# Amyloid-Like Protein Aggregates Combining Antifouling with Antibacterial Activity

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## Experimental

### 1. Materials.

Bovine serum albumin (BSA), thioflavin T (ThT), dimethyl sulfoxide (DMSO), ascorbic acid (Vc) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich. Tris (2-carboxyethyl) phosphine (TCEP) was purchased from TCI. ε-Polylysine (ε-PL) was purchased from Macklin. Hydrochloric acid (HCl), sodium hydroxide (NaOH), sodium chloride (NaCl), sulfuric acid (H2SO4), absolute alcohol and acetone were purchased from Sinopharm Chemical. Sodium diphenyl diazobis-*α*-naphthylamine-4-sulfonate (Congo red), phosphate buffered solution (PBS), Mueller-Hinton Agar (MHA), and Mueller-Hinton Broth (MHB) were purchased from Solarbio. Fluorescein isothiocyanate (FITC) labeled ε-PL (FITC-ε-PL) was provided by Fu Baike co., ltd (Beijing, China). Ultrapure water was used in all experiments and was supplied by Milli-Q Advantage A10 (Millipore, USA).

### 2. Preparation of PTB@ε-PL coating on various substrates.

The phase transition solution of PTB@ε-PL was freshly prepared by mixing BSA solution (10 mg $mL^{-1}$), TCEP (50 mM at pH 4.5, pH adjusted by 5M NaOH) and ε-PL solution (0.1-0.5 g $mL^{-1}$) with the volume ratio of 1: 1: 1. All the above substances were dissolved in ultrapure water.

To assemble the PTB@ε-PL nanofilm at the solid/liquid interface, a substrate was ultrasonically cleaned alternatively in an ethanol and acetone solution and then dried in air. The substrate was completely immersed in the freshly-prepared phase transition solution and left in an oven at 30ºC for 2 h. The coated material was then removed and rinsed with ultrapure water to wash away BSA, ε-PL and other salts adsorbed on the surface. The PTB@ε-PL coated substrate was then dried by nitrogen for the next purpose.

### 3. Characterization of the PTB@ε-PL nanofilm.

#### 3.1 MALDI-TOF test.

MALDI-TOF analysis was performed on Bruker MALDI-TOF Ultraflex II machine. In short, the mixture of native BSA and ε-PL or PTB@ε-PL nanofilms degraded by 500 mM vitamin C[1,2] was mixed with an equal volume of the saturated solution of α-cyano-4-hydroxycinnamic acid (CCA) in acetonitrile (MeCN). An aliquot (2 μl) of the resulting mixture was spotted onto a target plate and allowed to dry.

#### 3.2 ThT staining.

The mixed solution of BSA (10 mg $mL^{-1}$) and ε-PL (0.4 g $mL^{-1}$) in a volume ratio of 1: 1 was diluted 5 times by ultrapure water, and 100 μl of ThT (10 mM dissolved in ultrapure water) was added to 2 mL of the above diluted solution, followed by the addition of 1 mL TCEP (50 mM, pH 4.5). The fluorescence intensity of the solution in the quartz cuvette was then measured by fluorescence spectrophotometer with the excitation at 440 nm and emission at 484 nm. The excitation and emission slits have a bandwidth of 5 nm.

**3.3 Congo red staining.**

The PTB@ε-PL coating on various substrates was placed in Congo red solution (1 mg $mL^{-1}$ in ultrapure water) for 2 h, and then rinsed with deionized water for several times.

**3.4 Far-UV Circular Dichroism (CD) assay.**

The phase transition solution was obtained by mixing BSA (10 mg $mL^{-1}$ in water), TCEP (50 mM in water, pH 4.5) and ε-PL (0.4 g $mL^{-1}$ in water) with a volume ratio of 1:1:1. Then the quartz was immersed in the above reaction solution and incubated at 30ºC for 2 h to obtain the quartz covered by a PTB@ε-PL nanofilm. Since Far-UV CD spectrum requires low sample concentration, the concentration of native BSA and ε-PL was diluted to 0.1 mg $mL^{-1}$ respectively. Far-UV CD spectra were then collected under constant nitrogen flush at 25℃, and recorded from 190 nm to 260 nm with a 2.0 nm bandwidth.

**4. In vitro antimicrobial activity of PTB@ε-PL coating.**

Antibacterial activity of the PTB@ε-PL coating was evaluated based on colony counting method. Three bacteria were used in this study, including *Staphylococcus aureus* (*S. aureus*, ATCC 6538)*, Escherichia coli* (*E. coli*, ATCC 25922) *and Proteus mirabilis* (*P. mirabilis*, ATCC 51286). Prior to the in vitro antibacterial test, the bacteria were cultured aerobically in 50 mL MHB overnight at 37ºC under shaking at 70 rpm, so that the bacteria were in the logarithmic growth phase. 1 mL of the bacterial suspension was collected in a sterile eppendorf tube, centrifuged at 5000 rpm, and washed with PBS to remove the culture medium. This process was repeated three times. Finally, the test bacteria were re-suspended in PBS at a concentration of $10^7$ CFU $mL^{-1}$. As shown in Figure S8 (a), 10 μl of the above bacterial suspension was dropped on a bare glass or a glass coated with 5 layers of PTB@ε-PL, and cover with another piece of the same glass to evenly distribute the bacterial suspension between the two pieces of glass. After 8 h of incubation in a 37ºC humid chamber, the glass was carefully separated by tweezers and then completely submerged in a centrifugal tube containing 10 mL of sterile PBS. This centrifuge tube was ultrasonicated to fully transfer bacteria attached to the material into PBS. The bacterial suspension was serially diluted and plated on MHA plates. After these MHA plates were incubated at 37ºC for 24 h, the number of colonies was recorded. Antibacterial activity is represented by the killing ratio, which is calculated according to the following equation:

$$Killing\ ratio = \frac{C_0 - C}{C_0} \times 100\%$$

Where C is the CFU of the experimental group on the PTB@ε-PL (or other related coating) covered substrate, and $C_0$ is the CFU of the control group on bare substrate.

**5. Quantification of the average loading density of ε-PL in PTB@ε-PL coating.**

In order to explore the effect of different initial ε-PL concentration on the average loading density in PTB@ε-PL nanofilm, FITC-ε-PL (0.1-0.4 g $mL^{-1}$), BSA (10 mg $mL^{-1}$) and TCEP (50 mM, pH 4.5) were mixed in a volume ratio of 1: 1: 1 to prepare

the phase transition solution. The glass was immersed in the above solution and incubated at 30ºC for 2 h to obtain PTB@ε-PL coated glass with different ε-PL immobilization density. The multilayer coating, obtained by repeating this process 5 times, was then degraded by Vc solution (1 M in water) for 6 h. The average loading density of the ε-PL was estimated based on the fluorescence intensity of the solution measured by the fluorescence spectrophotometer.

For testing the effect of BSA concentration on the loading density of ε-PL immobilized in the PTB@ε-PL nanofilm, the phase transition solution was prepared by mixing 5 mg $mL^{-1}$, 10 mg $mL^{-1}$ or 20 mg $mL^{-1}$ BSA with FITC-ε-PL (0.4 g $mL^{-1}$) and TCEP (50 mM, pH 4,5) in a volume ratio of 1∶1, and the subsequent method was the same as above.

**6. Anti-biofilm formation property of PTB@ε-PL coating.**

The bacteria in the logarithmic growth phase was centrifuged and suspended in PBS, and 200 μl of the bacterial suspension at a concentration of $10^6$ CFU $mL^{-1}$ was evenly spread on bare glass or the PTB@ε-PL (or other related coating) coated glass. To prevent the bacterial suspension from evaporating, all samples were placed in a humid chamber. After 24 h incubation at 37ºC, the sample was washed with PBS to remove the floating bacteria, and then the glass was placed in a centrifuge tube containing 10 mL of PBS and ultrasonicated at 200 W/ 40 HZ for 5 min to re-suspend the bacteria adhered to the surface in PBS. The bacterial suspension was serially diluted and plated on MHA plates. After 24 h incubation at 37ºC, the number of clones was counted to evaluate the bacterial load in the biofilm on the material. For samples observed by SEM, after incubated with the bacteria, all samples were washed by ultrapure water and then fixed in 4% paraformaldehyde PBS buffer overnight. After successively dehydrating the samples with 90%, 70%, 50% and 20% ethanol aqueous solutions, gold sputtering was performed for 30 sec, and the surface of the samples was inspected using SEM.

