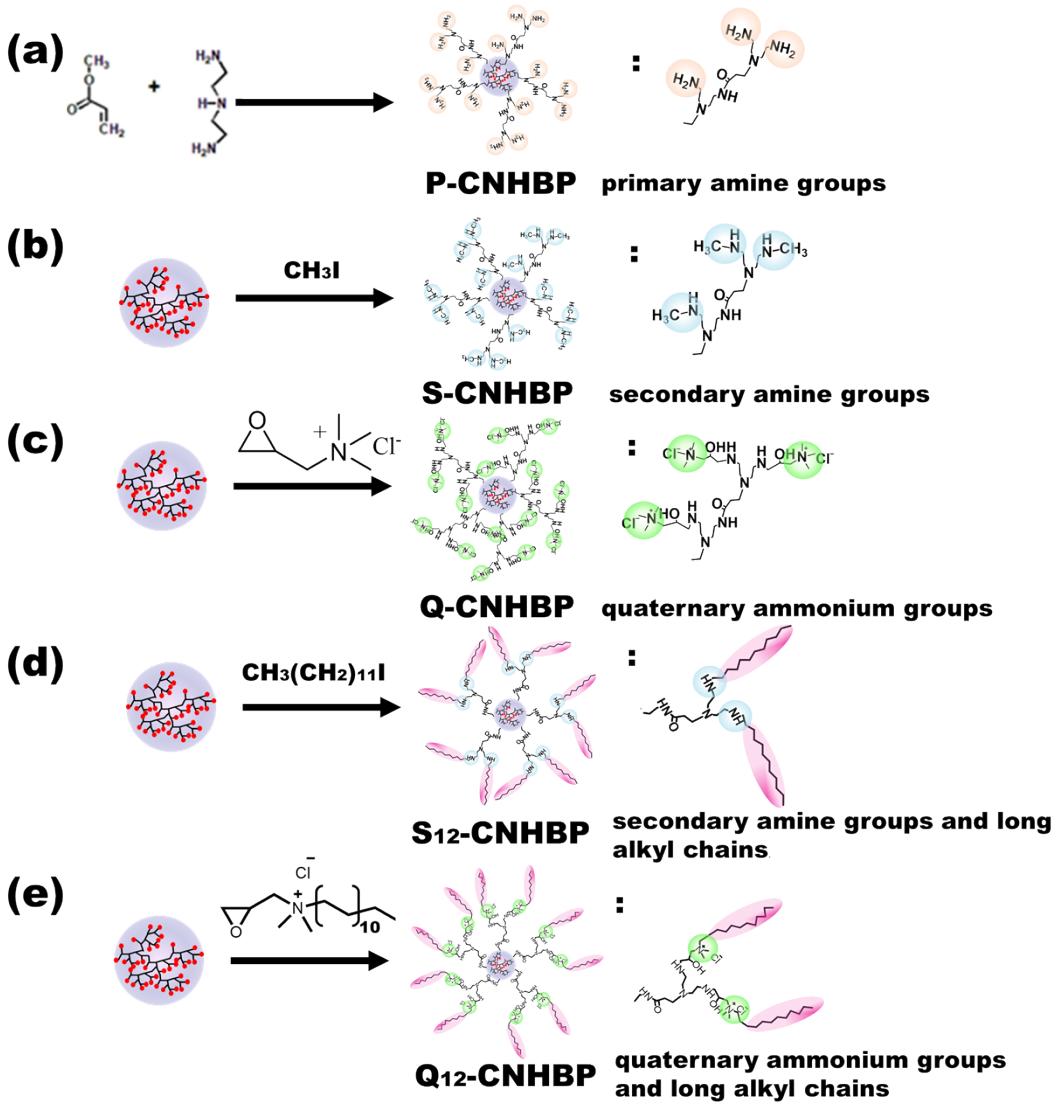
Firstly, 2.00 g P-CNHBP and 4.30 g GTA was added into a round-bottomed flask and then reacted at 60 °C for 6 h. After reaction, the product was washed with ethyl acetate three times and dried in vacuum. The yield of Q-CNHBP was 95.79%.

