Robust and Self-healable Antibiofilm Multilayer Coatings

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Abstract The infection induced by implantation of biomedical materials may result from the biofilm formation after bacteria attachment. Hence, the antibiofilm surface coating represents a novel technique to improve the antibacterial activity of biomedical materials. The traditional antibiofilm surface coatings exhibited some disadvantages and provided a limited service life. In this work, we used polyethyleneimine grafted 3-maleimidopropionic acid (PEIM) and poly(acrylic acid) grafted 2-furfurylamine (PAAF) to achieve robust and self-healable crosslinked multilayer coatings, employing Layer-by-Layer (LbL) self-assembly technique and Diels-Alder reaction. Then, thiol-terminated poly(3-acylamidopropyl) trimethylammonium chloride (PAMPTMA-SH) was grafted onto the crosslinked multilayer coating by thiol-ene click reaction to form a novel multilayer coating (PEIM/PAAF)\textsubscript{10}-PAMPTMA. We found that this coating showed robust and self-healable activity, and significantly inhibited the bacterial growth and biofilm formation after infection with \textit{Escherichia coli} (\textit{E. coli}) and \textit{Staphylococcus aureus} (\textit{S. aureus}) by \textit{in vitro} and \textit{in vivo} assays for 120 h. In addition, the multilayer coating did not induce significant hemolysis or affect the cell viability of red blood cells. In \textit{in vivo} studies also showed that (PEIM/PAAF)\textsubscript{10}-PAMPTMA coating efficiently blocked the infiltration of inflammatory cells and gene expression in the mouse skin challenged with \textit{E. coli} or \textit{S. aureus}. Taken together, these results showed that the prepared multilayer coating exhibited strong antibiofilm activity and provided a new strategy for the application of highly efficient antibiofilm surface coating of biomedical materials.

Keywords Layer-by-Layer assembly; Diels-Alder reaction; Self-healable; Antibiofilm coating; Thiol-ene click reaction

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INTRODUCTION

Microbial adhesion onto device/implant surfaces and formation of bacterial biofilms associated infections have become one of the challenging threats to biomedical devices in hospitals and implants in patients.\textsuperscript{[1−8]} Therefore, many scientists developed various methods for preparing antibacterial or antifouling surface coatings such as exclusion steric repulsion,\textsuperscript{[9,10]} electrostatic repulsion,\textsuperscript{[11]} low surface energy,\textsuperscript{[12]} biocide releasing,\textsuperscript{[13]} and contact-active antimicrobial.\textsuperscript{[14−18]} However, the occurrence of scratches and partial exfoliation is inevitable during the long-term application, leading to the surface devastation and failure of the biomedical devices and implants.\textsuperscript{[19−21]} Accordingly, the self-healing surface coating has been of great importance in improving the stability and durability of functional antibacterial coatings in long-term applications.\textsuperscript{[22−24]} As viewed from the predominant molecular mechanisms involved in the healing processes on functional polymer materials, the reported achievements consisted of two modes: (i) physical interactions (including hydrogen bond, hydrophobic interaction, host-guest interaction, crystallization, polymer-nanocomposite interaction, and multiple intermolecular interaction), and (ii) chemical interactions (including phenylboronate complexation, disulfide bonds, imine bonds, acrylhydrazone bonds, reversible radical reaction and Diels-Alder reaction).\textsuperscript{[25−28]}

Layer-by-layer (LbL) self-assembly technology is one of the convenient and flexible methods for the fabrication of self-healing polymer coatings on various substrates. LbL self-assembly is carried out through high diffusion by alternately depositing oppositely charged polyelectrolytes and generating high mobility of polyelectrolytes multilayer onto a charged substrate surface when subjected to external stimuli.\textsuperscript{[29−32]} However, the instinct of loose structures and weak non-covalent bonds between polyelectrolyte layers makes the multilayers separated from top to bottom of the surface coatings during the long-term application. In order to prevent top-down degradable multilayer, some scientists investigated crosslinked multilayers by condensation reaction\textsuperscript{[33,34]} or click chemistry\textsuperscript{[35]} to control the multilayer degradation rate. In these cases, the high temperature and long-time reaction of condensation reaction or high crosslinking density of click chemistry made the assembled multilayer mobility difficult, and thus the self-healing surface coating could not be achieved. Therefore, it seemed to be a contradiction between
robust and self-healing properties for the surface coating.

In this work, we developed a method for fabricating robust and self-healable antibiofilm surface coating. Firstly, polyethyleneimine (PEI) grafted 3-maleimidopropionic acid (PEIM) as a positive layer and poly(acrylic acid) (PAA) grafted 2-furfurylamine (PAAF) as a negative layer were used to fabricate robust and self-healable surface coating by LbL self-assembly technique and Diels-Alder reaction. Then thiol terminated poly((3-acrylamidopropyl) trimethylammonium chloride) (PAMPTMA-SH) as the antibacterial polymer prepared by supplemental activator and reducing agent atom transfer radical polymerization (SARA ATRP) was grafted onto the oxy-norbornene crosslinking group formed from Diels-Alder reaction of the multilayer coating by thiol-ene click reaction (Scheme 1). The as-prepared multilayer coating exhibited not only robust and self-healable properties, but also long-term antibiofilm efficacy, which could be applied in biomedical devices.