**7. Quartz crystal microbalance with dissipation monitoring (QCM-D) assay.**

QCM Au chips were cleaned by piranha solution (concentrated $H_2SO_4$: 35% $H_2O_2$ = 7: 3 v/v for 10 min). The bacterial lysate used in this experiment was obtained by crushing $10^8$ CFU $mL^{-1}$ *S. aureus* for 10 min using ultrasonic cell shredder (Ningbo Scientz Biotechnology Co,. Ltd). After centrifuging the whole blood at 1500 rpm for 10 minutes, the supernatant was collected and diluted with PBS solution ten times to obtain a human serum sample for testing.

**8. Cytotoxicity and hemolysis assay.**

The cytotoxicity of the PTB@ε-PL coating was evaluated by the MTT assay, according to ISO 10993-5: 2009. To prepare the extract, silicone rubber or PTB@ε-PL coated silicone rubber was extracted with Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) at 37ºC for 24 h (the ratio of the coated surface area to the volume of complete medium is 6 $cm^2$ $mL^{-1}$). NIH/3T3 murine embryonic fibroblasts were cultured in DMEM supplemented with 10% FBS

and 1% penicillin-streptomycin solution. The cells were isolated from culture flasks by adding a 0.25% trypsin-EDTA solution and re-suspending in fresh medium for subsequent experiments. Cells in complete medium (100 μL) of 2000 cells in each well were seeded into a 96-well plate and incubated at 37ºC for 24 h in a humidified atmosphere of 5% $CO_2$. After cell attachment, the complete medium in each well was replaced with 100 μL of extract. Every 24 h for the next 3 consecutive days, the culture medium in each well was carefully discarded before adding 200 μL of MTT (0.5 mg $mL^{-1}$) in DMEM, and incubated at 37ºC for 3 h. The MTT solution was then discarded and 150 μL DMSO was added to dissolve the formazan crystals. The optical absorbance of the samples was measured at 490 nm using a microplate reader.

Hemocompatibility of PTB@ε-PL coating was determined by hemolysis test, according to ISO 10993-4: 2017 (E). The fresh rabbit blood was diluted by normal saline at a ratio of 4: 5, and the PTB@ε-PL coated silicone rubber was dipped in a tube containing 2 mL of normal saline that was previously incubated for 72 h at 37ºC, while bare silicone rubber was used as control group. Then 40 μL of diluted blood was added to the tube and the mixture was incubated for 60 min at 37ºC. Similarly, normal saline solution and deionized water were used as negative and positive control, respectively. Finally, all the tubes were centrifuged for 5 min at 3000 rpm and the supernatant was measured by the UV/vis spectrometer to record the optical density (OD) at 540 nm. Hemolysis ratio was calculated using the equation.

$$\text{Hemolysis}(\%) = \frac{OD_{Sample} - \text{OD}_{Negative}}{\text{OD}_{\text{Positive}} - \text{OD}_{Negative}} \times 100\%$$

**9. In vivo assay of the antimicrobial activity of PTB@ε-PL coating.**

A total of 15 8-week-old female BALB / C mice were randomly divided into three groups. After each mouse was intraperitoneally anesthetized with 1% sodium pentobarbital, the prepared back skin was cut about 0.5 cm in length, and a subcutaneous bag was bluntly separated. Titanium materials with or without the PTB@ε-PL coating (5 layers) were implanted subcutaneously into the left and right lateral skin of a mouse, respectively. Then the incision was sutured discontinuously with 4-0 silk thread, and 150 μL of *S. aureus* containing $10^7$ CFU $mL^{-1}$ was injected around the implant using a syringe. In the non-implant group, after the skin was incised, the subcutaneous capsular bag was bluntly separated and sutured intermittently as a control.

Three days later, the mice were sacrificed after anesthesia, and all implants and surrounding tissues were collected. The bacteria on the surface of the titanium were suspended in PBS by sonication. The bacterial suspension was appropriately diluted and plated on MHA plates, and the number of clones was counted after 24 h incubation to determine the antibacterial effect in vivo of the PTB@ε-PL coating. In addition, a part of the implants were dehydrated after 4% paraformaldehyde fixation overnight, and the bacterial adhesion and biofilm formation on the surface were determined by SEM.

After fixing the collected skin tissue with 4% paraformaldehyde, the inflammation

of the local tissue was analyzed by hematoxylin and eosin (H&E) staining. Image Pro Plus Image Analysis Software (Media Cybernetics, USA) was used to analyze the infiltration of inflammatory cells.

**10. Characterization.**

Fourier transform infrared (FTIR) spectra were recorded on a Tensor 27 (Bruck). X-ray photoelectron spectroscopy (XPS) was obtained with AXIS ULTRA from Kratos Analytical Ltd., the binding energies were calibrated by setting C1s peak at 284.6 eV. Fluorescence spectrum was tested by F-7000 fluorescence spectrophotometer (Hitachi). Far-UV circular dichroism (CD) spectrum was collected by using Chirascan spectrophotometer (Applied Photophysics Ltd, England). Field emission scanning electron microscope (FE-SEM) observation was conducted on SU8220 (Hitachi). Optical microscopic observations were carried out on a Nikon Ti-U (Tokyo, Japan). Water contact angle (WCA) was performed by OCA 20 (Dataphysics, Germany). Surface zeta potential measurement was performed by SurPASS electrokinetic analyzer (Anton Paar GmbH, Austria).