1.3.5 Synthesis of Secondary Amine-functionalized CNHBP with Aliphatic Long Chains (S_{12}-CNHBP).

Firstly, P-CNHBP (2.00 g) was dissolved in methanol (8.00 g) and then added into a round-bottomed flask. 1-Iodododecane dissolved in methanol (30.00 g) with respect to the molar amount of primary amines was added. The mixture was reacted at 60 °C for 6 h with continuous magnetic stirring and N_2 atmosphere. After reaction, the product was washed with methyl tert-butyl ether, and the product was dried in vacuum and stored at room temperature. Various aliphatic long chains modified CNHBPs termed as CNHBP-A, respectively. The yield of S_{12}-CNHBP was 68.43%.

1.3.6 Synthesis of Quaternary Ammonium-functionalized CNHBP with Aliphatic Long Chains (Q_{12}-CNHBP)

Firstly, quaternary ammonium epoxide (QA-epoxide) was synthesized. Briefly, 0.04 mmol epichlorohydrin was reacted with 0.01 mmol N,N-dimethyldodecylamine at room temperature overnight (∼18 h). The mixture was washed with cold ether (8000 rpm, 5 min) three times. The QA-epoxide were dried in vacuum. Second, P-CNHBP was modified by using QA-epoxide via a ring-opening reaction. Briefly, P-CNHBP (2.00 g) and 4.50 g QA-epoxide (with respect to the total ammonia value of P-CNHBP) were added to a round-bottomed flask and then stirred at 60 ℃ for 6 h. After, the product was washed with ethyl acetate twice, and the product was dried in vacuum and stored at room temperature. The yield of Q_{12}-CNHBP was 97.89%.



Scheme S1. Functionalization scheme of cationic nano-sized hyperbranched polymers (CNHBPs) with different cationic functional groups.

1.4 Method for Molar Concentrations of CNHBPs.

Method used for calculating molar concentrations of CNHBPs: the number average molecular weight, Mn, obtained from GPC analysis was used to convert mass concentrations to molar concentrations. The reader is referred to Supplementary Figure S2 and Supplementary Table S1 for the Mn of CNHBPs.

According to above test, $M_{\text{primary amine values}}$ of CNHBP = 0.0092 mol/g;

Mn of P-CNHBP ≈ 35000 g/mol;

Number of active groups = $M_{\text{terminal primary values}}$ × Mn=0.0092 mol/g × 35000 g/mol ≈ 322;

Mn of S-CNHBP = 35000 g/mol + 322 × 65% × (142 - 128) g/mol ≈ 38000 g/mol;

Mn of Q-CNHBP = 35000 g/mol + 322 × 53% × 152 g/mol ≈ 61000 g/mol;

Mn of Q_{12}-CNHBP = 35000 g/mol + 322 × 64% × 306 g/mol ≈ 98000 g/mol;

Mn of S_{12}-CNHBP = 35000 g/mol + 322 × 42% × (296-128) g/mol ≈ 58000 g/mol;

Thus, molar concentrations of CNHBPs were calculated by eq. (2):

$$Molar\ concentration\ (\mu M) = Mass\ concentration\ (\mu g/mL)/Mn\ (g/mol)\ \times\ 1000 \quad (2)$$

The calculation was exemplified as below:

MBC of Q_{12}-CNHBP against *E. coli* was 78.13 μg/mL;

Mn of Q_{12}-CNHBP was 98000 g/mol;

Therefore,

MBC (μM) =78.13 (μg/mL) / 98000 (g/mol) × 1000 = 0.80 μM.

	Degree of end modification	Mn (g/mol)	Molar concentration (μM)	Zeta potential
P-CNHBP	/	35000	286	1.18±0.49
S-CNHBP	65%	38000	263	1.41±0.25
Q-CNHBP	53%	61000	164	11.10±0.72
S_{12}-CNHBP	42%	58000	172	4.97±0.32
Q_{12}-CNHBP	64%	98000	102	10.03±2.75

