

# Synthesis, Antifungal Activity, and Cytotoxicity of AgBr-NP@CTMAB Hybrid and Its Application in PMMA

Qiao-Jun Zhang<sup>1,2,\*</sup>

Yue Liu<sup>3,\*</sup>

Wen-Ting Zhang<sup>3</sup>

Jing-Jing Huang<sup>2</sup>

Hao-Hong Li<sup>3</sup>

You-Guang Lu<sup>1</sup>

Ming Zheng<sup>2</sup>

Da-Li Zheng<sup>1</sup>

<sup>1</sup>Fujian Key Laboratory of Oral Diseases, Fujian Biological Materials Engineering and Technology Center of Stomatology, School and Hospital of Stomatology, Fujian Medical University, Fuzhou, Fujian, 350004, People's Republic of China;

<sup>2</sup>Department of Prosthodontics, School and Hospital of Stomatology, Fujian Medical University, Fuzhou, 350001, People's Republic of China; <sup>3</sup>College of Chemistry, Fuzhou University, Fuzhou, Fujian, 350116, People's Republic of China

\*These authors contributed equally to this work

**Objective:** To synthesize and determine the antifungal activity of AgBr-nanoparticles (NP) @CTMAB (cetyltrimethyl-ammonium bromide) against *Candida albicans* (*C. albicans*) for use in the field of denture cleaning.

**Methods:** The morphology and structure of AgBr-NP@CTMAB were characterized by IR, UV-Vis, XRD and SEM. The antifungal potential of AgBr-NP@CTMAB against *C. albicans* was determined by colony formation assay and growth curve analysis. PMMA containing AgBr-NP@CTMAB was prepared, and the long-term antifungal efficacy was analyzed. The effect against *C. albicans* biofilm was analyzed by SEM and OD600, and the color changes of the specimens were observed by stereomicroscopy after 1 week of incubation. Cytotoxicity to human oral gingival fibroblasts and oral mucosal epithelial cells was detected by Cell Counting Kit-8 (CCK-8) in vitro.

**Results:** The compound showed a good crystalline phase, the presence of AgBr nanoparticles and the hybridization of CTMAB+ with AgBr-NPs. AgBr-NP@CTMAB showed significant antifungal activity against *C. albicans* at concentrations of 10 µg/mL and 20 µg/mL. PMMA specimens containing AgBr-NP@CTMAB showed no long-term antifungal effect against *C. albicans* biofilm. The clearance rate of *C. albicans* attached to PMMA was 44.73% after soaking in 10 µg/mL AgBr-NP@CTMAB solution for 30 min and 91.35% for 8 h. There was no significant residual cytotoxicity or visual color change after soaking.

**Significance:** AgBr-NP@CTMAB showed promising potential treatment for denture cleaners.

**Keywords:** *C. albicans*, AgBr-NP@CTMAB, polymethyl methacrylate, cytotoxicity, biofilm

## Introduction

Polymethyl methacrylate (PMMA) has become the most commonly used denture base because of its sufficient physical, mechanical and aesthetic properties.<sup>1</sup> However, PMMA also has defects such as multiple pores and high water absorption capacity.<sup>2</sup> When denture wearers maintain direct contact with oral mucosa for a long time, oral microorganisms easily adhere and accumulate in the unique microenvironment between denture and oral mucosa and form a biofilm on the surface of denture base resins.<sup>3</sup> *Candida albicans* (*C. albicans*) is one of the main factors contributing to infection, forming biofilms<sup>4-6</sup> and developing into common and recurrent oral disease-denture stomatitis (DS).<sup>7</sup> There are still problems related to the high cost of antifungal drugs and the side effects of microbial resistance to

Correspondence: Da-Li Zheng  
Fujian Key Laboratory of Oral Diseases,  
School and Hospital of Stomatology, Fujian  
Medical University, 88 Jiaotong Road,  
Fuzhou, 350004, People's Republic of China  
Tel +86-591-83720599  
Email dalizheng@fjmu.edu.cn

Ming Zheng  
Department of Prosthodontics, School and  
Hospital of Stomatology, Fujian Medical  
University, 246 Yangqiao Middle Road,  
Fuzhou, 350001, People's Republic of China  
Tel +86-591-83736431  
Email zmdentist@163.com

antibiotics.<sup>8</sup> More seriously, an immune response may occur from denture plaques, and virulence factors released from *C. albicans* biofilms are high risk factors for opportunistic infections and aspiration pneumonia in the elderly and individuals with weak immune systems.<sup>9</sup> Therefore, the prevention of *C. albicans* infection leads to a series of clinical problems related to PMMA, which is of great significance for the oral health of denture wearers.

The use of mechanical and chemical methods to control denture organisms or microorganisms is essential to maintain good oral hygiene in denture wearers. The usefulness of mechanical methods for cleaning denture plaque has been clearly demonstrated, but due to conditions such as Parkinson's disease or mental decline in the elderly, these denture wearers have limited motor skills due to neurological diseases, dementia, lack of flexibility and lack of vision<sup>10</sup> and may not be able to apply daily denture hygiene to the elaborate mechanical brushing of dentures.<sup>11</sup> It has been reported that the use of inappropriate brushing may cause denture surfaces to become rough and dent, resulting in more *C. albicans* colonizing the tiny pores of PMMA.<sup>12</sup> The usage of denture cleaners is recommended as a major chemical method and its use is becoming more widespread.<sup>3</sup> The chemical method is to soak dentures in denture cleaners, which easily removes dental plaque.<sup>13</sup> For example, neutral peroxide foaming tablets (commercially named Polident) are commonly used in clinical practice. Its antimicrobial mechanism is achieved by chemical cleaning through the oxygen release of neutral enzyme peroxide in water solution. However, this sanitary method is not sufficient to completely eliminate microbial biofilms.<sup>14</sup> The program of denture cleaning has always been an interesting field to researchers, but there is no ideal method to meet the clinical cleaning requirements.

In recent years, nanomedicine has become a major research field, and many types of nanomaterials have been explored for use in the medical field. Among these, Ag nanoparticles (AgNps) showed superior antibacterial, antifungal, antiviral and other effects.<sup>15</sup> They are widely used in many fields and are an integral part of many preparations, including soap, deodorant, textiles, electronics, household appliances, cosmetics, and water disinfectants. They are also useful in the food industry for food container production, food sterilization and packaging food supplements.<sup>16</sup>

Quaternary ammonium salts are widely studied in the synthesis of organic antimicrobial materials and have been used for more than 100 years.<sup>17</sup> According to many

previous studies, the preparation process is simple, the synthesis cost is low, and the yield and repetition rate are high. Because of their properties, quaternary ammonium salts are widely used in water treatment, textiles, coatings, medicine and health, as well as other fields.<sup>18</sup>

In the present study, we synthesized AgBr-nanoparticle @cetyltrimethyl-ammonium bromide (CTMAB) hybrids and explored their antifungal activity, safety and application in PMMA of AgBr-NP@CTMAB. Through the above experiments, we provide a theoretical basis and guidance for the application prospect of this kind of material. The benefit to denture wearers is a prospect of this study.

