The complex of tannic acid and cetylpyridinium chloride: An antibacterial and stain-removal cleaner for aligners

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Introduction: Effective aligner hygiene is recognized as an important part of orthodontic treatments and oral hygiene. However, there is no effective cleansing method for removable aligners. **Methods:** In this study, we incorporated tannic acid (TA) with cetylpyridinium chloride (CPC) to develop the TA-CPC complex. The antibacterial properties of 15.8 mg/mL TA-CPC against *Escherichia coli* and *Staphylococcus aureus* were evaluated in vitro, which were compared with 5.1 mg/mL TA, 10.7 mg/mL CPC, a commercial denture cleansing solution (YA; 15 mg/mL), and water. As for the assessment of stain-removal ability, the aligners stained by coffee were soaked in cleansing solutions, and the color changes (ΔE^*) were calculated on the basis of the CIE L*a*b* color system, and the National Bureau of Standards system was used for the clinical interpretation of the color change. Atomic force microscope examination, tensile property assessment, and wavelength dispersive x-ray fluorescence analysis were performed to investigate the material compatibility of TA-CPC, and Cell Counting Kit-8 assay and live/dead assay were used to test the cytotoxicity of TA-CPC. **Results:** The results showed that TA-CPC had a positive zeta-potential, and cation-π interaction changed the chemical environments of the phenyl group in TA-CPC, resulting in greater inhibition zones of *S. aureus* and *E. coli* than other cleaners. The quantification of the biofilm biomass and the fluorescent intensities also reflected that the TA-CPC solution exhibited better antibacterial ability. As for the ability of stain removal, ΔE^* value of group TA-CPC was 2.84 ± 0.55, whereas those of stained aligners immersed with deionized distilled water, TA, YA, and CPC were 10.26 ± 0.04, 9.54 ± 0.24, 5.93 ± 0.36, and 4.69 ± 0.35, respectively. The visual inspection and National Bureau of Standards ratings also showed that the color of stained aligners cleansed by TA-CPC was much lighter than those of the other groups. Meanwhile, TA-CPC had good compatibility with the aligner material and cells. **Conclusions:** TA-CPC is a promising strategy to inhibit the formation of biofilms and remove the stains on the aligners safely, which may disinfect the aligners to improve oral health and help keep the transparent appearances of aligners without impacting the morphology and mechanical properties. (Am J Orthod Dentofacial Orthop 2024;165:173-85)

Clear aligners for treating malocclusion were initially invented by Kesling in 1946 and developed rapidly because of the innovation and breakthrough of digital design and biocompatible materials in the last 20 years.[1,2] These thermoplastic orthodontic appliances are made entirely of transparent

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plastic and are removable for meals and brushing, which offer advantages of esthetics and comfort.[3,4] Consequently, clear aligners attracted more adult and teenage patients with malocclusion globally.

Although these thermoplastic orthodontic appliances proved less noticeable changes to the appearance of patients, it has been reported that some potential downsides come during their use.[5-8] Discoloration and stains of the aligners are typical occurrences over time, which are important clinical esthetic considerations.[9] Some studies have reported patients are in insufficient compliance with the recommendation that clear aligners should be removed before eating and drinking staining food, such as curry, tea, and coffee.[10] As a result, during orthodontic treatments, the staining agents from food accumulate on the aligners, changing their color and making them less aesthetically pleasing. This has become a serious clinical concern.[11,12]

In addition, many studies presented bacterial biofilms are localized on the surfaces of the thermoplastic aligners, especially on attachment dimples and cusp tips, which are more recessed and sheltered.[13,14] Increased plaque levels not only affect the esthetic aspect of clear aligners but are also a strong risk factor for developing bacteria-related diseases, such as dental caries and periodontal disease.[15,16] Therefore, aligner hygiene is recognized as an important part of orthodontic treatments and oral hygiene.

An appropriate method of thoroughly cleansing aligners can retard or impede the accumulation of bacteria and staining agents on the surfaces of aligners, thus keeping the color stability and transparency of aligners and reducing the risks of spreading cariogenic and periodontal pathogens in the oral cavity. According to the dental health professional recommendation, brushes are widely accepted as a cleansing method for removable appliances.[17] However, it is difficult to remove bacterial biofilms with mechanical cleansing alone, and mechanical cleaning could increase the surface roughness of these removable appliances, which might subsequently be more prone to pigment accumulation and microbial colonization.[18-20] Some commercial cleansing tablets are specifically designed to cleanse removable oral appliances. The antimicrobial action of cleansing tablets is typically based on peroxide-generating chemistry, and they also usually contain surfactants to aid cleansing.[21] However, their stain-removal potentials are less than clinically satisfactory.[22] Some wearers also use household bleach (sodium hypochlorite) to cleanse the removable oral appliances, which may risk damaging denture materials chemically and change the flexibility of the material.[21] Therefore, developing an effective cleansing method to clean and disinfect the removable aligners is important.

Cetylpyridinium chloride (CPC) is a kind of quaternary ammonium compound and a cationic surface-active agent. In clinical dental practice, CPC is mainly used as an antimicrobial ingredient in products marketed for diminishing plaque accumulation, including hard-surface cleaners, mouthwashes, and toothpaste.[23,24] However, it has not been used for stain removal. Tannic acid (TA) is a natural antibacterial polyphenol ubiquitous in various plants, such as Chinese nutgall and unripe fruit, which is widely applied to adsorption materials, coatings, biomedical materials, and food additives.[25] Because TA is a compound with multiple phenolic hydroxyl groups, it can chemically and physically interact with other materials, such as Michael addition, hydrogen bonds, coordination with metals, and Schiff-base reactions.[26-28] In addition, TA are excellent candidates to produce biosorbents, and it is reported that TA-based adsorbents have a natural affinity to various types of dyes in wastewater.[25]

Thus, we incorporated TA with CPC to develop a new cleansing solution and investigated its antibacterial activity, ability of bacteria and stain removal, and effects on morphology and mechanical property of thermoplastic material in the present study. It was hypothesized that TA-CPC could remove the biofilm and staining on the thermoplastic aligners more effectively and had good antibacterial activity, material compatibility, and cytocompatibility. Our results suggest a promising strategy to remove bacterial biofilms and stains on the aligners, which may reduce the bacterial infection of the aligners to improve oral health and help keep their transparent appearances.

MATERIAL AND METHODS

TA was obtained from Aladdin Chemistry (Shanghai, China). CPC and Tris(hydroxymethyl)aminomethane (Tris) were purchased from Solarbio Life Sciences (Beijing, China). A commercial denture cleansing tablet (YA) was obtained from Yakelin (Anhui, China). The Biolon polyethylene terephthalate glycol (PET-G) sheets (Φ120 mm, δ0.75 mm) were obtained from Dreve Dentamid GmbH (Belgium, Germany), and 9-mm-diameter discs were punched from these thermoplastic material sheets in this study. The aligners made of thermoplastic materials were manufactured by Invisalign (Align Technology, Santa Clara, Calif). The aligners were split, and canine, first premolar, and second premolar were chosen as the representative teeth.

Staphylococcus aureus was a gift from the First Affiliated Hospital of Ningbo University, and

Escherichia coli (ATCC 25922) was purchased from the American Type Culture Collection. Tryptic Soy Broth (TSB) and Luria-Bertani medium (LB) were obtained from Solarbio Life Sciences (Beijing, China). Phosphate-buffered saline was purchased from Phygene Life Sciences (Fujian, China). Coffee power (Pure Black Instant Coffee; UCC Coffee, Co, Ltd, Osaka, Japan) was used in this study. LIVE/DEAD BacLight Bacterial Viability Kits L13152 was obtained from Thermo Fisher Scientific (Waltham, Mass).

