Enhanced antibacterial activity of polyphenol-bound microtopography by synergistic chemical and micro/nanomechanical effects

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1. Introduction

Biomedical equipment [1,2], implants [3], marine apparatus [4], and textiles [5,6] are prone to bacterial contamination. Bacterial contamination usually commences with early bacterial attachment, and subsequent proliferation can give rise to biofilms that are quite difficult to treat. Therefore, early intervention is important in preventing bacterial infections, and in this respect, antibacterial surfaces are crucial to many applications [7] including biomedical engineering [8], food science, and environmental engineering. A common approach to prevent bacterial contamination is surface modification of materials and deposition of antibacterial coatings [9,10]. However, time-consuming growth and substrate-dependent coating design can make the process complicated and commercially impractical. Therefore, new antibacterial strategies and materials that can inhibit early bacteria attachment are highly desirable.

Deposition of a germicidal coating is an effective strategy to mitigate early bacteria attachment and various methods such as plasma treatment [11] and wet chemical processing [12] have been developed. However, most of these methods are either laborious or substrate-dependent thus hampering broad application. Besides, the adhesion strength is an important factor especially in load-bearing situations. The popularity of poly(dopamine) (PDA) coatings indicates that phenols can be used to form versatile and sticky coatings with strong adhesion. For instance, rare-earth elements have been incorporated into catechin coated on polyamide membranes to improve the bacterial resistance and adhesion [13]. In addition, introduction of metals [14] and biological molecules [15] can endow polyphenol-based materials with the desirable properties. Most reported polyphenols-based coatings are prepared by ion chelation which limits the accessibility and flexibility. Hence, developing an ion-independent polyphenol-based antibacterial coating is of great significance.
The antibacterial efficiency also depends on the antibacterial pathways. Diverse surfaces have been designed to kill bacteria based on release [16] and non-release [17] mechanisms. A common “release” practice is to use antibiotics or germicidal chemicals grafted onto surfaces, but they can enter the blood stream to produce deleterious side effects. In contrast, non-releasing mechanisms kill bacteria on contact without involving antibiotics or chemicals. In fact, coatings with specific surface morphologies can kill bacteria via mechanical stress on the nano-micro level. Herein, a geometric-based tannins coating is constructed by one-step electrostatic precipitation with soft micelles as the template. The strong viscosity of micelles improves the film attachment and coating stability and upon contact, bacterial membranes are ruptured mechanically at precise locations. This dual-functional tannin-based coating has large potential in clinical treatment and biomedical engineering.

2. Materials and methods

2.1. Materials

Tannic acid (TA), cetyltrimethylammonium chloride solution (CTAC, 25 wt% in H2O), and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich, and 8-amino-1-naphthalene sulfonic acid (ANS, 96 %) and crystal violet (CV, 98 %) were bought from Macklin. Nitrophenyl β-galactopyranoside (ONPG) was provided by Beyotime. Sodium chloride (NaCl, GR ACS 99.8–%), sodium hydrogen carbonate (NaHCO3, 99.99 %), potassium chloride (KCl, GR ACS 99.8–%), di-potassium hydrogen phosphate trihydrate (K2HPO4 · 3H2O, GR, 99–%), magnesium chloride hexahydrate (MgCl2 · 6H2O, GR, ACS 99–%), anhydrous calcium chloride (CaCl2, 95–%), anhydrous sodium sulfate (Na2SO4, GR, ACS 99.5 %), tris(hydroxymethyl) aminomethane (99 %), and hydrochloric acid (HCl, 36–38 %) were ordered from International Lab, USA. The sterile fiber-off sheep blood was brought from Nanjing Quan Long Biotech Co Lit. A silicon wafer with a diameter of 100 mm was cut into 1 cm × 1 cm pieces, and the ITO glass was purchased from South China Science & Tech Co Lit. The silicon substrates and glass slices were treated ultrasonically in alcohol and deionized (DI) water for 30 min successively and vacuum-dried. All the chemicals were used directly without purification.