**Supplementary data.**

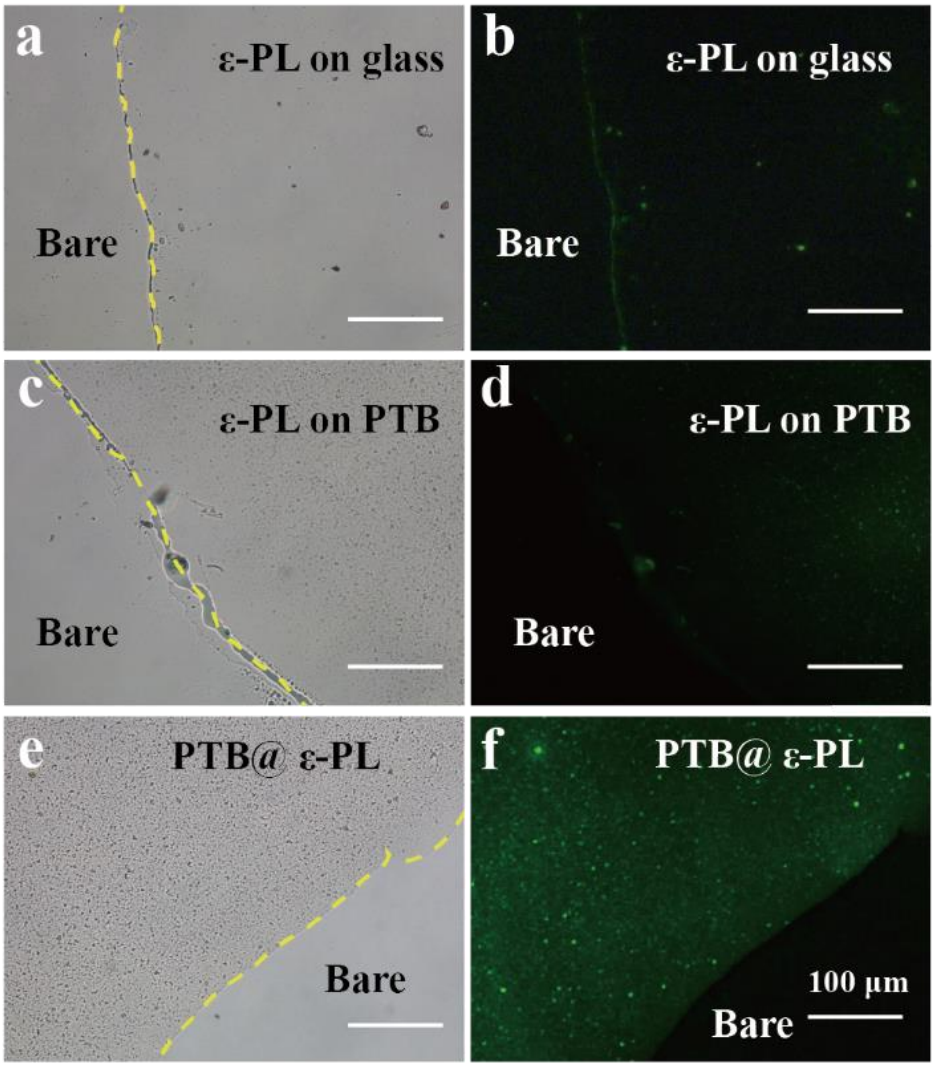


Figure S1. The microscopic optical (a, c, e) and fluorescent (b, d, f) images for simply adsorbing FITC-ε-PL on the surface of bare glass (a, b) and PTB nanofilm (c, d) as well as entrapping ε-PL in the PTB@ε-PL nanofilm (e, f). The PTB@ε-PL nanofilm was obtained by incubating the glass at 30ºC for 2 h in the phase transition solution, which was prepared by mixing BSA (10 mg $mL^{-1}$ water), TCEP (50 mM, pH 4.5 water) with FITC-ε-PL (0.1 g $mL^{-1}$ water) at a volume ratio of 1:1:1. The glass covered by the PTB nanofilm was obtained by replacing the FITC-ε-PL in the phase transition solution with an equal volume of ultrapure water. By immersing bare glass or PTB nanofilm-coated glass in FITC-ε-PL solution (40 mg dissolved in 1.2 mL water) for 2 h, the FITC-ε-PL can be simply adsorbed on the surface of bare glass or PTB nanofilm. The dotted line in each figure showed the boundary between the bare substrate and the surface after different treatments.

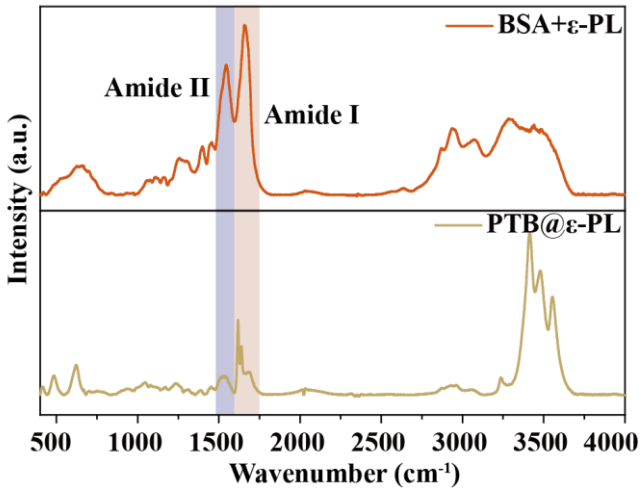


Figure S2. The comparison of the FTIR peaks between the PTB@ε-PL nanofilm and the mixture of native BSA and ε-PL.

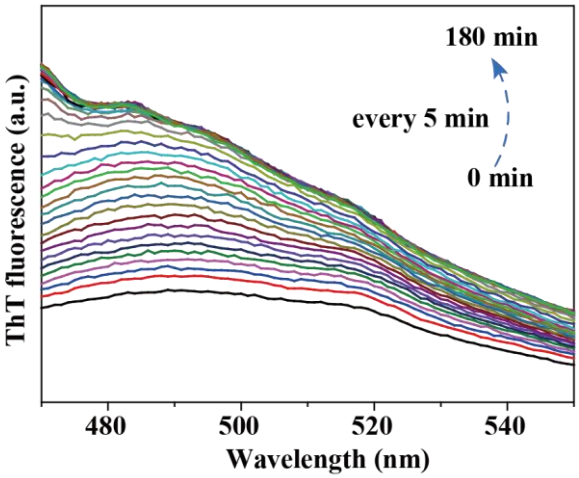


Figure S3. The ThT fluorescence spectra of the PTB@ε-PL samples with different reaction time. The arrow indicating the intensity enhancement with increasing the reaction time from the bottom curve to the top curve, at a time interval of 5 min within 180 min.

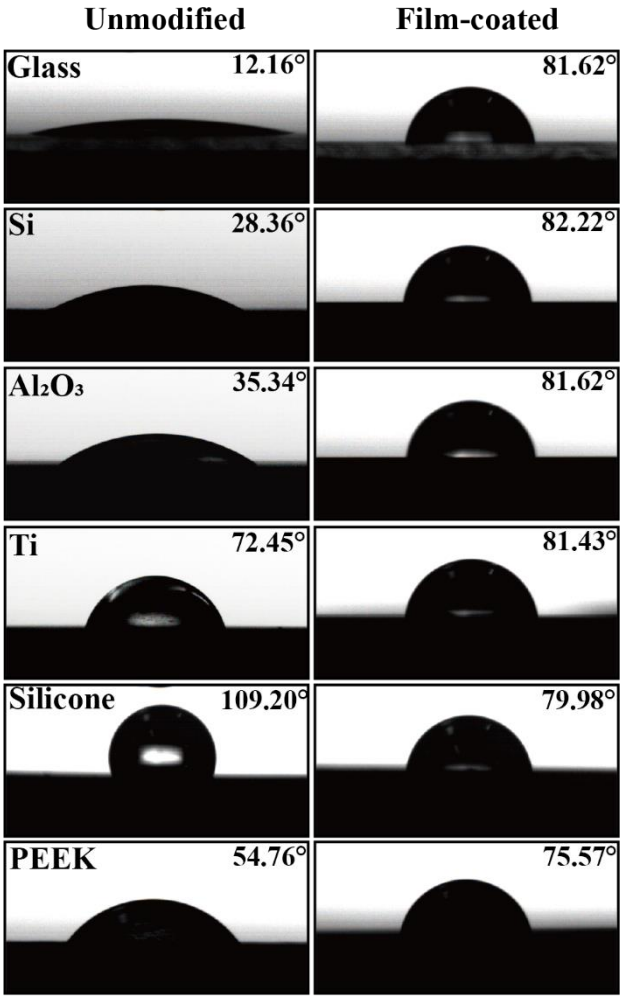


Figure S4. Images of water contact angle on the substrate before (left) and after (right) PTB@ε-PL coating. The reaction time was 120 min, and the phase transition solution was prepared as follows: BSA (10 mg $mL^{-1}$ in water), TCEP (50 mM, pH 4.5 in water) and ε-PL (0.4 g $mL^{-1}$ in water) were mixed in a volume ratio of 1: 1: 1.