Bone marrow mesenchymal stem cells (BMSCs) were obtained from Cyagen Biosciences (Guangzhou, China) and cultured in Minimum Essential Media α basic culture medium (Gibco, Grand Island, NY) supplemented with 1% antibiotic solution (penicillin and streptomycin) and 10% fetal bovine serum (Gibco). Cells from passages 3-4 were used for subsequent experiments. Cell viability was detected by Cell Counting Kit-8 (CCK-8) Cell Proliferation and Cytotoxicity Assay Kit (Solarbio Life Sciences, China) and Live/Dead kit (Calcein AM/PI; KeyGEN BioTECH, Nanjing, China)

TA-CPC was synthesized via a 1-step electrostatic assembly between TA and CPC. Fifty-one mg TA and 107 mg CPC were dissolved in 10 mL of 10 mM Tris buffer (pH 8.5) at room temperature. Fifteen minutes after the reaction, the TA-CPC complex formed in the solution was collected by lyophilization. TA, CPC, and TA-CPC powders were analyzed using ^{1}H nuclear magnetic resonance (NMR) (AVANCE NEO, 400 MHz, Bruker; Kontich, Belgium), using dimethyl sulfoxide-d6 as the solvent. Fourier transformation infrared (FT-IR) analysis of TA, CPC, and TA-CPC powders was performed using a Nicolet iS50 Spectrometer (Thermo Fisher Scientific). The 5.1 mg/mL TA, 10.7 mg/mL CPC, and 15.8 mg/mL TA-CPC powders were suspended in 10 mM Tris buffer (pH 8.5). The particle zeta-potential distribution of these samples and coffee solution (3 g coffee powder per 100 mL deionized distilled water [dH_2O]) was determined by the dynamic light scattering method (Zetasizer Nano ZS 90; Malvern Instruments Ltd, Worcestershire, United Kingdom) at 25°C.

The PET-G sheets, immersed in 10 mL of water and 15.8 mg/mL TA-CPC solution for 12 hours, were fixed on the stage respectively, and the surface topography of the samples was analyzed by Atomic Force Microscope (AFM) (Bruker Fast Analyst; Bruker) with tapping mode in air using a FastScan-b probe at 0.999 Hz scan rate for the measurements.

The mechanical property of the PET-G samples after being immersed in 10 mL of water and 15.8 mg/mL TA-CPC solution for 12 h was also tested by the Universal Testing Machine (CMT-1104; SUST, Xi'an China) as reported.[29] Rectangular specimens (5.00 mm × 40.00 mm × 0.75 mm) of PET-G sheets were prepared for tensile tests according to International Organization for Standardization standard 527-2. Each sample was stretched until ruptured at a speed of 5 mm/min at room temperature by the Universal Testing Machine in accordance with guideline GB/T1040.3-2006. The tensile stress-strain curve was recorded, and the elastic modulus was calculated. The mean value and standard deviation of 6 test specimens were calculated for each group.

Nitrogen in the TA-CPC complex and that remaining on the surface of PET-G discs after the cleansing process was measured by wavelength dispersive x-ray fluorescence (WD-XRF) spectrometer (S8 TIGER; Bruker). The discs were immersed in TA-CPC solution (10 mL, 15.8 mg/mL) for 12 hours, and then the discs were washed with deionized dH_2O and dried in air. The WD-XRF spectrometry used a 4 kW Rh anode x-ray tube with a proportional flow counter and scintillation counter detectors as the energy source.

CCK-8 and live/dead assays were used to test the cytotoxicity of TA, CPC, and TA-CPC according to the manufacturer's instructions. Briefly, BMSCs were seeded in 96-well plates (cell density of 3000 cells/well) and treated with 100 μL culture medium containing 5.1 mg/mL TA, 10.7 mg/mL CPC, and 15.8 mg/mL TA-CPC solutions, respectively, for 10 minutes. Then, 10 μL CCK-8 solution was added to each well and incubated in the dark under 5% carbon dioxide and 37°C environment for 2 hours. Cell viability was assessed by a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Scientific) at 450 nm. Furthermore, BMSCs were seeded in 24-well plates (cell density of 5×10^4 cells/well) and treated with 1 mL culture medium containing 15.8 mg/mL TA-CPC solutions for 10 minutes, and then the solution was removed, and cell viability was detected by the Live/Dead kit (Calcein AM/PI; KeyGEN BioTECH). The cell images were visualized and taken with a fluorescence microscope (Leica, Wetzlar, Germany). The cells treated with a culture medium were set as the controls.

S. aureus and *E. coli* were cultured in an appropriate medium (TSB for *S. aureus* and LB for *E. coli*) at 37°C, shaking at 100 rpm overnight to reach the midexponential phase (optical density at 600 nm of the bacterial culture reached 0.5). Fifty microliters of the culture were transferred onto an agar culture plate to produce a bacteria lawn. Filter paper discs (diameter of 8 mm) were fully swelled in solutions of TA (5.1 mg/mL), CPC (10.7 mg/mL), TA-CPC (15.8 mg/mL), and YA (15 mg/mL), and placed onto the agar plates. The plates were incubated at 37°C for 24 hours; then, the photographs of the plates were taken, and the diameters of bacterial

growth inhibition zones were measured by a Vernier digital caliper. All the measurements were performed in triplicate by the same operator. Representative graphs were shown, and the results were expressed as the mean ± standard deviation of the triplicates.

The overnight bacterial culture described above was collected and diluted 100 times using the appropriate culture medium (TSB for *S. aureus* and LB for *E. coli*). PET-G discs (diameter of 9 mm) were sterilized by ultraviolet light irradiation for 60 minutes and co-cultured with 5 mL bacterial suspension at 37°C for 24 hours with shaking at 100 rpm. The samples were then washed with phosphate-buffered saline 3 times to remove any planktonic bacteria and then immersed in 10 mL of aqueous solutions of TA (5.1 mg/mL), CPC (10.7 mg/mL), TA-CPC (15.8 mg/mL), YA (15 mg/mL), and deionized water for 10 minutes at room temperature under static condition, respectively. Discs without intervention (pristine group) were set as control.

Biofilm was observed and quantified by crystal violet (CV) assay and scanning electron microscopy (SEM). Briefly, the samples after the biofilm formation experiment were washed with deionized dH_2O and fixed by 2.5% glutaraldehyde solution, followed by stepwise dehydration using 50%, 70%, 80%, 90%, 95%, and 100% ethanol for each step. For the CV assay, each sample was immersed in 1 mL of 0.5% (w/v) CV solution for staining for 15 minutes. The samples with stained biofilms were gently rinsed with water again, air-dried, and observed under microscopy (Olympus, Japan). In addition, 1 mL of 33% (v/v) acetic acid was added to each sample to solubilize the dye of CV. Optical densities of the CV-dissolved acetic acid solution were tested using a SpectraMax 190 microplate reader at 570 nm. As for SEM observation, the samples were dried in air and coated with Au by ion sputtering (MC1000; Hitachi High-tech, Tokyo, Japan). The bacteria on the surface of the discs were observed by SEM (Regulus 8230; Hitachi), and the biofilm coverage was quantified by ImageJ software (version 2.1.0; National Institutes of Health and the Laboratory for Optical and Computational Instrumentation, University of Wisconsin, Madison, Wis).

Three-dimensional (3D) structures of the biofilms and live/dead bacteria were observed by confocal laser scanning microscopy (TCS SP8, Leica). The combined dye (LIVE/DEAD BacLight Bacterial Viability Kits, L13152; Thermo Fisher Scientific) was used to stain bacteria on the surface of discs as per the manufacturer's instruction. The samples were incubated with dye solutions in the dark for 15 minutes and washed with sterilized dH_2O. Microscopic observations were performed with confocal laser scanning microscopy with a 488 nm laser source and SYTO Green and propidium iodide dye channels. Three-dimensional images were reconstructed, and the means of fluorescence intensity in the SYTO Green channel and propidium iodide dye channel were quantified by Leica Application Suite X. Three replicates were assessed for each group. Representative graphs were shown, and the results were expressed as the mean ± standard deviation of the triplicates.

Liu et al reported that coffee can lead to severe stains on aligners made of thermoplastic materials.[12] According to previous studies, coffee solution (3 g coffee powder per 100 mL boiling deionized dH_2O) was used.[12] The lower aligners were immersed in 100 mL coffee solution in a water bath at 37°C for 1 day. The aligners were cleansed in water by ultrasound (40 kHz) for 5 minutes and dried in air. Then they were immersed in 100 mL solutions of TA (5.1 mg/mL), CPC (10.7 mg/mL), TA-CPC (15.8 mg/mL), YA (15.0 mg/mL), and deionized water for 10 minutes at room temperature with shaking at 200 rpm, respectively, and the stained aligners without intervention (named as pristine group) were as controls.

