Supporting Information

Surface charge switchable nanoparticles capable of controlled nitric oxide release for treatment of acidity-associated bacterial infection

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Materials

(Methacryloxy)-propyltrimethoxysilane (MPS), hydrofluoric acid, benzoyl peroxide (BPO) were purchased from Tianjin Fuyu Fine Chemical Co. Ltd. Ethyleneglycol dimethacrylate (EGDMA), tetraethyl orthosilicate (TEOS) were purchased from Alfa Aesar and used without purification. Acetonitrile (analytical grade, Tianjin Chemical Reagents II Co.) was dried over 4 Å molecular sieves and purified by distillation. Tetrahydrofuran was refluxed over sodium and then distilled. Trifluoroacetic acid, 2-(ethylamino)-ethanol, *di-tert*-butyl dicarbonate, hydrofluoric acid, methacryloyl chloride, *boc*-L-His(Dnp)-OH, thionyl chloride, methoxy polyethylene glycol amine, *N*-hydroxysulfosuccinimide sodium salt (NHS), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), and 2-mercaptoethanol were purchased from Tianjin Heowns Biochemical Technology Co., Ltd., China. Griess reagent was purchased from Shanghai Beyotime Institute of Biotechnology.

Synthesis and experimental section

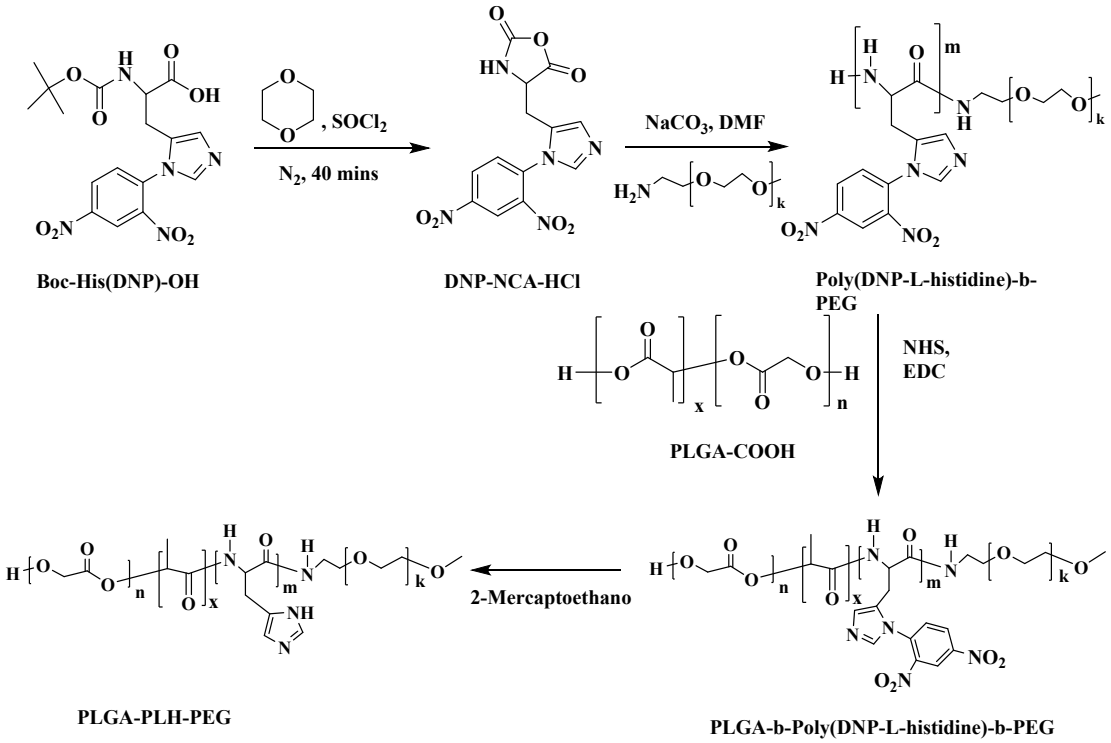
Synthesis of the Hollow P(AmEMA-co-EGDMA) nanoparticles.