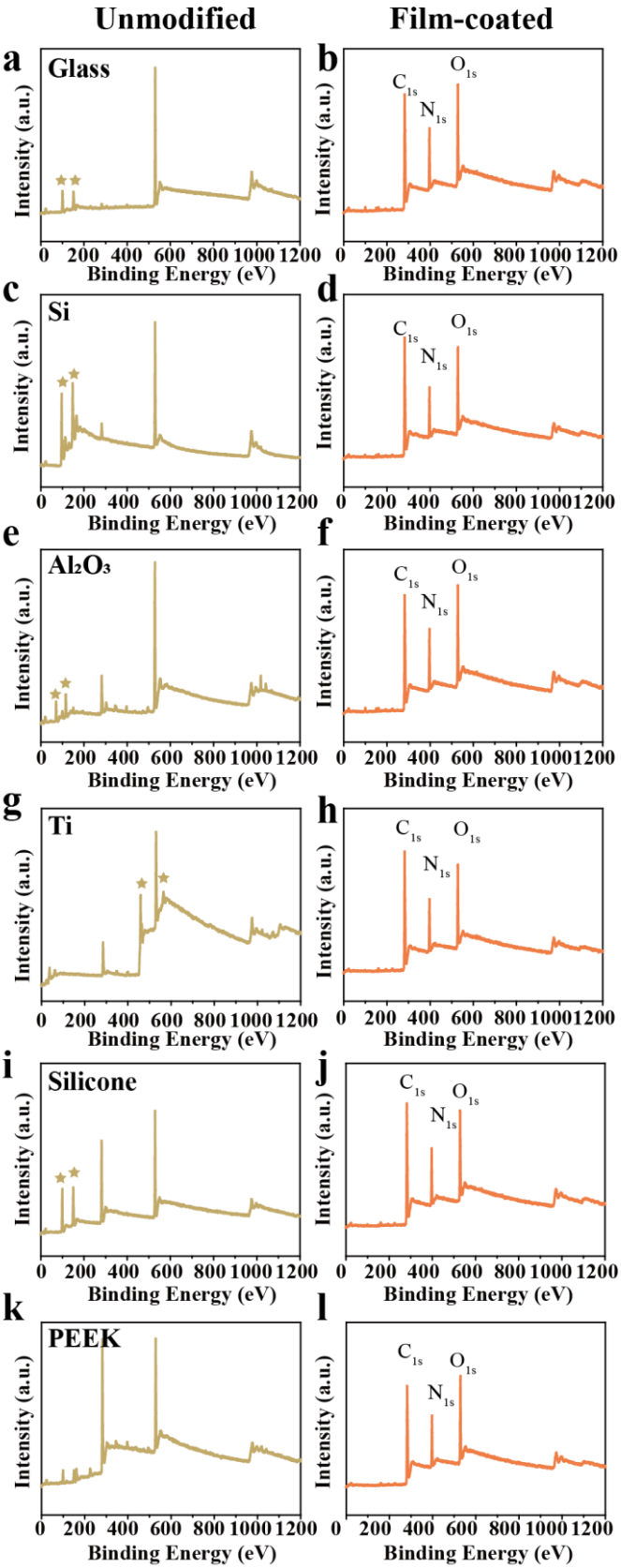


Figure S5. XPS characteristic of the PTB@ε-PL nanofilm adhered onto various substrates. (a, c, e, g, i, k) XPS wide scan of bare substrates. (b, d, f, h, j, l) XPS wide scan of various substrates with the coating of the nanofilm.

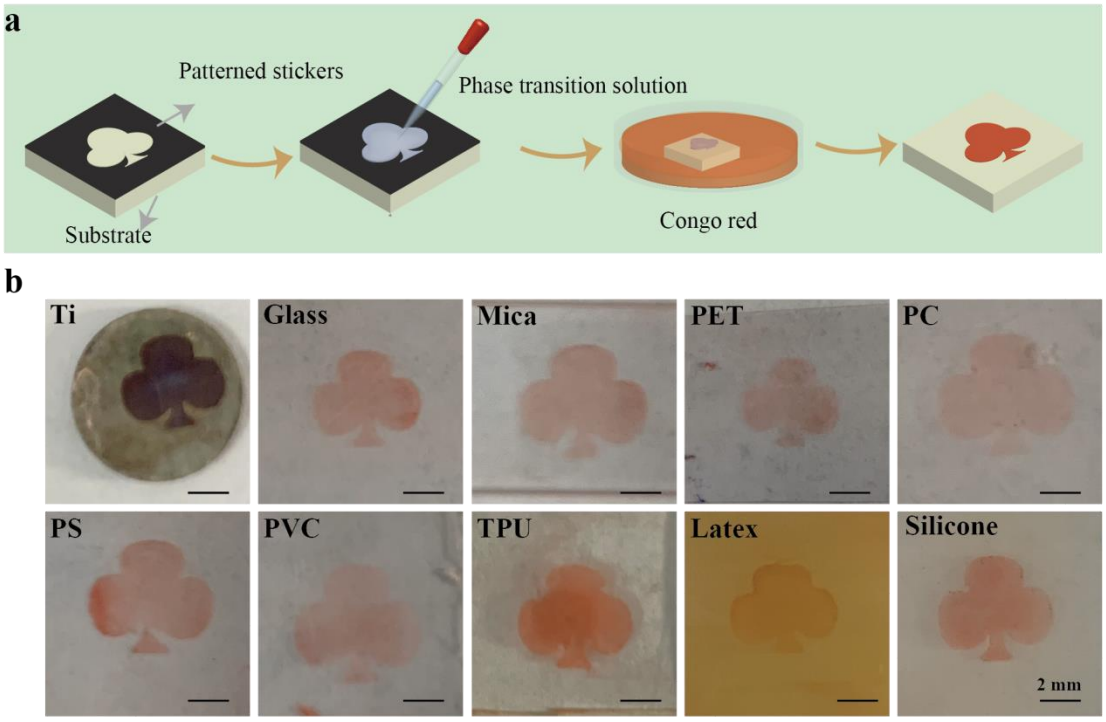


Figure S6. (a) The schematic cartoon showing the preparation and dyeing process of the PTB@ε-PL coating on various substrates. (b) Optical photographs showing the patterned PTB@ε-PL coating dyed with Congo red on inorganic, metallic and polymer materials. The pattern was obtained by using a sticker to control the dispersed region of the phase transition solution on the surface. PET: polyethylene terephthalate; PC: polycarbonate; PS: polystyrene; PVC: polyvinyl chloride resin; TPU: thermoplastic polyurethane; Latex: Polyisoprene emulsion (95%).

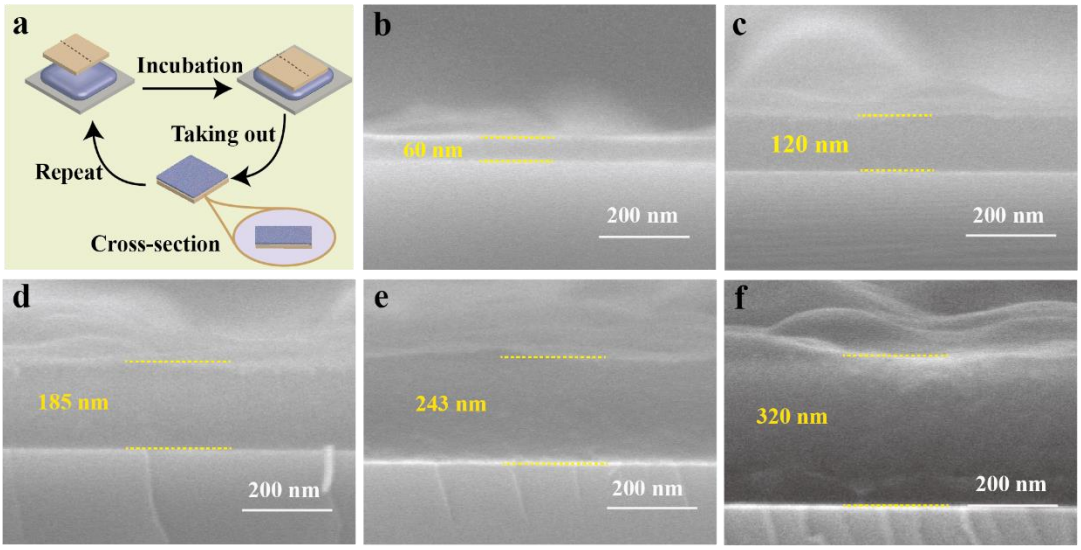


Figure S7. Cross-section SEM images of the PTB@ε-PL nanofilm coated on Si with different coating time. (a) Schematic cartoon showing the method of preparing multilayer coating. (b-f) The cross-sectional SEM images after preparing the 1-5 layers of the PTB@ε-PL coating on Si. The dotted line representing the upper and lower edges of the coating in cross-sectional view.