2.2. Characterization

The UV–vis spectra were acquired on a double-beam UV–visible spectrophotometer (Halo DB-20, Dynamica, Australia) and the Zeta potentials was measured by DLS on the Malvern zetasizer Nano ZS. The FTIR spectra were obtained on the PerkinElmer spectrum II FTIR spectrometer (Halo DB-20, Dynamica, Australia) and the Zeta potentials was measured by DLS on the Malvern zetasizer Nano ZS. The UV–vis spectra were acquired on a double-beam UV–visible spectrophotometer (Halo DB-20, Dynamica, Australia) and the Zeta potentials was measured by DLS on the Malvern zetasizer Nano ZS. The FTIR spectra were obtained on the PerkinElmer spectrum II FTIR spectrometer. Scanning electron microscopy (SEM) was performed on the QUATTRO S and fluorescence was detected by the Molecular Devices SpectraMax iDS microplate reader (USA).

2.3. Preparation of storable TC powders

The electrostatic precipitation method was used in the synthesis. TA (0.225 g) was dissolved in 9 mL of deionized (DI) water in a 50 mL round-bottom centrifuge tube, and the pH value of the TA solution was adjusted with NaOH (1 M). An equal volume of the diluted CTAC solution (70 mM, 9 mL) and cationic amphiphilic molecules was added dropwise under magnetic stirring to form the TC complex. The flocculent precipitate formed immediately. After the reaction, the TC complex was centrifuged at 11,000 rpm for 15 min and the supernatant was discarded. The solid was collected, washed twice with DI water, and lyophilized to form the TC powders. The samples prepared at pH 8 and 9 were denoted as TC8 and TC9. All the TC powders were sealed and stored in a drying cabinet before use.

2.4. Coating formation

The TC powders (0.06 g) were dispersed in 2 mL of methanol and treated ultrasonically for 15 min to obtain a yellowish suspension. 1 mL of the suspension was added to the wells of the 24-well plate with the clean silicon pieces placed in advance. The plates were kept still for 10 min so that coatings were formed on the upside. Afterward, the TC-coated silicon samples were taken out, dried at room temperature, rinsed twice with DI water, and vacuum-dried. The TC-coated ITO and normal glass were prepared by the same procedures.

2.5. Stability assessment

2.5.1. TA release assay

The concentration of released TA is measured by UV–vis spectroscopy to evaluate the chemical stability of the TC coatings. The TC-coated silicon samples were placed on a 35 mm tissue culture dish (FALCON 353001) and immersed in 1 mL of DI water at room temperature for 24 h and 48 h. A small amount of the DI water was collected at a specified time and the absorbance at 274 nm was monitored. The concentration of TA was determined with reference to the standard curve established with gradient TA solutions.

2.5.2. Static water contact angle (WCA) assay

The structural stability of the TC coatings was evaluated by the WCA assay. The TC-coated Si samples were immersed for one day in saline solution, DMEM medium and simulated body fluid (SBF) containing NaCl (7.996 g), NaHCO3 (0.350 g), KCl (0.224 g), K2HPO4 · 3H2O (0.228 g), MgCl2 6H2O (0.305 g), HCl (1 kmol/m3), 40 cm3), CaCl2 (0.278 g), Na2SO4 (0.071 g), (CH3OH)CNH2 (6.057 g). An appropriate amount of HCl (1 kmol/m3) was used to adjust the pH to 7.25. The static images were acquired before and after on the Rame-Hart imaging system in triplicate. The Image J software was used to determine the WCAs.

2.5.3. Scanning electron microscopy

The morphology was evaluated by immersion, tape adhesion, and ultrasonic tests. The TC-coated silicon samples were immersed in DI water for 2 days, ultrasonically treated for 30 min, torn with 3 M tape, and sputtered with gold prior to SEM. The bacteria were examined according to a previous protocol [18]. The E. coli cells were harvested in the exponential growth phase (OD600 = 0.6–0.7), and the diluted bacterial solution (100 μL, 106 CFU mL–1) was seeded onto the different samples for cultivation. The samples were rinsed three times gently with DI water and fixed by the glutaraldehyde solution (2.5 %) overnight at 4 °C. The bacteria were dehydrated with gradient ethanol solutions (30 %, 50 %, 75 %, 90 %, 95 %, and 100 %) and then vacuum-dried.

