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Application of silver nanoparticles in situ synthesized in dental adhesive resin

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ABSTRACT

Objective: The objective was to apply the silver nanoparticles (AgNPs) in situ synthesized in an adhesive resin.
Materials and methods: Silver 2-ethylhexanoate was added into Clearfil SE Bond (Kuraray Inc.) to form 0.0% Ag, 0.1% Ag, 0.2% Ag groups. The size and distribution of AgNPs formed in light-cured adhesive resin disks were observed by TEM. The degree of conversion was evaluated by FTIR. The cytotoxicity against human periodontal ligament fibroblasts was examined by CCK-8 assay. After each water-storage time (1 week, 4 weeks, 3 months, 12 months), biofilms of *Streptococcus mutans* on the resin disks were observed by SEM and tested for live/dead staining and colony-forming units.
Results: The average particle sizes of evenly dispersed AgNPs in the 0.1% Ag and 0.2% Ag groups were 10.9 ± 3.7 nm and 8.8 ± 4.2 nm, respectively. The incorporation of AgNPs did not compromise the degree of conversion and biocompatibility of the 0.1% Ag and 0.2% Ag groups. After 12 months of water storage, the antibacterial properties of 0.1% Ag and 0.2% Ag groups were much higher than that of 0.0% Ag group.
Conclusion: The in situ synthesis of AgNPs might be a promising technique in the antibacterial modification of dental adhesive resin.

1. Introduction

The advancement of bonding technology in the last two decades has created opportunities for the development of oral minimally invasive treatment [1]. However, the bonding interface between tooth structure and adhesive resin is still a weak link in restoration. In the oral environment, the tooth/restoration interface is constantly challenged by water, enzymes, chewing force, temperature, biofilms, and so on, thus becoming unstable. This can produce marginal deterioration and further lead to both a higher susceptibility to microleakage and secondary caries adjacent to restoration [2,3]. Therefore, adhesive resin materials with antibacterial properties to reduce the incidence of secondary caries and prolong the service life of restoration are necessary.

Improving the antibacterial properties of adhesive resin has been one of the hottest topics in the field of restorative materials. Researchers have incorporated a variety of antibacterial agents into dental adhesive systems to provide antibacterial activities in dental applications [4]. Antibacterial agents include leachable compounds (e.g. chlorhexidine), polymeric monomers (e.g. quaternary ammonium methacrylates), and filler particles (e.g. silver zeolite) [5–7]. However, leachable compounds are simply dispersed in the matrix phase and have limited efficacy due to burst release effects. Meanwhile, polymerizable antibacterial agents such as 12-methacryloyloxydodecylpyridinium bromide (MDPB) can effectively reduce biofilm formation [6], but are limited by the fact that immobilized antibacterial agents inhibit bacteria having direct contact with the surface, and the accumulation of biofilms or salivary proteins on the surface will weaken their long-term antibacterial properties [8].

Silver has a long history of application in medicine as an antimicrobial material for its broad-spectrum antimicrobial properties and good biocompatibility [9]. With the development of nanotechnology, the high surface area to mass ratio of AgNPs with a particle size below 100 nm makes it easier to release Ag^+ than metal silver [9], allowing better antibacterial activity at a lower concentration without significantly compromising the color or mechanical properties of materials [10]. Although the antibacterial mechanism of AgNPs is still unclear, AgNPs with resistance to multi-resistant bacteria are confirmed to exert the antibacterial effect by releasing Ag^+ or dissolving themselves [11]. However, when mechanically mixed into resin, AgNPs can form

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agglomerates due to their ultrafine size [12,13]. Recently, the in situ synthesis of AgNPs was applied in dental resin to solve this problem. A solution of silver salt was mixed into resin monomer, upon polymerization, which was accompanied with a simultaneous reduction of the silver salt, AgNPs were formed in situ in the resin [14–16]. Our previous study successfully incorporated AgNPs into dental resin matrix (BisGMA: TEGDMA = 1:1) via in situ synthesis and found that when the concentration of silver salt fell within the range of 0.05%–0.25%, the synthesized AgNPs were uniformly distributed in the resin matrix with an average size of 4-6 nm. The AgNPs in BisGMA/TEGDMA produced a weak anti *Streptococcus mutans* effect [16], which may be related to the high hydrophobicity of BisGMA/TEGDMA. Therefore, we speculated that the in-situ synthesis of AgNPs might have a stronger antibacterial effect in the self-etch adhesive resin containing hydrophilic monomers, such as 10-methacryloyloxydecyl dihydrogen phosphate and 2-hydroxyethyl methacrylate.