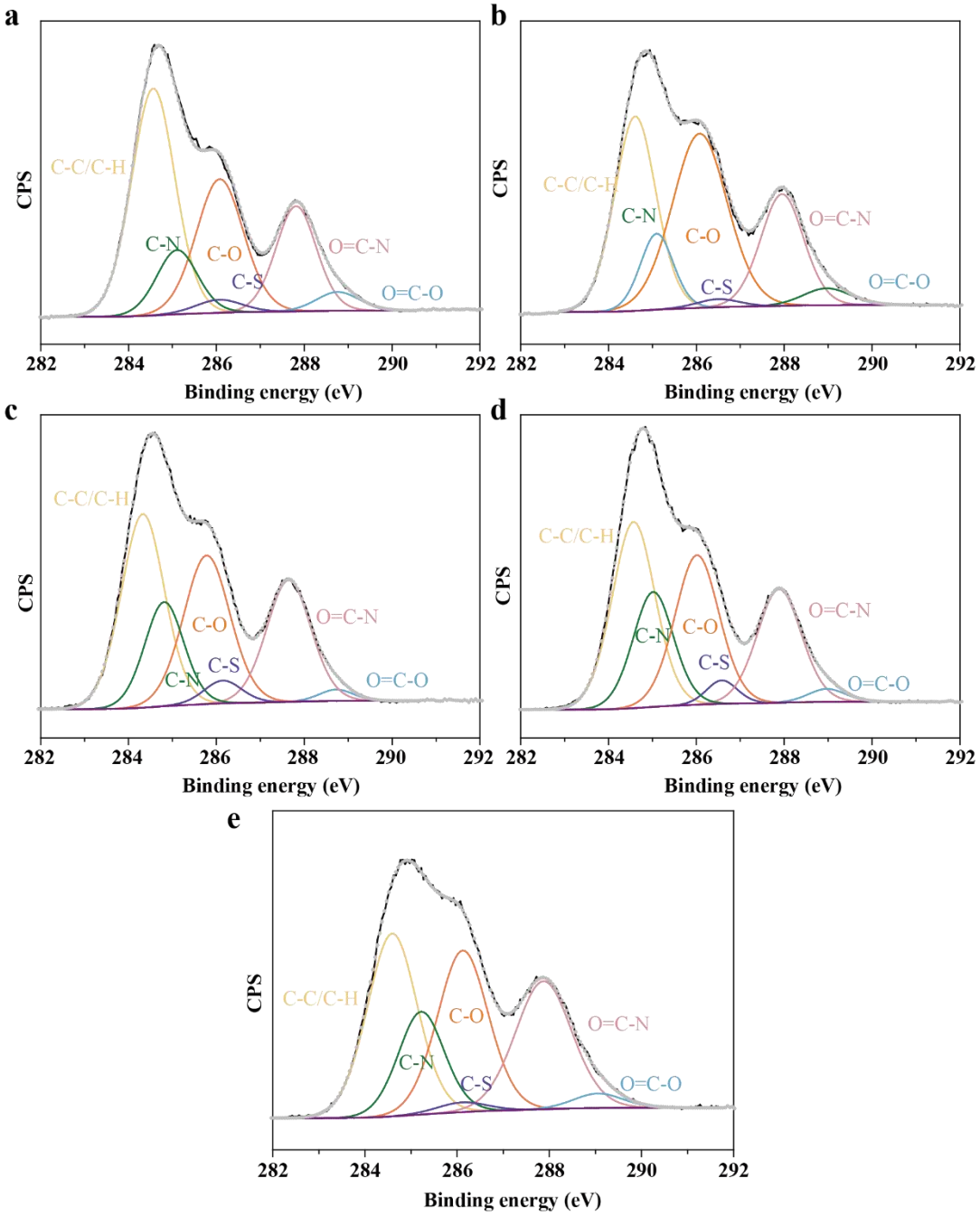


Figure S8. High-resolution C1s deconvolution spectra of 1 (a), 2 (b), 3 (c), 4(d) and 5 layers PTB@ε-PL coating on Si.

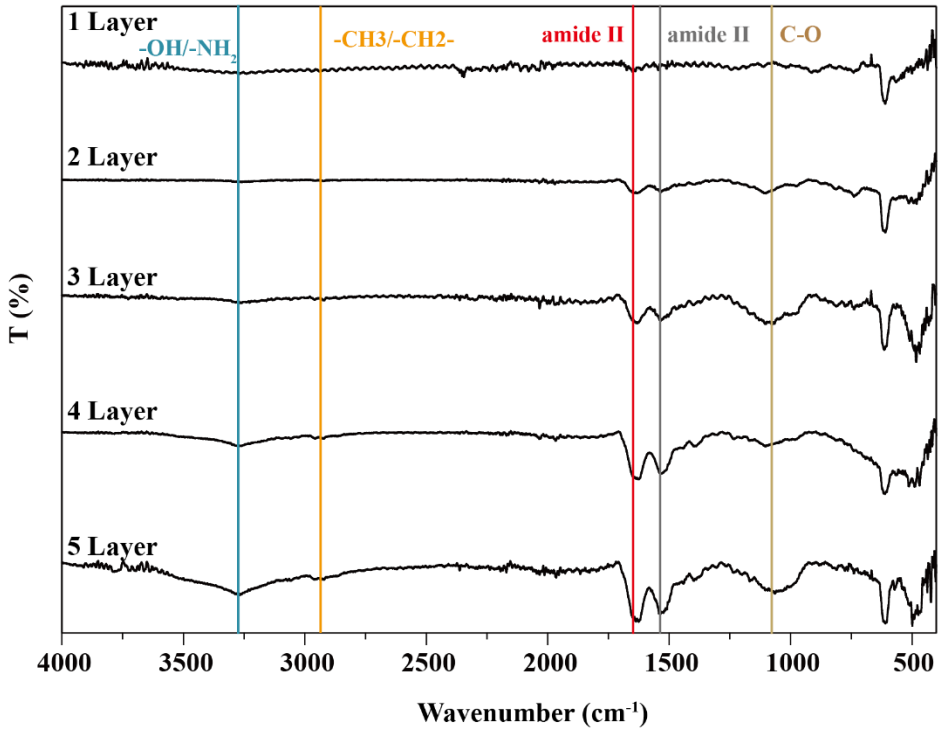


Figure S9. ATR- FTIR spectra of 1-5 layers PTB@ε-PL coating on Si.

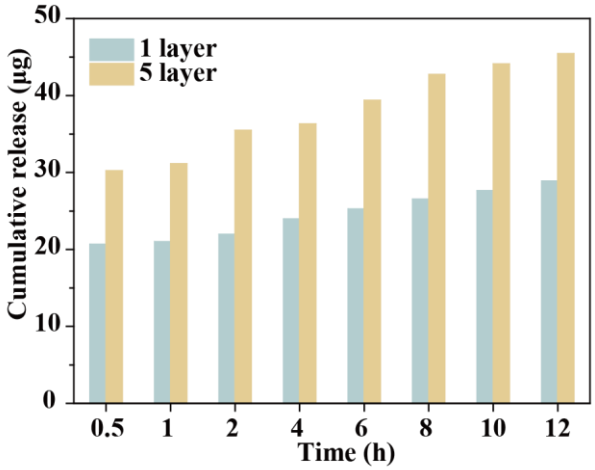


Figure S10. The cumulative release of ε-PL from 1 or 5 layers of PTB@ε-PL coating in PBS. The glass was coated with 1 or 5 layers of PTB@ε-PL coating, and then placed in 2 mL of PBS solution and released in a shaker at 37 ºC (70 rpm). A 200 μL sample was taken at a specific time point and then 200 μL of fresh PBS was added. By measuring the concentration of FITC-ε-PL in the release medium, the cumulative release rate of ε-PL was calculated.

