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pH Responsive Zwitterbian-to-Cationic Transition for Safe Self-defensive Antibacterial Application

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1. Materials

Polypropylene (PP) is cheap and easy to manufacture and has good mechanical properties. PP films were cut into 1.0 × 0.8 cm^2 piece and were conducted with plasma treatments using air as a carrier gas. 2-Isopropylthioxanthone (ITX), glycidyl methacrylate (GMA), *N*-(tert-butoxycarbonyl)-N'-methylethylenediamine (MED-Boc), methyl iodide (CH_3I), n-butyl bromide (C_4H_9Br), 1-Bromooctane ($C_8H_{17}Br$), 1-Bromododecane ($C_{12}H_{25}Br$), 2,3-dimethylmaleic anhydride (DMMA), 2-(*N*-morpholino) ethanesulfonic acid (MES), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS) and Trifluoroacetic acid (TFA) were purchased from Aladdin Chemistry (Shanghai, China). Gram-positive *Staphylococcus aureus* (*S. aureus*; ATCC 6538) were obtained from Nanjing Clinic Biological Technology Co. Ltd. Luria-Bertani (LB) broth, LB-agar and Phosphate buffered solution (PBS; 0.1 mol L^{-1}, pH 7.4) were provided by Dingguo Biotechnology (China). All other chemicals (AR grade) were used as received directly without further purification.

2. Synthesis of dual function antimicrobial surface on PP substrates

Schematic diagram of this scheme as shown in **Figure S1**. First, 10 μL of 30% ITX in acetone was added to the surface of the pristine PP, and the quartz piece was covered. Under the UV light of 254 nm, after 20 min of irradiation, ultrasonic cleaning with acetone for 10 min, and then dried in the dark. The GMA monomer was dissolved in n-butanol solvent (20 μL, 5 vol%), added to the surface of the PP grafted with ITX, and then subjected to UV irradiation (high-pressure mercury lamp, 400 W, main wavelength 365 nm) for 5 min. The resultant PP substrates with poly (GMA) brushes (denoted as PP-*g*-PGMA) were washed with ethanol to remove the physically adsorbed monomer, followed by drying in a vacuum oven. MED-Boc was chemically fixed on a poly (GMA) brush at 80 °C for 3 h to obtain PP-N-MED-Boc. The PP-N-MED-Boc films was placed into 10% *N*, *N*-dimethylformamide solvent of CH_3I, C_4H_9Br, $C_8H_{17}Br$ and $C_{12}H_{25}Br$, and stirred at 40 °C overnight. The surfaces obtained by the above reaction were named PP-N^+C_1-MED-Boc, PP-N^+C_4-MED-Boc, PP-N^+C_8-MED-Boc and PP-N^+C_{12}-MED-Boc. The above membrane was deprotected by a mixed solution of TFA and dichloromethane to obtain PP-N^+C_1-NH_2, PP-N^+C_4-NH_2, PP-N^+C_8-NH_2 and PP-N^+C_{12}-NH_2, respectively. In order to finally form the membrane surface into an zwitterionic antibacterial surface, the membrane is placed in a tetrahydrofuran solution containing 5% DMMA to finally obtain an zwitterionic antibacterial having a pH-responsive surface, then named PP-N^+C_1-DMMA, PP-N^+C_4-DMMA, PP-N^+C_8-DMMA and PP-N^+C_{12}-DMMA. The final film surface obtained above was ultrasonically cleaned with an ethanol solution for 10 min to

remove the physically adsorbed material and facilitate the next reaction. And vacuum drying and then proceeding to the next step.