## Materials and Methods

### Synthesis of AgBr-NP@CTMAB Hybrid Material

CTMAB·Br (0.07289 g, 0.2 mmol), KBr (0.0238 g, 0.2 mmol), and AgBr (0.0376 g, 0.1 mmol) were dissolved in 5 mL acetonitrile. After stirring for 2 h at room temperature, the solution was transferred to a small beaker and volatilized at room temperature for 5 days to obtain white needle crystals.

### Materials and Physical Measurements

All chemicals were commercial products and used without further purification. IR spectra were recorded on a Perkin-Elmer Spectrum-2000 FTIR spectrophotometer (4000–400  $\text{cm}^{-1}$ ) from powdered samples spread on KBr plates. Optical diffuse reflectance spectra were measured on a Perkin-Elmer lambda 900 UV/VIS spectrophotometer equipped with an integrating sphere at 293 K, and BaSO<sub>4</sub> plates were used as a reference. Powder XRD patterns were obtained using a Philips X'Pert-MPD diffractometer with CuK $\alpha$  radiation ( $\lambda = 1.54056 \text{ \AA}$ ). High-resolution scanning electron microscopy (HR-SEM) measurements of the surface morphologies of films were executed on a Verios G4 UC instrument.<sup>19,20</sup>

### Computational Methods

According to the EDS in SEM, a model with the formula (CTMAB)<sub>2</sub>(Ag<sub>2</sub>Br<sub>4</sub>) was constructed, and full geometry optimization was executed. During the optimization, first-principle density-functional theory (DFT) calculations were performed with the Cambridge Sequential Total Energy Package (CASTEP) program.<sup>21,22</sup> The exchange correlation functional was described by a generalized gradient approximation (GGA) with the PW91 function for

the solids scheme. A  $3 \times 2 \times 2$  Monkhorst-Pack grid with a total number of 6 k points in the irreducible Brillouin zone was used. The number of plane waves included in the basis was determined by a cutoff energy  $E_c$  of 489.8 eV. The other parameters and convergent criteria were the default values of the CASTEP code.

## Fungal Culture

The *C. albicans* strain from American Type Culture Collection (ATCC, #90029) was used as the model pathogen for the antifungal activity experiments. The fungi from stock cultures were cultivated in Sabouraud dextrose agar (SDA) plates and incubated at 37 °C for 48 h.<sup>23</sup> One loopful of this young culture was transferred to 10 mL of yeast extract peptone dextrose (YPD) medium and incubated at 37 °C for 24 h. The concentration of the bacterial liquid was determined by the absorbance at 600 nm (OD<sub>600</sub>).<sup>24</sup>

## Colony Formation Assay

Twenty microliters of fungal solution with approximately  $1 \times 10^4$  colony forming units per mL (CFU/mL) of *C. albicans* was spread on SDA agar plates containing different concentrations of AgBr-NP@CTMAB. After incubating at 37 °C for 48 h, the yeast colonies were photographed, and the number of colonies was counted manually.

## Growth Curve Assay

Two hundred microliters of the suspension of *C. albicans* ( $10^4$  CFU/mL) in YPD broth medium were added to each well of a 96-well plate, and different concentrations of AgBr-NP@CTMAB were added to the wells. The growth of *C. albicans* was assessed using a Bioscreen C Automated Microbiology Growth Curve Analysis System (Turku, Finland) at 37 °C under shaking at 200 rpm. The OD values at 600 nm were measured every hour for up to 48 h.<sup>25</sup>

## Cell Culture

Human oral mucosal epithelial cells (HOEC, Beijing Beina Biotechnology, BNCC340217) were cultured in Dulbecco's modified Eagle's medium containing 10% FBS. Primary human gingival fibroblasts (HGFs) were mechanically isolated from fragments of gingival tissues obtained from patients (ages 20 to 30) with healthy periodontium who were subjected to surgery for tooth extraction with informed consent. Tissues were

processed by enzymatic dispersion to produce primary cell cultures, and cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotic/antimycotic (penicillin, streptomycin, amphotericin; Gibco/BRL). Cells between passages 3 and 6 were used for experiments.<sup>26</sup> This study was approved by the Institutional Review Board of Fujian Medical University School and Hospital of Stomatology (Approval Number: FMUSS-18-031).

## Cytotoxicity Assay for AgBr-NP@CTMAB

HOECs and HGFs were cultured in a 96-well plate at a density of  $4 \times 10^3$  and  $2 \times 10^3$  cells/well, respectively, and 24 h later, different concentrations of AgBr-NP@CTMAB were added. After culture for 72 h, 10  $\mu$ L of CCK-8 reagent was added to each well, and the absorbance at 450 nm was measured after 1 h incubation.

## Fabrication of Specimen

The AgBr-NP@CTMAB particles were incorporated at 0.25, 0.50 and 1.00% (wt/wt) with the PMMA powder and mixed manually under constant agitation for 10 min to obtain an adequate particle distribution. Then, the mixture powders were dispersed in the MMA liquid and immediately mixed together under stirring by the same operator. The powder (g)-to-liquid (mL) ratio was maintained as recommended by the manufacturers.<sup>27</sup> After homogenization, the mixture was injected into a stainless-steel mold during the plastic phase.<sup>28</sup> After low-temperature polymerization ( $55 \pm 5^\circ\text{C}$ , 30 min, Multicure, Vertex, Zeist, Netherlands), specimens were polished with abrasive papers 600 up to 1000 grit under water cooling. Then, the specimens (10.0 mm  $\times$  10.0 mm  $\times$  2.0 mm) were scrubbed with water and ultrasonically cleaned for 30 min before immersion in PBS  $52 \pm 2$  h at 37 °C.<sup>29</sup> The morphology of the modified PMMA-based specimens was analyzed by a stereomicroscope (Magellan 400L; FEI Co) before testing.

## Surface Roughness Measurement

The surface roughness of all specimens was measured with a profilometer (DSF600, Kosaka Laboratory Ltd, Japan). Three measurements were made for each specimen. According to the JIS'13/ISO'97 standard, the

resolution was 0.01 mm, the interval (cut off length) was 0.8 mm, the transverse length was 2.0 mm, and the stylus speed was 0.5 mm/s. The Ra( $\mu\text{m}$ ) value of that specimen was selected as the decision parameter. All measurements were recorded by one operator.<sup>30</sup>

## Vickers Microhardness Measurement

Vickers microhardness was measured by using a hardness tester (HX-1000TM, Taiming, Shanghai, China). The diamond indenter point was kept on the surface of specimens for 30 seconds with a 300-gram load. Three points on the diagonal line of each sample are pressurized. The measured values were taken as the HV of the specimens.<sup>2</sup>

## Antifungal Effect of AgBr-NP@CTMAB/PMMA

The antifungal activity of the AgBr-NP@CTMAB/PMMA specimens was detected by direct contact test (DCT).<sup>31</sup> Conventional PMMA served as the negative control. The specimens were placed in 12-well plates, and 2 mL of  $1 \times 10^7$  CFU/mL of *C. albicans* suspension was added into each well. After incubation at 37 °C for 24 h, the fungal biofilms on the specimens were gently washed 3 times with PBS and placed in a centrifuge tube containing 2 mL PBS. Agitation of these tubes three times (2 min each time) in a vortex-type agitator allowed the dispersion of the *C. albicans* adhered to the surface of PMMA specimens. Two milliliters of fresh PBS were used in each agitation, and the PBS after 3 shocks was finally mixed.<sup>32</sup> To estimate colony formation, fungal suspensions from each specimen were diluted serially, and an aliquot of 20  $\mu\text{L}$  was inoculated onto SDA plates.