**EXPERIMENTAL**

**Materials**

Dopamine hydrochloride (DA, 99%), 2-hydroxyethyl disulfide (99%) and (3-acrylamidopropyl) trimethylammonium chloride (AMPTMA, 75%) were purchased from Sigma Aldrich (St. Louis, MO, USA); triethylamine (TEA, 99%), α-bromoisobutyryl bromide (BiBB, 98%), CuBr₂ (97%), tris base (Trizma base, Pharma grade), 4-dimethylaminopyridine (DMAP, 99%), methanol (99%),...


*N,N,N,N*-pentamethyldiethylenetriamine (PMDETA, 99%), dimethyl sulfoxide (DMSO), DL-1,4-dithiohreitol (DTT, 98%), 2-
furfurylamine (99%), maleic anhydride (MAH, 99%), β-alanine (99%), tetrahydrofuran (THF, 99%), N-hydroxysuccinimide (NHS, 99%), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydro-
chloride (EDC, 99%), and glutaaldehyde (3%) were purchased from 
Aladdin (Shanghai, China). PEI (*M*ₙ=1.0×10⁴) and PAA (*M*ₙ=1.0×10⁴) 
were purchased from Macklin (Shanghai, China); copper wire (Cu(0), 
d=1 mm) was washed with *HCl*, rinsed with methanol, and dried 
under a stream of *N₂* before use. Dulbecco’s phosphate buffered saline (PBS) was purchased from 
Sigma-Aldrich; Dulbecco’s Modified Eagle’s Medium, 3-
(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), L13152 LIVE/DEAD® Bac Light TM Bacterial Viability Kit 
were purchased from Thermo Fisher Scientific; Triton X-100 
(0.3%) was purchased from SAIES, China; phalloidin-TRITC and 
4’,6-diamidino-2-phenylindole (DAPI) were purchased from 
Sigma-Aldrich; 4% paraformaldehyde was purchased from 
Boster Biological Technology Co.

**Synthesis of PAMPTMA-SH by SARA ATRP**

Firstly, we synthesized disulfide initiator bis(2-(2-bromoisobu-
tryloxy)ethyl) disulfide (BiBOEDS) as bifunctional atom transfer radical polymerization (ATRP) initiator according to the previous 
paper.[36] Secondly, BiBOEDS (0.036 g, 0.08 mmol), AMPTMA 
(3.97 mL, 16 mmol), CuBr₂ (5.40 mg, 0.024 mmol) and PMDETA 
(5 μL, 0.024 mmol) were dissolved in 6 mL of a 50/50 volume 
mixture of water and methanol. A magnetic stirring bar and 
copper wire (1.00 g, d=1.00 mm) were added to the Schlenk 
flask, sealed with rubber stopper and deoxygenized with *N₂* 
under three freeze-vacuum-thaw cycles. Then the reaction 
was carried out at 50 °C and stirred at 940 r/min under nitrogen 
condition for 24 h. Finally, the reaction mixture was dialyzed 
against deionized water (DI *H₂O* for 3 days and disulfide 
(poly(3-acrylamidopropyl) trimethylammonium chloride) 
(DSPAMPTMA) was obtained after freeze drying. Proton nuclear 
magnetic resonance (¹H-NMR) (400 MHz, D₂O, δ, ppm): 1.07 (s, 
d=1.2 H₂O, 3H₃), 1.92 (s, 148H, CH₂CH₃), 3.05 (s, 451H, NCH₂CH₂CH₂), 
3.20 (s, 79H, NHCH₂CH₂CH₂), 3.45 (s, 77H, CH₂CH₃), 
3.72 (s, 145H, NCH₂CH₂CH₂), 5.94 (s, 2H, −CH=CH−).

The as-prepared DSPAMPTMA disulfide was dissolved 
in 20 mL of THF and purified with *N₂* for 30 min. DTT (0.77 g, 
5 mmol) was introduced into mixture solution. The mixture 
was stirred for 24 h under *N₂* condition at 50 °C. The resulting 
PAMPTMA-SH solution was dialyzed against DI *H₂O* for 2 days. 
The dry PAMPTMA-SH polymer was obtained after freeze dry-
ing.

**Synthesis of PAAF**

PAAF was synthesized by dissolve PAA (1 g, 0.01 mmol) in 
50 mL of 1×PBS (pH=7.4). NHS (0.32 g, 2.80 mmol) and EDC 
(0.54 g, 2.80 mmol) were added in the mixture and it was stirred 
for 6 h at 37 °C. Then, 2-furfurylamine (0.27 g, 2.80 mmol) was 
added dropwise into the above mixture. The reaction mixture 
was stirred for 48 h at 37 °C and dialyzed (7 kDa MWCO) against 
0.1 mol/L NaCl for 2 days, followed by further dialysis against DI *H₂O* for 2 days. The product was obtained after freeze drying. 
¹H-NMR (400 MHz, D₂O, δ, ppm): 2.32 (s, 347H, −CH=CH−), 5.68 (s, 
2H, −C=CH−CH=CH−).

**Synthesis of PEIM**

3-Maleimidopropionic acid was synthesized as previously described.[37] PEIM was synthesized by dissolving branched 
PEI (1 g, 0.10 mmol) in 50 mL of 1×PBS (pH=7.4). NHS (0.50 g, 
4.30 mmol) and EDC (0.82 g, 4.30 mmol) were added in the mixture 
and it was stirred for 6 h at 37 °C. Then 3-
maleimidopropionic acid (0.73 g, 4.30 mmol) was added dropwise into the above mixture. The reaction mixture was 
stirred for 48 h at 37 °C and dialyzed (4 kDa MWCO) against 0.1 
mmol/L NaCl for 2 days, followed by further dialysis against DI *H₂O* for 2 days. The product was obtained after freeze drying. 
¹H-NMR (400 MHz, D₂O, δ, ppm): 2.09−3.57 (m, 67H, −CH₂−), 5.94 
(s, 2H, −CH=CH−).