2.6. Antibacterial evaluation

The bacterial colony-forming units (CFU) counting method was adopted to evaluate the antibacterial ability. Before incubation, the TC-coated samples were sterilized by UV irradiation for 30 min. The Gram-negative strain (Escherichia coli, ATCC 25922) was chosen as the model microorganism. The E. coli cells were cultivated in LB broth overnight in a shaking incubator (220 rpm, 37 °C) and harvested in the exponential growth phase (OD600 = 0.6–0.7). The diluted bacterial solution (100 μL, 106 CFU mL–1) was seeded on the samples for a set time. Afterward, the bacterial solution (10 μL) was spread on solid agar plates and cultivated for another 18 h to count the CFUs. The antibacterial efficiency against S. aureus (29213) and S. epidermidis (pAO1) was derived.

The antibacterial effect of the A solutions against E. coli was evaluated as well. The diluted bacterial solution (500 μL, 106 CFU mL–1) was mixed with the TA solution with the highest concentration of 1.88 mM and half sequential dilution. The final highest concentration of TA solution incubated with bacteria was 0.94 mM. The TA solutions with different concentrations were mixed with an equal volume of LB broth.
set as the background. All the solutions were placed on a 96-well plate and cultivated for 16 h. The antibacterial rate was determined at OD_{600} after deducting the background as follows:

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\text{Antibacterial rate} = \frac{\text{OD}_{\text{control}} - \text{OD}_{\text{TA}} - \text{OD}_{\text{background}}}{\text{OD}_{\text{control}} - \text{OD}_{\text{background}}} \times 100\%.
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2.7. Adherence assays

The crystal violet (CV) assay was used to stain the bacteria attached to the samples. The TC suspensions were placed on the 96-well plate for 15 min and dried at room temperature. The wells were rinsed with DI water three times before use. The pure TA and CTAC solutions were subjected to the same procedures as TC to serve as the control. The bacterial solution treated with 10 μM Bacterial Membrane Potential Kit (Molecular Probes, Invitrogen, USA) was added to the bacteria solution and mixed ultrasonically with CCCP for 1 min under dark conditions. The bacterial solution treated with 10 μM ANS solution was added to the above solution and cultured for 20 min at 37 °C. The absorbance at 595 nm was determined on the microplate reader (USA) at λ = 595 nm. The fluorescence intensity was measured by the Molecular Devices SpectraMax IDS microplate reader (USA) at λ_{ex} = 380 nm and λ_{em} = 460 nm. In the bacterial membrane permeability test, 1 mM TC solution was added to the solution and mixed under dark conditions. The bacterial solution treated with 10 μM H_{2}O_{2} was the positive control. The bacteria solutions were transferred to a 96-well plate and the absorbance was monitored on the microplate reader at λ_{ex} = 488 nm and λ_{em} = 535 nm.

2.8. Integrity of bacterial membranes

Before the membrane permeability and potential test, the bacterial solution (100 μL, 10^{6} CFU mL^{-1}) was seeded on the coated samples and cultured for 1 h and 3 h. Afterward, normal saline (900 μL) was added and mixed. In the bacterial membrane permeability test, 1 mM TA solution was added to the above solution and cultured for 20 min at 37 °C. The fluorescence intensity was measured by the Molecular Devices SpectraMax IDS microplate reader (USA) at λ_{ex} = 380 nm and λ_{em} = 460 nm. In the bacterial membrane potential test, the BacLight™ Bacterial Membrane Potential Kit (Molecular Probes, Invitrogen, USA) was added to the bacteria solution and mixed ultrasonically with CCCP as the positive control (PC). Staining was carried out [17] before flow cytometry.

To detect the reactive oxygen species (ROS), the DCFH-DA (Beyotime) solution was added to the bacteria solution and incubated for 20 min under dark conditions. The bacterial solution treated with 10 μM H_{2}O_{2} was the positive control. The bacteria solutions were transferred to a 96-well plate and the absorbance was monitored on the microplate reader at λ_{ex} = 488 nm and λ_{em} = 535 nm.