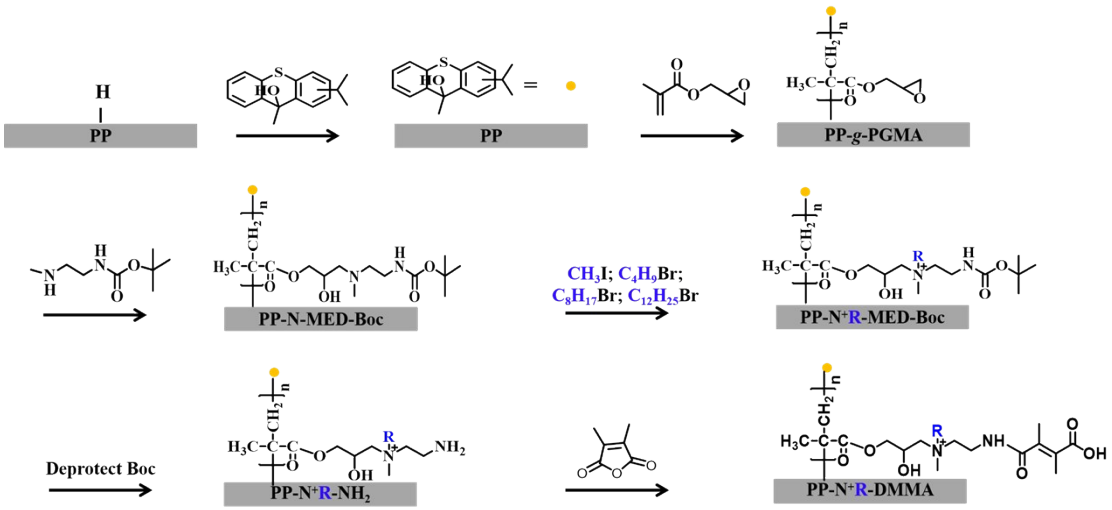


Figure S1. Preparation of PP substrates with self-adaptive antibacterial surface.

3. Surface characterization

FTIR-ATR spectra were obtained from a Fourier transform infrared spectrometer (FTIR; Bruker Vertex 70).

X-ray photoelectron spectroscopy (XPS, VG Scientific ESCA MK II Thermo Avantage V3.20 analyzer equipped with an Al Kα anode mono-X-ray source (hv = 1486.6 eV) was applied to determine the surface composition. Surface spectra were collected over a range of 0-1200 eV and high-resolution spectra of C 1s, N 1s, and O 1s regions were also provided. The binding of the C-C component of C 1s at 284.7 eV was used as a reference for all binding energies (BE). Under these conditions, the full-widths-at-half-maximum (fwhms) of the sample was set to 1.3 eV.[1]

The static water contact angle (WCA) of each sample was obtained by using a

contact angle goniometer (DSA KRÜSS GMBH, Hamburg 100) at room temperature. 2 μL of distilled water was injected on the surfaces and WCA was recorded. Three measurements were taken across each sample for calculating the average value.

The density of surface quaternary ammonium salt group was determined by fluorescein binding quantity per unit area. PP-N^+C_1-NH_2, PP-N^+C_4-NH_2, PP-N^+C_8-NH_2 and PP-N^+C_{12}-NH_2 were placed on the surface of 3 mL of 1 wt % fluorescein (Sodium salt) distilled water for 10 min. The PP-N^+C_1-NH_2, PP-N^+C_4-NH_2, PP-N^+C_8-NH_2 and PP-N^+C_{12}-NH_2 films were then rinsed with distilled water and placed in 0.1% cetyltrimethylammonium chloride solution of 3 mL.[2] The sample was shaken on a rail shaker for 15 min at 100 rpm to remove the dye. Finally, 100 mM phosphate (pH 8.0) of 10% v/v was added to determine the absorbance of the solution at 501 nm. The number of quaternary ammonium on the surface of each sample is the number of cation, which is quantified by Beer-Lambert's law with extinction coefficient of 77 $mM^{-1} \cdot cm^{-1}$.

4. DMMA released from the surface

DMMA hydrolysis of PP-N^+C_8-DMMA was monitored by fluorescent imaging. PP-N^+C_8-DMMA samples were activated in a MES solution (pH 5.5) containing 0.4 M EDC and 0.1 M NHS at 4 °C for 1 h, and then incubated with a solution of 5'-aminofluorescein in N,N-dimethylformamide (2 mg mL^{-1}) at 25 °C for 1 h. The sample was immersed in a buffer solution of pH 5.5, pH 6.0, pH 6.5, pH 7.0 and pH 7.4 for 4 h, and then washed 3 times with ultrapure water, followed by lyophilization.

The samples were observed by confocal laser scanning microscopy (CLSM; LSM 700, Carl Zeiss). The formula for calculating the percentage of 5'-aminofluorescein release on the PP-N^+C_8-DMMA:

Fluorescence Realeasing (%)=(sample-negative control)/(positive control-negative control)×100% E. 1

Negative control group is the reaction of PP surface with 5'-aminofluorescein. The positive control group is the reaction of PP-N^+C_8-DMMA surface with 5'-aminofluorescein.

