**ORIGINAL ARTICLE**

# Human Dentin Coated with Silver Nanoclusters Exhibits Antibacterial Activity against *Streptococcus mutans*

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**Synopsis**
Silver nanoclusters (AgNCs) are ultrasmall in size (< 2 nm) and are expected to be an effective antibacterial substance to combat oral infective diseases. In the present study, we synthesized AgNCs ($Ag_{\sim 75}$) as the main component for esthetic and antibacterial application against the caries pathogen *Streptococcus mutans*. The results showed that AgNCs significantly reduced the turbidity and viability of *S. mutans*. In addition, the bactericidal effects of AgNCs were confirmed by LIVE/DEAD staining. After AgNC application to human dentin, no discoloration of dentin was observed, as compared to silver diamine fluoride application, and AgNC-treated dentin showed an inhibitory effect on colony formation by *S. mutans*. Therefore, AgNCs appear to be beneficial for dental therapy as an antibacterial and/or esthetic substrate.

***Key words:*** *antibacterial effect, human dentin, silver nanoclusters (AgNCs), streptococcus mutans*

## Introduction

Oral bacterial infections cause various oral diseases, such as dental caries and periodontitis, which reduce oral function. In general, antimicrobial drugs are used for infective diseases [1-3]; however, bacterial biofilms diminish the antibacterial effects of these drugs [4]. Hence, novel antibacterial materials need to be developed for dental therapy. Heavy metals, such as silver (Ag), copper and zinc, have been frequently reported to inhibit bacterial cell growth [5-7]. Ag kills bacterial cells through the inhibition of DNA replication after Ag ion infiltration into the bacterial cell body [8]. Therefore, Ag application to oral bacteria would be beneficial for the treatment of oral diseases. Indeed, silver diamine fluoride (SDF) is clinically applied to treat or prevent dental caries [9]. However, SDF reportedly possesses high cytotoxicity and induces discoloration of the tooth surface [10]. Hence, biosafe and esthetic Ag application to teeth is required.

In this study, Ag nanoclusters (AgNCs; $Ag_{\sim 75}$) having a diameter below 2 nm, were created as an antibacterial substance. It was recently reported that the antibacterial activity of AgNCs is highly active when compared to the relatively large size of Ag nanoparticles [11]. In addition, Le Guével et al. reported that AgNCs do not affect mammalian cell viability [12]. Thus, we hypothesized that an antibacterial tooth coating could be created via the application of AgNCs. The aim of this study was to assess whether

AgNCs discolored tooth dentin and whether AgNC-treated dentin exerted antibacterial activity against the dental caries pathogen *Streptococcus mutans*.

## Materials and Methods

### 1. Reagents

All chemicals were used as received without further purification. Glutathione (Reduced Form, GSH, 97.0% purity), silver nitrate ($AgNO_3$, 99.9% purity), formic acid (abt. 99% purity), 1-buthanol (99.0% purity), ethanol (99.5% purity), acrylamide (99% purity), N,N'-methylenebis-(acrylamide) (97.0% purity), 1 mol/L hydrochloric acid, glycerol (99.0% purity), N,N, N',N'- tetramethyl - ethylenediamine (TEMED, 99.0% purity), ammonium peroxodisulfate (APS, 99% purity) and tris hydrochloride acid buffer (1 M Tris-HCl, pH 8.8) were purchased from FUJIFILM Wako Pure Chemical Corporation Ltd. (Osaka, Japan). Glycine was purchased from PEPTIDE Institute (Ibaraki, Japan). Tris(hydroxymethyl) aminomethane (TRIS) was purchased from SERVA Electrophoresis GmbH (Heidelberg, Germany) . Pure water was prepared with a water distillation apparatus (RFD250, ADVANTEC, Tokyo, Japan).

### 2. Synthesis of AgNCs

AgNCs (~$Ag_{75}$) were prepared using the modified method of Chakraborty et al. [13]. GSH (25 mg) and $AgNO_3$ (40 mg) were mixed in an aqueous solution of 20 mL, stirred at 300 rpm for 10 min at 50C. Next, 20 mL of 22.4 mM formic acid-aqueous solution was added to the mixture, and the aqueous solution was stirred at 50C for 3 h to completely reduce the thiolates to clusters. The red-brown solution was subjected to dialysis against pure water through a dialysis membrane (molecular weight cut off = 7,000 Da) for 24 h to remove by-product compounds. The resultant solution was dried by evaporation to obtain a solid powder.

