Development of an antibacterial surface with self-defensive and pH-responsive function

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Materials: Polypropylene (PP) film was used as pristine substrates. Acrylic acid (AA) was purchased from Innochem. Benzophenone (BP), 2-(Nmorpholino) ethanesulfonic acid (MES), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 2,3-Dimethylmaleic Anhydride (DMA), 5-aminofluorescein and melittin (MLT) was purchased from aladdin. Gram-negative *Escherichia coli* (*E. coil*; ATCC 25922), Gram-positive *Staphylococcus aureus* (*S. aureus*; ATCC 6538), phosphate buffered solution (PBS; 0.1 mol L^{-1}, pH 7.4), Luria–Bertani (LB) broth, and LB-agar were provided by Dingguo Biotechnology (China). ethylenediamine (EDA), tetrahydrofuran (THF) and N, N-dimethylformamide (DMF) were purchased from Innochem and Haodi Chemical Reagents Co., Ltd. (China), respectively. All other chemicals (AR grade) were used as received directly without further purification.

Immobilization of antimicrobial peptide on PP film: PP films were cut into 1.0 × 1.5 cm^2 pieces and washed with acetone and alcohol in the ultrasonic machine for 30 min and then were vacuum dried at 40 °C. The PP films were put into BP (1 wt%) of absolute ethyl alcohol for 30 min at the dark environment and dried at room temperature. Dropping 10 μL AA (5 vol%) of ultrapure water on the PP films and UV irradiation (high-pressure mercury lamp, 400 W, main wavelength 365 nm) for 8 min. The resultant samples, denoted as PP-*g*-PAA, were rinsed with water and dried in the vacuum oven at room temperature.

The PP-*g*-PAA brush samples were activated in a MES solution (pH = 5.5) containing 0.4 M EDC and 0.1 M NHS at 4 °C. The activated films were added to the mixture solution which were EDA of DMF solution (5 vol%) and stirred at 40 °C. The final sample ware named as PP-*g*-EDA.

The PP-*g*-EDA films were put into DMA of THF solution (5 vol%) and stirred for 12 h at 25 °C. The resultant samples of PP-*g*-DMA were washed in absolute ethyl alcohol for 15 min, followed by drying in a vacuum oven. The final antimicrobial peptide surface (PP-*g*-MLT) was obtained from the PP-*g*-DMA films after shaken at a table concentrator in the MLT of PBS solution.

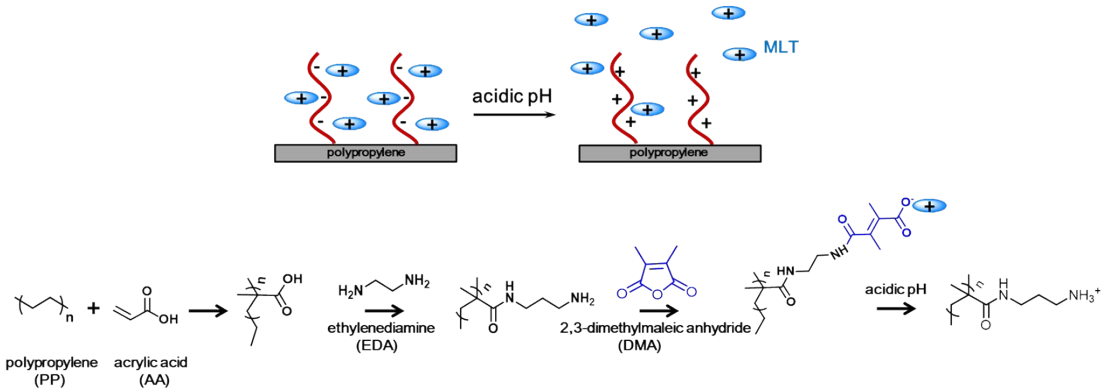

Figure S1. Schematic of the construction of pH-responsive antimicrobial surface was constructed. MLT immediately showed bactericidal activity when bacteria came into contact with the surface. In addition, DMA on the surface was released when bacteria adhered to the surface in large quantities or grow in the solution.