For the reusability test (cycling), the specimens after the first antifungal test were rinsed thoroughly with water and soaked in alcohol for 30 min. The specimens were air dried, sterilized under ultraviolet radiation for 30 min on each side<sup>33</sup> and used for the antifungal test as before.

## Cytotoxicity Assay for AgBr-NP@CTMAB/PMMA

The eluent was prepared by soaking each specimen in 1 mL of DMEM in 12-well cell plates at 37 °C for 72 h. The eluent was filtered for sterilization and then stored at 4 °C before use.<sup>34</sup> HGF and HOEC were seeded in the wells of 96-well plates at a density of  $4 \times 10^3$  cells and

$2 \times 10^3$  cells per well, respectively. Twenty-four hours later, the culture medium in the 96-well plates was removed and replaced with 100  $\mu\text{L}$  eluent of PMMA. The cells were cultured for 72 h, and the cell viability was measured using CCK-8 reagent.<sup>35</sup>

## Anti-Biofilm Activity of AgBr-NP@CTMAB

The PMMA specimens were then sterilized under ultraviolet radiation for 30 min on each side, immersed in a 24-well plate containing *C. albicans* suspension at a concentration of  $3 \times 10^6$  CFU/mL, incubated at a constant temperature of 28 °C with a rotating speed of 180 r/min for 90 min, and then placed in an incubator at 37 °C for 24 h.<sup>36</sup> After washing each side with PBS twice, the samples were placed into different concentrations of solutions, soaking for 30 min or 8 h each, with PBS as the control. The soaked PMMA specimens were washed, dried and placed in a centrifuge tube containing 2 mL PBS. Agitation of these tubes three times (2 min each time) in a vortex-type agitator allowed the dispersion of the *C. albicans* adhered to the surface of PMMA specimens.<sup>37</sup> Two milliliters of fresh PBS was used at each time of agitation, and the PBS after 3 shocks was finally mixed to determine the OD600.

## Scanning Electron Microscopy

Biofilms of *C. albicans* were grown in 24-well plates containing PMMA for 24 h and then treated with PBS, Polident, 10 and 20  $\mu\text{g/mL}$  of AgBr-NP@CTMAB for 8 h. At the end of the incubation period, specimens were removed and washed with PBS three times. Afterwards, biofilms were qualitatively analyzed by scanning electron microscopy<sup>4</sup> (Quanta 250, Thermo-Fisher Scientific, USA). Specimens were fixed in 2.5% glutaraldehyde at 4 °C and dehydrated in ascending ethanol ranging from 50 to 100% for 10 min. The specimens were then sputter coated with gold using a sputter coater and imaged via SEM with an accelerating voltage of 15 keV, and SEM images of surfaces were recorded after fixation.<sup>38</sup>

## Residual Cytotoxicity

Each sterilized specimen was immersed in PBS, Polident, 10  $\mu\text{g/mL}$  and 20  $\mu\text{g/mL}$  of AgBr-NP@CTMAB solution on a 12-well plate. After 8 h, each sample was washed three times in PBS to remove the excess soaking solution and then



placed in a new culture plate. The extract was prepared by soaking each specimen in 1 mL of DMEM in 12-well cell plates at 37 °C for 24 h.<sup>39</sup> An in vitro residual cytotoxicity assay was carried out by exposing HOEC and HGF cells as described above.

## Color Stability of Specimens

During the experiment, all specimens were immersed for 8 h in solutions and then for 16 h in artificial saliva, simulating night disinfection and daily use of the prosthesis. After the next immersion period cycle, the specimens were rinsed in water to remove excess disinfectant solution. The specimens were stored for 7 days, and the solutions were changed daily.<sup>40</sup> Color stability tested by stereomicroscopy (CMS; Leica Microsystems).

## Statistical Analysis

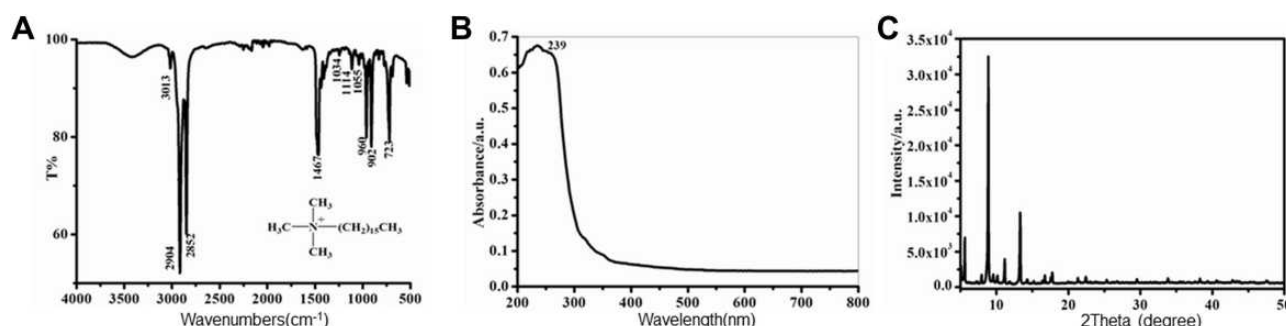
All experiments were performed three times to obtain representative results. Data were analyzed using Prism 5.0 software (GraphPad) by one-way analysis of variance (one-way ANOVA) followed by Tukey's multiple comparison test, with the value of significance set at  $p < 0.05$ .

## Results

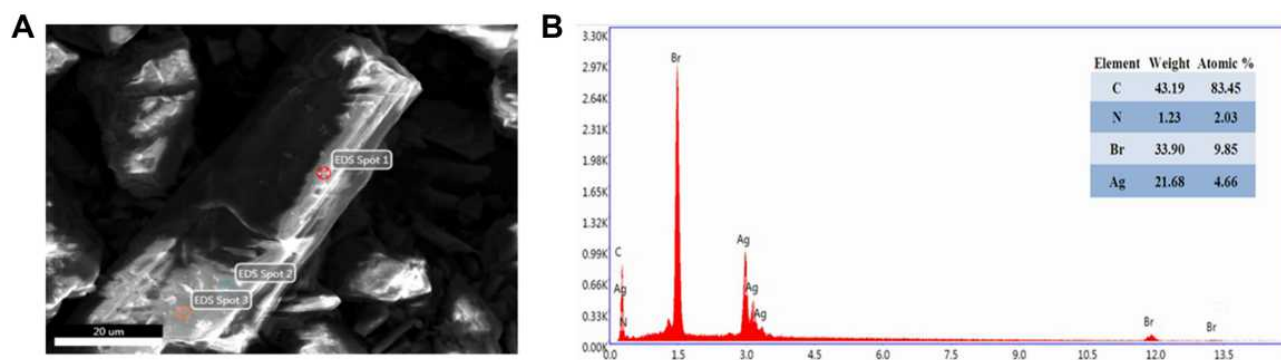
### Synthesis and Characterization of AgBr-NP@CTMAB

The AgBr-NP@CTMAB hybrid material was synthesized, and the material characterization was analyzed by IR spectroscopy. All IR peaks ( $\text{cm}^{-1}$ ): 3013(w), 2094(s), 2852(s), 1467(s), 1114(s), 1055(w), 1034(w), 960(s), 902(s), 723(s) corresponded to the characteristic vibrations of C-C, C-N and C-H bonds in CTMAB<sup>+</sup> (Figure 1A), respectively. UV-Vis adsorption at 239 nm also hinted at the presence of CTMAB<sup>+</sup> (Figure 1B). In the PXRD pattern (Figure 1C), the  $2\theta$  values of 25.16, 29.58, and 47.61° suggested the presence of AgBr nanoparticles. The HR-SEM diagram (Figure 2) illustrated its good crystalline phase, and surface EDS analysis further verified the hybridization of CTMAB<sup>+</sup> with AgBr-NP.