The color changes (ΔE) were characterized via the Commission Internationale de l'Eclairage L*a*b* color system (CIE L*a*b*).[30,31] The parameters L*, a*, and b* of aligners were measured with NR110 Precision Colorimeter (3nh, Shenzhen, China). The optical sensor tip of the Precision Colorimeter contacted firmly and vertically to the labial surface of the mandibular lateral incisors of each aligner, and white paper was used as the background reference. Standard measurements were performed in the same room and were conducted by one investigator blind to the arrangement of the group. The ΔE value, which represents the color change, was calculated according to the formula: $\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$. ΔL*, Δa*, and Δb* are the subtractions of the L*, a*, and b* color parameters measured before staining (as-received aligners) and after cleansing (staining in coffee solutions for 1 day and then immersing in cleansing solutions for 10 minutes), respectively.

The National Bureau of Standards (NBS) system was used to describe the perceptible color change (a clinical interpretation)[12,22]:

NBS = ΔE* × 0.92 (Trace: NBS <0.5, the color change is extremely slight; Slight: 0.5≤ NBS <1.5, the color change is slight; Noticeable: 1.5≤ NBS <3.0, the color change is perceivable; Appreciable: 3.0≤ NBS <6.0, the color change is marked; Much: 6.0≤ NBS <12.0, the color change is extremely marked; Very much: 12≤ NBS, the color changes to other color).

Representative graphs were shown, and the results were expressed as the mean ± standard deviation of the triplicates.

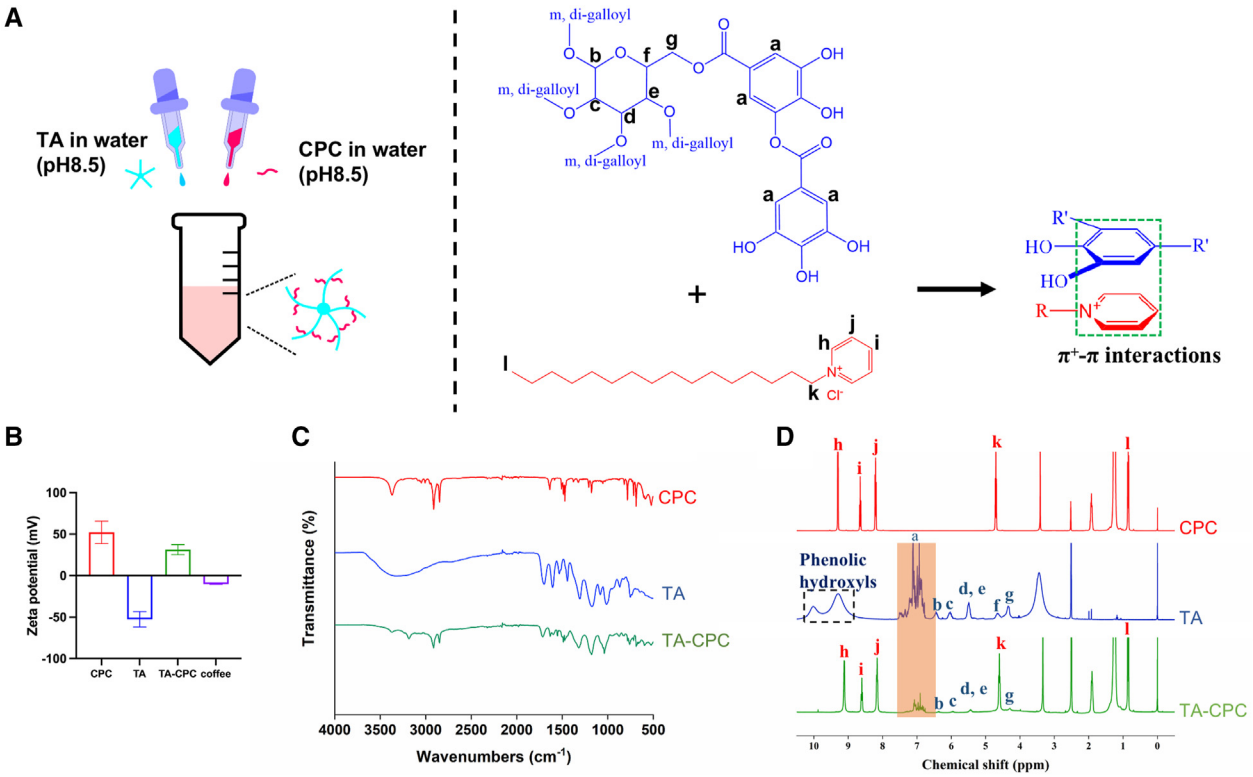

Fig 1. Synthesis and characterization of TA, CPC, and TA-CPC (molar ratio 1:10): **A,** Schematic of the synthesis of TA-CPC; **B,** Zeta-potential of the free CPC (denoted as CPC), free TA (denoted as TA), TA-CPC, and coffee, respectively; **C,** FT-IR spectra of the free CPC, free TA, and TA-CPC, respectively; **D,** ^{1}H-NMR spectra of the free CPC, free TA, and TA-CPC, respectively.

Data analysis

All data were displayed as the mean ± standard deviation. Statistical analysis was evaluated using SPSS (version 15.0; SPSS, Chicago, Ill). Analysis of 2 groups was performed via a 2-tailed Student *t* test, and analysis of ≥3 groups was conducted by One-way analysis of variance followed by Student–Newman–Keuls post-hoc tests for multiple comparisons. *P* values of <0.05 were considered significant statistically.

RESULTS

In this study, TA-CPC was synthesized via the assembly of TA and CPC (Fig 1, *A*). Zeta potential is an important surface property of nanoparticles. It can influence the interactions between nanoparticles and other substances by electrostatic repulsion or attraction. The results of zeta-potential measurement in this study showed that TA-CPC (31.3 ± 6.1 mV) and CPC (52.2 ± 13.6 mV) had positive zeta-potential, whereas TA (−52.7 ± 9.3 mV) and coffee (−10.3 ± 0.3 mV) had negative zeta-potential (Fig 1, *B*).

Results from FT-IR spectroscopy showed that there were typical absorption peaks of O-H stretching vibration at 3306 cm^{-1} in the spectra of TA, whereas the spectra of CPC presented aromatic C-H stretching vibration at 3362 and 3011 cm^{-1}, and C-N stretching vibration at 2161 cm^{-1}. After self-assembly of TA and CPC at a molar ratio of 1:10, the absorption intensity was decreased by 3000-3500 cm^{-1} accompanied by a shift of O-H peak to a low wavenumber (from 3380 to 3306 cm^{-1}) (Fig 1, *C*).

To further explore the formation mechanism, we examined the changes in ^{1}H NMR spectra during the TA-CPC formation. As shown in Fig 1, *D*, compared with the free form of TA, the chemical signal peak of phenolic hydroxyls in TA-CPC almost disappeared. Meanwhile, the peaks of chemical shifts of protons on an aromatic ring (a, h, i, and j peaks in Figure 1, *D*) shifted to low values in TA-CPC, which indicated that the chemical environments of the phenyl group in TA-CPC were changed. We suggested there exist noncovalent interactions between TA and CPC. Cation-π interactions can occur between positively charged groups and