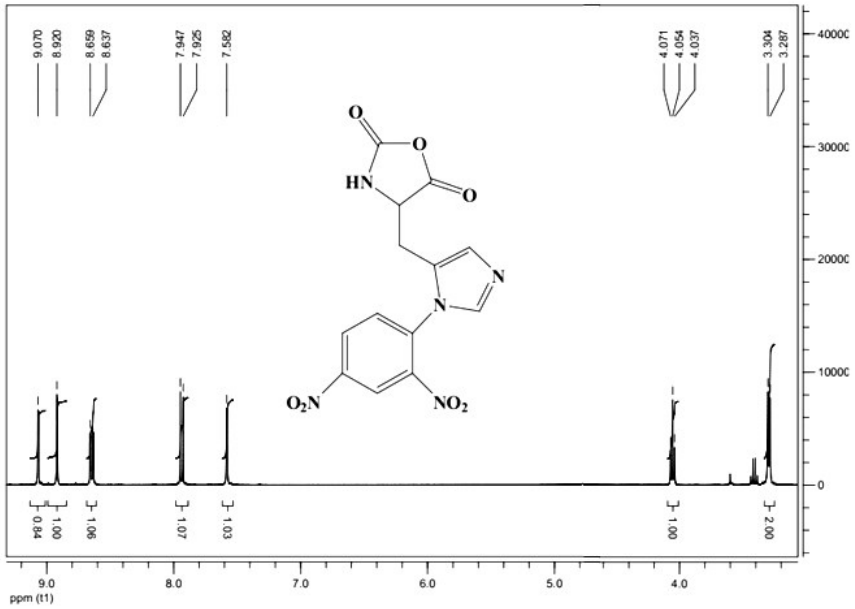
The detailed procedures for the synthesis of monomer 2-(ethyl(*boc*)amino)ethyl methacrylate (*boc*AmEMA), MPS SiO_2 modified nanoparticles, SiO_2-P(*boc*AmEMA-*co*-EGDMA) nanoparticles, and the hollow P(AmEMA-*co*-EGDMA) nanoparticles were described in our previous work (*Polymer Chemistry* **2015**,6, 1512-1520).

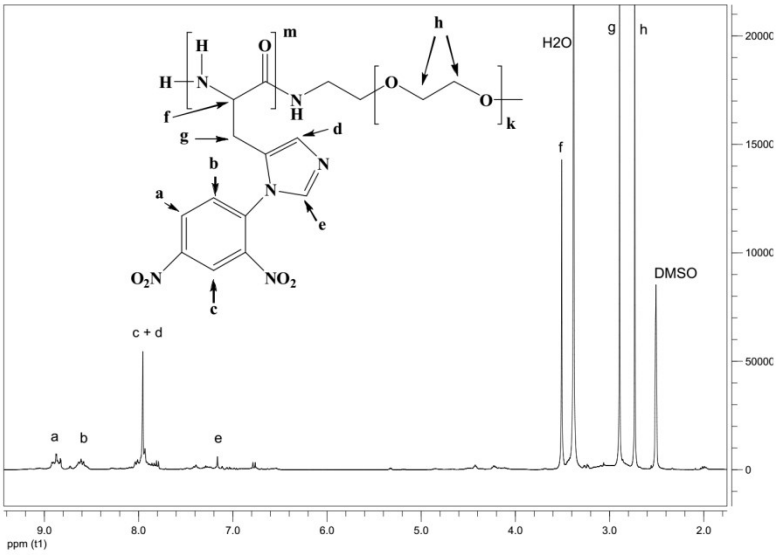
Synthesis of the PLGA-PLH-PEG tri-block copolymers