According to the EDS in SEM (Figure 2), a molecular simulation based on the DFT method using the CASTEP program was also conducted to disclose the electronic structure and geometry of AgBr-NP@CTMAB. Upon full optimization, the AgBr-NP@CTMAB hybrid can be described as the model of  $(\text{CTMAB})_2(\text{Ag}_2\text{Br}_4)$  (Figure



**Figure 1** IR spectrum (A), UV-Vis spectrum (B), and PXRD pattern (C) of nanohybrid material.



**Figure 2** SEM (A) and EDS (B) diagrams of the nanohybrid material.

3A). In this model, Ag is in two coordinated environments, the  $\text{AgBr}_3$  triangle and the  $\text{AgBr}_4$  tetrahedron. A  $\text{Ag}_4\text{Br}_8$  tetramer was built from edge-sharing of two  $\text{AgBr}_3$  triangles and  $\text{AgBr}_4$  tetrahedrons. Specifically, the  $\text{CTMAB}^+$  cations are generally linear, and this large steric hindrance leads to the poor adherence of the  $\text{AgBr-NP@CTMAB}$  hybrid with PMMA. The band structure and partial density of states (PDOS) are given in Figure 3B. A calculated direct band gap of 1.12 eV was observed. PDOS indicated that the valence-band maximum mainly results from the mixed nonbonding states of  $\text{Br-4p/Ag-4d}$  and the p-orbital of  $\text{CTMAB}^+$ , but at the bottom of the conductive band, the  $p^*$  anti-bonding orbitals of  $\text{CTMAB}^+$  dominate (right of Figure 3B).

### Solutions of $\text{AgBr-NP@CTMAB}$ Inhibit the Growth of *Candida albicans* Plankton

Next, we explored the effect of  $\text{AgBr-NP@CTMAB}$  on planktonic *Candida albicans*. In the colony formation assay,  $\text{AgBr-NP@CTMAB}$  showed excellent antifungal activity by decreasing the number and size of the colonies of *C. albicans* in a dose-dependent manner. The antifungal rates of 5, 10, 20 and 40  $\mu\text{g/mL}$   $\text{AgBr-NP@CTMAB}$  were  $31.32 \pm 4.95\%$ ,  $53.38 \pm 8.08\%$ , and  $97.36 \pm 2.21\%$ , respectively, which were statistically significant compared with the blank control group (Figure 4A and B,  $P < 0.05$ ). No colonies were observed when the concentration was greater than 20  $\mu\text{g/mL}$ . According to the automatic growth curve analyzer, 2  $\mu\text{g/mL}$   $\text{AgBr-NP@CTMAB}$  slightly extended the lag phase of *C. albicans*, while at 5  $\mu\text{g/mL}$ , the lag phase of *C. albicans* was obviously prolonged by 5 h (Figure 4C). When the concentration of  $\text{AgBr-NP@CTMAB}$  reached 10  $\mu\text{g/mL}$ , the OD600 value slightly increased only in the last several hours of the 48 h, and no significant microorganism growth was found at the concentration of 20  $\mu\text{g/mL}$ .

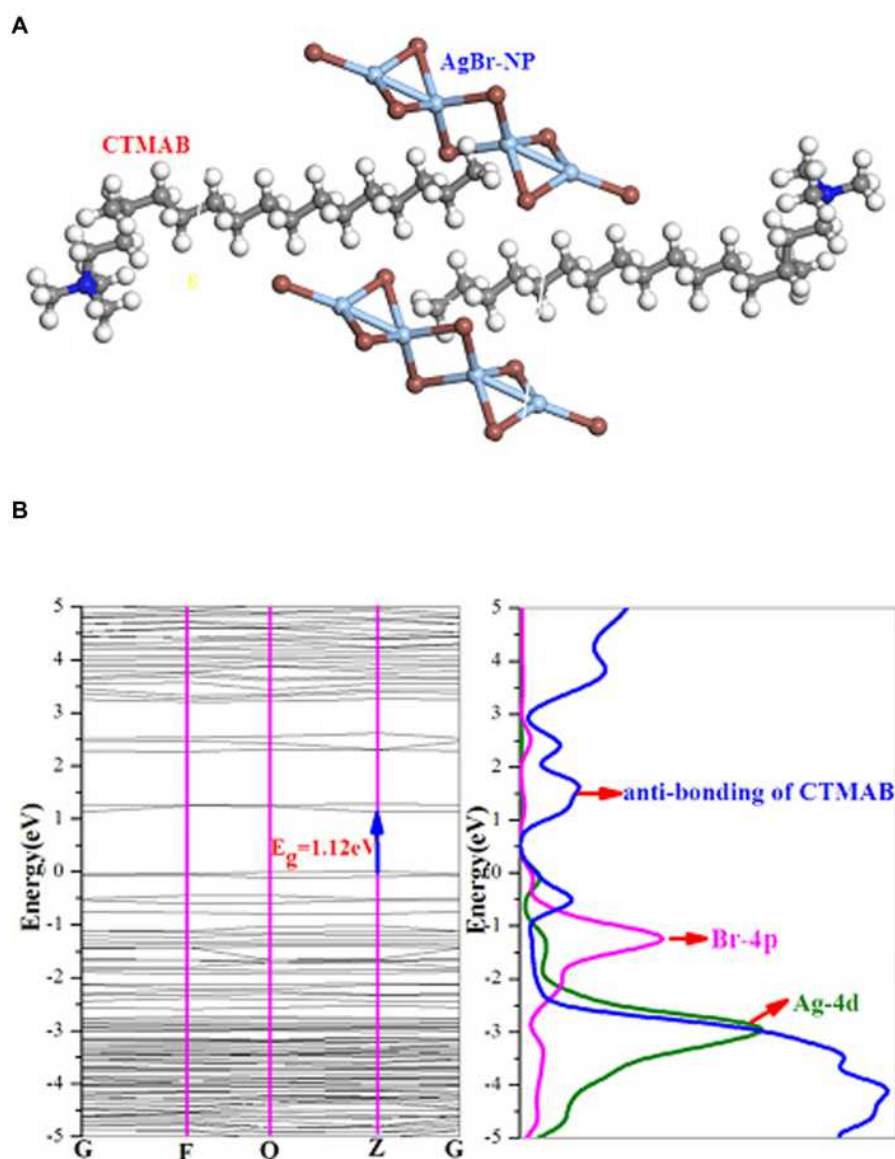
### $\text{AgBr-NP@CTMAB}$ Was Not Suitable to Be Incorporated into PMMA