5. Bacteria adhesion assay

S. aureus was incubated overnight at 37 °C on separated Luria-Bertani (LB) agar plates. A single colony was incubated in 50 mL LB broth overnight at 37 °C. *S. aureus* were collected and centrifuged at 3000 rpm for 10 min to remove the supernatant. The *S. aureus* was resuspended in sterile PBS solution and configured into a bacterial solution having a concentration of 10^8 cells of mL^{-1}. 20 μL of the *S. aureus* suspension was pipetted onto the sample and covered with a sterile PE membrane and incubated at 37 °C for 1.5 h. After the incubation, the sample was gently washed with PBS, and the sample was placed in PBS (2 mL) and sonicated for 3 min to release the adhered bacterial cells into PBS. The resulting suspension was serially diluted, and after incubation at 37 °C for 24 h, the number of colony forming units (CFU) was determined by plate counting on agar.

500 μL of 10^8 cells of mL^{-1} bacterial solution was placed into a well-plate

containing different samples, incubating for 1.5 h at 37 °C. The sample was gently washed with ultrapure water, stained for 15 min in the dark with 20 μL of LIVE/DEAD BacLight Viability Kit, and then washed three times with ultrapure water. CLSM is used to detect dead and living bacteria on the surface.

The morphology of the adherent *S. aureus* was evaluated using field emitted scanning electron microscopy (SEM, XL 30 ESEM FEG, FEI Company, USA). 500 μL of bacterial solution was placed into a well-plate containing different samples, incubating for 1.5 h at 37 °C. Then the samples were immobilized by 4% paraformaldehyde for 12 h, dehydrated with a serial of ethanol/water mixtures (30%, 50%, 70%, 90%, and 100%) and observed bacterial morphology by SEM.

6. Antibacterial assay

A colony from a LB agar plate that was prepared as described above was grown in LB liquid medium overnight at 37 °C. The *S. aureus* was diluted in LB to a concentration of 10^6 cells mL^{-1}. The number of surface live *S. aureus* cells was determined using a spread plate method. On the surface of each sample, 10 μL 10^6 cells mL^{-1} bacterial solution was added, and then covered with sterilized PE film, incubating at 37 °C for 24 h. After 24 h, the two membrane colleagues were placed in PBS (2 mL), treated with ultrasound for 3 min, and coated with 100 μL bacterial suspension and agar plate. After culture at 37 °C for 24 h, the number of CFUs was determined by agar plate counting method.

500 μL of bacterial solution was placed into a well-plate containing different

samples, and then incubated for 24 h at 37 °C. After incubation, the substrates were washed thrice with PBS. The samples were observed under CLSM and SEM as described above. CLSM images were analyzed using Image J software. The killing efficiency was determined by dividing the area of dead bacteria by the total area of both live and dead *S. aureus*.

7. Surface transformation assay

The *S. aureus* was diluted in LB to a concentration of 10^6 cells mL^{-1}. 500 μL of bacterial solution was placed into a well-plate containing different samples, and then incubated for 2 h, 4 h, 8 h, 12 h, 24 h at 37°C. After incubation, the substrates were washed thrice with ultrapure water. The samples were observed under CLSM and SEM as described above.

8. Platelets and red blood cells (RBC) adhesion

Fresh blood was obtained from a healthy rabbit in accordance with the guidelines issued by the ethical committee of the Chinese Academy of Sciences. The blood was anticoagulated with a 3.8 wt % sodium citrate solution at a dilution ratio of 9:1, and centrifuged at 1000 rpm 15 min to separate platelet rich plasma (PRP) and red blood RBC. The collected RBC was further diluted to a concentration of 2 vol% with PBS. 20 μL of PRP or RBC solution was respectively added onto the samples, and incubated at 37 ºC for 1 h. Samples with adhering platelets were additionally rinsed twice with PBS, then all the samples were fixed with 4% paraformaldehyde for 12 h, and dehydrated by sequential immersion steps as mentioned before. Finally, the

morphologies of adhered PRP or RBC on the surfaces were visualized by SEM.

9. Hemolytic assay on surface

The collected rabbit RBC for hemolytic assay was diluted to 5 vol% with PBS. 200 μL of RBC solution was added onto the top of samples, and each sample was then covered with an identical sample to ensure the solution fully spread over the surface. The samples were incubated at 37 °C for 1 h to allow for the hemolysis process to take place. The RBC solution was carefully collected and centrifuged at 3000 rpm for 5 min. The supernatant was transferred to a 96-well plate, and hemoglobin release was measured by UV-absorbance at 545 nm using a microplate reader (TECAN, Switzerland). The hemolysis ratio (HR) was calculated according to the following formula:

$$HR\ (\%) = [(OD_{test}-OD_{neg})/(OD_{pos}-OD_{neg})]\times 100\% \qquad \text{E. 2}$$

where OD_{test}, OD_{pos}, and OD_{neg} are absorbance value of the test sample, positive and negative control, respectively. Results were given as the mean of triplicate experiments and standard deviation.