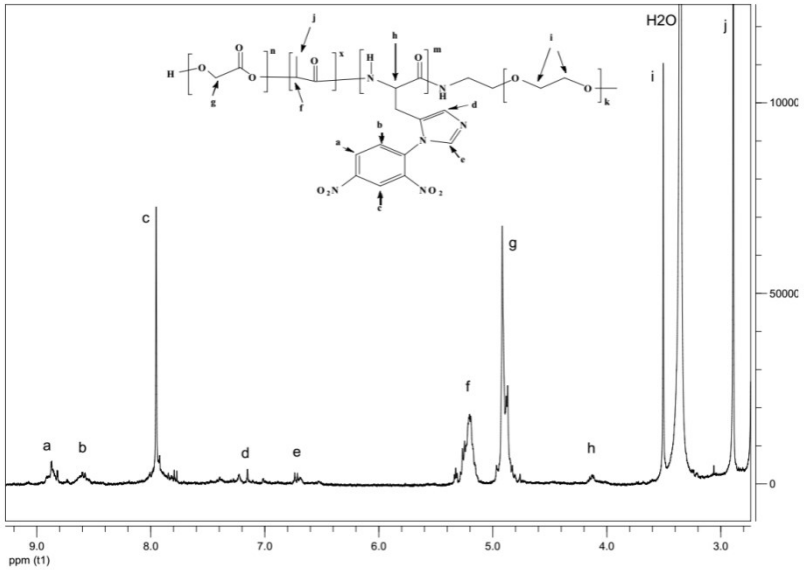
The PLGA-PLH-PEG *tri*-block copolymers were prepared according to the

literature (*International Journal of Pharmaceutics* **2015**, 487, 81-90) with some modification as following:






H2O
DMSO
c + d
a
b
e
f
g
h
ppm (t1)


H2O
a
b
c
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e
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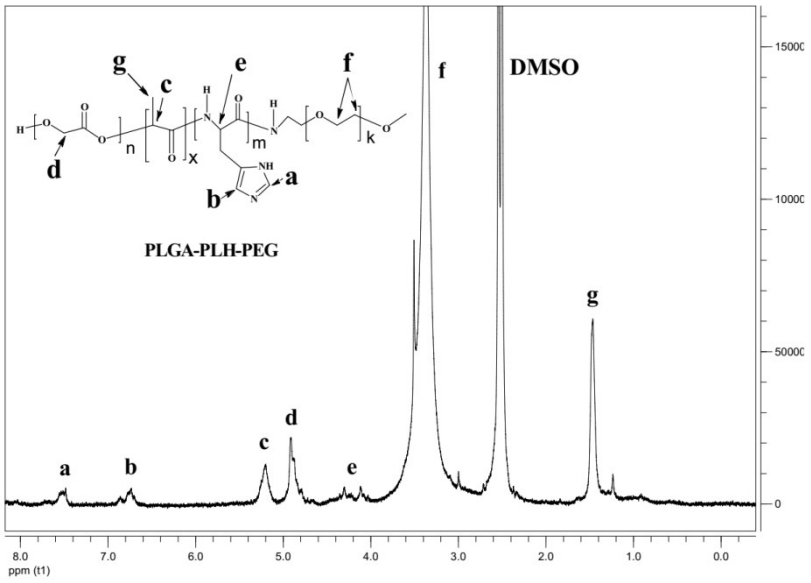
h
i
j
ppm (t1)