Therefore, the objective of this study was to apply the AgNPs in situ synthesized in dental adhesive resin and put forward the null hypothesis that modified adhesive resin has no antibacterial ability.

2. Materials and Methods

2.1. Adhesive resin disk preparation

0.08 g silver 2-ethylhexanoate (LOT# A1918077, Strem chemicals Inc., Newburyport, MA, USA) was dissolved into 0.92 g 2-(tert-butylamino)ethyl methacrylate (LOT# MKBF9770V, Sigma-Aldrich Inc., Saint Louis, MO, USA) to form silver salt solution, sonicating for 1 min to dissolve completely. The obtained solution was stored in dark for 40 min and mixed in the proportion of 0%, 0.1% and 0.2% into the bond part of a two-step self-etching adhesive resin (Clearfil SE Bond, LOT# AE0180, Kuraray Inc., Tokyo, Japan), comprised of 10-methacryloyloxydecyl dihydrogen phosphate, bis-phenol A diglycidyl methacrylate, 2-hydroxyethyl methacrylate, silanated colloidal silica and photoinitiators, to obtain three experimental groups named as 0.0% Ag, 0.1% Ag and 0.2% Ag groups. The bond part of an antibacterial two-step self-etching adhesive resin (Clearfil Protect Bond (CPB), LOT# CD0060, Kuraray Inc., Tokyo, Japan) was regarded as the positive control group, the compositions of which embrace 10-methacryloyloxydecyl dihydrogen phosphate, 2-hydroxyethyl methacrylate, bis-phenol A diglycidyl methacrylate, sodium fluoride, silanated colloidal silica and photoinitiators.

Plexiglass rings made by a low-speed diamond saw (IsoMet 1000, Buehler Inc., Lake Bluff, IL, USA) with 6 mm diameter and 1 mm thickness were served as a mold. After that, resin disks were prepared in the plexiglass rings between two glass slides separated by two polyester films (thickness = 0.1 mm). Resin disks were photopolymerized by a LED light unit with a light intensity of 800 mW/cm^2 (Bluephase C8, Ivoclar Vivadent Inc., Schaan, Principality of Liechtenstein) for 20 s on each side. Then resin disks were removed from rings without damage. A total of 50 resin disks were prepared for each group of resin cement. All the cured resin disks were cleaned by sonicating for 5 min to remove the stains and unpolymerized resin monomers on the surfaces. After that, these disks were stored in sterile water for 24 h and dried before being used in the following experiments.

2.2. AgNPs in adhesive resin observation

Five cured resin disks in each group with 70 nm thickness slices were obtained using an ultramicrotome (Leica UC7, Leica Inc., Weztlar, Hessen, Germany) and were placed on 200 copper grids for transmission electron microscope (TEM, JEM-2100, JEOL Inc., Tokyo, Japan) analysis. The average size of AgNPs was calculated by measuring 100 particles randomly from the TEM images via an analysis software, Nano Measure, Version 1.2. The particle size distribution of AgNPs in adhesive resins was analyzed by Origin (Origin 9.0, OriginLab Inc., Northampton, MA, USA).

2.3. Degree of conversion of adhesive resin

Droplets of unpolymerized resin solution and polymerized resin disks (n = 3) for each group were placed and vertically pressed against a horizontal diamond element of an attenuated total reflectance (ATR) unit of the Fourier transform infrared spectroscopy (FTIR, NEXUS870, Thermo Nicolet Inc., Waltham, MA, USA). The resin solution and the resin disks were placed in the sample holder of the device, and spectra were recorded. Thirty-two scans were conducted in the mid-IR region (4000 cm^{-1} to 500 cm^{-1}) at a resolution of 4 cm^{-1}. The absorption band locating at 1609 cm^{-1} (aromatic C=C) was used as an internal reference peak and 1639 cm^{-1} was worked for the aliphatic C=C double bonds. The degree of conversion (DC) was calculated by determining the FTIR bands area using the following formula:

$$DC = [1\text{-}(A_1\text{cured} / A_0\ \text{cured}) / (A_1\ \text{uncured} / A_0\ \text{uncured})] \times 100\%$$

where A_1 represents the absorbance intensity of aliphatic C=C at 1639 cm^{-1} and A_0 represents the absorbance intensity of aromatic C=C at 1609 cm^{-1}.