Table S1 Parameters of CNHBPs**.**

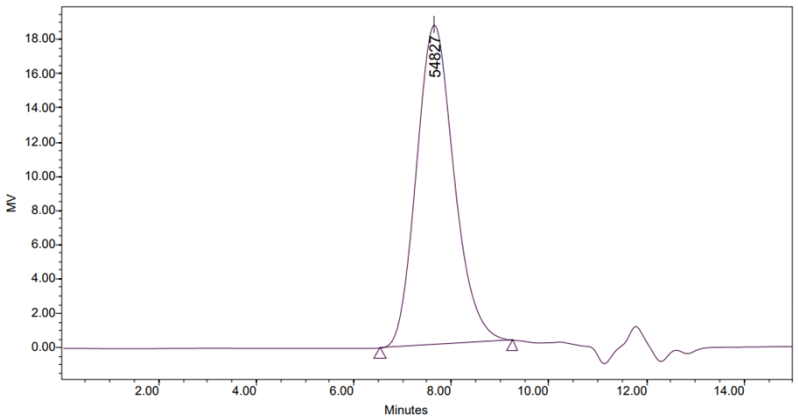


Figure S2. GPC analysis of P-CNHBP.

1.5 Antibacterial Assay.

Firstly, a single colony of bacteria was inoculated into Luria-Bertani broth (LB) agar plates and incubated at 36 °C for 18-24 h. Overnight cultures of test bacteria solution were diluted by LB liquid medium to the optical density (OD) reading of 0.065~0.08 at 660 nm. A stock solution of test CNHBPs solutions were added to polypropylene tubes and diluted with LB liquid medium to the desired concentration. Negative controls consisting of cells without any treatment were used. Positive controls only consisting of LB culture without any cells. After incubation for 22-24 h at 37 °C, the minimum inhibitory concentration (MIC) was judged visually by the transparency of the liquid. The MIC values were listed in Table 1. MBC was taken as the lowest CNHBPs concentration required to eliminate at least 99.99% of viable bacteria. A minimum of three independent experiments (biological replicates) of the assay were conducted, and two technical replicates were used in each experiment.

According to the same method in the MBC measurement described above, for bactericidal dynamics experiments, bacteria were cultured with CNHBPs at MBC concentration, respectively. After gentle shaking at 37 °C for 1, 2, 3, 4, 5, 6, 7, 8, and 24 h, the bacteria samples were taken for a series of tenfold dilutions, and plated out in LB agar plates. The plates were incubated for 24 h at 37 °C and counted for colony-forming units (CFU).

1.6 Hemolysis Assays.

The fresh pig blood with sodium citrate was supplied by Beijing Bersee Science and Technology CO. LTD (Beijing, China). The red blood cells (RBCs) were collected by centrifugation (1500 rpm, 10 min) followed by washing with sterile saline until the supernatant is colorless. RBCs were resuspended in sterile saline (10% v/v RBC) to obtain the RBC suspension. Twofold serial dilutions of test compound were prepared in stroke-physiological saline solution in 2 ml Polypropylene tubes. Tubes with distilled water as positive group. Tubes with sterile saline as negative group. An equal volume (900 uL) of washed erythrocytes was added. Tubes were incubated for 1 hours at 37

°C, at which point tubes were centrifuged and supernatant was transferred to an optically clear, flat, white-wall 96-well plate, taking care not to resuspend unlysed cells. The absorbance of supernatants was then read at an optical density (OD) of 540 nm. Percent hemolysis was calculated using the following eq. (3):

$$Hemolysis\ (\%) = \frac{OD_{540\ of\ sample} - OD_{540\ of\ negative}}{OD_{540\ ofpositive} - OD_{540\ of\ negative}} \times 100$$

(3)

The percent hemolysis was plotted against CNHBPs concentration, and linear regression analysis was used to determine the hemolytic concentration needed to lyse50% (HC50) of RBCs. Two indenpendent runs of the assay were conducted and two replicates were used in each run for each polymer.

1.7 In Vitro Cytotoxicity Assay.

Cell Cultivation.

Mouse embryonic fibroblast cell line L929 cells were cultured in sterilized cell culture flasks supplemented with MEM containing 1% penicillin−streptomycin and 10% FBS at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air, and these L929 cells were passaged by trypsinization before confluence. After several passages, the cultured cells were used for the follow experiments.

Cytocompatibility investigation.