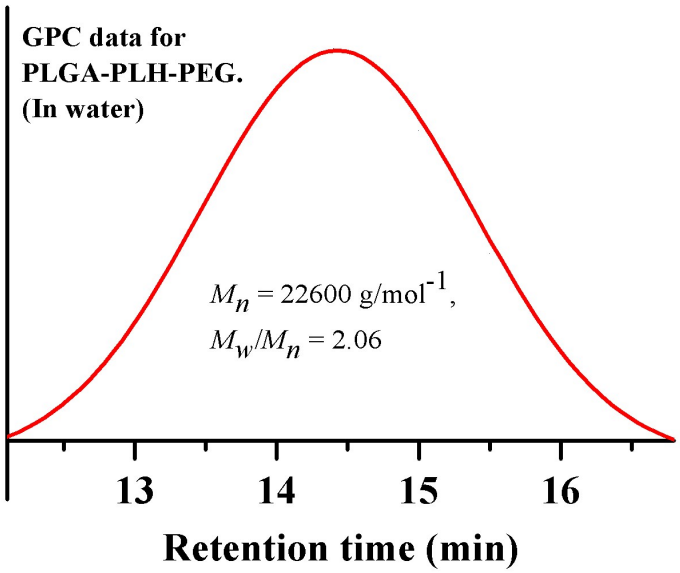


Fig. S1 The synthetic route, NMR, and GPC data of the monomers or polymers. (GPC data, M_n value was calculated relatively to the mono disperse poly-(methyl methacrylate) calibration standards)

4 g of *boc*-His(DNP)-OH was dispersed in 50 mL of 1,4-dioxane. Under the nitrogen protection, 4 mL of thionyl chloride was added into the mixture and stirred for 40 mins. After the reaction, the mixture was added into the ice cold diethyl ether

to get the crude DNP-NCA-HCl products. The crude products were dissolved in acetone to remove the insoluble impurities, and drip added at ice cold diethyl ether for purification. The pure DNP-NCA-HCl products were vacuum dried for further utilization.

1 g of DNP-NCA-HCl and 0.41 g $NaCO_3$ was added into 20 mL of dry DMF and stirred for 1 hour. Then, 130 mg of methoxy polyethylene glycol amine (M_W ~ 2000) was added into the mixture and stirred for 3 days. After the reaction, the solvent was distilled and the crude products were added into ice cold diethyl ether to obtain poly (DNP-*L*-histidine)-*b*-PEG product.

100 mg of PLGA-COOH (M_W ~ 5000) was added into 5 mL of dichloromethane. Then, 175 mg of EDC-HCl and 126 mg NHS was added into the mixture and stirred for 60 mins at room temperature. After the reaction, the mixture was precipitated in ice cold ethanol and dried under vacuum to obtain PLGA-NHS. Then, 50 mg PLGA-NHS and 50 mg of poly (DNP-*L*-histidine)-*b*-PEG was added into the 5 mL DMF, following with the addition of 12 μL *N, N*- diisopropyl ethylamine. After 12 hours, the mixture was added into ice cold methanol/ether (v/v, 1/1) to obtain PLGA-*b*-Poly(DNP-*L*-histidine)-*b*-PEG which was vacuum dried.

400 mg of PLGA-*b*-poly(DNP-*L*-histidine)-*b*-PEG was added into 20 mL of dry DMF. Then, with the nitrogen protection, 2-mercaptoethanol was dropped into the mixture and stirred for 12 hour at room temperature. After the reaction, the mixture was dropwise add into methanol to precipitate the PLGA-*b*-Poly(*L*-histidine)-*b*-PEG *tri*-block copolymers.

Preparation of the Hollow Bilayer PLGA-PLH-PEG-P(AmEMA-co-EGDMA) nanoparticle

50 mg of PLGA-*b*-Poly (*L*-histidine)-*b*-PEG *tri*-block copolymers were dispersed and swelled in 5 mL DMSO. To this mixture, 10 mL deionized water containing 100 mg of hollow P(AmEMA-*co*-EGDMA) nanoparticles was added into dropwise. After the reaction was stirred for DMSO physical evaporation in two days, the mixture was centrifugated to obtain the bilayer hollow PLGA-PLH-PEG-P(AmEMA-*co*-EGDMA) nanoparticles.