Surface Characterization: FTIR-ATR spectra were obtained using a Fourier transform infrared spectrometer (FTIR; BRUKER Vertex 70) in Attenuated Total Reflection (ATR) mode.

The surface composition was determined by X-ray photoelectron spectroscopy at room temperature (XPS, VG Scientific ESCA MK II Thermo Avantage V3.20 analyzer equipped with an Al Kα anode mono-X-ray source (hν = 1486.6 eV)). Surface spectra were collected over a range of 0-1200 eV and high-resolution spectra of C1s, N1s, and O1s regions were also provided. The atomic concentrations of the element were calculated from the peak area ratio.

The static contact angles of water (WCAs) on the surfaces of the virgin and modified PP membranes were carried out at room temperature using a contact angle goniometer (DSA KRÜSS GMBH, Hamburg 100) by injecting 2 μL of distilled water on the membrane surfaces. The value of water contact angle was recorded after 10s. Five measurements were made on each sample to obtain the average value of contact angles. Five measurements were taken across each sample for calculating the average value.

The surface morphology of the samples was examined by atomic force microscopy in contact mode (AFM; Olympus). The surface morphology and root-mean-square (rms) roughness were provided by AFM analysis[1].

For the preparation of modified surface, PP film was cut into 1 cm × 1.5 cm^2 pieces and ultrasonically cleaned in ethanol and doubly distilled water for 5 min in each step. The grafting density (GD)[2] of modified surface was calculated using the following equation:

$$GD = (W_1 - W_0) / S$$

Here, W_0 and W_1 are the weights (μg) of the virgin and modified samples, respectively. S represents the surface area (cm^2) of the sample. Each result is an average of at least three parallel experiments.

The removal of DMA from the surface of PP-*g*-DMA membrane was detected by ultraviolet spectrophotometer and visualizing FITC-labeled**. The PP-*g*-DMA was respectively immersed in pH 5.5, pH 6.0, pH 6.5, pH 7.0 and pH 7.5 buffer**

solution and cultured at 37 °C. DMA released in solution was detected in a gradient at the same time. The second method used to detect whether DMA generates a release is mainly through visualizing FITC-labeled testing**.** First, the pristine PP and **PP-*g*-DMA** surfaces were reacted with 5-aminofluorescein for 12 h, then fluorescence measurements on the membrane surface were performed after 10 minutes of ultrasonic cleaning. Then, the membrane surface was cleaned by a buffer solution of pH 5.5 for 6 h. After 10 minutes of ultrasonic cleaning, fluorescence measurements were again performed on the membrane surface. After cleaning under acidic conditions, fluorescence intensity of the membrane surface was compared.

Bactericidal assay: *S.ureus* and *E. coli* were incultivated on LB-agar for 24 h at 37 °C, respectively. A single colony of agar plate was selected into 50 mL Luria Bertani (LB) broth and incultivated for 12 h at 37 °C. An appropriate amount of overnight culture of *S.ureus* and *E. coli* centrifugation was carried out with sterile PBS and LB liquid medium diluted to 10^8 cells mL^{-1} and 10^6 cells mL^{-1}, respectively. The 20 μL bacteria of 10^6 cells mL^{-1} of LB bacteria solution were added to the surface of PP, PP-*g*-PAA and PP-*g*-MLT films, then covered with PE film and incubated for 24 h at 37 °C. The experimental films was placed in 2 mL sterile PBS and ultrasonic oscillation for 3 minutes. The ultrasonic liquid planking was carried out.

The diluted bacterial liquid of 500 μL of 10^6 cells mL^{-1} were dropped on PP , PP-*g*-PAA and PP-*g*-MLT films, which were then incubated for 24 h at 37 °C.[3] The antibacterial capabilities were investigated by both confocal laser scanning microscopy (CLSM, LSM 700, Carl Zeiss) and field-emitted scanning electron microscopy (SEM, XL 30 FESEM FEG, FEI Co.).