2.9. Mechanical study of bacterial membranes

The nanoindentation measurement was conducted on the AFM (Veeco MultimodeV, Veeco, USA) to determine the mechanical properties of the bacterial membranes. After interacting with the coating for 3 h, the bacteria were immobilized on the TC-coated Si sample with 2.5 % glutaraldehyde for 30 min before mounting on the AFM stage. The tapping mode enabled real-time imaging to screen the area of interest before obtaining the distance-force curves. The pyramidal ACTA cantilever with a spring constant of 40 N/m was used. The sensitivity of the cantilever was first measured on a standard sapphire sample assumed to be absolutely hard. The trigger threshold was set as 700 nN for the indentation F = − kx. The raw data for the force-indentation curves were processed by Matlab R2022b. In the mechanical analysis, the Young’s modulus was fitted based on the non-linear part of the line with a Poisson ratio (ν = 0.5) for biological samples. The tip was considered conical with an opening angle (α) of 35° based on the Sneddon model:

\[
F = \frac{2}{\pi} \sin^{-1} \left( \frac{F}{k} \right) \rho. \]  

The stiffness was calculated from the linear part of the line [20].

2.10. Hemolysis assay

The whole sheep blood was diluted with 4 % phosphate buffer saline (PBS) and a 4 % RBC suspension (1 mL) was added to a 24-well plate with the sterile TC-coated samples. The RBC suspension incubated on clean silicon was the negative control (NC) and the 1 % Triton-X treated RBC suspension for cell lysis was the positive control (PC). All the samples were incubated for 1 h at 37 °C. The absorbance at 576 nm was monitored, and the hemolysis rate was calculated as follows: Hemolysis rate (%) = \left( \frac{\text{OD}_{\text{samples}} - \text{OD}_{\text{NC}}}{\text{OD}_{\text{PC}} - \text{OD}_{\text{NC}}} \right) \times 100 \%.

2.11. Cytocompatibility

The cytotoxicity of the TC-coatings was assessed by the Cell Counting kit-8 (CCK-8, Beyotime) with MC3T3-E1 osteoblasts obtained from the cell bank of the Chinese Academy of Sciences. Dulbecco’s modified eagle medium (DMEM) containing 10 % fetal bovine serum (FBS) was used to incubate the cells at an incubator with 5 % CO_{2} at 37 °C. The medium was replaced every day. The cells in their logarithmic growth phase were harvested and seeded on a 24-well plate incubated with the sterile sample with a density of 2 × 10^{4} cells mL^{-1}. The cells incubated on pure silicon was the negative control. The CCK-8 solution was added to each well after 24 h and incubated for another hour. The solution (100 μL) was transferred to a 96-well plate, and the intensity of OD_{450} was detected by a micro-reader (Bio Tek, US). Each sample was tested three times.

3. Result and discussion

3.1. Materials characterization

The electrostatic force-based assembly technique is powerful for the design of drug delivery systems, bioreactors, or functionalized cells with a wide range of sizes and patterns. Moreover, the desired microstructures with the designated components can be prepared. Herein, cationic amphiphilic molecules are chosen as the soft micelle template support as the inner layer with positive potentials. The ionized tannin acts as the external block with multiple bonding forces to facilitate assembly. The deprotonated tannin is the key component in the electrostatic self-assembly system. Generally, when the pH of the medium is higher than pKa, tannic acid tends to be deprotonated or protonated otherwise. However, an excessively high pH can result in full oxidation of phenolic hydroxy to quinone [21], which is no longer electronegative to maintain the electrostatic force in self-assembly.

The synthesis process is illustrated in Fig. 1a. The proper pH for ionization is screened by monitoring the products formed at different pH by UV–vis spectrophotometry (Fig. S1a). The fresh TA solution shows absorption peaks at 212 nm and 274 nm representing the typical isobestic points of TA due to the non-ionized phenolic groups [22]. As the pH increases from 7 to 10, two new absorption peaks appear at 235 nm and 320 nm for p-n conjugation of ionized TA. When the pH exceeds 10, a new peak appears at 290 nm because ionized TA is fully oxidized and rearranges into various conjugated quinone derivatives. The pictures showing the reactions at different pH are depicted in Fig. S1b. The solution remains light yellow when the pH is increased to 6, but the white flocculent precipitate emerges at pH between 7 and 9. At a high pH, the yellow solution becomes light green, and no precipitate is formed indicative of full oxidation. The change in the solution color and status is corroborated by the UV–Vis spectra. Based on these results, the optimal pH for self-assembly is determined to be 8 and 9.