**Fabrication of Self-healing Surface Coating by Layer-
by-layer Self-assembly and Diels-Alder Reaction**

Firstly, stainless steel (SS) slides (d=10 mm) were washed with 
ethanol and DI *H₂O* three times in ultrasonic bath and dried with 
*N₂*; secondly, the SS slides were pre-treated with piranha solvent 
(*V*₅*H₂SO₄*:*V*₅*H₂O₂)=7:3) for 40 min, washed with DI *H₂O*, and dried with 
*N₂*; thirdly, the pre-treated SS slides were immersed into 
Tris-HCl solution (pH=8.5, 10 mmol/L), which contained 
4 mg/mL DA, and strongly stirred for 24 h at 37 °C until the SS 
slides showed an evident color change from metallic to dark 
brown. The SS coated poly(dopamine) (SS-PDA) slides were 
then rinsed with DI *H₂O* and dried overnight in a vacuum dryer 
at 50 °C.

With the as-prepared PEIM as positive charge layer, PAAF as 
negative charge layer, the PEIM/PAAF layers coated onto SS-
PDA slides were fabricated by LbL self-assembly. The SS-PDA 
slides were alternately immersed in aqueous solutions of 
PEIM (1 mg/mL, pH=8.4) and PAAF (1 mg/mL, pH=3.5) for 
10 min each time, followed by rinsing three times for 1 min 
each in DI *H₂O* baths, respectively. By repeating the above 
deposition and rinsing steps in a cyclic fashion, multilayer coat-
ing (PEIM/PAAF)₁₀ with 10 deposition cycles can be fabric-
ated. The drying step with *N₂* was only conducted after de-
positing the last layer. Diels-Alder reaction of multilayer coating 
(PEIM/PAAF)₁₀ on SS-PDA slides surface was carried out 
in oven at 50 °C for 4 h.

The (PEI/PAA)₁₀ multilayer coatings as control samples 
were fabricated on SS-PDA slides surface in the same way 
with the (PEIM/PAAF)₁₀ slides, but without crosslinking pro-
cedure.

**Fabrication of Antibiofilm Surface Coating Based on 
(PEIM/PAAF)₁₀ Multilayer by Thiol-ene Click Reaction**

The as-prepared PAMPTMA-SH (50 mg) was dissolved in DI *H₂O* to form the concentration of 3 mg/mL, containing 0.5% UV 
initiator (IRGACURE 2959). SS-PDA slides coated with (PEIM/
PAAF)₁₀ multilayer coating were immersed into the polymer 
solution under *N₂*. The thiol-ene click reaction was carried out 
under UV reaction (wavelength 365 nm, MXGain-LAB40A, 
Shanghai, China) for 15 min at 25 °C. Finally, the (PEIM/PAAF)₁₀-
PAMPTMA coated SS slide was washed by DI *H₂O* three times 
and dried by *N₂*.

**Characterization**

Functional groups of polymers were characterized by ¹H-NMR 
(AVANCE III 400 MHz, Bruker) using the solvent signal for

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calibration and Fourier transform infrared spectrometry (FTIR) (Nicolet-460, Thermo Fisher). The molecular weight of polymers was characterized by gel permeation chromatography (GPC) measurements, performed on a Waters GPC system, equipped with a Waters 1515 isocratic HPLC pump, a Waters 717 plus Autosampler injector, and a Waters 2414 refractive index detector, using water as the eluent at a flow rate of 1.0 mL/min.

The surface structures of SS and modified SS slides were characterized by X-ray photoelectron spectroscopy (XPS) measurements, carried out on a Kratos Axis Ultra HSA spectrometer equipped with a monochromatized Al Kα X-ray source (1486.6 eV photons). Static water contact angles were measured using JC2000D1 goniometer (Powereach, China) and employing the sessile drop method at room temperature. Surface morphology of LbL self-assembly multilayer coatings on SS slides was characterized by field emission scanning electron microscopy (FE-SEM) (Zeiss Sigma 500, Germany). Thickness of LbL self-assembly multilayer coatings on SS slides was measured by spectroscopic ellipsometry (VASE J.A. Woollam). The surface charge of multilayers on surfaces was characterized by surface zeta potential measurement (Anton Paar Electro Kinetic Analyzer).

Quartz crystal microbalance (QCM) (QCM-Z500) measurements were carried out with an Au-coated resonator. The crystal was mounted in a fluid cell with one side exposed to the solution. A measurement of LbL deposition was initiated by switching the liquid exposed to the resonator. Then, PEI and PAA solutions were alternately introduced with buffer rinsing for 10 min. Both PEI and PAA were 1 mg/mL in DI water and injected into the fluid cell at a rate of 200 μL/min to fabricate multilayer coating (PEI/PAA)_{10}. The QCM measurement of (PEIM/PAAF)_{10} followed the same procedure.