2.4. Cytotoxicity assay of adhesive resin extract

Human periodontal ligament fibroblasts (HPDLFs) were obtained from healthy premolars freshly extracted during orthodontic treatment, and proper informed consent was obtained from these donors. The protocol was approved by the local Ethical Committee the Affiliated Stomatological Hospital, Medical School of Nanjing University, Nanjing, China. The extracted teeth were washed twice with phosphate-buffered saline (PBS, Thermo Fisher Scientific Inc., Waltham, MA, USA).

Then the periodontal ligaments attached to the middle third of the roots of the tooth were gently scraped off with a scalpel. The periodontal tissue was incubated with 3 mg/mL collagenase type I (Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA) at 37 °C for 1 h. After that, cell suspensions were filtered through a 70 μm strainer (BD Cell Strainer 352350, Becton, Dickinson Inc., Franklin Lakes, NJ, USA) and centrifuged at 300 g for 5 min. After removing the upper liquid, the cells were incubated with 1 mL Dulbecco's modified Eagle's medium (DMEM, Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, ExCell Bio Inc., Suzhou, Jiangsu, China). The cell suspensions were pipetted sufficiently and the cells were plated in 25-cm^2 flasks at a density of 4 × 10^5 cells per cm^2. Then the cells were incubated with 4 mL DMEM supplemented with 10% FBS and 5% penicillin/streptomycin (HyClone Laboratories, GE Healthcare Life Sciences, Pittsburgh, PA, USA) at 37 °C in 5% CO_2 incubator and the culture medium was changed every two days until the cells reached 80% confluency. The cells were collected by 0.25% trypsin (Gibco, Life Technologies Inc., Waltham, MA, USA) and 0.1% EDTA (pH 7.3) until they grew to 80% confluency, and were transferred to culture dishes as passage one (P_1). Then the confluent cells were detached by being incubated with 0.25% trypsin and 0.1% EDTA at 37 °C for 2 min for further passaging.

Extracts from polymerized resin disks were incubated with HPDLFs to evaluate the cytotoxicity of polymerized resin disks. According to ISO 10993-12 [17], ten resin disks sterilized in an ethylene oxide sterilizer (Anprolene AN 74i, Andersen Inc., Haw River, NC, USA) from each group were randomly selected for immersion in 2.5 mL of complete DMEM medium and left at 37 °C for 24 h under reciprocal shaking. The DMEM medium was changed every day and the extracts on the 1st day, 3rd day, 5th day, 7th day were collected for further research. The extract medium was filtered through a sterile 0.2 μm syringe filter (Millex-HPF, Merck Millipore Inc., Billerica, MA, USA) and then stored at 4 °C before being used.

Cell viability was measured by the Cell Counting Kit-8 (CCK-8,

Dojindo Laboratories Inc., Tokyo, Japan). Purified HPDLFs at passage six (P_6) were transferred to 96-well culture plates (100 μL per well) at a density of 5000 cells per well and incubated for 24 h. After that, the wells were exposed to extracts collected on the 1st day, 3rd day, 5th day, 7th day, and incubated for another 24 h. HPDLFs treated with fresh DMEM alone were served as the negative control group. After incubation, the wells were rinsed twice with PBS. Then, 100 μL of fresh DMEM without FBS and 10 μL of CCK-8 solution were added to each well, followed by an additional 4 h of incubation at 37 °C. After that, the optical density value was measured at 450 nm absorbance by an automated microplate reader (Spectra-Max M3, Molecular Devices Inc., San Francisco, CA, USA). Each condition was analyzed in triplicate. Cell relative growth rate (RGR) was calculated by the following formula:

RGR (%) = (average absorbance of samples / average absorbance of negative controls) × 100%.

2.5. Antibacterial activity of adhesive resin

Resin disks were submerged in distilled water at 37 °C for 1 week, 4 weeks, 3 months and 12 months for water-aging. Ten resin disks of each group were placed in sterile water in a sealed polyethylene container. The water was changed once a week. At the end of each period, resin disks were taken out from the container to assess the antibacterial activity. Sterile polyester films (diameter = 6 mm, thickness = 1 mm) were used as the blank control group. All resin disks and polyester films were dried and then sterilized in an ethylene oxide sterilizer, then measured by the following antibacterial methods: Colony-forming unit (CFU) assay, live/dead bacterial staining assay, and observation of surface biofilm morphology.