The chemical structure of the TC powder is determined by FTIR. As shown in Fig. 1b, the absorption bands are assigned to the phenol hydroxyl stretching (3230–3480 cm^{-1}), stretching of C–O of carboxylic esters (1715 cm^{-1}), and aromatic C=C resonance vibration of TA (1605 cm^{-1}). The peaks of TA at 1715 cm^{-1}, 1600 cm^{-1} and 1317 cm^{-1} can be observed from TC8 and TC9. The peak of the phenol hydroxy groups

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\text{Antibacterial rate} = \frac{\text{OD}_{\text{control}} - \text{OD}_{\text{TA}} - \text{OD}_{\text{background}}}{\text{OD}_{\text{control}} - \text{OD}_{\text{background}}} \times 100\%.
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widens in TC8 and TC9 due to the disruption of hydrogen bonds. The new saturated fat C-H stretching peak appears at 2930 cm⁻¹ during electrostatic assembling between TA and CTAC. Compared to TA, the peak at 1470 cm⁻¹ from TC may arise from the aromatic C-C stretching of TA, which is usually found in the polyphenolic structure caused by deprotonation [23]. FTIR confirms that TA is deprotonated and electrostatic assembly is successful. The zeta potentials (Fig. 1 c) show that TA and CTAC are the anion and cation at 11.3 mV and 60 mV, respectively, as shown in Fig. 1 a. The template is supported as the inner layer, and the anion is assembled on the outside layer to show the final negative potential.

3.2. Application assessment

3.2.1. Storability and film-forming properties

The practicability of TC powders as raw materials for coating formation is investigated. Firstly, the lyophilized TC powders can be stored well at room temperature and deposited on various substrates, including ITO, glass slices, and silicon wafers by simple immersion (Fig. S2). Here, the silicon substrate is chosen for further characterization. The morphology of the TC-coated Si sample is displayed in Fig. 1 d1, d2 and e1, and e2. The uniformly distributed spherical microparticles have average sizes of 8.2 μm for TC8 (Fig. 1 d2) and 4.3 μm for TC9 (Fig. 1 e2). The cation surfactant, as a soft micelle template, forms the nucleation centers to attract ionized tannin with a negative potential. The appropriate electrostatic force fosters the formation of the outside layer composed of the ionized tannin, and excessive amounts of anions result in a negative zeta potential on the final TC8 and TC9. The assembly process does not affect the versatility of tannin. The hydrogen bonds and intermolecular forces of the TC powders make it easy to coat on various substrates firmly (Fig. S2). Besides, the linkage between TA on the outside layer and proteins in microorganisms is established to trap bacteria in the vicinity.

3.2.2. Stability

The above results demonstrate that the adhesive structure of the outer tannin fulfills the one-step formation of the coatings by simply immersing different substrates in the solution (Fig. S2). The stability is evaluated from the morphological and compositional perspectives. The morphological durability of the TC8 and TC9 coatings on silicon is assessed by immersion, ultrasound, and adhesion tests. Insignificant morphology changes are detected from TC8 and TC9 after immersion in static water for 2 days (Fig. 2 a1 and 2a2) or ultrasonic treatment in water for 30 min (Fig. 2 b1 and 2b2), indicating that the coating is water-stable and ultrasound-resistant. The anti-adhesive ability is evaluated by tearing with 3 M tape. As shown in Fig. 2 c1-c4 and 2d1-d4, the surface morphology does not change after tearing indicating strong adhesion. The lyophilized TC powders are re-dispersed to produce the TC coatings after storage for six months and good morphology is observed (Fig. S3). Moreover, the coating remains hydrophilic after immersion in saline, DMEM, and SBF for one day (Fig. S4), further corroborating the morphological stability under physiological conditions.