Because of the excellent antifungal activity of  $\text{AgBr-NP@CTMAB}$ , we next explored whether it can be incorporated into PMMA specimens to improve the antifungal effect and prevent biofilm accumulation. PMMA specimens were fabricated with and without the incorporation of 0.25%, 0.5% or 1.0%  $\text{AgBr-NP@CTMAB}$ . The polymerization and the surface roughness values of PMMA specimens were not affected by the incorporation of  $\text{AgBr-NP@CTMAB}$

(Figure 5A), while the microhardness values of PMMA with higher concentrations of  $\text{AgBr-NP@CTMAB}$  (0.5, 1.0 wt%) were slightly higher than those of the specimens without  $\text{AgBr-NP@CTMAB}$  (Figure 5B,  $P < 0.05$ ). The incorporation of  $\text{AgBr-NP@CTMAB}$  inhibited biofilm accumulation on the PMMA surface (Figure 5C and E), but when we reused the PMMA specimens for antifungal assays, we found that the inhibition effect was attenuated (Figure 5D and F) the second time and disappeared after 3 times of usage (data not shown), which indicated that the effective antifungal component was quickly released from the PMMA specimens. Furthermore, the incorporation of  $\text{AgBr-NP@CTMAB}$  significantly decreased the viability of HGFs and HOEC cells (Figure 6A and B), which also indicated its release from PMMA specimens and the high cytotoxicity of  $\text{AgBr-NP@CTMAB}$ . Additionally, there were some white visible spots on the  $\text{AgBr-NP@CTMAB}$ -containing PMMA specimens due to the uneven mixture (Figure 6C). Based on the short-term antifungal activity results, high cytotoxicity and uneven mixture, we concluded that  $\text{AgBr-NP@CTMAB}$  was not suitable for incorporation into PMMA.

### $\text{AgBr-NP@CTMAB}$ Solution Efficiently Inhibited Biofilm Formation on PMMA Specimens

Next, we explored whether  $\text{AgBr-NP@CTMAB}$  solution can serve as denture cleaner similar to Polident. The PMMA specimens with a biofilm formed by *C. albicans* were soaked for 30 min or 8 h (overnight) in different concentrations of  $\text{AgBr-NP@CTMAB}$ , as well as PBS for the negative control and Polident (freshly prepared as recommended by the manufacturer) for the positive control. The results showed that  $\text{AgBr-NP@CTMAB}$  inhibited biofilm formation on PMMA in a dose-dependent manner (Figure 7A and B), and the biofilm clearance rate was 76.00% when treated with 20  $\mu\text{g/mL}$   $\text{AgBr-NP@CTMAB}$  for 30 min, similar to that of Polident (79.44%,  $P > 0.05$  compared with 20  $\mu\text{g/mL}$   $\text{AgBr-NP@CTMAB}$ ). After soaking for 8 h, the *C. albicans* biofilm was inhibited by 91.35%, 92.57% and 92.73% by solutions of 10  $\mu\text{g/mL}$  and 20  $\mu\text{g/mL}$   $\text{AgBr-NP@CTMAB}$  and Polident, respectively ( $P > 0.05$  between these 3 groups by one way ANOVA).  $\text{AgBr-NP@CTMAB}$  at concentrations of 10  $\mu\text{g/mL}$  and 20  $\mu\text{g/mL}$  almost completely diminished the biofilm formed on the PMMA surfaces compared with the control. The SEM images (Figure 7C) showed that the *C. albicans* biofilm layer effectively spread on the specimens by penetrating into their notched surfaces



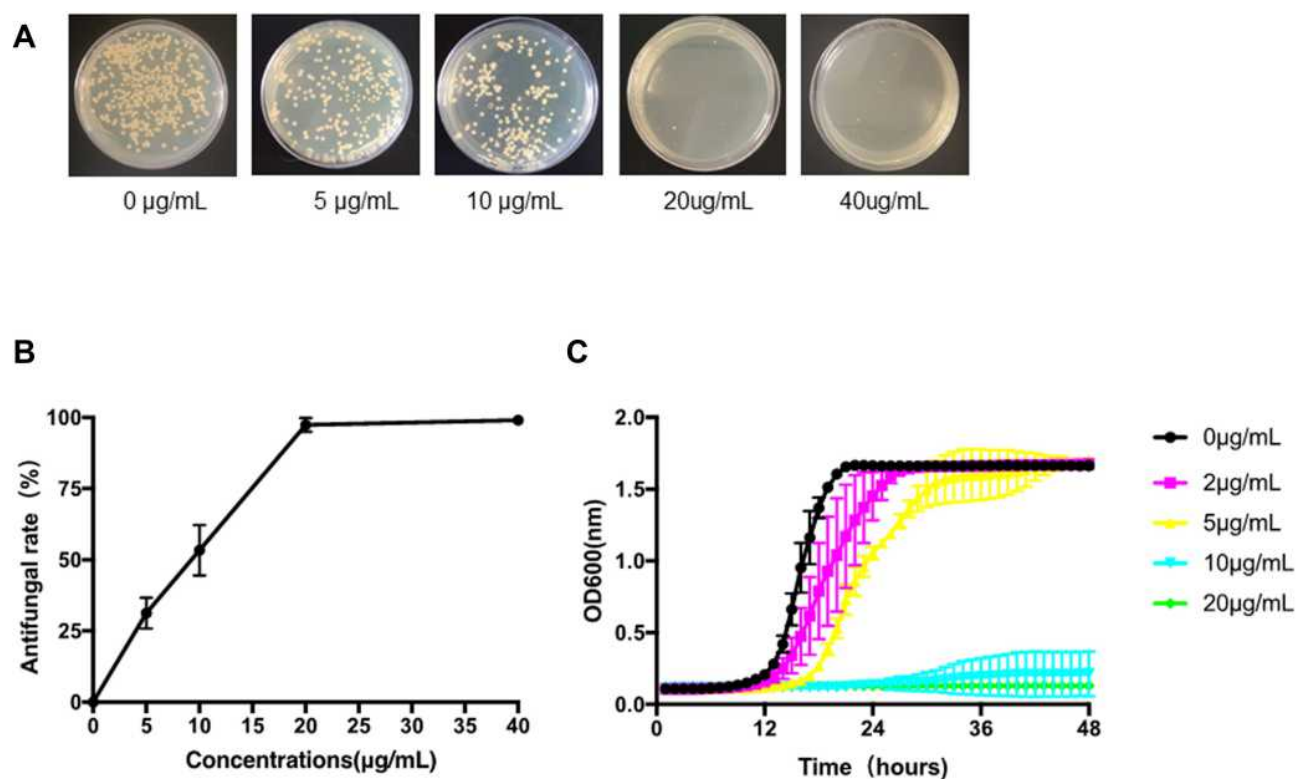
**Figure 3** The geometry of  $(\text{CTMAB})_2(\text{Ag}_2\text{Br}_4)$  (A); band structure (left) and projected density of states (right) for  $(\text{CTMAB})_2(\text{Ag}_2\text{Br}_4)$  (B).

in the control group, whereas the biofilm was destroyed by the AgBr-NP@CTMAB solutions and Polident, and there were only some small colonies attached on the surfaces of PMMA. Although directly added to culture medium, AgBr-NP@CTMAB at the effective concentrations (10  $\mu\text{g/mL}$  and 20  $\mu\text{g/mL}$ ), as well as Polident, showed high cytotoxicity to HGFs and HOEC cells (Figure 8A and B,  $P < 0.01$  when compared these groups with control), but there was no detectable residual cytotoxicity left after washing 3 times with PBS (Figure 8C and D,  $P > 0.05$  among all the groups). To detect whether the immersion in the solution containing AgBr-NP@CTMAB cause surface deterioration of denture-base resin, the PMMA specimens were immersed for 8 h in solutions

and then for 16 h in artificial saliva, and the results showed there was no obvious visual color change after soaking for one week (Figure 8E).