The Stability and Degradation of Multilayer Coatings
The stability of surface multilayer coatings was measured by UV-Vis absorption spectra (Shimadzu UV-2450 spectrophotometer, Japan) and surface nanoindentation test instrument (Bruker, Hysitron Ti 980 Tribolindenter, U.S.). Firstly, the multilayers were coated onto the quartz glass and immersed in PBS at 37 °C for different time periods, and then the multilayer coated quartz glass was dried by N₂ and measured by UV-Vis absorption spectra in the wavelength range from 190 nm to 700 nm. Secondly, the stiffness of surface multilayer coatings was tested by nanoindentation test instrument according the reported method.[38]

Self-healing Procedure of Multilayer Coatings on SS Slide
The self-healing process of scratches (made by a scalpel) on the surface of each sample was conducted in open air at room temperature (25 °C). The morphologies of samples during the self-healing process were observed by an atom force microscope (AFM) with tapping mode (Bruker, U.S.) and a polarizing optical microscope (Nikon, ECLIPSE E200).

In Vitro Antimicrobial Activity of Multilayer Coatings on SS Slide
SS and modified SS slides (d=10 mm, previously washed with water then dried and UV sterilized for 1 h) were put into a 24-well plate, and added with 1 mL of 10^6 CFU/mL for E. coli and 10^7 CFU/mL for S. aureus bacteria suspensions into each well, respectively (LB for E. coli and MHB for S. aureus). The plate was incubated at 37 °C for 24, 72 and 120 h, respectively (added with fresh medium every two days). Each sample was washed gently thrice with PBS buffer to remove unattached bacteria. Each sample was then placed in 10 mL of PBS buffer. Then the slides were placed in ultrasonicator bath for 30 min to remove the attached bacteria from the surface. CFU number was counted by dilution of the stripped bacteria suspension in PBS and culture on LB Agar and MHA plates, respectively. Log reduction was calculated using Eq. (1):

\[
\text{Number of log reduction} = \log_{10} \left( \frac{\text{CFU}_{\text{control}}}{\text{CFU}_{\text{sample}}} \right)
\] (1)

Fluorescence Microscopy
SS and modified SS slides (d=10 mm, previously washed with water then dried and UV sterilized for 1 h) were put into a 24-well plate, respectively, and then 1 mL of MC3T3-E1 suspension was seeded in each well with a density of 1.0×10^4 cells/mL. After 24 h incubation in growth medium, the samples were rinsed carefully with PBS buffer solution. Then 4% paraformaldehyde was used to fix cells for 10 min. After SS and modified SS slides were washed with PBS three times (5 min each wash), the cells were permeabilized with 0.5% Triton X-100 in PBS for 5 min, and the microfilaments were labeled with 1 mg/mL phalloidin-TRITC for 30 min. The cells were treated with 2 mg/mL DAPI for 5 min to stain cell nuclei, and finally all the stained samples were rinsed with DI H₂O thoroughly. Cell images were recorded on an inverted fluorescence microscope (EVOSTM™ FL Imaging System AMF4300, ThermoFisher, U.S.).

SS and modified SS slides (d=10 mm, previously washed with water then dried and UV sterilized for 1 h) were put into a 24-well plate, and added with 1 mL of 10^6 CFU/mL for E. coli and 10^7 CFU/mL for S. aureus bacteria suspensions into each well, respectively. The plate was incubated at 37 °C for 120 h. SS and modified SS slides were washed gently thrice with PBS buffer to remove unattached bacteria. Stain containing L13152 LIVE/DEAD® Bac Light TM Bacterial Viability Kit was used by consulting the manufacturer’s protocol. Stain solution (40 μL, the final concentration of each dye will be 6 μmol/L SYTO 9 stain and 30 μmol/L propidium iodide) was added and spread on each sample and incubated in the dark for 15 min before examination under a fluorescence microscope. Fluorescence imaging was done with 480/500 nm filter for SYTO 9 stain and 490/635 nm filter for propidium iodide in the microscope optical path by Zeiss LSM 710 for the live/dead bacteria imaging.

Biofilm Morphology Check under FE-SEM
SS and modified SS slides (d=10 mm, previously washed with water then dried and UV sterilized for 1 h) were put into a 24-well plate, and added with 1 mL of 10^6 CFU/mL for E. coli and 10^7 CFU/mL for S. aureus bacteria suspensions (LB for E. coli and MHB for S. aureus) into each well, respectively. The plate was incubated at 37 °C for 120 h. SS and modified SS slides were washed gently thrice with PBS buffer to remove unattached bacteria. Each sample was immersed in 2 mL of 3% glutaraldehyde in PBS and refrigerated at 4 °C overnight. The samples were dehydrated with a graded concentration series of ethanol/water mixtures (25%, 50%, 75%, 100%). Dehydrated samples were further dried under nitrogen before characterized by FE-SEM.

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Hemolytic Assays of Multilayer Coatings on SS Slide

Fresh human blood (5 mL) was collected from a healthy donor (age 25, male). Erythrocytes were separated by centrifugation at 1200 r/min for 10 min, washed thrice with PBS buffer, and diluted to a final concentration of 5 vol%. SS and modified SS slides (d=10 mm, respectively) and negative control SS slide were immersed into 1 mL of erythrocytes stock placed in a 24-well cell culture plate. All measurements were performed in triplicate. The plate was incubated at 37 °C for 1 h with shaking at 200 r/min. Subsequently, the slides were centrifuged at 1500 r/min for 10 min. All aliquots (100 μL) of supernatant were transferred to a 96-well plate. Hemolytic activity was determined by absorbance measurement at 540 nm with a 10 fold dilution of the stripped bacteria suspension in PBS and culture on LB Agar for E. coli and MHA for S. aureus, respectively. Log reduction was calculated using Eq. (1).