The bacterial suspensions of *Streptococcus mutans* (*S. mutans*, ATCC 700610, China general microbiological culture collection center, Beijing, China) in brain-heart infusion broth (BHI, Thermo Fisher Scientific Inc., Waltham, MA, USA) was adjusted to an optical density of 0.5 at 600 nm (approximately 10^8 CFU/mL). The resin disks of each group and the polyester films were placed into each well of 24-well plate filled with 1.5 mL of inoculum and incubated in 5% CO_2 at 37 °C for 24 h. Then, the disks were transferred to a new 24-well plate filled with 1.5 mL fresh BHI medium and incubated for another 24 h. After 48 h of incubation, these resin disks with biofilms were transferred to a new 24-well plate and gently rinsed with 200 μL PBS before being measured. All assays were performed in triplicate to ensure reproducibility.

2.5.1. Colony-forming unit (CFU) count of adhesive resin surface biofilm

The biofilms on resin disks and sterile polyester films were harvested by sonication (3510R-MTH, Branson Ultrasonics Inc., Denbury, CT, USA) at a frequency of 40 kHz for 5 min, and by vortexing at the maximum speed for 20 s using a vortex mixer (HMS-350, Hengao Technology Inc., Tianjin, China). The collected bacterial suspensions thus were serially diluted and cultured on the BHI agar plates, which were incubated at 5% CO_2 and 37 °C for 48 h, and the number of colonies of the *S. mutans* was counted to calculate the CFU. After that, the antibacterial rate was also calculated, the calculation formula is displayed as follow:

Antibacterial rate (%) =(N_0-N)/ N_0 × 100%

where N_0 represents the average number of CFU recovered in the blank control group; N represents the average number of CFU recovered in four test groups (n = 3).

2.5.2. Live/dead bacterial staining assay of adhesive resin surface biofilm

Resin disks with biofilms of four test groups (n = 3) were stained with a live/dead bacterial viability kit (L7012, Thermo Fisher Scientific Inc., Waltham, MA, USA) for 15 min. Live bacteria were stained green with syto-9, whereas dead bacteria were stained red with propidium iodide. The biofilms on the specimens were rinsed moderately and watched by confocal laser scanning microscopy (CLSM, TE2000-S, Nikon Inc., Tokyo, Japan).

2.5.3. Morphology observation of adhesive resin surface biofilm

Resin disks with biofilms of four test groups (n = 3) were fixed with 2.5% glutaraldehyde overnight at 4 °C, dehydrated with a graded ethanol series, dried in a critical-point drier, and sputter-coated with gold. The resin disks were observed by scanning electron microscope (SEM, S-3400 N, Hitachi Inc., Tokyo, Japan) and representative pictures were selected to evaluate biofilm morphology.

2.6. Statistical analysis

Statistical analysis was performed with SPSS (SPSS Statistics 26.0, International Business Machines Inc., Armonk, NY, USA). Data of RGR of HPDLFs and antibacterial rate were analyzed via two-way ANOVA and Tukey's test (variables: group and time). Meanwhile, one-way ANOVA followed by SNK-q test was applied to evaluate the results of DC among four test groups (α = 0.05).

3. Results

3.1. The characterization of AgNPs in adhesive resin

Fig. 1 exhibited a darkening color from light yellow to dark brown as the Ag concentration increased. The agglomerated fillers were noticed in resin disks of all groups, while AgNPs were observed only in 0.1% Ag and 0.2% Ag groups, which were spherically and homogeneously distributed. As shown in Fig. 2, the average particle sizes in the 0.1% Ag and 0.2% Ag groups were 10.9 ± 3.7 nm and 8.8 ± 4.2 nm, respectively.

3.2. Degree of conversion of adhesive resin

The DC of four groups were 76.8 ± 2.2 (0.0% Ag), 75.1 ± 4.9 (0.1% Ag), 74.1 ± 4.4 (0.2% Ag), 80.2 ± 2.3 (CPB), respectively. There was no statistically difference among 0.0% Ag, 0.1% Ag and 0.2% Ag groups ($p > 0.05$).

3.3. Cytotoxicity assay of adhesive resin extract

The RGR data of HPDLFs for all groups were exhibited in Table 1. Two-way ANOVA revealed that both the groups ($F = 1.67$, $p > 0.05$) and the incubation time ($F = 1.56$, $p > 0.05$) or the interaction of the two factors ($F = 1.28$, $p > 0.05$) had no effect on RGR. No significant difference in RGR was observed both in the same group after different incubation time ($p > 0.05$) and among four groups at the same incubation time ($p > 0.05$).