The compositional stability is assessed by the release behavior of TA with time. The cumulative amount of released TA in one day is evaluated based on the standard curve of TA at OD₂₇₄ (Fig. S5a). Less than 7 μM (Fig. S5b) is detected, implying that the coating does not decompose in water. A previous study demonstrates that tannic has the antibacterial ability [24], but the survival rate of E. coli is more than 70 % for a TA concentration of 7 μM (Fig. 3 b). Hence, the antibacterial ability should arise from other factors other than released TA.
Fig. 2. Morphological and compositional stability of the TC coatings prepared on Si after different treatments: (a) Immersion in DI water for two days, (b) Ultrasonic treatment for 30 min, (c) Detachment test with 3 M type. The scratch is made on one sample and the 3 M type is applied to one side of the coating (c1, d1) while leaving the other side untreated (c3, d3). The scale bars of TC8 and TC9 are 50 μm and 5 μm, respectively.

Fig. 3. Antibacterial activity and chemical effects on the bacterial membrane. (a) Antibacterial rates of TC8 and TC9 for 1 h and 3 h; (b) Survival rates for E. coli in the TA solutions; (c) CV-based adhesion tests of different samples; (d) SEM images of different treated groups with the scale bar being 5 μm for the above and 1 μm for below; (e) Permeability of bacterial membranes after different treatments; (f) Bacterial membrane potentials of different treated samples. The statistical analysis is based on the Student t-test and calculated with the control group at the same time point with *, **, and *** denoting $p < 0.05$, $p < 0.01$, and $p < 0.001$ respectively. The error bar indicates ±SD with $n = 3$. 
3.3. Antibacterial ability

The antibacterial ability is evaluated. Based on the CFU counting method, the antibacterial rates are 67 % after 1 h and almost 85 % after 3 h (Fig. 3a and Fig. S6). The antibacterial efficacy against *S. epidermidis* and *S. aureus* is more than 90 % after 3 h (Fig. S6 and Fig. S7). Similarly, the survival rate of *E. coli* against the TA solution is evaluated. The antibacterial rate is about 95 % when the concentration of TA solution is 0.94 mM after incubation for 16 h (Fig. 3b). This is far more than the released amount of less than 7 µM, but it is time consuming. As the initial attachment determines bacterial survival, tannins alters the bacterial membranes [25] and both the short-time and non-release behavior is investigated starting with initial interactions.

3.4. Interactions with bacteria

3.4.1. Adhesion assay

Initial attachment is the key step for subsequent proliferation. Compared to the TA, CTAC, and control groups, the TC group shows higher bacterial adherence (Fig. 3c), in which both the enhanced stickiness and attenuated stiffness of the outside layer are important [26, 27]. Although TA is supposed to inhibit the growth of microorganisms, by disrupting the bacterial membrane and producing metabolic disorder [28,29], the detached state makes it hard to enable full interactions with bacteria. On the contrary, the stable microparticles in TC enable full exposure of active site to produce mechanical distortion.

3.4.2. Morphology

The morphology of the coatings incubated with bacteria is examined by SEM (Fig. 3d). The bacteria incubated on pure silicon have a round and full shape and so the influence of the substrate can be ignored. In contrast, the bacteria on the TC samples exhibit obvious morphological change with lacunose membranes observed. Although TA in a high concentration can also damage the bacterial membrane, bacteria tend to adhere to the microparticles regardless of size, and the unique surface morphology twists the bacteria membrane by physical stress.

3.4.3. Bacterial membrane

The permeability of the bacterial membrane is evaluated semi-quantitatively. Although TA (0.94 mM) decreases the bacterial viability to a similar degree as TC8 and TC9 after incubation for 16 h, the TA samples exhibit similar fluorescence as the control group at the same time point of 3 h, indicating little loss in the membrane integrity (Fig. 3e). On the other hand, fluorescence from the TC samples increases significantly with time. It is much higher than that of the control after 3 h indicating sustained and increased membrane permeability. Changes in the cell membrane permeability affect the activity of ion channels and ion flow and alter the membrane potentials. Here, the membrane potential is also evaluated to provide evidence for the permeability change (Fig. 3f). After 1 h, the TA samples show a similar red/green ratio in agreement with the unchanged permeability. However, the red/green ratio decreases after reacting with TC for 1 h, and the state is maintained for as long as 3 h, suggesting impaired membrane potentials similar to the positive control. The TA samples show limited effects on the bacterial membranes in a short time. Moreover, the ROS level has very little influence (Fig. 3g), implying that the influence of TC coatings on ROS regulation is small and can be ignored. Both TC8 and TC9 show similar antibacterial efficiency and morphological change, thereby supporting the hypothesis that the mechanical pressure at the interface plays a major role in the antibacterial process.