The PP and PP-*g*-MLT films were dyed with LIVE/DEAD BacLight Viability Kit for 15 min and washed 3 times with ultrapure water. The SYTO 9 dye yields green fluorescence which represents live bacteria and the Propidium Iodide yields red fluorescence which represents dead bacteria. The dyed membranes were freeze-dried overnight and detected with CLSM. For SEM examination, the bacterial adherence to membranes surface were fixed with 4% paraformaldehyde for 4h, then dehydrated with a serial of ethanol/water mixtures (10%, 30%, 50%,70%, 90%, and 100%) and were analyzed with SEM.

Quantification of *S.ureus* and *E. coli* growth in solutions. PP, PP-*g*-PAA and PP-*g*-MLT films were placed in 24-well culture plates dropped with 300 μL of *S.ureus* and *E. coli* bacterial suspension (10^6 CFU mL^{-1}) and incubated at 37 °C for different periods of time to achieve different pH values. The optical densities of these solutions were then measured at 570 nm using a UV-vis Spectrometer Lambda 40. [4]

Hemolysis evaluation: Fresh blood extracted from a healthy rabbit was immediately mixed with 3.8 wt % sodium citrate solution at a dilution ratio of 9:1. The whole blood was centrifuged at 1000 rpm for 15min and the supernatant which were plasma and buffy coat layers (platelets and white cells) were discarded. The remaining whole blood cells were resuspended with PBS. Finally the whole blood cells were diluted to 5% with PBS. The MLT was diluted into a serial solution with different concentrations in a gradient, and then added to 96-well plates after mixing with 5% blood cells of PBS at 1:1 for 1h, aiming to verify the solution state of MLT hemolytic. The pristine PP, PP-*g*-PAA, PP-*g*-EDA, PP-*g*-DMA and PP-*g*-MLT films were cut into 1.0 × 1.5 cm^2 pieces and equilibrated with PBS at 37 °C for 12 h. Then the membranes were put into 12-well plates with 100 μL rabbit blood cells dropped, and incubated at 37 °C for 4 h. The positive and negative controls were produced by adding 0.1 mL of blood to 0.1 mL of Triton and PBS, respectively.

Preserved RBCs were removed in centrifuge tube and centrifuged (8000 rpm for 5 min) to get the supernatant, which then was transferred to 96-well plates. Optical density (OD) of the supernatant was measured using the TECAN absorbance reader at 540 nm. The hemolysis ratio (HR) was calculated according to the following equation:

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

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

Platelets adhere on the modified PP film surface: The pristine PP, PP-*g*-EDA, PP-*g*-DMA and PP-*g*-MLT films were equilibrated with PBS at 37 °C for 12 h. The membranes were put into 12-well plates and dropped with 20 μL platelet, then incubated at 37 °C for 1 h. The membranes were carefully rinsed twice with PBS and fixed with 4% paraformaldehyde for 4 h afterward. The membranes were dehydrated with a serial of ethanol/water mixtures (10%, 30%, 50%, 70%, 90%, and 100%). Finally, the membranes were freeze-dried and the morphologies of adhered PRP or RBCs on the membranes surfaces were visualized by SEM (SEM, JEOL, JSM-7500F, JP)

Cell viability assay: NIH-3T3 mouse fibroblasts in RPMI 1640 medium (1 mL, 10^4 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 (TECAN SUNRISE, Swiss). The parallel experiment without the samples was conducted as a blank control. The results were expressed as percentages relative to the control experiment.

Mouse Infection Model: All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Jilin medical University and approved by the Animal Ethics Committee of Jilin medical University. To

evaluate the in vivo antibacterial effect of PP-*g*-MLT films, the *S. aureus* infection mice model was built. Female BALB/c mice (6-8 weeks, 18-22 g) were prepared. Approximately 10^8 CFU mL^{-1} of *S. aureus* washed with sterile PBS and resuspended in sterile LB (50 μL) were injected by subcutaneous injection and infected in vivo for 24 h before treatment. The three groups of mice (three mice per group) were implanted PP and PP-*g*-MLT films, respectively. [5] After 5 d implantation, the PP and PP-*g*-MLT films were removed and surrounding tissues for quantification of bacterial number. [6] Hematoxylin and eosin (H&E) was used to analyze the antibacterial effect of the PP-*g*-MLT films on the infected mouse model.