3.4. Antimicrobial activity assessment of adhesive resin

The antibacterial rate data for all groups were displayed in Table 2. Two-way ANOVA revealed that both the groups ($F = 206.40$, $p < 0.05$) and the immersion time ($F = 25.51$, $p < 0.05$) or the interaction of the two factors ($F = 11.31$, $p < 0.05$) had significant impacts on antibacterial rate. 0.1% Ag, 0.2% Ag and CPB groups had no statistically significant difference in the antibacterial activity after different immersion periods ($p > 0.05$). Nevertheless, the antibacterial rate of 0.0% Ag group was statistically different with time aging ($p < 0.05$). Meanwhile, 0.0% Ag group was significantly different from the other three groups at the same immersion period ($p < 0.05$).

The live/dead staining confocal images of adhesive resin surface biofilms are shown in Fig. 3. After each water storage time, virtually full coverage of live bacteria was determined in 0.0% Ag group, and there were substantial numbers of dead bacteria on the resin surfaces of 0.1%

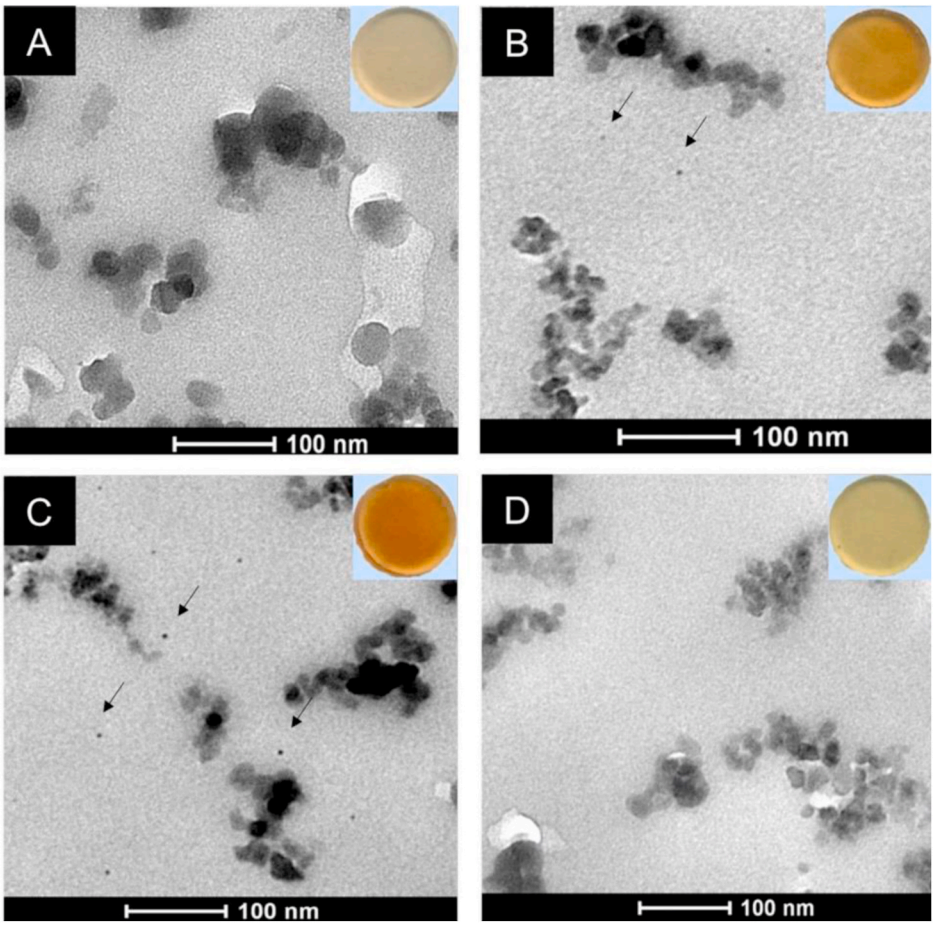

Fig. 1. TEM observation and specimen images of polymerized 0.0% Ag (A), 0.1% Ag (B), 0.2% Ag (C) and CPB (D) groups. Pointer: AgNPs.

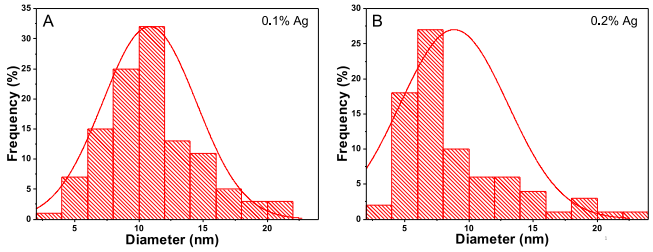

Fig. 2. Particle size distribution histograms of 100 AgNPs extracted from the TEM images in 0.1% Ag (A) and 0.2% Ag (B) groups.