3.4.4. Mechanical analysis

With more non-covalent bonds in polyphenols and non-covalent forces greatly impacting the mechanical quantity of biomacromolecules, the interactions between abundant non-covalent bonds and bacterial membranes are investigated by biophysical analysis. The area selected by the AFM tapping mode is shown in Fig. 4a. The force curves of the tip approaching and retracting from the top of the selected bacteria are recorded by the nanoindentation mode. The elasticity-related information, such as Young’s modulus (E), is calculated by fitting the non-linear part based on the Sneddon model (Fig. 4b and c), and the stiffness of the bacterial membrane bears a positive correlation with the slope of the linear region [30]. The E value of the control is 1.43 ± 0.11 GPa and it increases in the order of TA (3.05 ± 1.50 GPa) < TC8 (5.56 ± 1.88 GPa) < TC9 (8.85 ± 5.67 GPa), indicating that the membrane behaves mechanically as it is in contact with the coatings. Compared to the control, the stiffness of TA and TC increases by 30 % (20.98 ± 0.96 N/m) and more than 60 % (26.16 ± 7.17 N/m, 24.43 ± 6.38 N/m), respectively. It can be explained by that the detached TA encounters bacteria randomly and interferes with the bacterial envelope chemically. On the TC samples, the bacteria are trapped by the sticky coating and then the microparticles reinforce the non-covalent mechanical stress, as shown by the altered Young’s modulus and stiffness. The distribution of the membrane stiffness is more concentrated (coefficient of variation, COV = 0.08) for the control and TA groups revealing homogeneous mechanical effect on the bacteria. However, the stiffnesses of TC8 and TC9 with COV of 0.27 and 0.26 are more dispersed, implying that the nano-topography attracts the bacteria and exerts mechanical stress at multiple sites.

3.5. Biocompatibility assessment

The hemolysis assay is conducted to evaluate the biocompatibility of mammalian cell membranes. Due to the lack of complicated structures such as nuclei and organelles, red blood cells (RBC) are usually used to investigate the interactions between external entities and mammalian cell membranes. Negligible hemolysis is found from the TC samples (Fig. S9). The biocompatibility is evaluated further by the CCK-8 test, and there is little influence on the growth of MC3T3-E1 osteoblasts on the TC samples compared to pure silicon (Fig. S10).

4. Discussion

Polyphenols with good viscosity and bacterial-killing properties have large potential in antibacterial coatings. Among the various polyphenols, dopamine has recently gained significant attention due to its multifunctionality. However, the high cost and dark color of PDA coatings may limit applications. Besides, there are still remaining issues regarding the formation of coatings and antibacterial modes. The polyphenol coatings, including PDA used in our experiments here, are usually fabricated by polymerization. In spite of good adhesion, PDA coatings are prone to oxidation and lost activity after storage for an extended period of time. In this study, an electrostatic adsorption process is utilized to achieve self-assembly of stable micro/nanoparticles using CTAC as a cationic template and negatively charged TA as the functional outer layer. From the perspective of coating formation, the adhesion strength of TA is retained while boasting a large number of exposed hydroxyl groups. The self-assembled micro/nanoparticles can be stored without stringent storage conditions after freeze-drying. The powder can be re-dispersed by simple immersion. The TC coatings show morphological and compositional stability under external forces and physiological conditions. As a proof, the lyophilized TC powders are redispersed to produce TC coatings after storing for six months and the favorable properties including morphology are maintained. Therefore, electrostatic assembly of cationic templates and TA represents a significant advancement in the preparation of durable coatings.