### 3. Characterization of AgNCs

UV-vis (absorption) and fluorescence (excitation and emission) spectra were recorded using a UV-vis-NIR spectrophotometer (V-670, JASCO, Tokyo, Japan) and spectrofluorometer (FP-6300, JASCO), respectively. FT-IR spectra of the dried samples were recorded using the FTIR 4200 spectrometer (JASCO) using an attenuated total reflection technique (ZnSe, 650-4000 $cm^{-1}$). Dynamic light scattering (DLS) measurements were performed with Zetasizer Nano ZSP (Malvern, UK) for the evaluation of the size of AgNCs. Polyacrylamide gel electrophoresis (PAGE) was performed on a five-lane electrophoresis system (Mini-Protean, BIO-RAD, Hercules, CA, USA). The concentrations of acrylamide monomer and cross-linker in the resolving gels were 47 and 3 wt% [acrylamide/bis(acrylamide)], respectively. The eluting buffer contained 1.4 M Tris-HCl (pH 8.8). The as-synthesized AgNCs were dissolved in 5% (v/v) water, after which 0.3 mL of the sample was loaded and subjected to elution at 150 V for 5 h.

### 4. Evaluation of dentin color change after application of AgNCs or SDF

Dentin blocks (4 mm × 6 mm size, with 1 mm thickness) were prepared from extracted vital third molars of patients (20-40 years of age) at Hokkaido University Hospital. The use of human teeth in this study was approved by the Institutional Review Board of Hokkaido University Hospital for Clinical Research (approval no. 12-46). The tooth root was trimmed with a sterilized diamond disc (87xFSI, Horico, Berlin, Germany) and sandpaper (#240 and #600) to prepare dentin blocks. After sonication with 3% ethylenediaminetetraacetic acid (Smeaclean, Nippon Shika Yakuhin Co., Ltd., Shimonoseki, Japan) for 3 min and washing with distilled water, dentin blocks were immersed in phosphate buffered saline (PBS; FUJIFILM Wako Pure Chemical Corporation Ltd.) applied with AgNCs (500 μg/mL) or SDF (Saforide, Bee Brand Medico Dental Co., Ltd., Osaka, Japan) for 5 min and then washed twice with PBS. After 0 and 24 h, digital photographs of the dentin surface were taken and the intensity of the color was measured using software (ImageJ 1.41, National Institutes of Health, Bethesda, MD, USA).

### 5. Preparation of cell suspensions

*S. mutans* strain ATCC 35668 was incubated in brain heart infusion (BHI) broth (Pearlcore®, Eiken Chemical, Co., Ltd., Tokyo, Japan) supplemented with 0.1% antibiotics (gramicidin D and bacitracin, FUJIFILM Wako Pure Chemical Corporation Ltd.) and 1% sucrose (FUJIFILM Wako Pure Chemical Corporation Ltd.). The stock suspension was stored frozen until analysis.

**6. Antibacterial properties of AgNCs**

AgNCs (final concentration: 0 or 500 μg/mL) were dissolved in the suspension of *S. mutans* (final concentration: $5.5\times10^6$ colony-forming units [CFU]/mL). The mixture was dispensed into 48-well microplates and incubated for 24 h under anaerobic incubation at 37°C. The suspension was stained using a LIVE/DEAD BacLight Bacterial Viability Kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Live bacteria were stained with SYTO 9 to produce green fluorescence and bacteria with compromised membranes were stained with propidium iodide to produce red fluorescence. Samples were observed using confocal laser scanning microscopy (FluoView, Olympus Corporation, Tokyo, Japan).

For morphological observations, some cultured samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and then dehydrated in increasing concentrations of ethanol. After Pt-PD coating, the samples were analyzed using scanning electron microscopy (SEM; S-4000, Hitachi Ltd., Tokyo, Japan) at an accelerating voltage of 10 kV.

After *S. mutans* suspensions treated with AgNCs (0, 50, 100, 250 and 500 μg/mL) were incubated for 24 h, the turbidity of each suspension was measured using a turbidimeter (CO7500 Colourwave, Funakoshi Co. Ltd., Tokyo, Japan) at 590 nm. In addition, suspensions containing AgNCs (0, 100 or 500 μg/mL) were cultured for 24 h. Viability of *S. mutans* was assessed using measuring kits with water-soluble tetrazolium salt (WST)-8 (Cell Counting Kit-8, Dojindo Laboratories, Mashiki, Japan), according to the manufacturers' instructions. Absorbance was measured using a microplate reader (ETY-300, Toyo Sokki, Yokohama, Japan) at 450 nm.