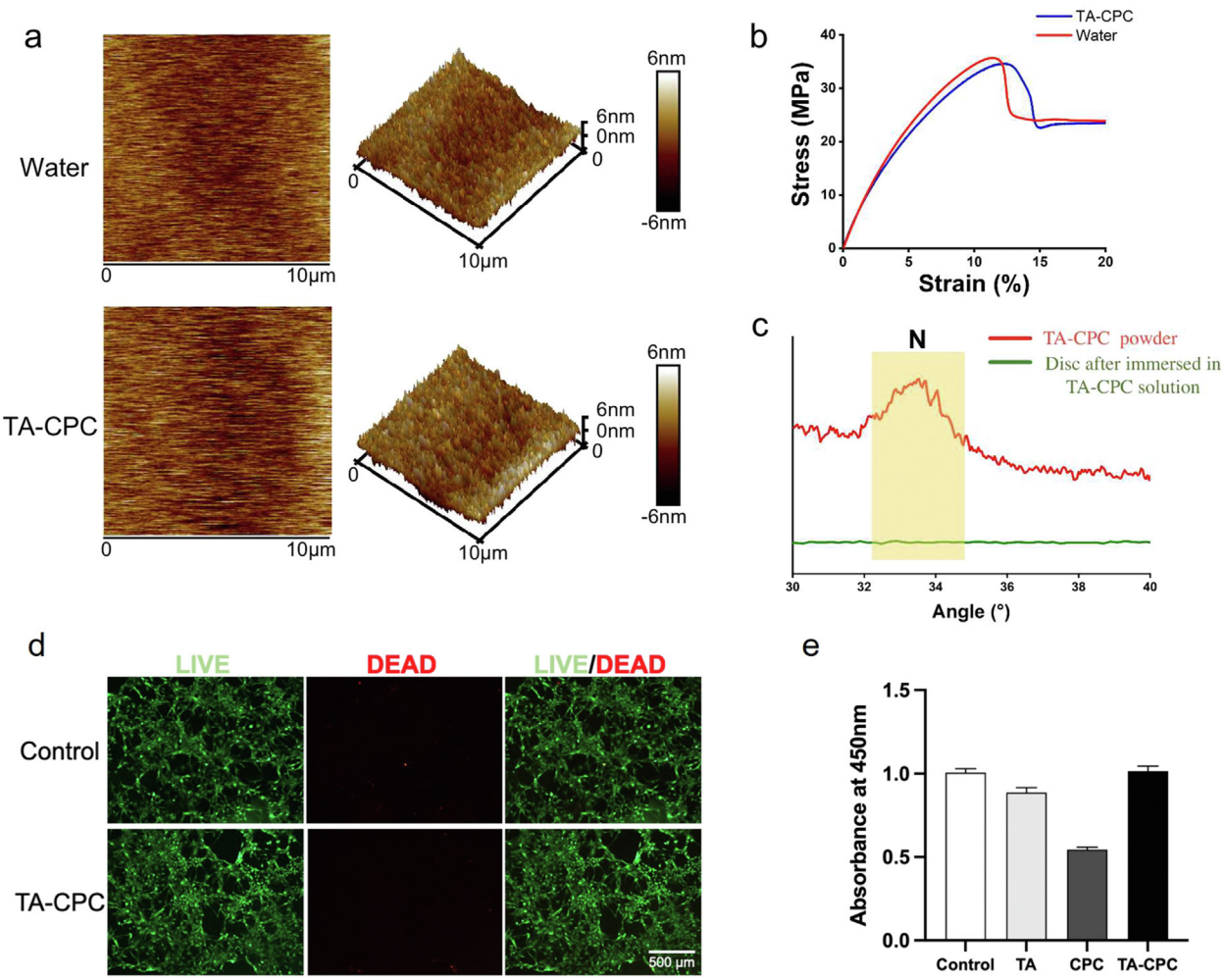

Fig 2. Compatibility of TA-CPC with thermoplastic materials and cells: **A,** AFM height images and 3D reconstruction surface topography pictures; **B,** Stress-strain curves of the PET-G discs immersed in Water (denoted as Water) and those immersed by TA-CPC (denoted as TA-CPC); **C,** WD-XRF spectrums of TA-CPC powder and the PET-G discs after immersed in TA-CPC solution; **D,** Live and dead staining images of cells treated with culture medium (named as controls) and TA-CPC solution; **E,** Results of CCK-8 assay of culture medium (named as controls), and TA, CPC and TA-CPC solutions, respectively.

aromatic rings.[32-34] In this work, we envisioned that cation-π interactions probably formed between the aromatic rings of TA and the cation of the quaternary ammonium group of CPC (Fig 1, *A*).

The effect of TA-CPC treatment on the morphology of thermoplastic material discs was investigated via AFM. The representative height images and pictures of 3D reconstruction surface topography are displayed in Figure 2, *A*. According to the roughness results of AFM measurement, the Rq values of discs immersed in 15.8 mg/mL TA-CPC solution (group TA-CPC) and in deionized dH_2O (group water) were 1.60 ± 0.20 nm and 1.34 ± 0.12 nm, respectively. It showed that the surface roughness of PET-G discs was not influenced significantly by the immersion treatment of the TA-CPC solution.

The tensile performance of PET-G after soaking in water and TA-CPC solution for 12 hours was evaluated respectively in this study, and the representative stress-strain curves were revealed with different colors in Figure 2, *B*. After specimens were immersed in TA-CPC solution, the tensile fracture stress of samples was 33.3 ± 1.63 MPa, which was no statistical significance compared with specimens immersed in water (33.1 ± 2.35 MPa; $P > 0.1$).

Any element of the TA-CPC complex remaining on thermoplastic materials after the TA-CPC cleansing

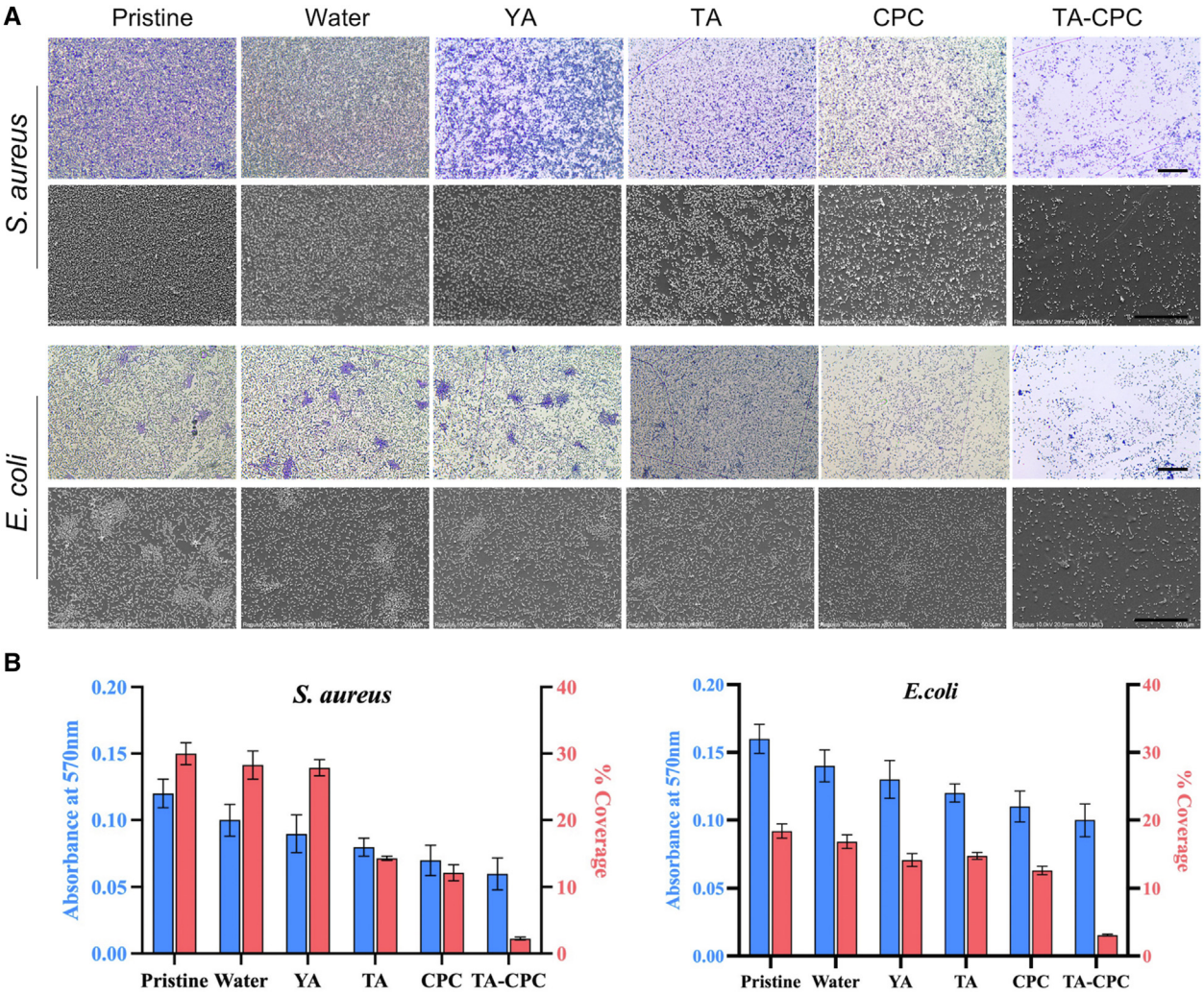

Fig 3. Removal of *S. aureus* and *E. coli* biofilm biomass: **A,** CV staining and SEM images; **B,** CV assay and coverage percent qualified by SEM of samples immersed in TA, CPC, TA-CPC, YA, and water for 10 minutes at room temperature, respectively, and the samples without intervention (pristine group) were as controls. Images were representative of 3 different samples, and the means and standard deviation values were calculated by 3 replicates. Scale bars indicated 50 μm.

process was analyzed by WD-XRF. The typical WD-XRF spectra of TA-CPC powder and the PET-G discs after being immersed in TA-CPC solution are shown in Figure 2, *C*. The spectrum of TA-CPC powder clearly showed the presence of nitrogen (because of the quaternary ammonium group in CPC), whereas no nitrogen signal was found in the spectrum of the disc sample after TA-CPC treatment. It is suggested there was no remaining content of TA-CPC on PET-G after being immersed in the TA-CPC solution.