10. Cytotoxicity test

The standard methyl thiazolyltetrazolium (MTT) assay was applied to investigate the cytotoxicity of the samples. NIH-3T3 mouse fibroblasts in RPMI 1640 medium (1 mL, 10^5 cells mL^{-1}) were seeded in each well of a 48-well plate, and incubated in a humidified atmosphere of 5% CO_2 at 37 °C for 24 h. After refreshing the medium, the

samples were added into the wells. After 24 h of incubation at 37 °C, 100 μL of MTT solution (0.5 g mL^{-1} in PBS) were added into the wells. After 2 h of incubation, 100 μL of the supernatant was transferred to a 96-well plate and the absorbance of the solution at 490 nm was measured using a microplate reader. The parallel experiment without the samples was conducted as a blank control. The results were expressed as percentages relative to the control experiment.

11. *In vivo* implantation

All animal experiments were conducted in accordance with the guidelines of the Animal Care and Ethics Committee of Changchun Institute of Applied Chemistry, Chinese Academy of Sciences. Pristine PP and PP-N^+C_1-DMMA were cut into of 0.1×0.3 cm^2 rectangular membrane and aseptically processed, implanted into the back tissue of female BALB/c mouse (6-8 weeks) to evaluate the biocompatibility of the material. Five days after the implantation, the mice were dissected, and then the tissue inflammation reaction of the mice was observed with eyes, followed by H&E staining by soaking the tissue of the back contact material with 4% paraformaldehyde for 24 h. The slides were visualized using fluorescence microscope.

For the infectious mouse model, the Pristine PP and PP-N^+C_1-DMMA membranes were incubated with 500 μL of 10^8 cells of mL^{-1} *S. aureus* for 6 h in vitro and then implanted into mice. Five days after implantation, the extent of the associated inflammation was visually examined, the relevant tissue around the implant site was removed, and histological analysis was performed as described above. In order to

detect the numbers of bacteria in the tissue, the implanted sample and surrounding tissue were immersed in PBS buffer and sonicated for 3 min, 100 μL of the bacterial solution was applied to the agar plate and cultured at 37 °C for 24 h. Bacteria were counted and statistically analyzed.

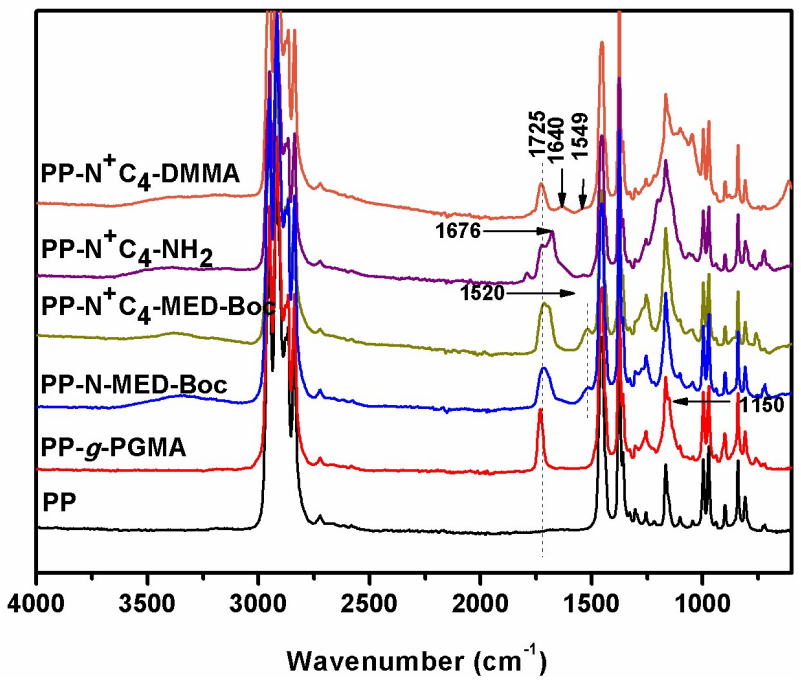


Figure S2. The process of ATR-FTIR spectrum from PP, to grafting GMA, fixing n-butyl bromide and finally forming an internal salt structure.