Part of the harvested tissues were fixed in 4% phosphate-buffered paraformaldehyde for 12 h and embedded in paraffin. Serial sections (5 μm thick) were cut from the paraffin embedded blocks and underwent H&E staining. Photographs were analyzed using a polarizing optical microscope (Nikon, ECLIPSE E200) from three randomly selected views of each specimen, respectively.

RNA Extraction and Real-time RT-PCR of mRNA

The total RNA was extracted by TRIzol reagent (Takara, Japan) according to the manufacturer’s protocol from part of the tissue. Then, 1 μg of mRNA was reversely transcribed to cDNA with Fast Quant RT Super Mix (TIANGEN, China) by Veriti 96 well thermal cycler (Applied Biosystems, USA). Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis was performed by using the SYBR Premix Ex Taq II kit (Takara, Japan) and tested by Step One Plus real-time PCR system (Applied Biosystems, USA). Cyclophilin A (PPIA) was used as the internal control for quantitation of the inflammation expressed mRNA (TNF-α, IL-6, IL-1β) in Table S1 in the electronic supplementary information, ESI). The setting of program was 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min.

RESULTS AND DISCUSSION

Synthesis of Functional Polymers

DSPAMPTMA as the antibacterial polymer was synthesized by SARA ATRP. GPC was used to determine the molecular weights and polydispersity (PDI) of the polymers in aqueous solution. From GPC result in Fig. 1, the estimated Mw of DSPAMPTMA was 14000 g/mol with a PDI of 1.4. DSPAMPTMA was reduced to PAMPTMA-SH using DTT, and the molecular weight of PAMPTMA-SH decreased to 8000 g/mol (PDI=1.4) (Fig. 1).

PAAF was synthesized by amination reaction of PAA and 2-furfurylamine with the aid of NHS and EDC. From 1H-NMR in Fig. S1(b) (in ESI), a weak signal at 6.18 ppm corresponding to...
to double bond protons of furan groups confirmed that the 2-furfurylamine groups were grafted onto PAA backbone successfully; furan substitution ratio of the area under the furan proton signal (6.18 ppm) to the area under the signal for ethylene group protons on PAA (2.32 ppm) determined that the 16 furan groups were grafted onto per PAA polymer chain. FTIR spectra of PAA and PAAF are shown in Figs. 2(a) and 2(b). PAA exhibited a characteristic band in the region of 1700 cm\(^{-1}\) due to C=O stretching vibration. A new band at 1631 cm\(^{-1}\) in PAAF assigned to O=C=N-H was attributed to N-H bending vibration and also confirmed that 2-furfurylamine was grafted onto PAA successfully.

![FTIR spectra of (a) PAA, (b) PAAF, (c) PEI, (d) PEIM.](https://example.com/fig2)

**Physical Properties of Multilayer Coatings**

After fabrication of the LbL multilayer coatings onto SS-PDA slides, we compared the difference of physical properties between traditional multilayer (PEI/PAA)\(_{10}\) and novel multilayer (PEIM/PAAF)\(_{10}\). Firstly, the wettability of (PEI/PAA)\(_{10}\) and (PEIM/PAAF)\(_{10}\) was evaluated by static water contact angle analysis (Fig. 4a). The SS substrate was found to be hydrophilic, with a contact angle of 48\(^\circ\)\pm1\(^\circ\). The contact angle decreased to 29\(^\circ\)\pm2\(^\circ\) after coating PDA onto SS. Subsequently, PEI layer was coated, and the contact angle was changed to 35\(^\circ\)\pm5\(^\circ\). For the PAA layer self-assembled, the contact angle decreased to 0\(^\circ\)\pm1\(^\circ\) in a short time, which is because that the contact angle shows a distinct time dependence with the droplets in PAA out-most layers. For the (PEIM/PAAF)\(_{10}\) multilayer coating, the contact angle was changed to 20\(^\circ\)\pm5\(^\circ\) after PEIM layer was coated, and the contact angle was still 0\(^\circ\)\pm1\(^\circ\) after PAAF layer was covered.

Afterwards, the thickness of multilayer (PEI/PAA)\(_{10}\) increased rapidly in a nearly linear manner (Fig. 4b) as revealed by QCM, while the thickness of (PEI/PAA)\(_{10}\) reached up to 1300 nm. However, the thickness of (PEIM/PAAF)\(_{10}\) increased in a slow but almost linear way with increasing numbers of coating deposition cycles, and reached a maximum thickness of 500 nm. This is because PAA contained ~5% negatively charged carboxylate groups in pH 3.5 aqueous solution. However, PAAF had fewer carboxylate groups than PAA due to PAA grafting furan groups. Similarly, PEIM provided fewer primary and secondary amine groups than PEI due to polymer chain grafting maleimide groups. Carboxylate groups of PAA could have stronger electrostatic interactions with protonated primary and secondary amine groups of PEI in (PEI/PAA)\(_{10}\) than the interaction between PAAF and PEIM in...
The thicknesses of (PEI/PAA)_{10} and (PEIM/PAAF)_{10} were 50.2 and 40.1 nm, respectively, as measured by spectroscopic ellipsometry, smaller than the QCM measuring results. This is because wet and swelled multilayer coatings were measured by QCM, while dry multilayer coatings were measured by spectroscopic ellipsometry. The surface roughness and morphology of multilayer coatings were characterized by AFM and FE-SEM as shown in Fig. 4(c) and Fig. 5, respectively. The roughness of SS-PDA slide coated with LbL multilayer coatings decreased as compared to SS slide and SS-PDA slide, because the multilayer coatings absorbed water and covered the substrate uniformly. The surface morphology characterization by FE-SEM also confirmed that LbL multilayer coatings were uniform on the SS slide.