The biocompatibility of TA-CPC was assessed in this study. Representative images of BMSCs treated with TA-CPC and culture medium are displayed in Figure 2, *D*. Live cells were stained *green*, whereas dead cells were stained *red*. Therefore, most of the BMSCs in group TA-CPC were alive but rarely dead, almost the same as those treated with culture medium (named as control). The results of the CCK-8 assay also showed that the viability of BMSCs was not affected by TA-CPC (Fig 2, *E*).

The biofilms were analyzed qualitatively and quantitatively using CV assay and SEM (Fig 3, *A*). The numbers of *S. aureus* colonies decreased after immersion with TA, CPC, and TA-CPC, and they were mildly reduced after immersion with water and YA. Among these cleansing solutions, the number of *S. aureus* colonies on the discs immersed with TA-CPC was the smallest, and the percent of biofilm coverage area decreased to 2.26% ± 0.21%

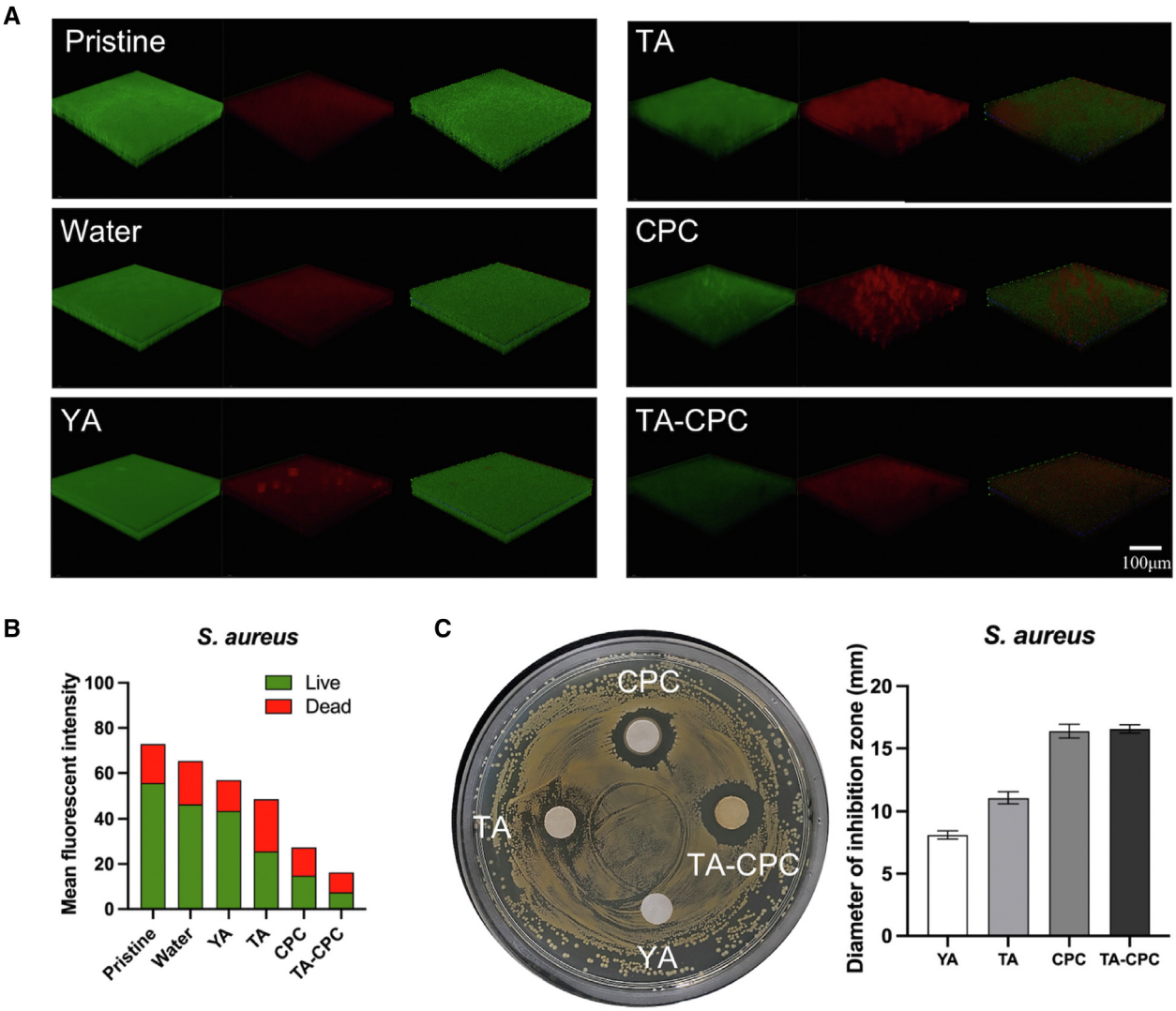

Fig 4. Antibacterial properties of cleansing solutions for *S. aureus*: **A,** Live and dead staining images; **B,** Fluorescence intensity quantification of *S. aureus* incubated with PET-G discs and immersed by TA, CPC, TA-CPC, water, and YA 10 minutes, respectively, in which live bacteria stained with sytox green staining were *green* and dead bacteria stained with propidium iodide were *red*, used those incubated with PET discs without immersion as control (pristine group); **C,** Representative plate photograph of the agar diffusion method and presented the mean and standard deviation values of the diameter of growth inhibition zone for each sample in the groups against *S. aureus*. The scale bar indicated 100 μm.

(Fig 3, *B*). Regarding *E. coli*, the numbers and coverages of colonies on the representative images of CV staining and SEM were also decreased after immersion with TA-CPC compared with other groups (Fig 3, *A*). The quantification of the biofilm biomass also reflected that the TA-CPC solution exhibited better antibacterial ability (Fig 3, *B*).

Figures 4, *A* and 5, *A* show representative images of *S. aureus* and *E. coli* biofilms on the discs which were washed by TA, CPC, TA-CPC, deionized dH_2O, and YA, respectively, and the discs without intervention (pristine group) were as controls. Live bacteria were stained green, whereas dead bacteria were stained red, and their mean fluorescent intensities were shown in Figures 4, *B* and 5, *B*. Among the cleansing solutions, the fluorescent intensities of *S. aureus* and *E.coli* colonies on the discs immersed with TA-CPC were the lowest, consistent with the CV assay and SEM observation results. TA-CPC also exhibited high bactericidal properties, especially against *S. aureus* (gram-positive). Therefore, these results