The cytotoxicity of CNHBP series were evaluated by Cell Counting Kit-CCK8 method. Firstly, the samples were autoclave 121 °C for 20 min. Afterword, the samples were immersed in fresh HEM at a ratio of 6 cm2/mL, and then incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air for 24 h and the resulting solutions for use. For testing, the L929 cells were seeded into 96-well plates with 200 μL of fresh medium (MEM) at an initial cell density of 1×10^4 per well. Cultured 1 day, each well of 96-well plates was replaced with 200 μL of the resulting solutions as test group. Blank group only with CCK8 solution. After another day incubated under the same culture condition, the cell viability of difference samples was evaluated by the CCK8

kit at the absorbance of 450 nm. The Cell Viability (%) was calculated by following eq. (4):

$$Cell\ Viability\ (\%) = \frac{OD_{450\ of\ test\ groups} - OD_{450\ of\ blank\ groups}}{OD_{450\ of\ negative\ groups} - OD_{450\ of\ blank\ groups}} \times 100$$

(4)

The percent cell viability (%) was plotted against CNHBPs, and linear regression analysis was used to determine lethal concentration (LC_{50}) that reduced mammalian cell viability by 50% in culture media.

1.8 Cell Membrane Integrity Assay.

Cell membrane integrity was measured by LIVE/DEAD BacLight Viability Kit (ThermoFisher Scientific) and the loss of cytoplasmic constituents. Washed bacteria suspension was incubated with CNHBPs were introduced into the suspensions at 37 °C for 1 h. For tomography imaging, the bacteria were exposed to the mixture stain for 20 min. And then, fluorescence measurements were performed by confocal laser scanning microscopy (CLSM, Leica SP8). For the leakage of cytoplasmic constituents, the bacterial supernatant was obtained via centrifugation (10000 rpm, 5 min) for different intervals (10, 20, 30, 45 and 60 min). The absorbance of resulting supernatant at 260 nm were measured using an UV–vis spectrophotometer (UV2600, Shimadzu, Japan).

1.9 Scanning Electron Microscopy (SEM).

After treatment with CNHBPs, the cell morphologies of microorganisms were observed by FESEM (FESEM, S-4800, Japan). Briefly, the bacterial suspensions (~10^8 CFU/ml) were incubated with CNHBPs for 1 h at 37 °C, the mixtures were collected by centrifuged (10000 rpm for 5 min). The bacterial pellets were resuspended in PBS. Twenty microliters of the resulting suspensions were directly placed onto clean silicon slices. And then, the naturally dried samples were fixed with paraformaldehyde (4%) for 45 min at 4 °C. The fixed samples were washed with PBS three times and dehydrated by 30(0.5 h), 50(0.5 h), 70(0.5 h), 90(0.5 h), 100 % (0.5 h) ethanol. Finally,

the samples were dried and coated with platinum for FESEM observation.

1.10 Transmission Electron Microscopy (TEM).

E. coil or *S. aureus* that was exposed to CNHBPs was washed in PBS (0.1 M, pH 7.4). The exposed bacterial suspension was centrifuged down to a pellet and fixed in 2.5% glutaraldehyde overnight. Removed the glutaraldehyde solution, washed three times with PBS for 15 min, and fixed with 1% osmium acid solution for 1.5h. Fixed cell pellets were washed with PBS for 15 min, dehydrated with increasing concentrations of ethanol solutions (30, 50, 70, 80, 90, 100 and 100% EtOH in water) for 30 min, rinsed with EtOH/acetone (1:1) for 30 min, and rinsed with pure acetone 30 min for two times. Then, the pellets were soaked in a 1:1 acetone/epoxy resin for 2 h, a 1:3 acetone/epoxy resin overnight, a fresh epoxy resin for 8 h, and then finally fixed in pure resin in a vacuum oven. Slices of the pellet were microtomed with a Leica uC6 microtome for imaging. Sections were stained with uranyl acetate and lead citrate and placed on copper TEM grids (Ted Pella Inc.) for imaging.

1.11 The Effect of CNHBPs on Genomic DNA

All samples in this experiment used 0.01 mol/L NaBr aqueous solution as a constant ionic strength solvent to prepare a mixed solution of DNA and CNHBPs, respectively. The blank ionic solution solvent was used as a reference. The absorbance values at 260 nm and 280 nm of the DNA solution were measured to calculate the DNA concentration by using *Lambert Beer's law*, $A = \varepsilon bc$, $\varepsilon = 6600 \text{mo1L-1cm}^{-1}$, and the width of the quartz cuvette b=1cm. If the value of 1.7<A260/A280<1.8, indicating that the DNA concentration meets the requirements of experimental purity. In this experiment, A260/A280=1.73. Twofold serial dilutions of CNHBP solutions were added into PP tubes, and then equal of DNA solution was added. After incubated for 30 min at room temperature, the absorbance of the resulting solutions was measured using an UV–vis spectrophotometer (UV2600, Shimadzu, Japan) at the range of 200 nm-340 nm.