Preparation of the PLGA-PLH-PEG@N-diazeniumdiolated bilayer nanoparticles (PNBNPs)

In the presence of 100% excess (with respect to the secondary amine site concentration) of sodium methoxide in dry THF, the bilayer hollow PLGA-PLH-PEG-P(AmEMA-*co*-EGDMA) nanoparticles were stirred strongly under pure NO gas at 5 atm at room temperature for 24 h after being decentralized by ultrasonic-irradiation in dry THF. During the experiment, pure nitrogen was bubbled through the reaction system for 10 min to remove oxygen. NO with a high purity was then bubbled through the system for 10 min, further pressurized till 5 atm and sealed for diazeniumdiolation. After the reaction, the resulting microspheres were purified by three cycles of centrifugation, decantation, and resuspension in dry solvents. The diazeniumdiolated microspheres (PNBNPs) were dried in a vacuum oven at 50 °C for 2 h, and stored under nitrogen in the freezer.

Characterization and analysis of the nanoparticles

The morphology, particle size, and size distribution of the nanoparticles were analyzed by TEM (JEOL JEM-2100, Japan). All of the TEM size data reflected the averages of the particles, which were calculated by the formulae as follow.

$U = D_W / D_n$,

$$D_n = \sum_{i=1}^{k} n_i D_i / \sum_{i=1}^{k} n_i ,$$

$$D_w = \sum_{i=1}^{k} n_i D_i^4 / \sum_{i=1}^{k} n_i D_i^3$$

where, U is the poly dispersity index, D_n is the number-average diameter, D_w is the weight-average diameter, D_i is the particle diameter of the determined nanoparticles.

Fourier transform infrared spectra (FTIR) were carried out by a Bruker Alpha FTIR spectrometer over potassium bromide pellets and the diffuse reflectance spectra were scanned.

The elemental analyses (EA) were determined by a Perkin Elmer 2400 to determine the carbon, hydrogen, and nitrogen contents of the nanoparticles.

Dynamic light scattering (DLS) characterization was performed using a laser light scattering spectrometer (BI-200SM) equipped with a digital correlator (BI-9000AT) at 532 nm.

The zeta potential was determined with a Zeta Pals (Brookhaven Instrument Co., US) by measuring the electrophoretic mobility of the nanoparticles using different pH buffers as the solvents.

NO release behavior investigation

The kinetics of nitric oxide release in aqueous solutions in different buffer (pH =

6.0 or 7.4) was determined using the Griess kit assay. A typical procedure was as follows: 5.0 ml buffer with 5 mg of diazeniumdiolated microspheres were stirred strongly at 37 °C after ultrasonic-irradiation in a sealed bottle. After pre-determined time intervals, 1.0 mL supernate was taken out from the suspension after ultra-centrifugation. 1.0 mL of aqueous solutions was added sequentially. Then 100 μl of the supernate was added to 100 μL Griess I and 100 μL Griess II. An azo compound of purple color was formed. The absorbance at a wavelength of 540 nm is measured at suitable concentrations using a UV spectrometer. The total amount of NO release ($T_{[NO]}$) and the NO release kinetics were obtained.

Bacteria binding investigation experiments

The flow cytometry was utilized to detect the fluorescence intensity which revealed the nanoparticles binding capacity with the bacteria.

For the bacterial culture, *E. coli* (ATCC11229) was cultured in LB broth (BD244620). Then, colonies were streaked on LB-agar plate, selected, inoculated into growth medium (5 mL), and allowed to grow overnight in incubated shaker at 37 ^{o}C. *S. aureus* (ATCC25923) was cultured in a similar fashion using tryptic soy broth (TSB, BD211825).

After the culture, the bacteria was spun down, washed in saline solution, and resuspended in buffer with different pH (pH = 5.0, 6.0, or 7.4). The mixture was then added 1 mg of PNBNPs or the bare hollow *N*-diazeniumdiolated P(AmEMA-*co*-EGDMA) nanoparticles dispersed in 100 μL of buffer, and incubated for 30 mins.

Then, the bacteria were spun down, unbound nanoparticles in the supernatant were removed. Bacteria were resuspended in PBS of pH = 7.4 and their total fluorescence were measured by BD FACSVerse flow cytometry. In each experiment, 10 000 events per sample and the median of the fluorescence intensity in the FITC channel was calculated for each population. The relative intensity of fluorescence was determined as the relative nanoparticles binding capacity with the bacteria.