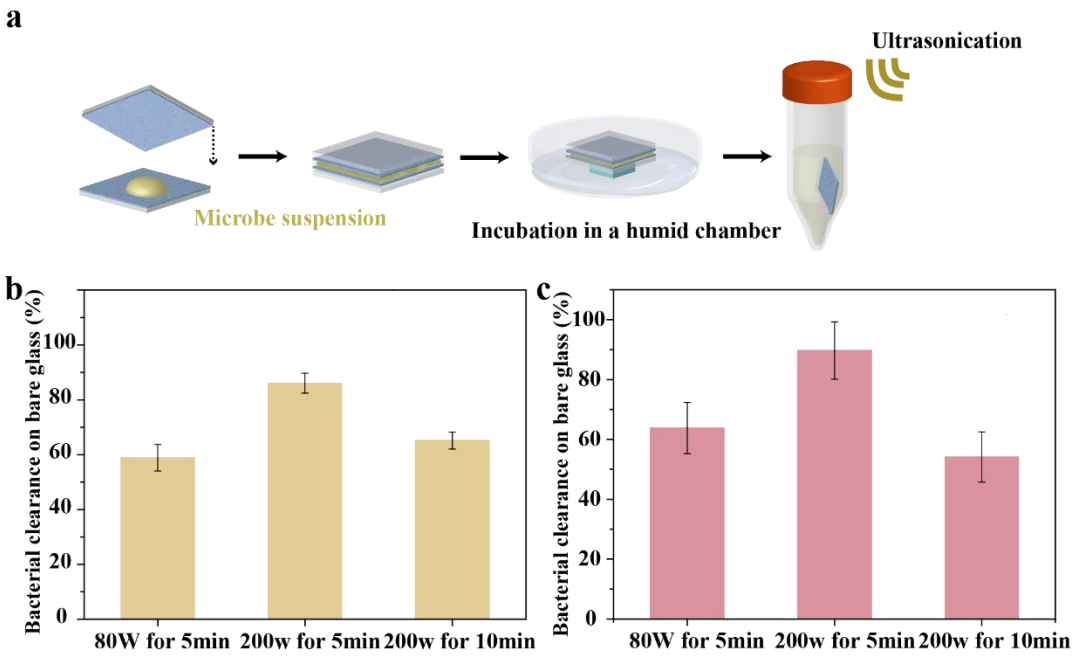


Figure S11. (a) Schematic showing the method for detecting antibacterial efficiency of the PTB@ε-PL coating. (b, c) Effect of ultrasound with different intensity and time to remove adhesion of *S. aureus* (b) and *E. coli* (c) on the surface of substrates.

**Supplementary Discussion:** The characteristics of biofilms as viscoelastic polymer fluids[3] and the non-covalent interaction[4] between extracellular polymeric substances (EPS) enable biofilms to withstand environmental mechanical stress and maintain stability. Ultrasound is widely used to disrupt the biofilm on the material surface and quantify the bacteria in it[5,6], but there is no uniform operating standard in the existing literature. In this study, we validated the efficiency of varying ultrasonic intensity to peel off adherent bacteria, and established a method for accurately quantifying the bacterial load in biofilms. It was found that after ultrasonic treatment with a power of 200W/40HZ for 5 min, 89.7% *Escherichia coli* (*E. coli*) and 86.1% *Staphylococcus aureus* (*S. aureus*) colonized in the biofilm could float in the PBS buffer, while excessive ultrasound (200w/40HZ for 10 min) would demage bacterial cells and affect viable clone counts.

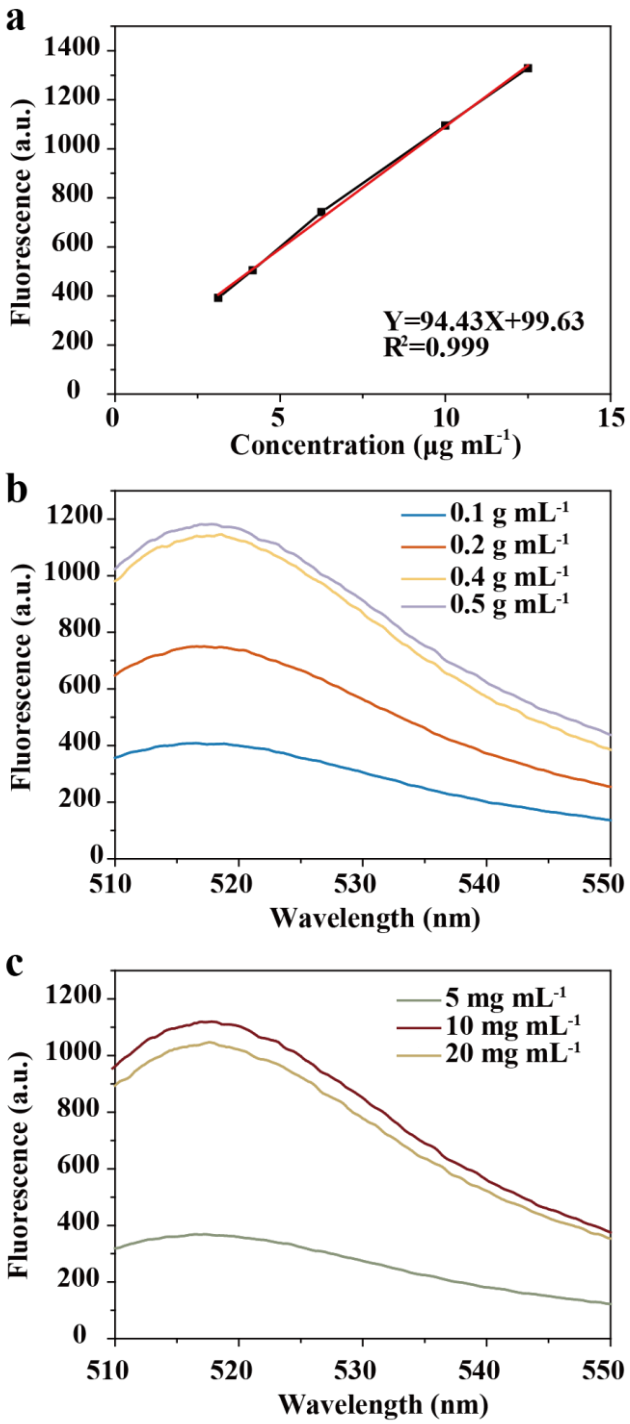


Figure S12. (a) Standard curve of FITC-ε-PL in Vc solution (1 M in water). (b) The fluorescence spectra of the solution after the PTB@ε-PL coating disassembled by 1M Vc aqueous solution at different ε-PL feeding concentration in the phase transition solution. (c) The fluorescence spectra of the solution of PTB@ε-PL coating disassembled by 1 M Vc aqueous solution at different BSA concentration in the phase transition solution. The hydrogen bonding formed between the -OH group in Vc molecule and the exposed hydrogen atom of the N-H group in the β-sheet backbone of amyloid could interrupt the hydrogen bonding between the β-sheets, thus making the amyloid structure unstable and being disassembled[2,7].