The Stability of Multilayer Coatings
The stability of LbL multilayer coating is essential for biomedical materials application. The long-term stability of coating onto

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**Table 1** Surface characterization of multilayer coatings.

| Coating samples | XPS atomic percent (%) | Layer thickness (nm) | Surface zeta potential (mV) |
|-----------------|-------------------------|-----------------------|-----------------------------|
| SS              | Fe:C:N:S:Br             | 3.13±2.87:92.87:4.01:−:− | 6.18±0.83                   | −37.80±2.83                  |
| SS-PDA          |                         | 2.74±9.07:8.19:−:−        | 29.82±3.38                  | −50.22±3.21                  |
| (PEI/PAA)_{10}  |                         | 1.88±9.07:7.40:−:−        | 50.22±2.73                  | −56.10±4.79                  |
| (PEIM/PAAF)_{10} |                         | 0.40±9.06:8.95:−:−        | 44.11±5.94                  | −42.17±2.10                  |
| (PEIM/PAAF)_{10}-PAMPTMA |         | 0.23±8.64:12.60:4.01.12  | 52.84±5.71                  | 3.80±1.36                    |

(PEIM/PAAF)_{10}. The thicknesses of (PEI/PAA)_{10} and (PEIM/PAAF)_{10} were 50.2 and 40.1 nm, respectively, as measured by spectroscopic ellipsometry, smaller than the QCM measuring results. This is because wet and swelled multilayer coatings were measured by QCM, while dry multilayer coatings were measured by spectroscopic ellipsometry. The surface roughness and morphology of multilayer coatings were characterized by AFM and FE-SEM as shown in Fig. 4(c) and Fig. 5, respectively. The roughness of SS-PDA slide coated with LbL multilayer coatings decreased as compared to SS slide and SS-PDA slide, because the multilayer coatings absorbed water and covered the substrate uniformly. The surface morphology characterization by FE-SEM also confirmed that LbL multilayer coatings were uniform on the SS slide.

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Fig. 4  (a) Water contact angles and sliding-angle changes of multilayer coatings (PEI/PAA)$_{10}$ and (PEIM/PAAF)$_{10}$ during 20 cycles; (b) Layer thickness change from QCM measurement for the formation of (PEI/PAA)$_{10}$ and (PEIM/PAAF)$_{10}$ multilayer; (c) Surface roughness of SS, SS-PDA and multilayer coatings resolved by AFM.

Fig. 5  FE-SEM images of (a) SS, (b) SS-PDA coating, (c) (PEI/PAA)$_{10}$ multilayer coating, (d) (PEIM/PAAF)$_{10}$ multilayer coating, and (e) (PEIM/PAAF)$_{10}$-PAMPTMA multilayer coating.
the biomedical material surface could make the device durable in service. In Figs. 6(a)−6(c), the UV-vis spectra show the stability of multilayers coated onto quartz glass surface after being immersed in PBS (pH=7.4) solution for 5 days. The degradation ratios of multilayer coatings \(\text{(PEI/PAA)}_{10}\) and \(\text{(PEIM/PAAF)}_{10}\)−PAMPTMA were slower than that of \(\text{(PEI/PAA)}_{10}\). This is because the crosslinked multilayers not only contained noncovalent bonds (such as ionic and hydrogen bond), but also contained oxy-norborne covalent bonds which were generated from Diels-Alder reaction between the positive layer and the negative layer. The surface nanoindentation characterization (Fig. 6d) also confirmed that the storage moduli of multilayer \(\text{(PEI/PAA)}_{10}\) and \(\text{(PEIM/PAAF)}_{10}\)−PAMPTMA were higher than that of \(\text{(PEI/PAA)}_{10}\) with increase of contact depth. Thus, the crosslinked multilayer coatings \(\text{(PEIM/PAAF)}_{10}\) and \(\text{(PEIM/PAAF)}_{10}\)−PAMPTMA by LbL self-assembly and Diels-Alder reaction exhibited long-term anchoring onto the surface.

Self-healing Property of Multilayer Coatings

The ability to heal damage is critical to extending the service life of the antibacterial coating. A 5–10 \(\mu\)m wide crack, which exposed the underlying substrate, was made on the multilayer coatings \(\text{(PEI/PAA)}_{10}\), \(\text{(PEI/PAA)}_{10}\), and \(\text{(PEIM/PAAF)}_{10}\)−PAMPTMA with a scalpel, respectively. After the damaged coatings had been immersed in PBS, the crack of coating \(\text{(PEI/PAA)}_{10}\) disappeared after 1 h, and the cracks of coatings \(\text{(PEIM/PAAF)}_{10}\) and \(\text{(PEIM/PAAF)}_{10}\)−PAMPTMA disappeared after 1.5 h, indicating complete healing of the crack (Fig. 7).