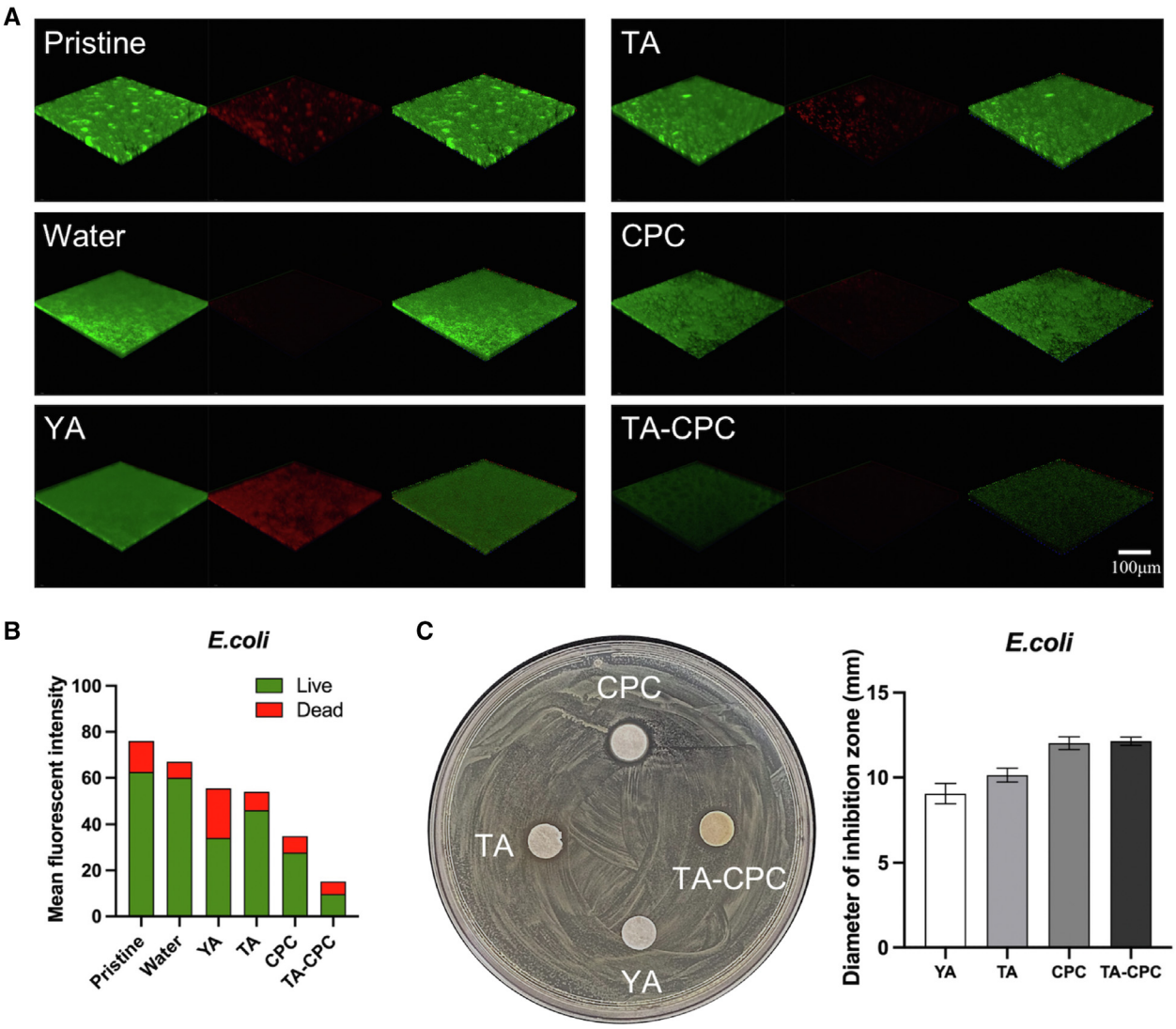

Fig 5. Antibacterial properties of cleansing solutions for *E. coli*: **A,** Live and dead staining images; **B,** Fluorescence intensity quantification of *E. coli* incubated with PET-G discs and immersed by TA, CPC, TA-CPC, water, and YA 10 minutes, respectively, in which live bacteria stained with sytox green staining were *green* and dead bacteria stained with propidium iodide were *red*, used those incubated with PET discs without immersion as control (pristine group); **C,** Representative plate photograph of the agar diffusion method and presented the mean and standard deviation values of the diameter of growth inhibition zone for each sample in the groups against *E. coli*. The scale bar indicated 100 μm.

demonstrated that TA-CPC showed promising efficacy in bacteria removal and killing gram-positive and gram-negative bacteria.

Figures 4, *C* and 5, *C* show representative plate photographs of the agar diffusion method and present the mean and standard deviation values of the diameter of the growth inhibition zone for each sample in the groups against *S. aureus* (gram-positive) and *E. coli* (gram-negative), respectively. The paper discs impregnated with TA-CPC, TA, and CPC solutions showed growth inhibition zones of *S. aureus* and *E.coli*. In addition, the antibacterial efficacy of TA-CPC against *S. aureus* (gram-positive) was more extraordinary than *E.coli* (gram-negative). As for YA, which is one kind of commercial cleansing solution, no obvious inhibition zones appeared around the YA sample against the *S. aureus* strain, whereas small inhibition zones were shown in the *E.coli* lawn, which was almost similar to those around TA samples.

Photographs in Figure 6, *A* showed the color change of the stained aligners after being immersed in 5.1 mg/mL

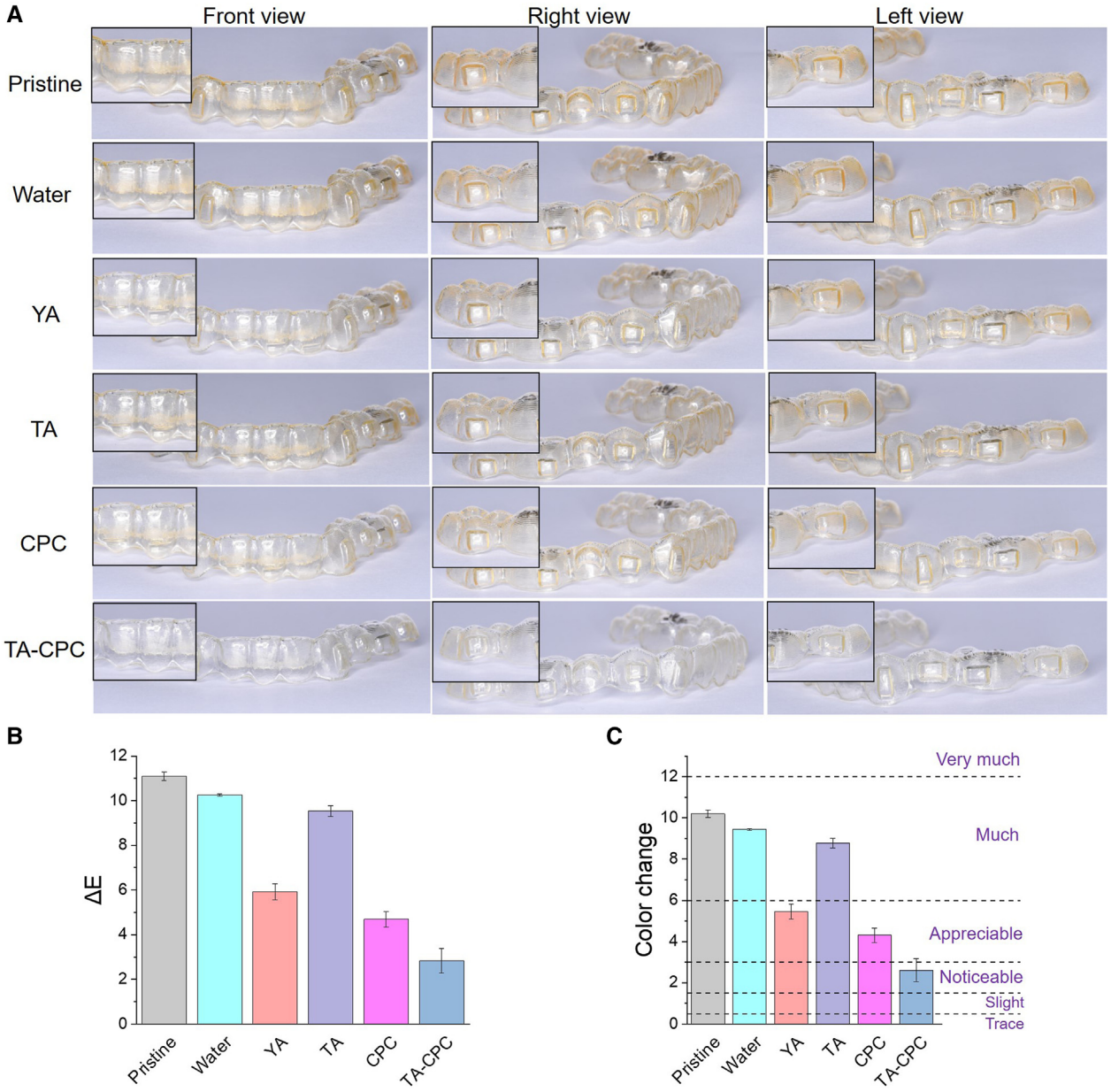

Fig 6. Analysis of stain-removal ability: **A,** Representative photographs; **B,** Mean and standard deviation values of ΔE*; **C,** NBS ratings of the stained aligners before and after immersion in TA, CPC, TA-CPC, YA, and water for 10 minutes, respectively, and the stained aligners without intervention (pristine group) were as controls.