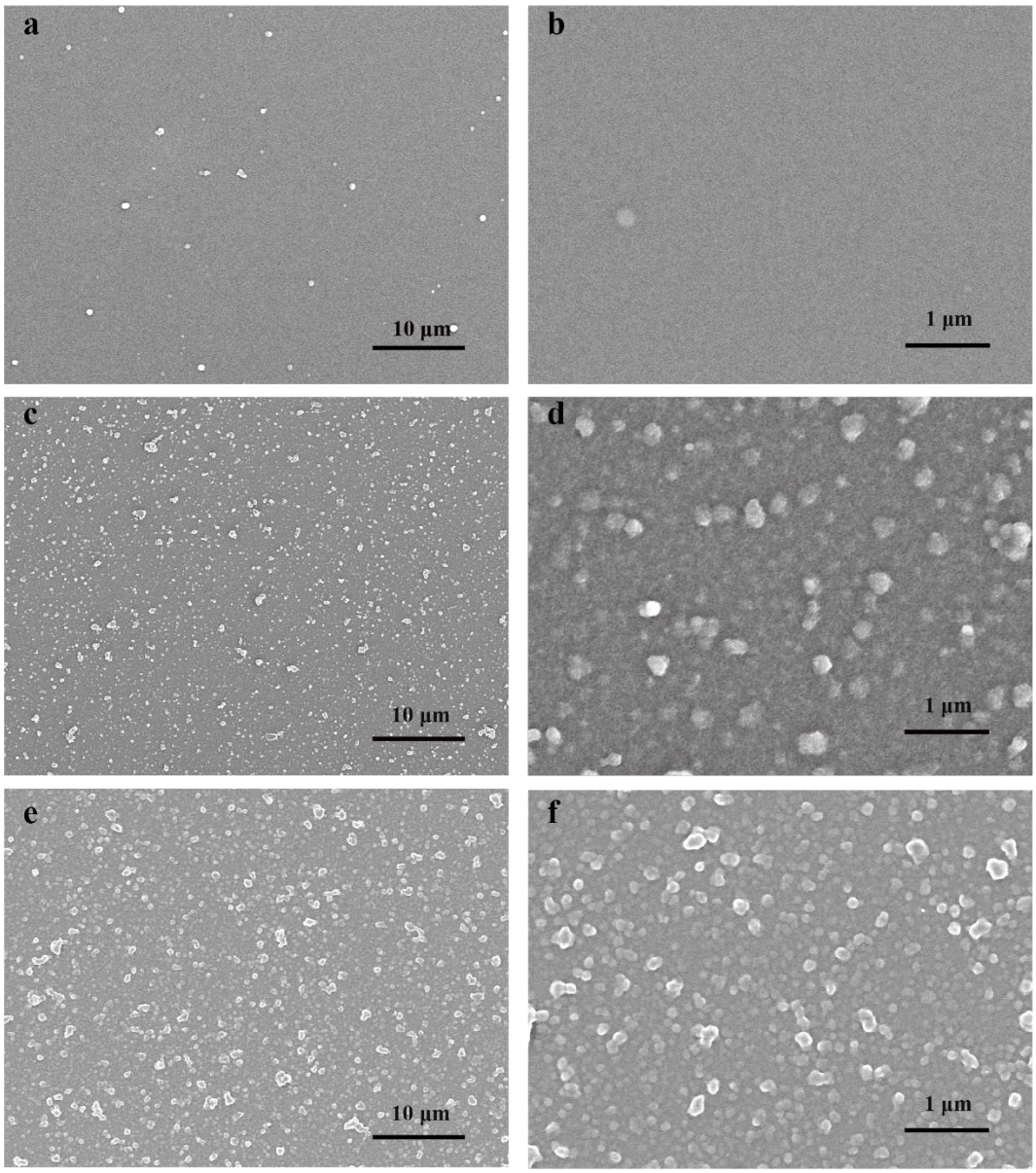


Figure S13. SEM images showing the surface morphology of PTB@ε-PL coating at different BSA concentration in the phase transition solution. (a, b) BSA 5 mg $mL^{-1}$, (c, d) BSA 10 mg $mL^{-1}$, (e, f) BSA 20 mg $mL^{-1}$. The reaction time is 2 h, and the phase transition solution was prepared by mixing BSA aqueous solution, TCEP (50 mM, pH 4.5 in water) and ε-PL (0.4 g $mL^{-1}$ in water) in a volume ratio of 1: 1: 1.

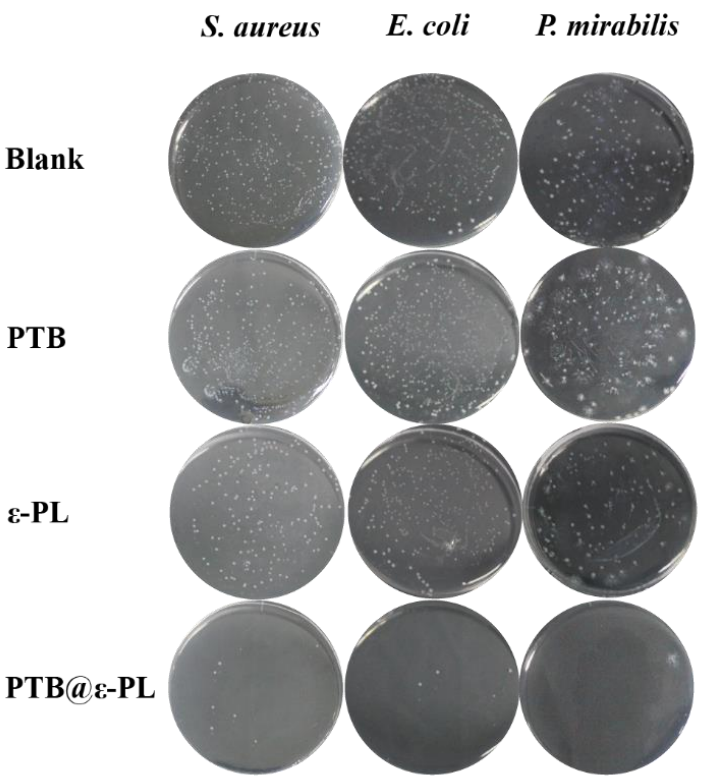


Figure S14. Photographs of colonies formed on MHA plates after the bacterial suspension between two pieces of glass was diluted 1000 times with PBS.

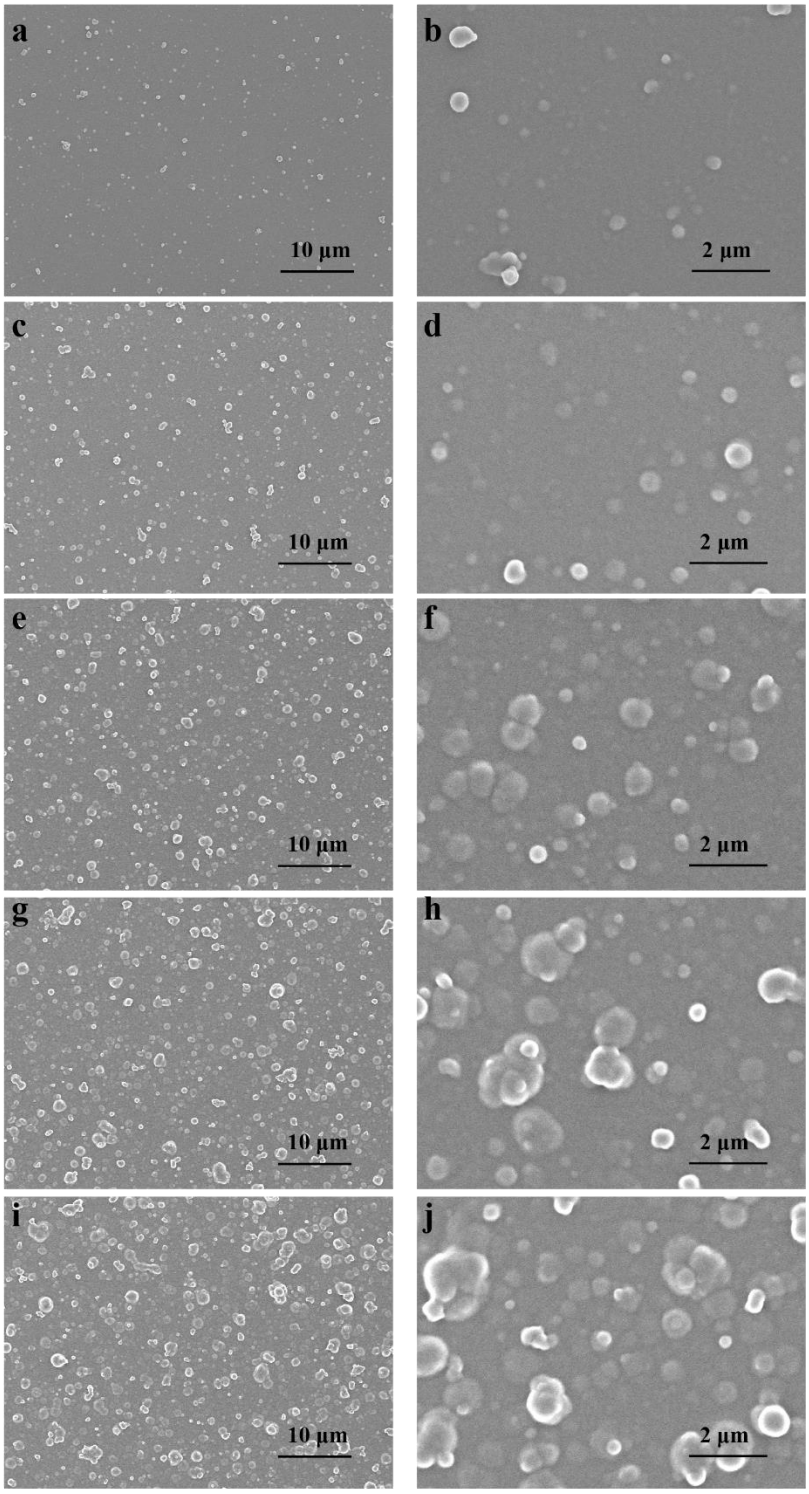


Figure S15. SEM images showing the surface morphology of 1 (a, b), 2 (c, d), 3 (e, f), 4 (g, h) and 5 (i, j) layers of PTB@ε-PL coating. The reaction time is 2 h, and the phase transition solution was prepared by mixing BSA aqueous solution (10 mg $mL^{-1}$), TCEP (50 mM, pH 4.5 in water) and ε-PL (0.4 g $mL^{-1}$ in water) in a volume ratio of 1: 1: 1.