Statistical Analysis: All experiment data were presented as mean ± standard deviation (SD). Each result was an average of at least three parallel experiments. The statistical significance was assessed by analysis of variance (ANOVA), * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

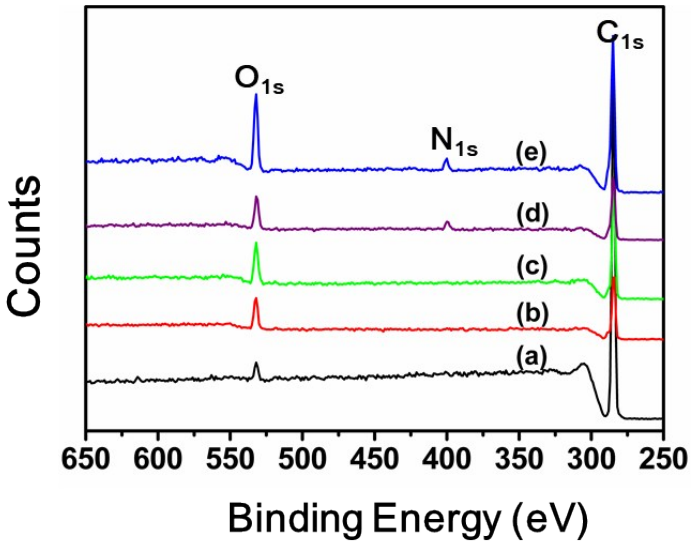

Figure S2. Wide-scan XPS spectra of (a) pristine PP, (b) PP-*g*-PAA, (c) PP-*g*-EDA, (d) PP-*g*-DMA and (e) PP-*g*-MLT.

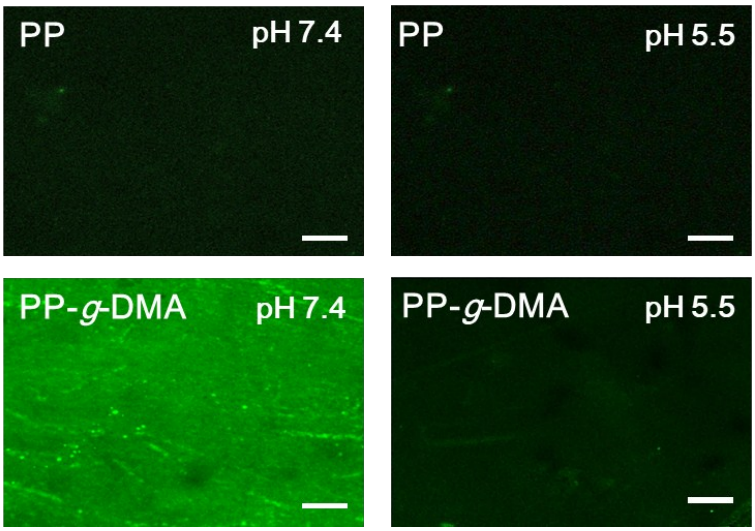

Figure S3. Fluorescent image detected DMA relasing from the PP-*g*-DMA membrane at different pH.

Table S1. Summary of surface characterization data for the samples.

Samples	Composition (at.%)				
	C	N	O	O/C	N/O
PP	97.23	-	2.77	0.02	-
PP-*g*-PAA	82.62	-	17.38	0.02	-

PP-*g*-EDA	84.62	1.11	14.27	0.17	0.08
PP-*g*-DMA	78.99	4.56	16.45	0.21	0.28
PP-*g*-MLT	75.75	5.06	19.19	0.25	0.26

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