2 Results and Discussion

2.1 Synthesis and Characterization of P-CNHBP, S-CNHBP, and Q-CNHBP.

To verify that CNHBPs were synthesized as expected, ^{1}H NMR spectra of P-CNHBP, S-CNHBP, and Q-CNHBP were characterized. In ^{1}H NMR spectrum of P-CNHBP (Figure S3a), P-CNHBP showed peaks at 3.20 ppm for -C$\underline{H}_2$-CO-NH- group, 2.57 ppm for -C$\underline{H}_2$-NH-CO- group, 2.30 ppm for -C$\underline{H}_2$-NH_2 group, and the peaks at 1.08 ppm for the -(CH_2)n groups. In ^{1}H NMR spectrum of S-CNHBP (Figure S3b), T-CNHBP showed peaks at 4.00 ppm for -C$\underline{H}_3$NH-, 3.20 ppm for -C$\underline{H}_2$-CO-NH- group, 2.57 ppm for -C$\underline{H}_2$-NH-CO- group, 2.30 ppm for -C$\underline{H}_2$-NH_2 group, and the peaks at 1.08 ppm for the -(CH_2)n groups. In Figure S3c, the characteristic chemical shifts of Q-CNHBP occurred at 4.22 ppm for -C$\underline{H}$(OH)-, 3.18 ppm for -N^+(C$\underline{H}_3$)$_3$, 2.01 ppm for -C$\underline{H}_2$-CH-OH and 0.82 ppm for -C$\underline{H}_3$ group, which demonstrated that the correlated functional groups have attached to P-CNHBP, S-CNHBP, and Q-CNHBP surface, respectively. Calculated from ^{1}H NMR data (Figures S3), 65% and 53% of the terminal amine groups were converted for S-CNHBP and Q-CNHBP, respectively.

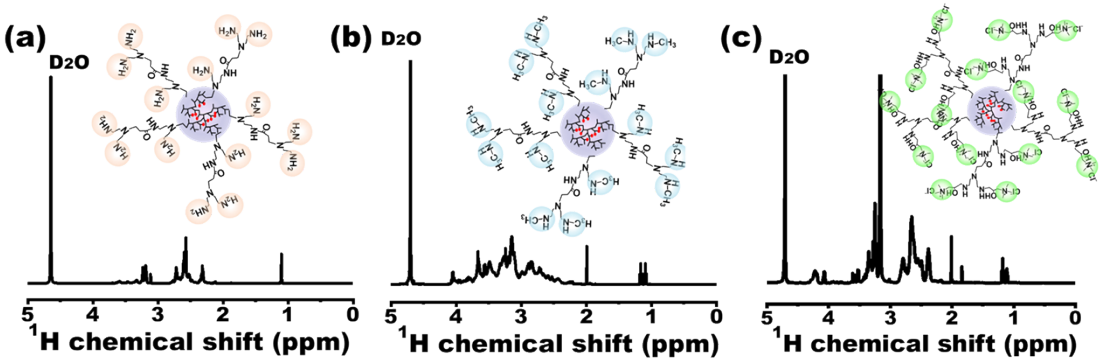


Figure S3. ^{1}H NMR spectra of (a) P-CNHBP, (b) S-CNHBP, and (c) Q-CNHBP.

2.2 Synthesis and Characterization of S_{12}-CNHBP and Q_{12}-CNHBP.

In Figure S4a, S_{12}-CNHBP showed peaks at 3.20 ppm for -C$\underline{H}_2$-CO-NH- group, 2.57 ppm for -C$\underline{H}_2$-NH-CO- group, 2.30 ppm for -C$\underline{H}_2$-NH_2 group, 1.08 ppm for the -(CH_2)n groups, and 0.82 ppm for -C$\underline{H}_3$ group. In Figure S4b, the characteristic chemical shifts of Q_{12}-CNHBP occurred at 4.22 ppm for -C$\underline{H}$(OH)-, 3.18 ppm for -N^+(C$\underline{H}_3$)$_3$, 2.01 ppm for -C$\underline{H}_2$-CH-OH and 0.82 ppm for -C$\underline{H}_3$ group. Above results demonstrated that which demonstrated that the correlated functional groups have attached to the CNHBPs

surface through epoxide-opening reactions and substitution reactions with terminal reactive amine. Calculated from ^{1}H NMR data (Figures S4), 42% and 64% of the terminal amine groups were converted for S_{12}-CNHBP and Q_{12}-CNHBP, respectively.