Table 1
Cell relative growth rate of four groups on the 1st, 3rd, 5th, 7th day of incubation.

	0.0% Ag	0.1% Ag	0.2% Ag	CPB
1st day	1.07 ± 0.03^{Aa}	1.05 ± 0.08^{Aa}	0.68 ± 0.16^{Aa}	1.05 ± 0.05^{Aa}
3rd day	1.01 ± 0.09^{Aa}	1.13 ± 0.07^{Aa}	0.99 ± 0.18^{Aa}	1.06 ± 0.08^{Aa}
5th day	1.00 ± 0.11^{Aa}	1.03 ± 0.08^{Aa}	0.99 ± 0.14^{Aa}	0.98 ± 0.14^{Aa}
7th day	0.92 ± 0.18^{Aa}	0.92 ± 0.13^{Aa}	0.96 ± 0.17^{Aa}	0.88 ± 0.14^{Aa}

Identical superscripts (uppercase: within column; lowercase: within row) represent no statistically significance in biocompatibility ($p > 0.05$).

Ag, 0.2% Ag and CPB groups.

The morphology of adhesive resin surface biofilms appeared in Fig. 4. After each water storage time, 0.0% Ag group was primarily full covered with bacteria, which was significantly more than the other three groups.

Table 2
Antibacterial rate of all groups after 1 week, 4 weeks, 3 months and 12 months of water storage.

	0.0% Ag	0.1% Ag	0.2% Ag	CPB
1 week	84.54 ± 6.05^{Aa}	95.19 ± 2.08^{Ab}	96.67 ± 1.73^{Ab}	96.48 ± 0.70^{Ab}
4 weeks	59.16 ± 7.93^{Ba}	95.17 ± 0.71^{Ab}	96.16 ± 0.94^{Ab}	94.82 ± 0.67^{Ab}
3 months	53.98 ± 8.59^{Ba}	87.67 ± 1.17^{Ab}	93.92 ± 0.94^{Ab}	89.72 ± 0.41^{Ab}
12 months	44.42 ± 7.92^{Ca}	90.36 ± 2.36^{Ab}	95.45 ± 0.11^{Ab}	91.87 ± 1.79^{Ab}

Dissimilar letters (uppercase: within column; lowercase: within row) stand for significant differences in antibacterial activity ($p < 0.05$).

4. Discussion

The two-step self-etch adhesive resin includes the primer and bond parts. The bond part modified with AgNPs in situ is the main component of bonding interface that directly contacts the external bacteria. The principle of the in-situ synthesis method is to take advantage of the free radicals generated from resin monomers during the photo-polymerization process to reduce the silver ions into AgNPs [12]. Some studies have reported that the formation of AgNPs in resin may reduce the DC of the material [18,19], since the silver ion reduction process may consume the initiator or terminate the propagating polymer chains [18,20]. However, our study presented that the DCs of 0.1% Ag and