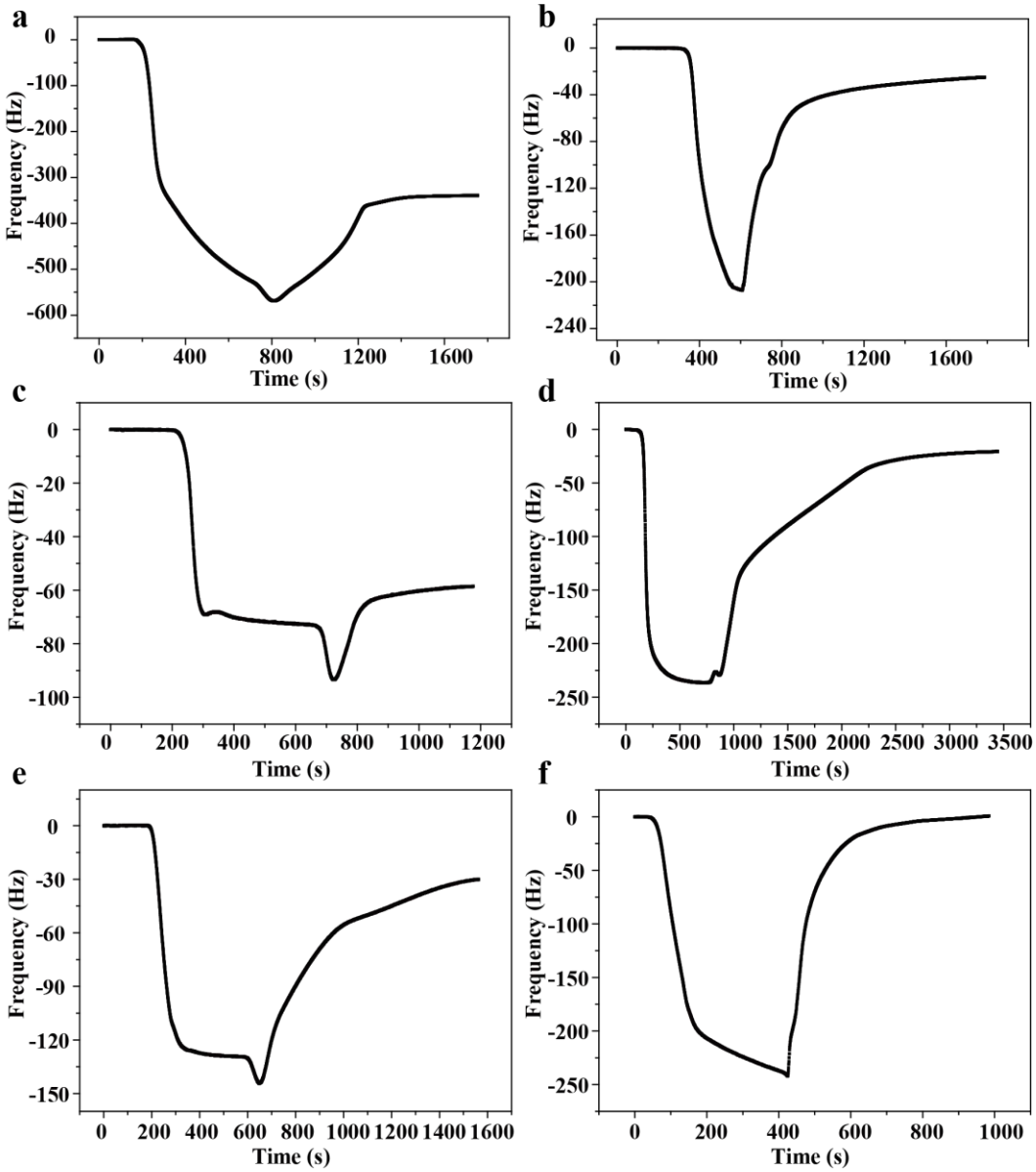


Figure S16. QCM frequency shift as function of time during the adsorption of human serum (a, b), FBS (c, d), and bacterial lysate (e, f) on bare Au chips (a, c, e) or the PTB@ε-PL -coated Au chip (b, d, f).

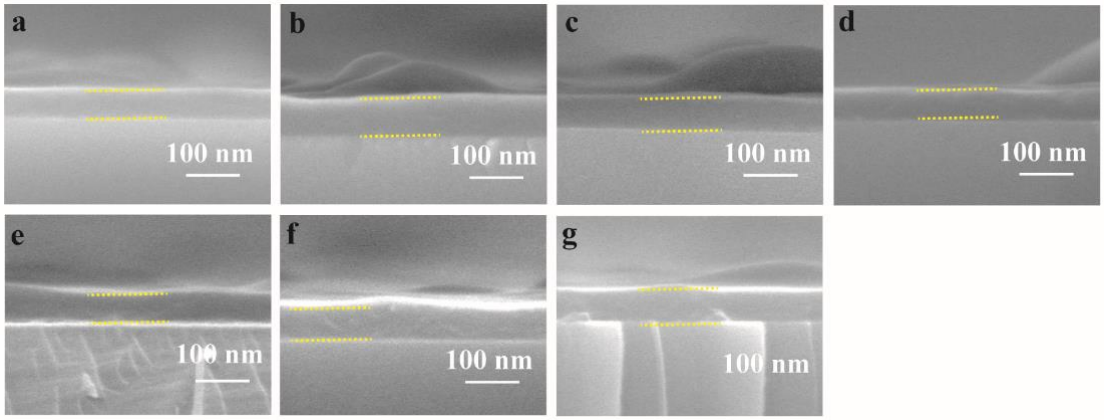


Figure S17. Cross-section SEM images of the PTB@ε-PL nanofilm coated on silicon wafer before (a) and after challenged by ultrasound (b), 3M adhesive tape peeling (c), autoclaving (d), DMSO (e), artificial saliva (f) and artificial urine (g). The dotted line representing the upper and lower edges of the coating in cross-sectional view.

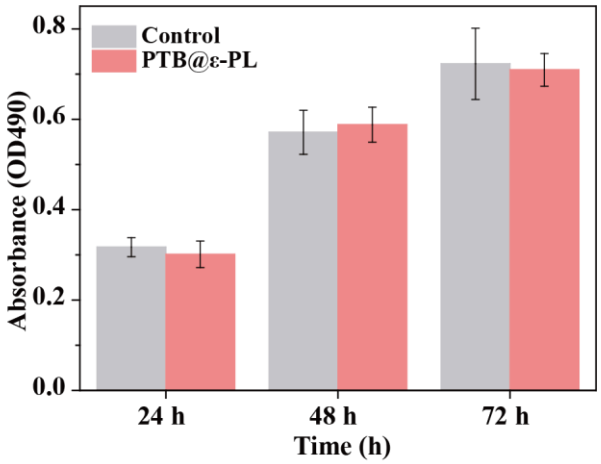


Figure S18. The cytotoxicity assay of the bare silicone rubber and PTB@ε-PL-coated silicone rubber as conducted in vitro via MTT assay.

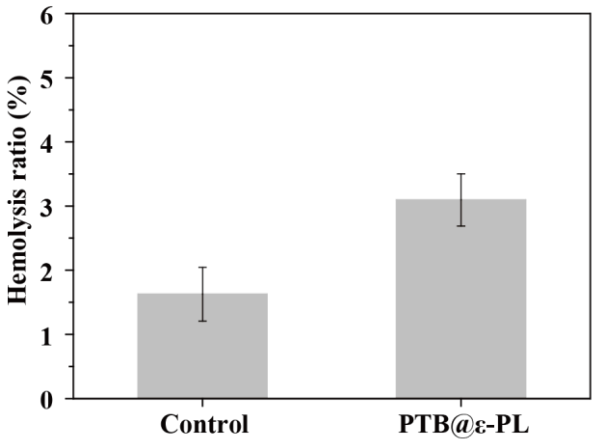


Figure S19. Hemolysis assay of bare silicone rubber and the PTB@ε-PL coated silicone rubber against erythrocyte cells.

## References


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