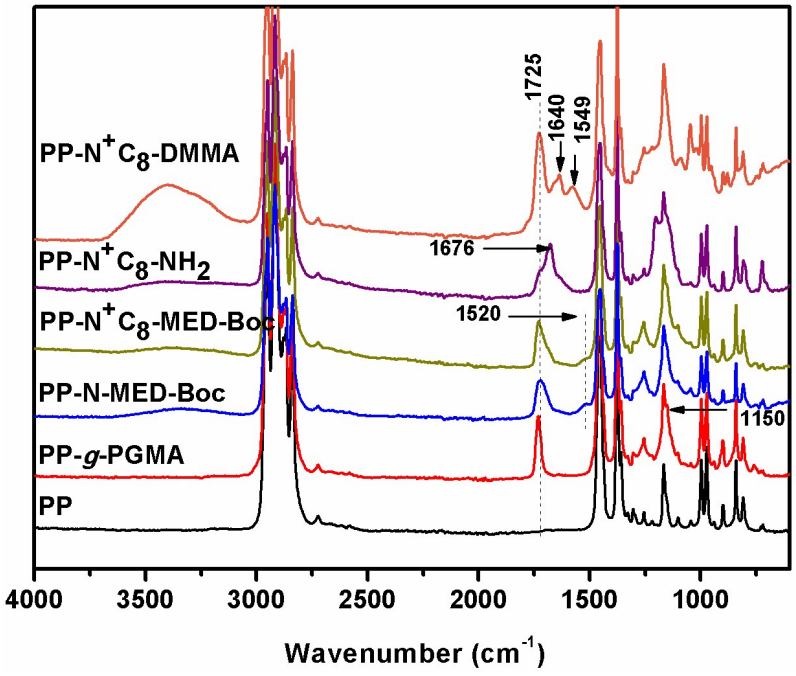


Figure S3. The process of ATR-FTIR spectrum from PP, to grafting GMA, fixing 1-Bromooctane and finally forming an internal salt structure.

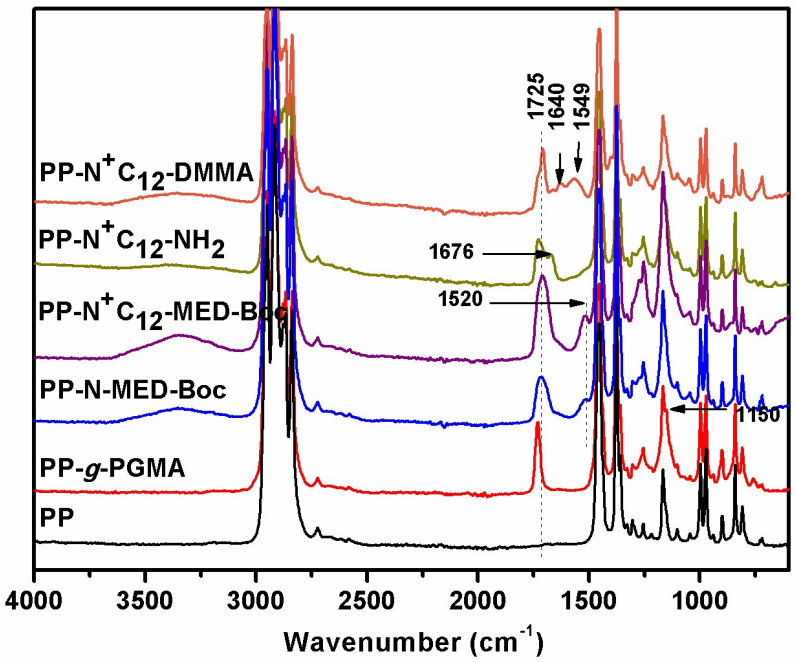


Figure S4. The process of ATR-FTIR spectrum from PP, to grafting GMA, fixing 1-

Bromododecane and finally forming an internal salt structure.