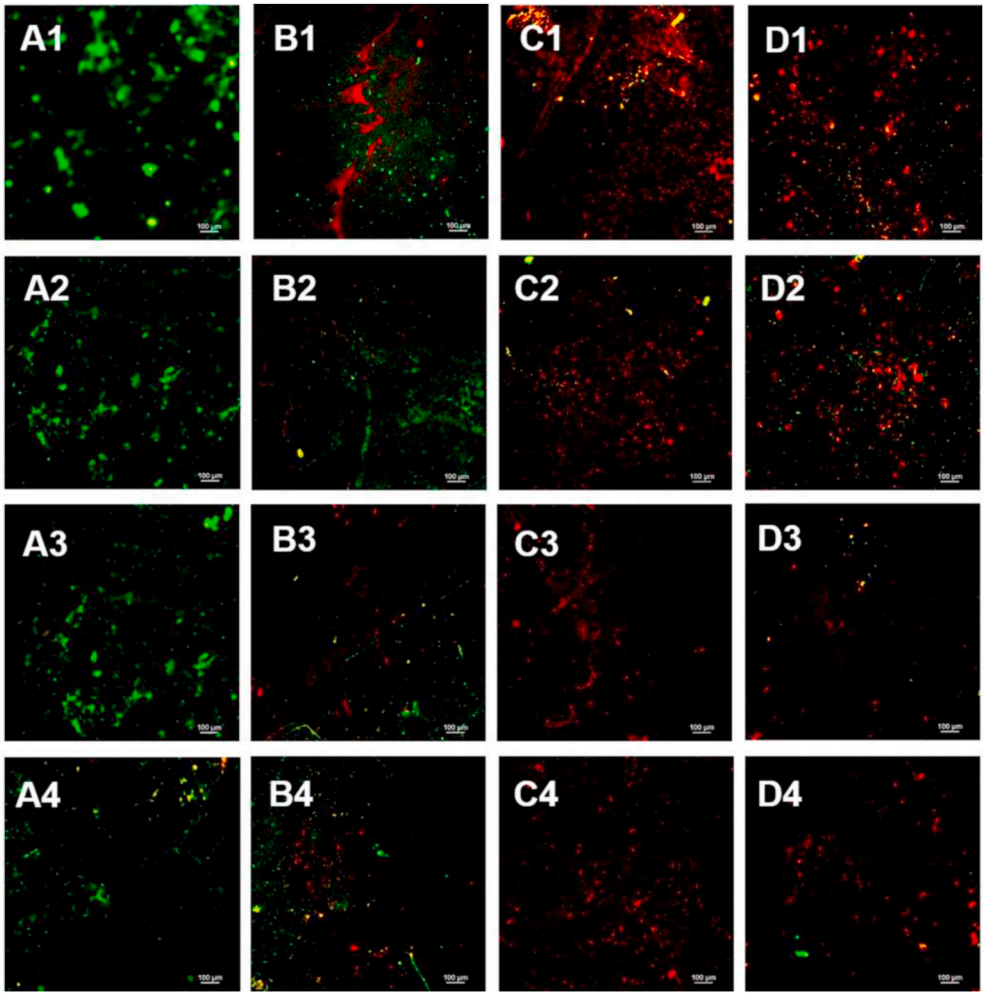

Fig. 3. Live/dead CLSM images of *S. mutans* biofilms grown on adhesive resin after different water-storage periods (live-green; dead-red; from A to D: 0.0% Ag, 0.1% Ag, 0.2% Ag, CPB groups, respectively; from 1 to 4: 1 week, 4 weeks, 3 months, 12 months, respectively). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

0.2% Ag groups were not significantly different from that of 0.0% Ag group, suggesting the added silver source did not have a destructive effect on the polymerization. In view of the positive correlation between the DC of adhesive resin and its bond strength [21–23], we did not further investigate the bond strength.

Previous research has found that more than 90% of extracts of polymers eluted within 24 h [24]. Thus, our study focused on the cytotoxicity of adhesive resin within 1 week after polymerization. In the oral environment, the extracts of adhesive resin enter the sulcus, which can affect the activity of periodontal ligament cells and even the health of periodontal tissues. Therefore, HPDLFs cells were selected to study the cytotoxicity assay of adhesive resin containing AgNPs. According to ISO 10993-5 [25], the tested materials have no obvious cytotoxic effect when the cell RGR is higher than 70%. On the 1st, 3rd, 5th and 7th day, the cell RGRs of all groups were stable and higher than 70%, indicating the incorporation of AgNPs did not increase the cytotoxicity of adhesive resin.

The cytotoxicity assay results also indicated that the extracts of adhesive resin could not play an effective antibacterial role. Therefore, this study evaluated the biofilms on the surface of specimens. *S. mutans* was used because the colonization of *S. mutans* on tooth surface has been recognized in the etiology and pathogenesis of human dental caries [26].

Although 0.0% Ag did not contain antibacterial agents, it also demonstrated some antibacterial activity against *S. mutans* after 1 week of water storage. The antibacterial activity might result from toxicity from the dissolved products accumulating on the adhesive resin surface [27]. The antibacterial effect of 0.0% Ag decreased with the increase of water storage time, supporting the above conjecture.

CPB is the first commercially available antibacterial two-step self-etching adhesive resin. MDPB in the primer part and sodium fluoride in the bond part endowed CPB with dual antimicrobial ability. Many studies have emphasized the antibacterial activity of MDPB against *S. mutans* [6,28,29], however in the current study, the bond part of CPB only containing sodium fluoride also has stable and lasting antibacterial activity. Since 1940, sodium fluoride has been used as the anti-caries agent [30]. The antibacterial mechanism of sodium fluoride could be related to the slow release of fluorine [31]. And the suppositions about the antibacterial process include impeding acid formation through obstruction of the microbial metabolism [32], disrupting intracellular pH regulation due to action as the transmembrane proton carrier [33] and enhancing remineralization [34].