TA, 10.7 mg/mL CPC, 15.8 mg/mL TA-CPC, 15 mg/mL YA, and deionized dH_2O, respectively, and the stained aligners without intervention (pristine group) were as controls. It is known that clear aligners are transparent and colorless. Visual inspection indicated that the stained aligners exhibited marked color change after immersion in coffee solutions for 1 day. The color of stained aligners immersed in YA, CPC, and TA-CPC became lighter.

Quantitative assessments were further performed for the color measurements. The color change values (presented as ΔE*) before staining and after immersion in cleansing solutions are displayed in Figure 6, *B*. NBS ratings were used to describe the perceptible color change for clinical interpretation (see Fig 6, *C*). It is showed that the color change of the stained aligners immersed with deionized dH_2O (ΔE* value, 10.26 ± 0.04) or TA

(ΔE^* value, 9.54 ± 0.24) rated "Much," which were at the same level as the stained aligners without intervention (named as pristine group) and the color changes were extremely marked. The stained aligners immersed with YA (ΔE^* value, 5.93 ± 0.36) and the aligners immersed with CPC (ΔE^* value, 4.69 ± 0.35) exhibited appreciable color change, whose color was lighter, but the color change was still marked. As for the stained aligners immersed with TA-CPC, the ΔE^* was 2.84 ± 0.55, significantly smaller than those of the other groups. It is suggested that TA-CPC had a better stain-removal ability.

DISCUSSION

Clear aligners are a fast-moving field mainly driven by patients seeking more comfortable, esthetic, and less intrusive orthodontic treatment.[35] Because aligners are nearly invisible and highly esthetic removable orthodontic treatment options, they have enjoyed great popularity and are accepted widely by orthodontics and patients. Better compliance with oral hygiene was displayed in patients treated with removable aligners, and thus, they had less plaque and lower risks of developing gingival inflammation than those treated with fixed appliances.[36] This is not only attributed to the fact that removable clear aligners allow unimpeded oral hygiene and give the patients the possibility to better control the plaque on the teeth but also correct hygiene for the removable aligners can inhibit the accumulation of bacteria on the surfaces and thus reduce the risk of spreading bacteria back on teeth and periodontium.[37] Therefore, cleaning and disinfecting the removable aligners before and after use is important.

The bacterial membrane carries a net negative charge, composed of a phospholipid bilayer with embedded proteins. The phospholipid bilayer is stabilized by divalent cations, such as Ca^{2+}, and forms a hydrophobic environment, which is critical to moderate the functionality of the embedded proteins. CPC is an amphoteric surfactant assembled by a hexadecane chain (the lipophilic side) and a positively charged pyridine (the hydrophilic headgroup).[38] Because of this molecular structure, CPC could initially interact with the cytoplasmic membrane by substituting divalent cations in the membrane with a positively charged pyridine ion, and then the hexadecane tail has the opportunity to integrate into the microbial cell membrane and disorganize it,[38] which is pathogen independent and unlikely to be affected by microorganism mutations.[39,40] Many studies have investigated the antibacterial potential of CPC in the oral environment.[41] TA is one kind of water-soluble polyphenol found naturally in some plants. In this study, we first incorporated TA with CPC to develop a new cleansing solution (TA-CPC) for thermoplastic clear aligners and investigated its antibacterial activity. Results from FT-IR spectroscopy and ^{1}H-NMR spectra showed that the absorption intensity of TA-CPC was decreased among 3000-3500 cm^{-1} accompanied by a shift of O-H peak to a low wavenumber, and the peaks of chemical shifts of protons on an aromatic ring shift to low values in TA-CPC, which indicate that the chemical environments of the phenyl group in TA-CPC were changed. Based on the direct evidence of the vibrational shift for the aromatic ring, we suggested there exists a noncovalent interaction between the aromatic rings of TA and the cation of the quaternary ammonium group of CPC probably because of cation-π interactions.[32-34]

Our study showed that biofilms immersed in TA-CPC solution for 10 minutes were primarily dead with red staining, especially against *S. aureus* (Gram-positive), which exhibited higher bactericidal properties than TA and CPC alone. TA-CPC also resulted in the greatest inhibition zones. Furthermore, the bacteria-removal property of TA-CPC against *E. coli* and *S. aureus* was evaluated in vitro in this study, which showed that TA-CPC solution exhibited better bacteria-removal ability. It is reported that TA could inhibit the enzyme glycosyl-transferase, which is critical for bacterial adherence, and thus, TA can suppress bacteria to adhere to the pellicle and alter the pellicle's ultrastructure.[42,43] CPC is a cationic surfactant that can enhance particle removal from the surface and diminish bacteria accumulation.[23,24,44] Therefore, the TA-CPC complex was suggested to integrate the advantages of TA and CPC, contributing to better bacteria-removal ability.

In addition to the importance of disinfecting the removable aligners before and after use, the removal of staining agents also should be of considerable critical attention. The "invisible" orthodontic treatment is a prominent feature of transparent clear aligners and an essential reason for its popularization. However, the light transmittance of the thermoplastic material can be compromised from repetitive use and cleansing cycles,[45,46] and thermoplastic clear aligners are vulnerable to pigment adsorption from staining beverages and food in the oral cavity, which affects the esthetic property of aligners.[12] Therefore, effective cleansing methods to remove the staining can increase the life span of the aligners and promote patients' better compliance, but there is no ideal cleansing method for thermoplastic material orthodontic appliances. In this study, we stained the aligners with coffee, and then the stained aligners were grouped randomly and soaked in TA, CPC, TA-

CPC, YA, and water, respectively. The results suggested that TA-CPC has a better stain-removal ability. The electrostatic force is important for bacteria and staining agents to adhere to the clear aligners. Zeta-potential is a parameter of the charges at the surface-liquid interfaces that substantially determines the adsorption/repulsion of particles on the surface. The different zeta potentials of TA, CPC, and TA-CPC can affect their interaction and adsorption on the surface of the clear aligners. The TA-CPC complex has a higher content of CPC molecules, interacts with the bacterial cells and stain particles (negatively charged) more easily, and removes them from the aligner surface by electrostatic repulsion. Therefore, the formation of a complex between TA and CPC is important for the effects of bacteria and stain removals.

CONCLUSIONS

In summary, this study designed the TA-CPC complex via cation-π interactions and revealed its promising effects to act as a new cleaner for thermoplastic clear aligners. TA-CPC showed excellent antibacterial activity and good ability for bacteria and stain removal. Meanwhile, it had good compatibility with the materials and cells. Remarkably, these results suggested a promising approach on the basis of TA-CPC in the disinfecting and stain-removal application for thermoplastic clear aligners.

AUTHOR CREDIT STATEMENT

Xiao Cen contributed to conceptualization, software, investigation, and original draft preparation; Xuefeng Pan contributed to visualization and resources; Rong Wang contributed to methodology, resources, and manuscript review and editing; Xinqi Huang contributed to formal analysis, supervision, and manuscript review and editing; and Zhihe Zhao contributed to methodology, resources, and supervision.