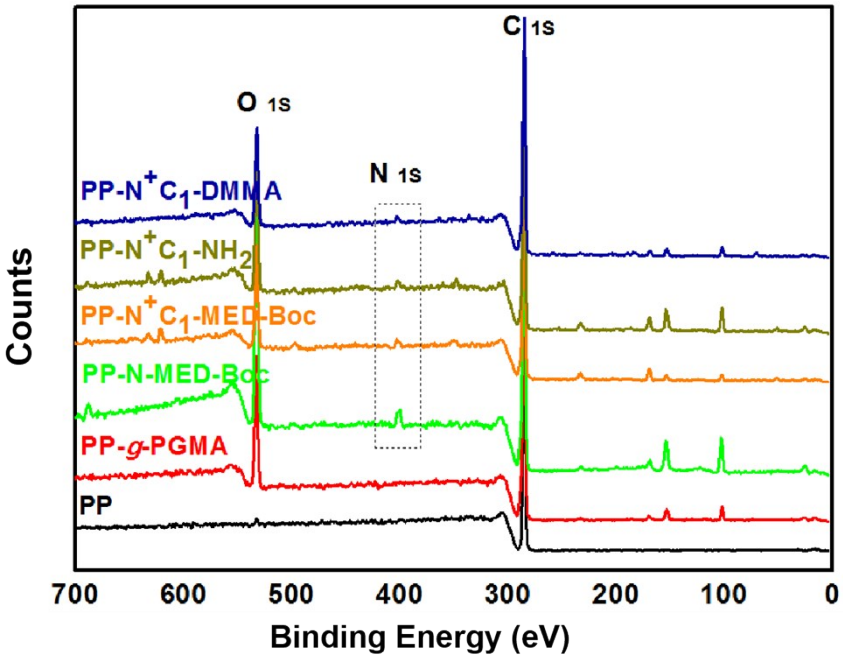
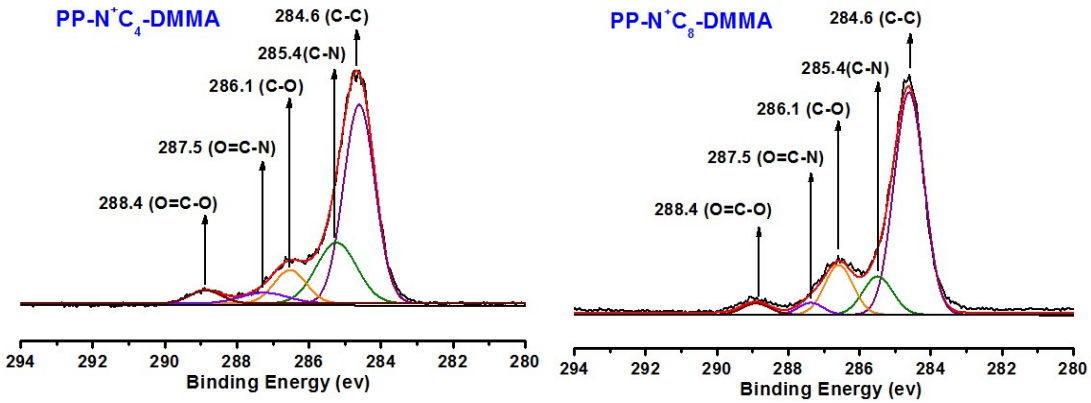


Figure S5. High-resolution C 1s XPS spectra of the samples and their peak-fitting curves.



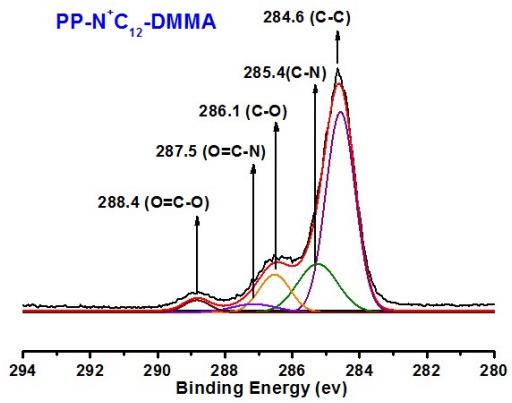


Figure S6. High-resolution C 1s XPS spectra of the samples and their peak-fitting curves.

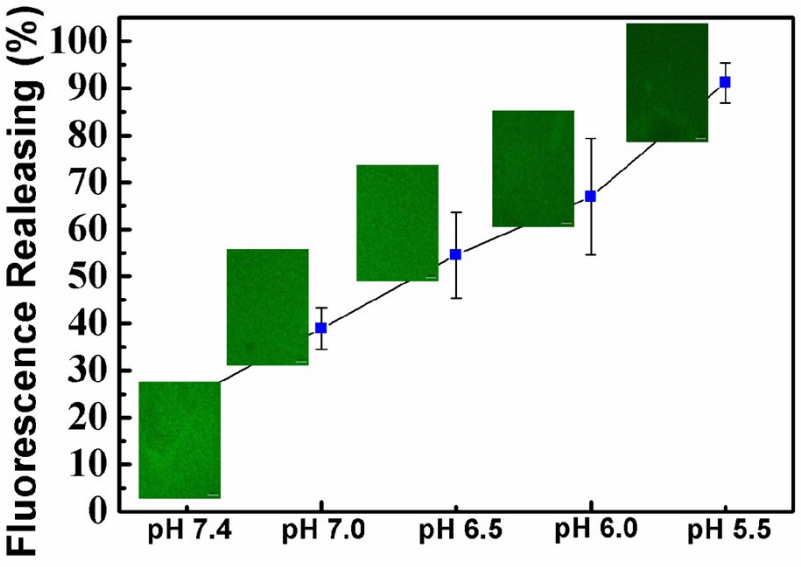


Figure S7. Release curves of PP-N^+C_8-DMMA surface at different pH values and was corresponding fluorescence pictures.