The healing procedure of the multilayer coating \(\text{(PEI/PAA)}_{10}\) was mainly ascribed to the dynamic nature of hydrogen bonds and the water absorption ability. When immersed in PBS, \(\text{(PEI/PAA)}_{10}\) multilayer absorbed a large amount of PBS that can affect the hydrogen bonds between amide and carboxylic acid groups of PEI and PAA. Because the absorbed PBS acted as plasticizer to decrease the storage modulus of the multilayer and facilitated the mobility of PAA and PEI chains, the multilayer coating \(\text{(PEI/PAA)}_{10}\) swelled and became soft and flowable, which enabled the fractured surfaces of the crack to come into intimate contact.[48] Next, the hydrogen-bonding interactions between PAA and PEI can be dynamically re-constructed in the fractured surfaces to heal the coating completely in 1 h when PBS was removed. However, it took 1.5 h for the self-healing procession of multilayer coatings \(\text{(PEIM/PAAF)}_{10}\) and \(\text{(PEIM/PAAF)}_{10}\)−PAMPTMA onto SS-PDA slides. This is because the coatings were not only influenced by hydrogen bonds and PBS absorption ability but also the low flexible movement of polymer chains caused by Diels-Alder crosslinked reaction in the multilayer coatings. Moreover, \(\text{(PEIM/PAAF)}_{10}\)−PAMPTMA multilayer coating could undergo self-healing procedure under three cycles in Fig. S2 (in ESI).

Antibiofilm Assay in Vitro

Fig. 8 shows the \textit{in vitro} antibiofilm activities of multilayer coatings.
Fig. 7  Self-healing procession of multilayer coatings by AFM.

Fig. 8  Cell counts of biofilms grew in the presence (CFU) of multilayer coatings after different time periods: (a) *E. coli*, (b) *S. aureus*. * means $P<0.05$; ** means $P<0.01$; NS means not significant ($P>0.05$).
coatings onto SS-PDA slide compared with unmodified SS slide. The cell counts of *E. coli* and *S. aureus* biofilms on the SS slide, SS-PDA slide and multilayer coating (PEI/PAA)$_{10}$ grew more rapidly than those of (PEIM/PAAF)$_{10}$ and (PEIM/PAAF)$_{10}$-PAMPTMA in 120 h. The biofilm grew on multilayer coatings (PEIM/PAAF)$_{10}$ and (PEIM/PAAF)$_{10}$-PAMPTMA surfaces relatively slowly as compared with other samples. Especially (PEIM/PAAF)$_{10}$-PAMPTMA coating obviously showed antibiofilm and contact killing effect against the bacteria, with the log reduction of 1.69 (killing ratio 97.9%) for *E. coli* and 1.59 (killing ration 97.4%) for *S. aureus* in 120 h, respectively.

To further explain the antibiofilm mechanism of surface coating, we compared the antibiofilm property of SS and modified SS slides in micromorphology. In Fig. 9, thick and dense *E. coli* and *S. aureus* biofilms attached onto the SS slide and SS-PDA surfaces from the bacterial live/dead fluorescence and FE-SEM images. This is attributed to their relatively hydrophobic and anionic surface. Though the multilayer coating (PEI/PAA)$_{10}$ was hydrophilic, the noncovalent bond LbL self-assembled multilayer was not stable and could be detached from the SS-PDA slide in 120 h (5 days), leading to the formation of *E. coli* and *S. aureus* biofilms on the SS-PDA surface. While the crosslinked multilayer coating (PEIM/PAAF)$_{10}$ onto SS-PDA slide had stable coating, it could only repel the

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**Fig. 9** Live/dead fluorescence and FE-SEM images of *E. coli* and *S. aureus* on SS slide, SS-PDA, and multilayer coatings after 120 h (the red dash lines represented magnified of biofilms).

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**Fig. 10** Live/dead fluorescence of multilayer coating (PEIM/PAAF)_{10}-PAMPTMA: before healing for (a) *E. coli* and (c) *S. aureus*, after healing for (b) *E. coli* and (d) *S. aureus*.

**Fig. 11** (a) Hemolysis and (b) MTT assay of the cell viability for the multilayer coatings onto SS slide; (c) Fluorescent images of MC3T3-E1 and stained with rhodamine phalloidin.

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bacteria from the coating surface due to the negative surface zeta potential and electrostatic repulsion.\(^\text{[49]}\) There were few *E. coli* and *S. aureus* biofilms attached onto the \((\text{PEIM}/\text{PAAF})_{10}\)-PAMPTMA coating after 120 h and dead bacteria on the surface (killing ratio 97.9% for *E. coli* and 97.4% for *S. aureus*, respectively), because PAMPTMA was a strong cationic polymer which had low minimum inhibitory concentrations (MIC) for both *E. coli* and *S. aureus*,\(^\text{[50]}\) and made the surface zeta potential of the multilayer coating change into positive.

To further investigate surface antibiofilm property of the healed \((\text{PEIM}/\text{PAAF})_{10}\)-PAMPTMA coating, the \((\text{PEIM}/\text{PAAF})_{10}\)-PAMPTMA coating damaged with a ~3 μm wide crack and the healed \((\text{PEIM}/\text{PAAF})_{10}\)-PAMPTMA coating were incubated with *E. coli* and *S. aureus* for 6 h, respectively. Figs. 10(a) and 10(c) show that a large amount of *E. coli* and *S. aureus* appeared on the crack area of the damaged coating. No obvious *E. coli* or *S. aureus* appeared on the healed regions of the coating in Figs. 10(b) and 10(d). Therefore, the healed \((\text{PEIM}/\text{PAAF})_{10}\)-PAMPTMA coating could restore its antibiofilm function in a short time.