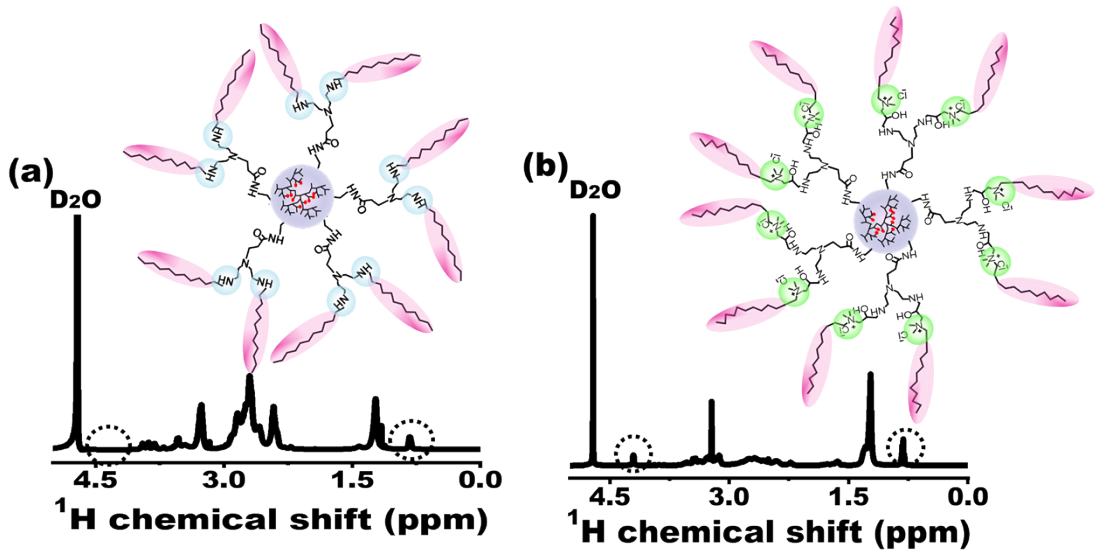


Figure S4. ^{1}H NMR spectra of (a) S_{12}-CNHBP and (b) Q_{12}-CNHBP.

2.3 Synthesis and Characterization of S_{12}-CNHBP and Q_{12}-CNHBP.

Although ^{1}H NMR analysis showed that the correlated functional groups have attached to the CNHBP surface as depicted in Figure S4, further insight into the specific chemical structures of the functional groups is needed. Then the X-ray photoelectron spectroscopy (XPS) of CNHBP was investigated (Figure S5). The nitrogen region (Figure S5a, S5b) for P-CNHBP and S_{12}-CNHBP showed two peaks at 398.4 and 399.2 eV which was often assigned as -C-N/-N-C=O and -C-N/-NH_2, [1] respectively. While the nitrogen region (Figure S5c) for Q_{12}-CNHBP not only showed the nitrogen peaks at 398.4 and 399.2 eV, but also the peaks at higher binding energies (401.8 eV) which have been reported as amines in their protonated states (-NH_3^+, -$N(CH_3)_3^+$). [1–3] The C 1s spectra of all samples showed the peak at 284.6, 285.8, and 287.3 eV, which was corresponded to C-C/C-H [1], C-N/C-O-C [2] and N-C=O [3], respectively. Q_{12}-CNHBP also showed the peaks at 285.2 eV that was assigned as C-O-H. The carbon 1s spectra showed consistent results with the N 1s spectra for S_{12}-CNHBP and Q_{12}-CNHBP surfaces, which further verified the presence of CO-NH, C-N and -$N(CH_3)_3^+$ moieties

in CNHBPs.