REFERENCES

1. Kesling HD. Coordinating the predetermined pattern and tooth positioner with conventional treatment. Am J Orthod Oral Surg 1946;32:285-93.
2. Putrino A, Barbato E, Galluccio G. Clear aligners: between evolution and efficiency-a scoping review. Int J Environ Res Public Health 2021;18.
3. Rosvall MD, Fields HW, Ziuchkovski J, Rosenstiel SF, Johnston WM. Attractiveness, acceptability, and value of orthodontic appliances. Am J Orthod Dentofacial Orthop 2009;135: 276.e1: 12; discussion 276.
4. Alansari RA, Faydhi DA, Ashour BS, Alsaggaf DH, Shuman MT, Ghoneim SH, et al. Adult perceptions of different orthodontic appliances. Patient Prefer Adherence 2019;13:2119-28.
5. Talic NF, Almudhi AA. The effect of dietary pigmentation on the esthetic appearance of clear orthodontic elastomeric modules. J Orthod Sci 2016;5:70-3.
6. Fang D, Li F, Zhang Y, Bai Y, Wu BM. Changes in mechanical properties, surface morphology, structure, and composition of Invisalign material in the oral environment. Am J Orthod Dentofacial Orthop 2020;157:745-53.
7. Porojan L, Vasiliu RD, Porojan SD, Bîrdeanu MI. Surface quality evaluation of removable thermoplastic dental appliances related to staining beverages and cleaning agents. Polymers (Basel) 2020;12.
8. Ihssen BA, Willmann JH, Nimer A, Drescher D. Effect of in vitro aging by water immersion and thermocycling on the mechanical properties of PETG aligner material. J Orofac Orthop 2019;80: 292-303.
9. Lombardo L, Arreghini A, Maccarrone R, Bianchi A, Scalia S, Siciliani G. Optical properties of orthodontic aligners—spectrophotometry analysis of three types before and after aging. Prog Orthod 2015;16:41.
10. Tsomos G, Ludwig B, Grossen J, Pazera P, Gkantidis N. Objective assessment of patient compliance with removable orthodontic appliances: a cross-sectional cohort study. Angle Orthod 2014;84: 56-61.
11. Wriedt S, Schepke U, Wehrbein H. The discoloring effects of food on the color stability of esthetic brackets—an in-vitro study. J Orofac Orthop 2007;68:308-20.
12. Liu CL, Sun WT, Liao W, Lu WX, Li QW, Jeong Y, et al. Colour stabilities of three types of orthodontic clear aligners exposed to staining agents. Int J Oral Sci 2016;8:246-53.
13. Low B, Lee W, Seneviratne CJ, Samaranayake LP, Hägg U. Ultrastructure and morphology of biofilms on thermoplastic orthodontic appliances in 'fast' and 'slow' plaque formers. Eur J Orthod 2011;33:577-83.
14. Dutra D, Pereira G, Kantorski KZ, Valandro LF, Zanatta FB. Does finishing and polishing of restorative materials affect bacterial adhesion and biofilm formation? A systematic review. Oper Dent 2018;43:E37-52.
15. Papadopoulou AK, Cantele A, Polychronis G, Zinelis S, Eliades T. Changes in roughness and mechanical properties of Invisalign® appliances after one- and two-weeks use. Materials (Basel) 2019;12.
16. Peng X, Cheng L, You Y, Tang C, Ren B, Li Y, et al. Oral microbiota in human systematic diseases. Int J Oral Sci 2022;14:14.
17. Axe AS, Varghese R, Bosma M, Kitson N, Bradshaw DJ. Dental health professional recommendation and consumer habits in denture cleansing. J Prosthet Dent 2016;115:183-8.
18. Lefever D, Perakis N, Roig M, Krejci I, Ardu S. The effect of toothbrushing on surface gloss of resin composites. Am J Dent 2012;25: 54-8.
19. Verran J, Jackson S, Coulthwaite L, Scallan A, Loewy Z, Whitehead K. The effect of dentifrice abrasion on denture topography and the subsequent retention of microorganisms on abraded surfaces. J Prosthet Dent 2014;112:1513-22.
20. Jackson S, Coulthwaite L, Loewy Z, Scallan A, Verran J. Biofilm development by blastospores and hyphae of Candida albicans on abraded denture acrylic resin surfaces. J Prosthet Dent 2014; 112:988-93.
21. Kiesow A, Sarembe S, Pizzey RL, Axe AS, Bradshaw DJ. Material compatibility and antimicrobial activity of consumer products commonly used to clean dentures. J Prosthet Dent 2016;115: 189-98.e8.
22. Bernard G, Rompré P, Tavares JR, Montpetit A. Colorimetric and spectrophotometric measurements of orthodontic thermoplastic

aligners exposed to various staining sources and cleaning methods. Head Face Med 2020;16:2.
23. Khalil RK. Selective removal and inactivation of bacteria by nanoparticle composites prepared by surface modification of montmorillonite with quaternary ammonium compounds. World J Microbiol Biotechnol 2013;29:1839-50.
24. Van der Weijden FA, Van der Sluijs E, Ciancio SG, Slot DE. Can chemical mouthwash agents achieve plaque/gingivitis control? Dent Clin North Am 2015;59:799-829.
25. Bacelo HAM, Santos SCR, Botelho CMS. Tannin-based biosorbents for environmental applications - a review. Chem Eng J 2016;303:575-87.
26. Xu LQ, Neoh K-G, Kang E-T. Natural polyphenols as versatile platforms for material engineering and surface functionalization. Prog Polym Sci 2018;87:165-96.
27. Shim G, Ko S, Park JY, Suh JH, Le QV, Kim D, et al. Tannic acid-functionalized boron nitride nanosheets for theranostics. J Control Release 2020;327:616-26.
28. Li R, Tan Y, Dai T, Zhang R, Fu G, Wan Y, et al. Bioaccessibility and stability of β-carotene encapsulated in plant-based emulsions: impact of emulsifier type and tannic acid. Food Funct 2019;10:7239-52.
29. Fang K, Gu Q, Zeng M, Huang Z, Qiu H, Miao J, et al. Tannic acid-reinforced zwitterionic hydrogels with multi-functionalities for diabetic wound treatment. J Mater Chem B 2022;10:4142-52.
30. Johnston WM. Color measurement in dentistry. J Dent 2009;37(Suppl 1):e2-6.
31. Hollis S, Eisenbeisz E, Versluis A. Color stability of denture resins after staining and exposure to cleansing agents. J Prosthet Dent 2015;114:709-14.
32. Pillai KV, Renneckar S. Cation-pi interactions as a mechanism in technical lignin adsorption to cationic surfaces. Biomacromolecules 2009;10:798-804.
33. Leduskrasts K, Kinens A, Suna E. Cation-π interactions secure aggregation induced emission of planar organic luminophores. Chem Commun (Camb) 2019;55:12663-6.
34. Geng H, Zhang P, Peng Q, Cui J, Hao J, Zeng H. Principles of cation-π interactions for engineering mussel-inspired functional materials. Acc Chem Res 2022;55:1171-82.
35. Flores-Mir C, Brandelli J, Pacheco-Pereira C. Patient satisfaction and quality of life status after 2 treatment modalities: Invisalign and conventional fixed appliances. Am J Orthod Dentofacial Orthop 2018;154:639-44.
36. Abbate GM, Caria MP, Montanari P, Mannu C, Orrù G, Caprioglio A, et al. Periodontal health in teenagers treated with removable aligners and fixed orthodontic appliances. J Orofac Orthop 2015;76:240-50.
37. Levrini L, Mangano A, Margherini S, Tenconi C, Vigetti D, Muollo R, et al. ATP bioluminometers analysis on the surfaces of removable orthodontic aligners after the use of different cleaning methods. Int J Dent 2016;2016:5926941.
38. Gilbert P, Moore LE. Cationic antiseptics: diversity of action under a common epithet. J Appl Microbiol 2005;99:703-15.
39. Popkin DL, Zilka S, Dimaano M, Fujioka H, Rackley C, Salata R, et al. Cetylpyridinium chloride (CPC) exhibits potent, rapid activity against influenza viruses in vitro and in vivo. Pathog Immun 2017;2:252-69.
40. Matsuo K, Yoshihara K, Nagaoka N, Makita Y, Obika H, Okihara T, et al. Rechargeable anti-microbial adhesive formulation containing cetylpyridinium chloride montmorillonite. Acta Biomater 2019;100:388-97.
41. Busscher HJ, White DJ, Atema-Smit J, Geertsema-Doornbusch G, de Vries J, van der Mei HC. Surfactive and antibacterial activity of cetylpyridinium chloride formulations in vitro and in vivo. J Clin Periodontol 2008;35:547-54.
42. Wu-Yuan CD, Chen CY, Wu RT. Gallotannins inhibit growth, water-insoluble glucan synthesis, and aggregation of mutans streptococci. J Dent Res 1988;67:51-5.
43. Hertel S, Pötschke S, Basche S, Delius J, Hoth-Hannig W, Hannig M, et al. Effect of tannic acid on the Protective Properties of the in situ Formed Pellicle. Caries Res 2017;51:34-45.
44. Free ML. Chapter 13. The use of surfactants to enhance particle removal from surfaces. In: Kohli R, Mittal KL, editors. Developments in surface contamination and cleaning. 2nd ed. Oxford: William Andrew Publishing; 2016. p. 595-626.
45. Agarwal M, Wible E, Ramir T, Altun S, Viana G, Evans C, et al. Long-term effects of seven cleaning methods on light transmittance, surface roughness, and flexural modulus of polyurethane retainer material. Angle Orthod 2018;88:355-62.
46. Wible E, Agarwal M, Altun S, Ramir T, Viana G, Evans C, et al. Long-term effects of various cleaning methods on polypropylene/ethylene copolymer retainer material. Angle Orthod 2019;89:432-7.