In vitro antibacterial activity experiments

S. aureus was selected as a model to test the antibacterial activity of the PNBNPs, bare hollow *N*-diazeniumdiolated P(AmEMA-*co*-EGDMA) nanoparticles, hollow PLGA-PLH-PEG-P(AmEMA-*co*-EGDMA) nanoparticles scaffolds, or free vancomycin in media with different pH (pH = 6.0 or 7.4).

Briefly, *S. aureus* bacteria were inoculated into 5 mL of tryptic soy broth (TSB). After 2 hours, when the enter log phase (OD_{600}) achieved around 0.3, different reagents diluted into a final volume of 100 μL of sterile water in triplicate at a clear flat-bottom 96-well plates. Bacteria in log phase were diluted to a theoretical OD_{600} of 0.001 in TSB with pH 7.4 or 6.0 and seeded onto the microplates to produce a final volume per well of 200 μL. The OD_{600} was measured immediately before placing into an incubated shaker at 37 ^{o}C, and then again around 4 h later. The relative *S. aureus* bacterial viability and the MIC (Minimum Inhibitory Concentration) was determined. No change in pH with time was detected.

In vitro normal cells cytotoxicity assay

We selected normal mouse fibroblasts cells lines as model to evaluate the cytotoxicity of PNBNPs and the bare hollow *N*-diazeniumdiolated P(AmEMA-*co*-EGDMA) nanoparticles at normal physiological pH.

In brief, the normal mouse fibroblasts cells were cultured in DMEM with 10 % FBS, 100 μg/mL streptomycin, and 100 U/mL penicillin. The cells were seeded in 96-well plate with concentration of 1×10^4 cells/well and incubated in humidified incubator (5 % CO_2, 37 °C) for 12 h. Then, the cell medium was replaced by fresh medium containing PNBNPs or Bare Hollow *N*-diazeniumdiolated P(AmEMA-*co*-EGDMA) nanoparticles with concentration of 2.5 mg/mL and incubated for 24 h. DMEM without cell sample was set as the control group. After incubation, the cell medium was removed and 100 μL of the 10 % CCK8 (Cell Counting Kit-8) solution were added into for 30 min incubation. The absorbance was measured by an ELISA Reader at 450 nm. The cell viability was calculated using the equation: cell viability (%) = 100 * (A-B)/(C-B).where A, B, and C were the absorbance value of the sample solution, background, and fresh medium aperture control group respectively, which were measured by a microplate reader.

In vivo antibacterial activity experiments

Poly-dimethylsiloxane slices (PDMS) were chosen and incubated in the *S. aureus* bacterial medium (10^8 CFU/mL) for 24 h at 37 °C under 5 % CO_2 atmosphere. After the bacterial biofilms were formed at the surface, the PDMS slices were implanted into the mice back between the epidermis and the muscle by surgery. After 12 hours, 0.5 mL of saline solution (Control 1), bare hollow *N*-diazeniumdiolated P(AmEMA-

co-EGDMA) nanoparticles dispersion (3.6 μmol/mL of NO-loading amount, *MIC* at pH = 7.4.), free vancomycin (2.0 μg/mL, *MIC* at pH = 7.4. Control 2), or PNBNPs dispersion (3.6 μmol/mL of NO-loading amount) were intravenously injected every 24 hours. After 3 days' administrations, the implanted slices were taken out by surgery and placed in 5 mL saline solution. After ultrasonic dispersion for 2 mins, the solution was diluted and cultured on solid medium. The total bacteria amount in every dish was calculated by a colony number (the bacteria number was the colonies number multiplied by the corresponding dilution factor).

The muscle tissues around the implanted site were also taken and handled for bacteria spread investigation by H&E staining images under an Olympus fluorescence microscope (Japan, IX71).