The coatings have enhanced antibacterial ability due to the micro-topography and synergistic chemical/mechanical interactions, which is another revealing discovery. Traditional polyphenol-based antibacterial materials are of the release type and the mechanism is usually the chemical effects on the bacterial membrane and interior components such as ROS/RNS, DNA/RNA, and proteins. However, the non-covalent
bonds in polyphenols enable interactions with the bacterial membrane. As the non-covalent forces impact the mechanical behavior of biomacromolecules, the abundant non-covalent forces in polyphenols suggest an important direction to study bacterial behavior and design antibacterial materials.

In this study, the self-assembled micro/nanoparticles form a unique morphology while retain the functional ligands outside. The interaction between the TA sample and bacteria takes a long time of 16 h and a large concentration (0.94 mM) is needed to reach an antibacterial effect of 95%. However, the amount of released TA from the TC coatings is less than 7 μM, and the antibacterial rate reaches 85% in only 3 h, suggesting the non-release antibacterial mode for the TC-coatings. The TA samples are then used to investigate the mechanism. The membrane permeability and potentials are studied. After a short time (3 h), the damage of the TA samples is limited but that of the TC samples is more pronounced. Accordingly, we speculate that micromorphological polyphenols interact more robustly with bacteria. The non-covalent forces in polyphenols affect the mechanical behavior of biomacromolecules as confirmed by AFM. Compared to the control, the TA samples have larger Young’s modulus and a small coefficient of variation in the stiffness, indicating that the non-covalent forces influence the membrane stiffness homogeneously. The calculated Young’s modulus of the bacterial membrane of the TC group increases more than that of the TA group. The differential membrane stiffness distribution also exhibits larger variations indicative of inhomogeneous and more effective damage. The SEM and AFM images show that the bacteria adhere to the microparticles and so it can be inferred that the micromorphology of bound polyphenols plays an important role. In fact, the synergistic chemical and micro-mechanical effects improve the antibacterial properties. Compared to previous articles on antibacterial polyphenols, this study represents a significant breakthrough in the antibacterial efficiency by combining chemical and physical interactions while imparting information about...
the underlying mechanism.

5. Conclusion

Storable microparticles are prepared by self-assembly of the cationic template and functional anionic TA. The microparticles are the desirable raw materials for the formation of coatings on various substrates by the dispersion-immersion process. The coatings are stable after storage for six months. TA on the outer layer of the microparticles produce firm adhesion to the substrate and affinity to bacteria. Besides, the microparticles exert mechanical stress on the bacterial membrane to cause physical damage. The combined chemical/mechanical damage caused by TA enhances the stress response as evidenced by the elevated Young’s modulus and differentiation of membrane stiffness, consequently improving the antibacterial properties greatly. The results disclose a simple and viable strategy to design multi-functional surfaces to combat bacterial infections.

CRediT authorship contribution statement


Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.compositesb.2024.111498.

References

Supporting Information

Enhanced antibacterial activity of polyphenol-bound microtopography by synergistic chemical and micro/nanomechanical effects

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**Figure S1.** (a) UV-vis spectra of TA at different pH and (b) Photograph of the TC complex during the initial interaction at different pH.
**Figure S2.** SEM images of the TC coating on ITO, glass slice, and silicon wafer. The scale bars is 500 µm for the upper image and 50 µm for the lower image.
Figure S3. SEM images of TC coatings on Si immersed for different periods and TC powders (insets) lyophilized and stored in a drying cabinet for six months. The TC coatings are produced by a redispersion/immersion process for different periods.
Figure S4. Water contact angles on the TC coatings immersed in saline, DMEM, and SBF for one day.
Figure S5. (a) Standard curve of TA determined at OD$_{274}$ and (b) Release of TA of different groups for two days.
**Figure S6.** Images of LB-agar plates with TC coatings treated with different strains.
Figure S7. Antibacterial efficiency against different bacterial strains after incubation for three hours.
Figure S8. ROS levels of different groups.
Figure S9. Hemolysis rates of different groups.
**Figure S10.** Cell viability of MC3T3-E1 cells on TC-coated substrates after incubation for 24 h.