## Discussion

Ag-based nanoparticles are common inorganic antimicrobial agents. Kumar<sup>41</sup> reported the antibacterial potential of Ag and Ag compound nanoparticles against a variety of foodborne pathogenic bacteria and different *Candida* species. Moreover, because of its small size, scanning transmission electron microscopy (STEM) has proven that Agnp has penetrated bacteria and fungi, causing damage in microorganisms by interacting with



**Figure 4** Agar plates of *C. albicans* cultured for 48 h in SDA with various concentrations of AgBr-NP@CTMAB (A). The anti-*C. albicans* activities evaluated via colony-counting methods (B). The growth curves of *C. albicans* in the presence of AgBr-NP@CTMAB were recorded at an OD600 value (C). The data are shown as the means  $\pm$  SD. Bars represent standard deviations of three independent experiments.

phosphorus and sulfur-containing compounds such as DNA. Petr<sup>42</sup> further compared the antibacterial activity of Ag nanoparticles and AgBr nanoparticles against gram-positive and gram-negative bacteria and several *Candida* species. The results showed that Ag nanoparticles had the best antibacterial activity against gram-positive bacteria, while AgBr-NPs had better antibacterial activity against gram-negative bacteria and experimental yeast strains.

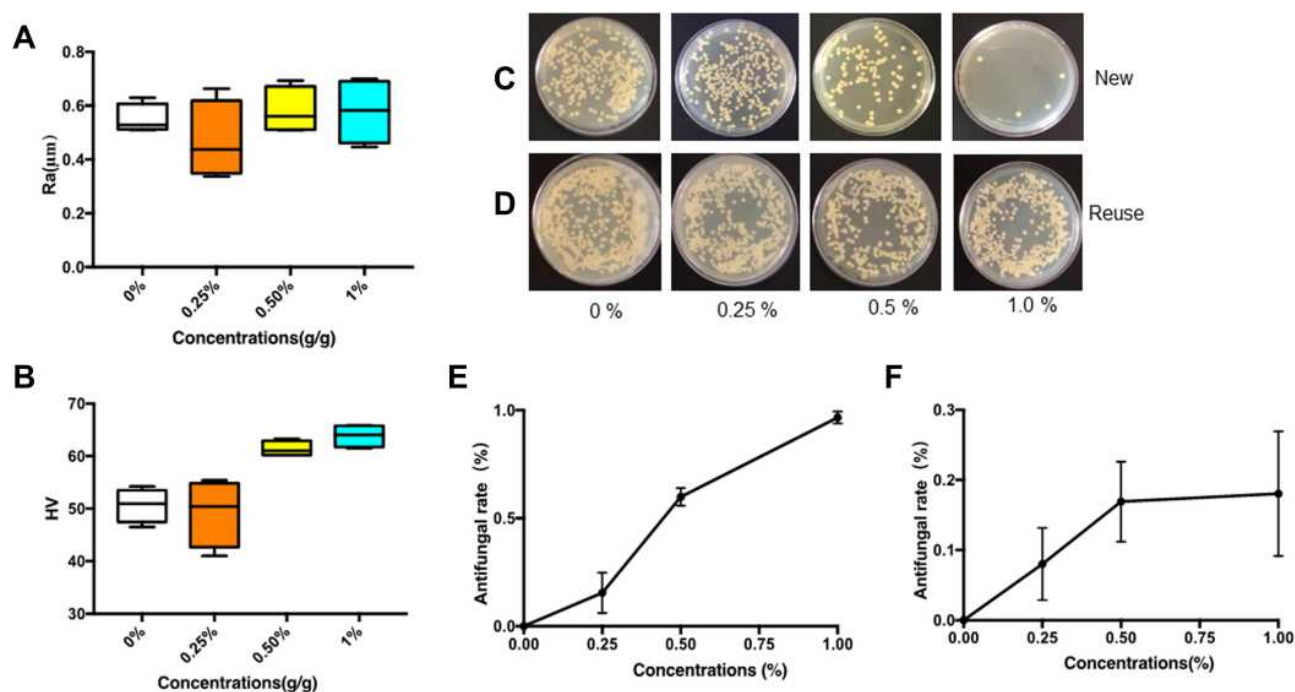
Quaternary ammonium salt compounds in organic antimicrobial agents have antibacterial and antifungal properties. CTMAB is a surfactant that belongs to the quaternary ammonium salts of organic antimicrobial agents. Previous investigations have proven that it is a promising material for the remediation of contaminated farmland.<sup>43</sup> The mixtures of CTMAB reduce the transfer of pollutants from soil to plants, which ensures the safety of agricultural production growing in contaminated soil. However, there are few reports on the application of CTMAB in the field of medicine.

In the present study, we synthesized AgBr-nanoparticle@CTMAB hybrid compounds and found that the composite materials show excellent antifungal

efficiency by inhibiting both the growth of planktonic cells and the formation of *C. albicans* biofilms, but the mechanism of how nanoparticles cause the death of *C. albicans* should be further studied. The electronic structure (Figure 3) suggests that the antifungal activity of the hybrid is the synergistic effect involving CMTAB + and AgBr-NP.

Although PMMA is widely used in denture base resin, it has limitations due to its lack of antimicrobial and antifungal activity. Several attempts to incorporate different compounds into PMMA to provide antifungal effects have been made for clinical use. Zhang<sup>44</sup> evaluated the antifungal properties, cytotoxicity to human dental pulp cells (HDPCs) and mechanical properties of silver bromide/cationic polymer nanocomposites added to PMMA. The experimental data showed that the addition of AgBr/NPVP gives a strong and lasting antifungal effect on *C. albicans* with low toxicity to HDPCs and no significant change in mechanical properties compared with the control group. After revealing the excellent antifungal activity of AgBr-NP@CTMAB, we next tried to incorporate it into PMMA specimens to improve the antifungal effect. The incorporation of up to 1%