### Hemolytic Activity and Cytocompatibility Assay in Vitro of Multilayer Coatings

Fig. 11(a) shows the hemolysis of unmodified and modified SS slides. The hemolysis of SS slide, SS-PDA, \((\text{PEIM}/\text{PAAF})_{10}\) and \((\text{PEIM}/\text{PAAF})_{10}\)-PAMPTMA coating was about 0.3%, which means nonhemolytic for biomaterials according to the American Society for Testing and Materials (ASTM) standard (less than 2%).\(^\text{[51]}\) The hemolysis of \((\text{PEI}/\text{PAA})_{10}\) was the highest (7%), because there was no covalent bond crosslinking in the multilayer. PEI layers and PAA layers detached from the SS slide surface and as a result, PEI layers could make the blood cell break. To evaluate the potential cytotoxicity of multilayer coatings, the MC3T3-E1 cells with the extracted liquid of each sample were immersed in the culture medium and measured by MTT testing. After 24 h incubation with samples, the MC3T3-E1 cell viability was all above 95% (Figs. 11b and 11c) and was not significantly different from that of SS slide. Thus, surface LbL multilayer coatings were non-cytotoxic.

### In Vivo Antimicrobial Activity of Crosslinked Multilayer Coating

The *in vivo* experiments were performed on mice after subcutaneous implantation of the slides seeded respectively with *E. coli* and *S. aureus* to evaluate the antimicrobial ability of multilayer coating \((\text{PEIM}/\text{PAAF})_{10}\)-PAMPTMA in a real bio-system. *E. coli* (10 μL of \(10^6\) CFU/mL) and *S. aureus* (10 μL of \(10^6\) CFU/mL) were respectively spotted on the SS slide \((d=5 \text{ mm})\) or

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**Fig. 12** *In vivo* essay of CFU of coatings biofilm after 120 h: (a) *E. coli* \((P<0.01)\), (b) *S. aureus* \((P<0.01)\).

**Fig. 13** Histological sections from SS slide and SS coated with \((\text{PEIM}/\text{PAAF})_{10}\)-PAMPTMA stained by H&E assay after 120 h.
multilayer coating (PEIM/PAAF)10-PAMPTMA on SS-PDA (d=5 mm) and dried for 15 min. The slide was implanted into each incision of mice’s back respectively for 120 h culture. As seen from Figs. 12(a) and 12(b), the log reduction of multilayer coating (PEIM/PAAF)10-PAMPTMA onto SS-PDA slide for E. coli was 1.01 (killing ratio 90.3%), while the log reduction of the multilayer coating for S. aureus was 1.19 (killing ratio 93.5%) after 120 h, which was consistent with the in vitro results.

**In Vivo Anti-inflammation Evaluation**

The skin tissues around the implantation were sectioned and stained by hematoxylin and eosin for histological evaluation (Fig. 13). A lot of inflammatory cells (stained by the blue color of arrow area) were visible in the tissue from SS slide, demonstrating serious infection by E. coli and S. aureus, respectively. However, there were few inflammatory cells visible in the tissue from multilayer coating (PEIM/PAAF)10-PAMPTMA onto SS-PDA slide infected by E. coli and S. aureus, respectively. The mechanism of anti-inflammation of the multilayer coating would be further investigated. In Figs. 14(a) and 14(b), by extracting the RNA from local skin tissue, we found that proinflammation related genes like TNF-α, IL-6, and IL-1β were shown to be significantly down-regulated in SS-PDA slide coated with (PEIM/PAAF)10-PAMPTMA for S. aureus than that for E. coli. Consistent with histological study, it is worth noting that the multilayer coating (PEIM/PAAF)10-PAMPTMA had good effect on anti-inflammation, which could significantly avoid the risk of biomedical implants from microbial contamination.

**CONCLUSIONS**

Herein, we reported robust and self-healable antibiofilm surface coatings via LbL self-assembly and Diels-Alder reaction. The surface structure and physical properties of the coatings were confirmed by XPS, water contact angle, QCM, UV-Vis spectra, nanoindentation and AFM. The multilayer coating (PEIM/PAAF)10-PAMPTMA obtained by the crosslinked LbL self-assembly exhibited unique stability and self-healability. In vitro data of the multilayer coating indicated obvious long-term antibiofilm property; a log reduction for E. coli was 1.69 (killing ratio 97.9%) and for S. aureus was 1.59 (killing ration 97.4%) in 120 h. Furthermore, the multilayer coating also inhibited the growth of E. coli and S. aureus biofilms after the crack healed. (PEIM/PAAF)10-PAMPTMA coating also exhibited low hemolysis and high cell viability. The in vivo assay confirmed that the multilayer coating exhibited not only excellent antibiofilm effect (log reduction of 1.01 for E. coli and 1.19 for S. aureus in 120 h), but also good efficacy on inflammation suppression. All these results revealed that the self-healable antibiofilm coating was a promising strategy for bacterial infection control of various implants in practical applications.

**Electronic Supplementary Information**

Electronic supplementary information (ESI) is available free of charge in the online version of this article at https://doi.org/10.1007/s10118-021-2513-3.

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