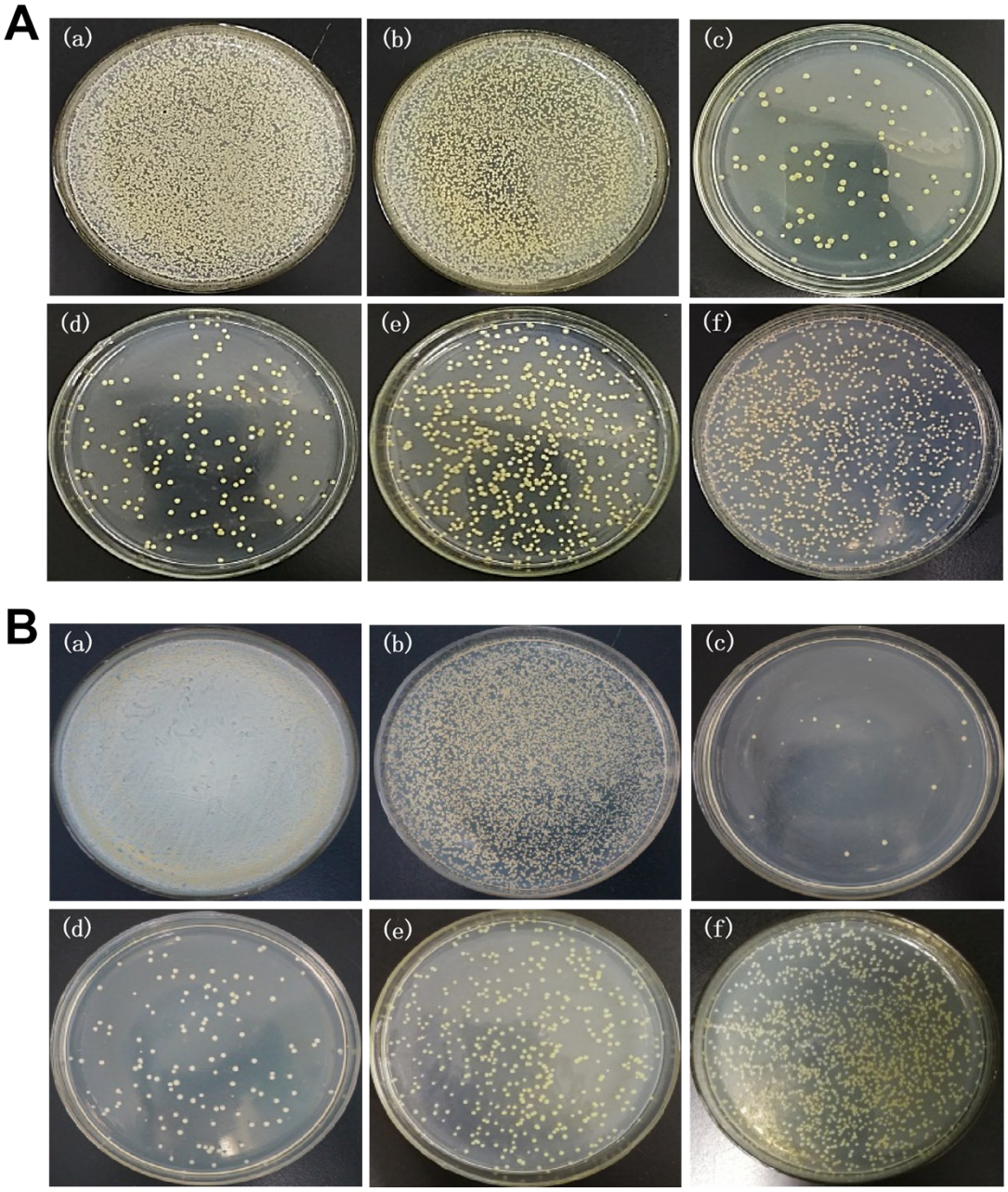


Figure S8. (A) and (B) are photographic images on agar plates of anti-adhesion and bactericidal assay of *S. aureus*, respectively. (a) pristine PP, (b) PP-N-DMMA, (c) PP-N^+C_1-DMMA, (d) PP-N^+C_4-DMMA, (e) PP-N^+C_8-DMMA and (f) PP-N^+C_{12}-DMMA.