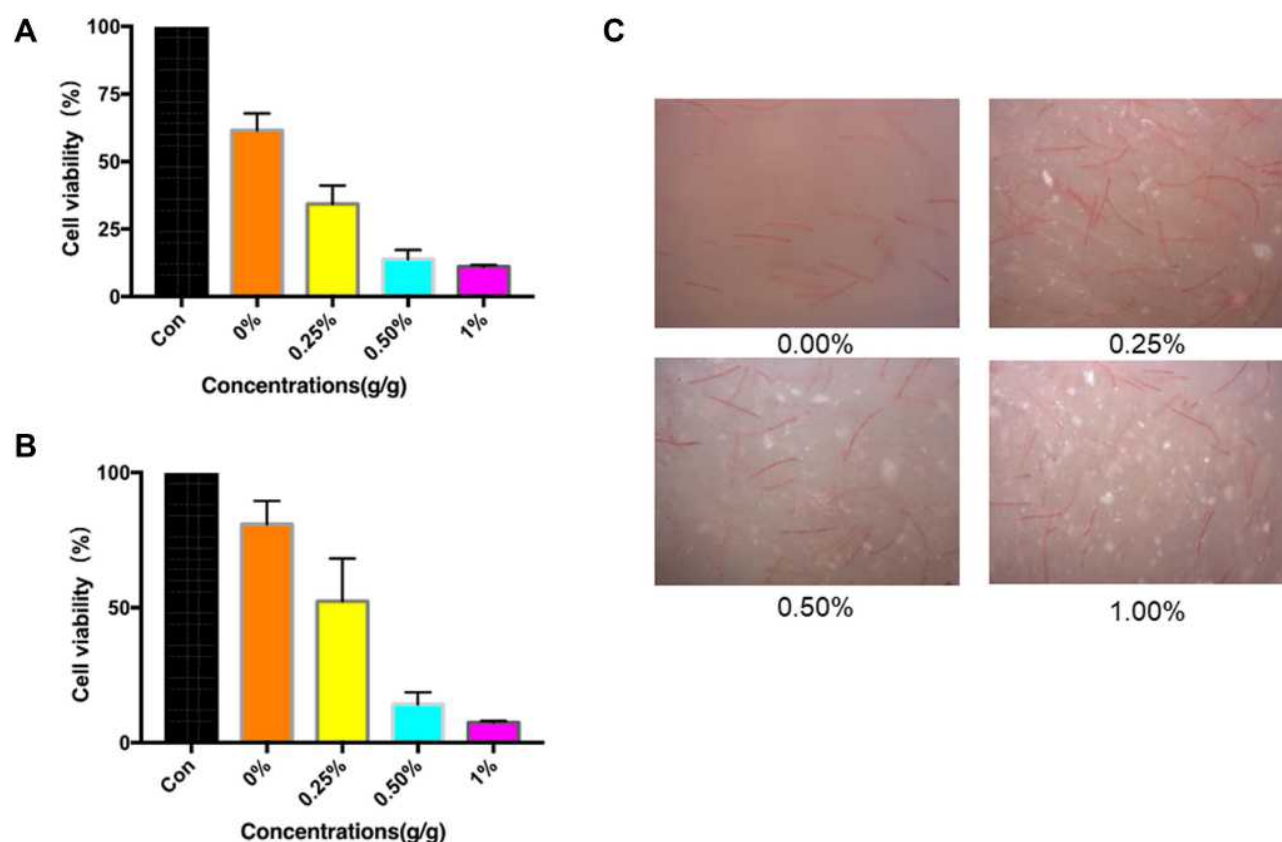
**Figure 5** Incorporation of AgBr-NP@CTMAB into PMMA resin and its antifungal activity. Mechanical properties of PMMA resin with or without AgBr-NP@CTMAB such as surface roughness (A) and Vickers microhardness value (B) were measured. The antifungal activity of 0.25, 0.50, and 1.00 wt% AgBr-NP@CTMAB incorporated into PMMA on *C. albicans* biofilm was tested using the freshly prepared specimens (C and E) and reused ones (D and F). The data are presented as the means  $\pm$  SD. Error bars show the standard deviation (SD).

AgBr-NP@CTMAB did not affect the polymerization of PMMA and did not decrease the surface roughness and microhardness of the PMMA specimens. The results also indicated that AgBr-NP@CTMAB containing PMMA showed excellent antifungal activity in the beginning but failed to yield long-term antifungal effects, which may due to the quick release of effective antifungal components from PMMA specimens. The eluent of PMMA specimens containing AgBr-NP@CTMAB also showed high cytotoxicity to HGFs and HOEC cells. The defects in the long-term antifungal activity and cytotoxicity of AgBr-NP@CTMAB dictated that it was not suitable for incorporation into PMMA.

There are many existing methods to clean dentures, such as mechanical, chemical or a combination of methods.<sup>10</sup> Mechanical procedures include toothpaste brushing the denture base, which is the most commonly used method. Because of the characteristics of irregular tissue surfaces and holes in the denture base, the cleaning effect of simple mechanical methods such as brushing teeth is limited. However, effective removal of biofilms requires a certain degree of artificial dexterity, which is often lacking in the elderly.<sup>11</sup> Therefore, chemical cleaning, such as soaking in

denture cleaner solution, is considered to be a feasible alternative.<sup>3</sup> The effectiveness of chemical cleansing to control *C. albicans* biofilms has been shown in many studies. Polident is available as commercial products, which can easily provide enough cleansing without causing damage to denture resins.<sup>14</sup> According to the manufacturer's instructions, 5 min of soaking or overnight soaking can kill most microbes. In the present study, we found that the solution of 20  $\mu\text{g/mL}$  AgBr-NP@CTMAB showed similar antifungal activity to inhibit biofilm formation as Polident. When added directly to cell culture medium, AgBr-NP@CTMAB, as well as Polident, showed high cytotoxicity to HGFs and HOEC cells. However, there was no detectable residual cytotoxicity left after washing 3 times with PBS, which indicated its safety when used correctly.

Chemical cleaners may cause acrylic bleaching and metal corrosion of dentures.<sup>45</sup> Color stability is an important physical and mechanical property factor of a resin base, indicating its applicability, aging or damage over a period of time. In the present study, we found no unacceptable discoloration of PMMA specimens after soaking these solutions for one week.



**Figure 6** Incorporation of AgBr-NP@CTMAB into PMMA specimens and its cytotoxicity and appearance. **(A and B)** The cell viability of HOECs **(A)** and HGFs **(B)** were measured after culture for 72 h with the extracts of AgBr-NP@CTMAB/PMMA incorporated specimens. The representative means and SDs were recorded after independent triplicate experiments. **(C)** The representative images of the PMMA specimens incorporated with AgBr-NP@CTMAB/PMMA.

## Conclusions

AgBr-NP@CTMAB could effectively suppress both the growth of planktonic cells and the formation of *C. albicans* biofilms. Due to its quick release and cytotoxicity, AgBr-NP@CTMAB is not suited to be incorporated into PMMA specimens, but it may be an effective alternative measure for cleansing biofilms from denture surfaces, with nearly equal efficiency as Polident in the elimination of vital cells from biofilms. In addition, immersion in the solution containing AgBr-NP@CTMAB does not cause surface deterioration of denture-base specimens, and it was deemed safe as a soak solution, as the experiment showed no toxic residue on the denture surface. The single ingredient of AgBr-NP@CTMAB can reach the equal efficiency of Polident, which contains four active ingredients, indicating that AgBr-NP@CTMAB has great potential as a new component of denture cleanser.