The results revealed that adhesive resin containing AgNPs exhibited similar antibacterial behavior to CPB. Both 0.1% Ag and 0.2% Ag groups had good antimicrobial properties, which were verified by the CLSM and SEM images. Therefore, the null hypothesis of this study was rejected. Morones et al. [35] inferred that the bactericidal activity of AgNPs is size-dependent. Silver nanoparticles with a diameter of 1-10 nm were found to alter the cell membrane permeability and cause cell damage due to higher reactive surfaces [35]. Fan et al. [14] added 0.5 wt% AgBz

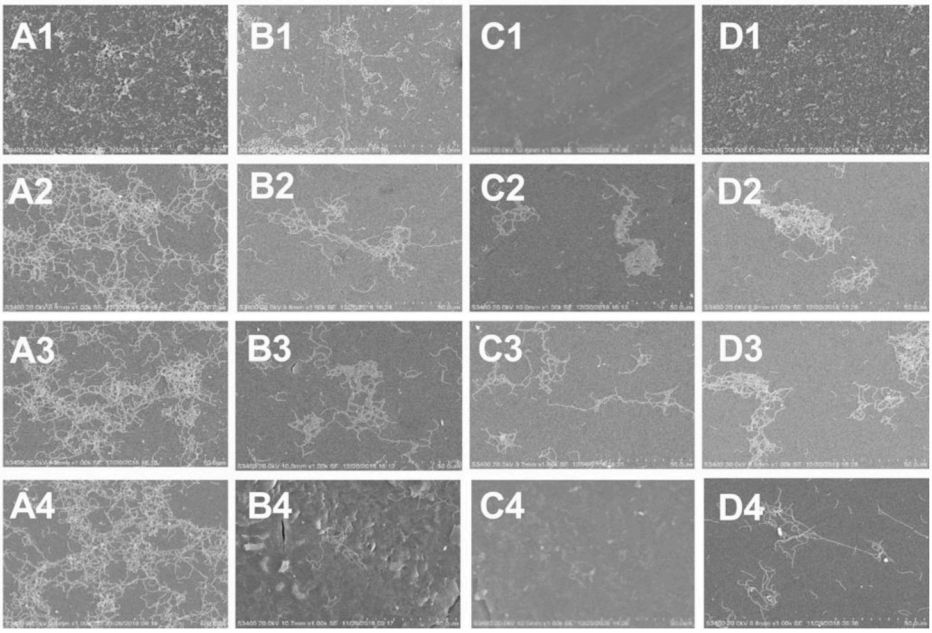

Fig. 4. SEM images of biofilm morphology on the specimens after different water-storage periods (from A to D: 0.0% Ag, 0.1% Ag, 0.2% Ag, CPB groups, respectively; from 1 to 4: 1 week, 4 weeks, 3 months, 12 months, respectively).

to acrylic dental resin and found that AgNPs with a diameter of 2-18 nm, synthesized in situ in dental acrylic resin, could inhibit the growth of 97.5% *S. mutans*. Cheng et al. [36] added 0.08 wt% silver 2-ethylhexanoate into 2-(tert-butylamino)ethyl methacrylate to generate AgNPs with a size of 2.7 ± 0.6 nm in situ, which had good antibacterial effects against *S. mutans*. Our results were consistent with past findings, in that the in situ synthesis of AgNPs in 0.1% Ag and 0.2% Ag groups, which were on average roughly 10 nm, exerted effective antibacterial properties.

Although many studies have found an antimicrobial effect of AgNPs, the antibacterial mechanism of AgNPs is not yet conclusive. Several studies proposed that silver ions inactivate respiratory chain enzymes, leading to the generation of reactive oxygen species (ROS) and impacting mitochondrial function [37,38]. Some scholars reported that DNA fails to replicate when silver ions interact with the bacteria [39]. Other experimental evidences showed the ionic silver causes the leakage of intracellular components by destroying the cell membrane [40,41]. The relative hydrophilicity of adhesive resin means that the direct dissolution of AgNPs is not easy, while in the water medium condition, it is possible that AgNPs acted as an "Ag^+ reservoir" and killed bacteria attached to the surface of the specimen by slowly releasing Ag^+ into the cell membrane [40,41].

5. Conclusion

The in situ synthesis of AgNPs might be a promising technique in the antibacterial modification of dental adhesive resin.

Author contributions

Yuqiong Yang: conceptualization, methodology, data curation, writing-original draft preparation. **Yashuang Ding:** software, methodology, visualization, validation, writing-original draft preparation. **Yue Fan:** methodology, data curation. **Lingyan Ren:** formal analysis, project administration. **Xuna Tang:** software, validation. **Xiangfeng Meng:** conceptualization, resources, writing-review and editing, supervision, funding acquisition.

Declaration of competing interest

The authors declare no potential conflict of interest.

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