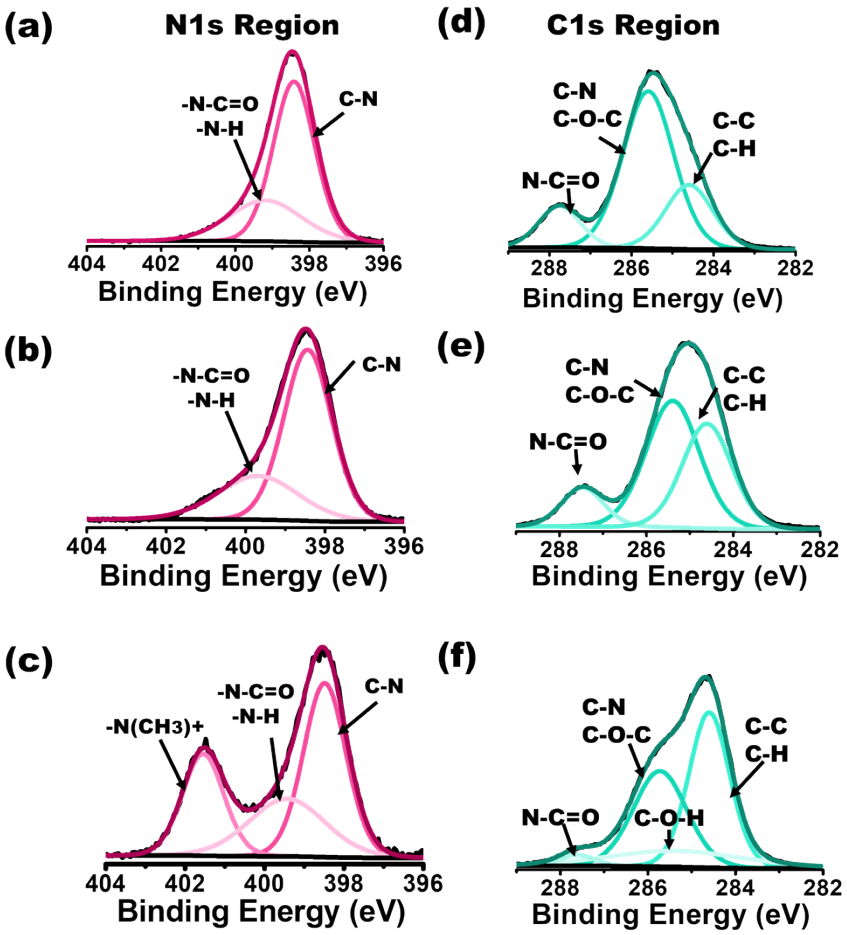


Figure S5. XPS spectra of (a-c) N 1s regions and (d-f) C 1s regions of (a, d) P-CNHBP, (b, e) S_{12}-CNHBP and (c, f) Q_{12}-CNHBP.

2.4 Biocompatibility of Q_{12}-CNHBP

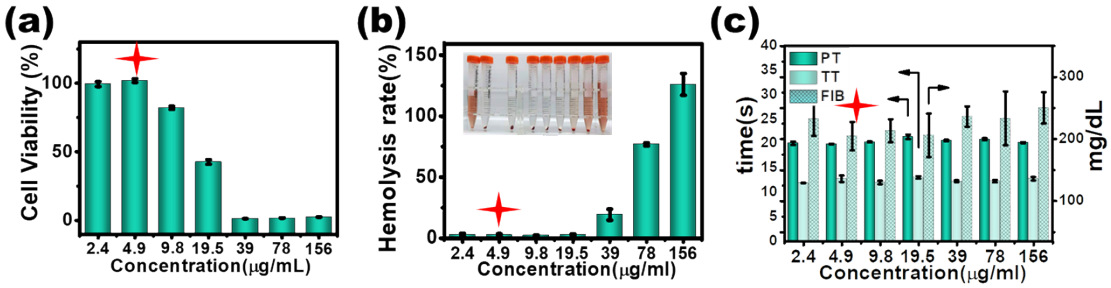


Figure S6. (a) Cell viability of Q_{12}-CNHBP; (b) Hemolysis rate of Q_{12}-CNHBP; (c) Whole blood coagulation test of Q_{12}-CNHBP. Note: red star was represented as MIC of Q_{12}-CNHBP for MRSA.

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