## Data Sharing Statement

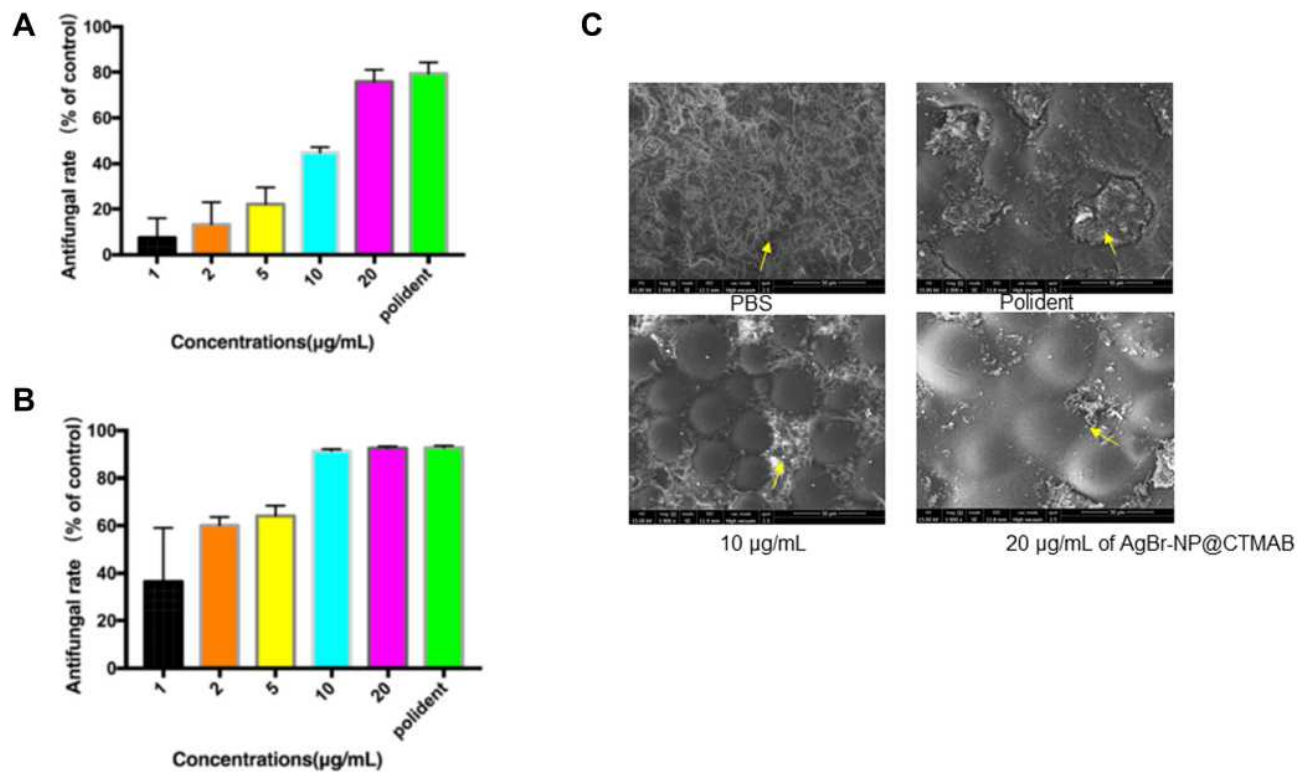
All data generated or analyzed during this study are included in this published article.

## Ethics Approval and Consent to Participate

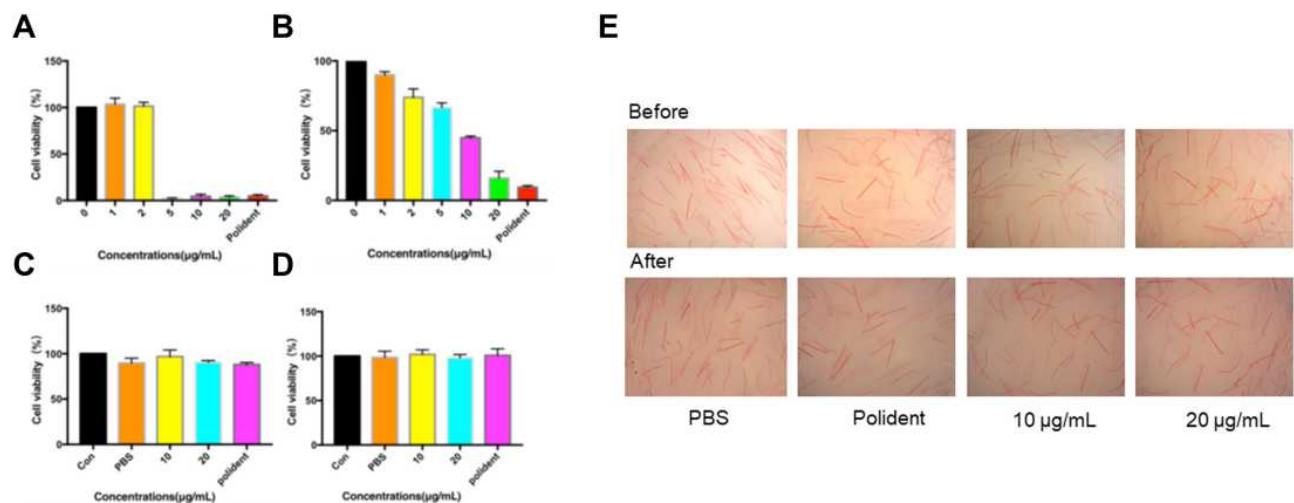
This study was approved by the Institutional Review Board of Fujian Medical University School and Hospital of Stomatology (Approval Number: FMUSS-18-031), and written informed consent was obtained from each participant. The study was carried out in accordance with the guidelines for the care and use of human specimens and animals, including in the approved protocol.

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**Figure 7** *C. albicans* biofilm on PMMA soaked in PBS, dental cleaner (Polident), and AgBr-NP@CTMAB (concentrations of 1, 2, 5, 10, 20 μg/mL) for different times: 30 min (A) and 8 h (B). SEM (C) images (1000×) show the anti-biofilm action on PMMA incubated in different solutions after 8 h. The scale bar is 50 μm and the yellow arrow showed the remained *C. albicans* biofilm.



**Figure 8** Cytotoxicity of AgBr-NP@CTMAB and color change of PMMA specimens. (A and B) Different concentrations of AgBr-NP@CTMAB were added directly to the culture medium and the cell viability of HOECs (A,  $P < 0.001$  for 5 μg/mL, 10 μg/mL, 20 μg/mL AgBr-NP@CTMAB, and Polident when compared with control) and HGFs (B,  $P < 0.01$  for 2 μg/mL, 5 μg/mL, 10 μg/mL, 20 μg/mL of AgBr-NP@CTMAB, and Polident when compared with control) was measured by CCK-8 assay. (C and D) The residual cytotoxicity of AgBr-NP@CTMAB after washing 3 times with PBS on HOECs (C,  $P > 0.05$  among all groups by one way ANOVA) and HGFs (D,  $P > 0.05$  among all groups by one way ANOVA) was measured by CCK-8 assay. (E) Color change of PMMA specimens after a repeated immersion cycles in AgBr-NP@CTMAB solutions for 7 days.

## Disclosure

The authors declare no potential competing interests in this work.

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