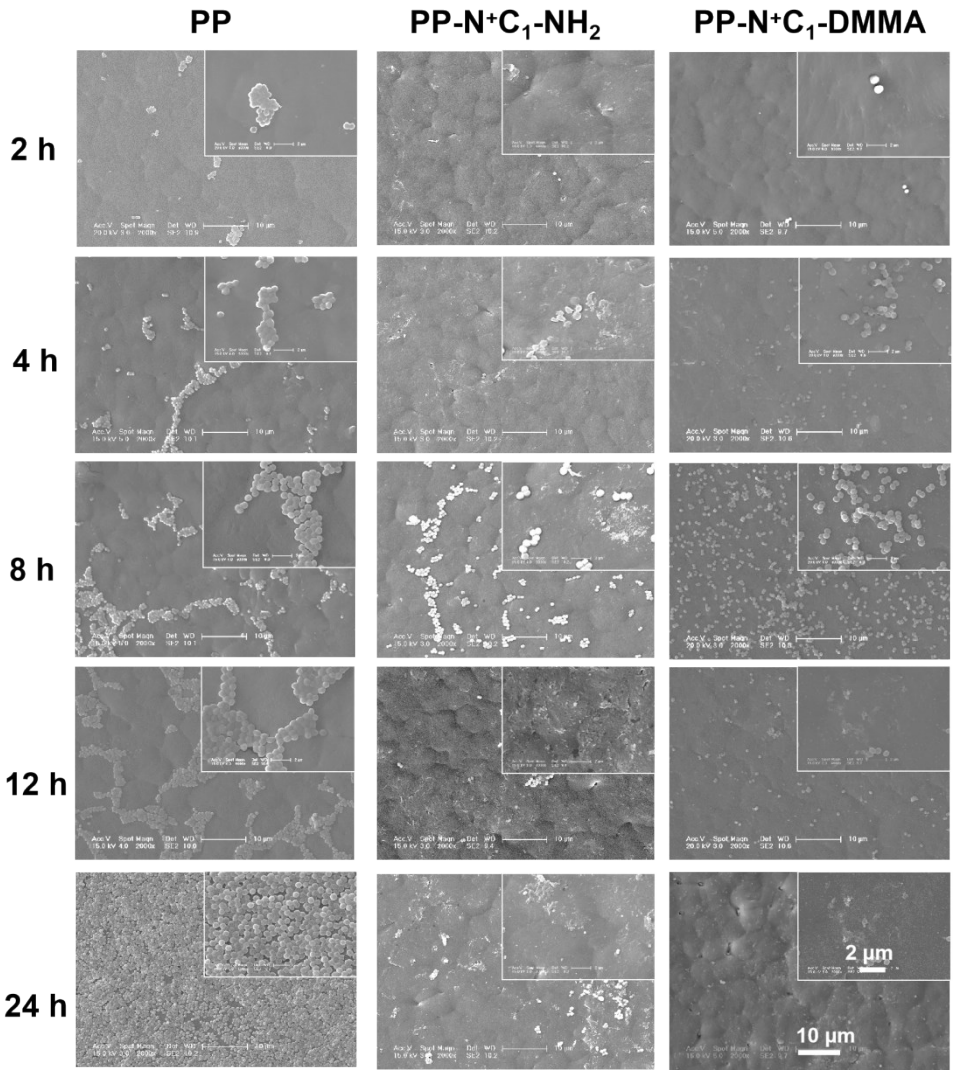


Figure S9. Representative SEM images of *S. aureus* after 2, 4, 8, 12, 24 h incubation on the samples.

Reference

1. Faure, E.; Falentin-Daudré, C.; Lanero, T. S.; Vreuls, C.; Zocchi, G.; Van De Weerdt, C.; Martial, J.; Jérôme, C.; Duwez, A.-S.; Detrembleur, C., Functional Nanogels as Platforms for Imparting Antibacterial, Antibiofilm, and Antiadhesion Activities to Stainless Steel. *Advanced Functional Materials* **2012,** *22* (24), 5271-5282.

2. Lim, K.; Chua, R. R.; Saravanan, R.; Basu, A.; Mishra, B.; Tambyah, P. A.; Ho, B.; Leong, S. S., Immobilization studies of an engineered arginine-tryptophan-rich peptide on a silicone surface with antimicrobial and antibiofilm activity. *ACS Applied Materials & Interfaces* **2013,** *5* (13), 6412-6422.