**7. Antibacterial assessments of human dentin applied with AgNCs**

Human dentin blocks (4 mm × 6 mm size, with 1 mm thickness) were immersed in BHI broth containing AgNCs (0 or 500 μg/mL) for 5 min and washed twice with PBS. Suspensions of *S. mutans* (final concentration: $5.5\times10^6$ CFU/mL) were dispensed onto dentin placed into 48-well microplates. After anaerobic incubation at 37°C for 24 h, the surface of the dentin blocks was observed by SEM. In addition, dentin blocks cultured with *S. mutans* were washed with fresh BHI broth using a vibrator to collect attached bacterial cells. Collected media were diluted 10-fold in fresh BHI broth, spread onto BHI agar plates (Eiken Chemical Co., Ltd.), and incubated at 37°C for 24 h to determine *S. mutans* colony counts.

**8. Statistical analysis**

Statistical analysis was performed by Student's t-test and Scheffé's test. $p$ values $< 0.05$ were considered statistically significant. All statistical procedures were performed using a software package (SPSS 11.0, IBM Corporation, Armonk, NY, USA).

## Results and Discussion

**1. Characterization of AgNCs**

The UV-vis spectrum of the as-prepared AgNCs showed a peak at 485 nm and a shoulder peak at 420 nm, while the emission intensity at 650 nm was observed upon excitation at 400 nm (Figure 1A). These optical properties are consistent with those of the reported AgNCs ($\sim Ag_{75}$) [13]. We confirmed the small size of AgNCs with about 3 nm with DLS measurement (Figure 1B) and the monodispersed AgNCs with the PAGE separation of one band (Figure 1C). The FT-IR spectrum of AgNCs is similar to that of GSH ligand alone, but the S-H band of GSH around 2520 $cm^{-1}$ disappeared for the AgNCs due to the Au-S bonding of AgNCs (arrow in Figure 1D). This indicates the AgNCs were protected by glutathione.

**2. Dentin color change**

Digital photographs and the color intensity of dentin blocks applied with PBS, AgNCs and SDF are shown in Figure 2A. When the intensity of the 0-h sample was set to 1, the intensity of the 24-h samples for PBS, AgNCs and SDF was 1.00, 0.96 and 2.93, respectively. Hence, dentin treated with SDF was darkened when compared to dentin treated with AgNCs. In general, dental treatment requires a positive esthetic outcome; however, SDF frequently blackens the tooth surface when applied to caries [10]. The chemical reaction of SDF has been proposed to be as follows:

$$Ag(NH_3)2F(aq) \rightarrow Ag(s) + 2NH_3(g) + F^-(aq) \quad (1)$$

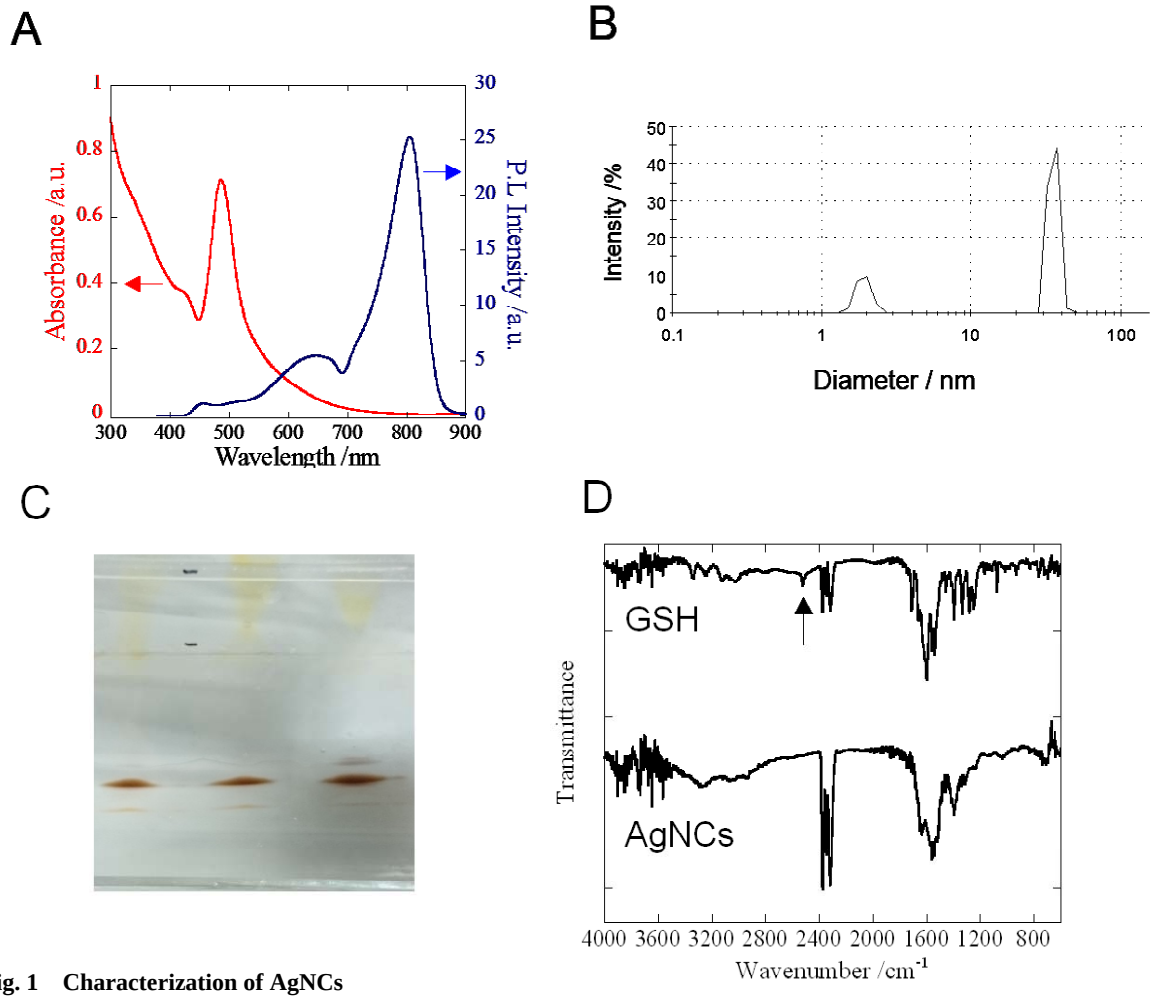


**Fig. 1 Characterization of AgNCs**
(A) UV-vis absorption/emission spectra of AgNCs in water. (B) Dynamic light scattering measurement of AgNCs. (C) Photograph of polyacrylamide gel electrophoresis separation of AgNCs. (D) Fourier transform infrared spectroscopy spectra of glutathione-protected AgNCs and GSH. Upper arrow indicates 2580 $cm^{-1}$.
Abbreviations: AgNCs, silver nanoclusters; au, arbitrary unit; GSH, glutathione; PL, photoluminescence; UV, ultraviolet.

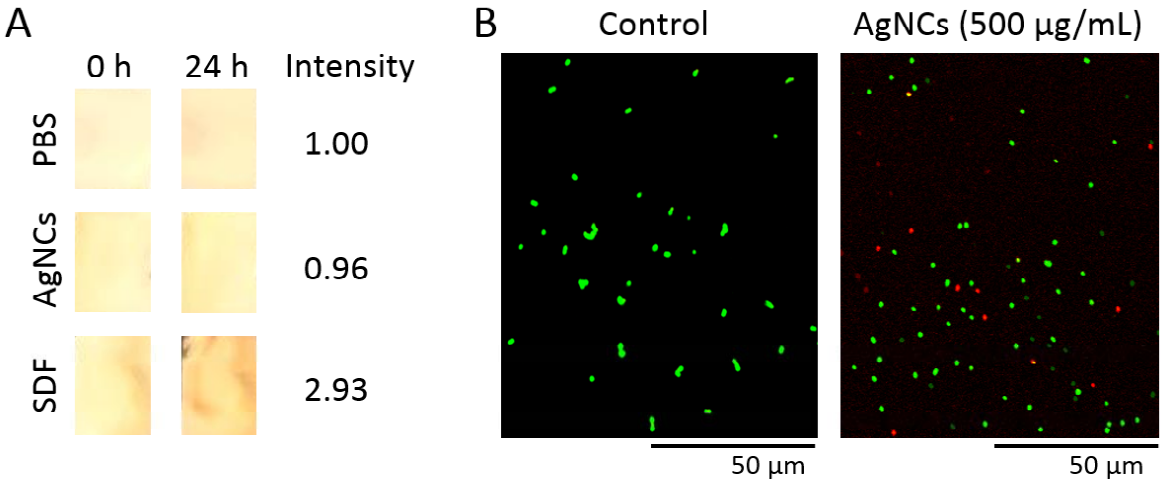


**Fig. 2 Assessment of color intensity of human dentin surface and LIVE/DEAD staining of *S. mutans***
(A) Digital photographs of dentin blocks treated with PBS, AgNCs and SDF after 0 or 24 h. (B) LIVE/DEAD BacLight staining of *S. mutans* after 24 h of incubation in control and AgNCs groups.
Abbreviations: AgNCs, silver nanoclusters; PBS, phosphate buffered saline; SDF, silver diamine fluoride.

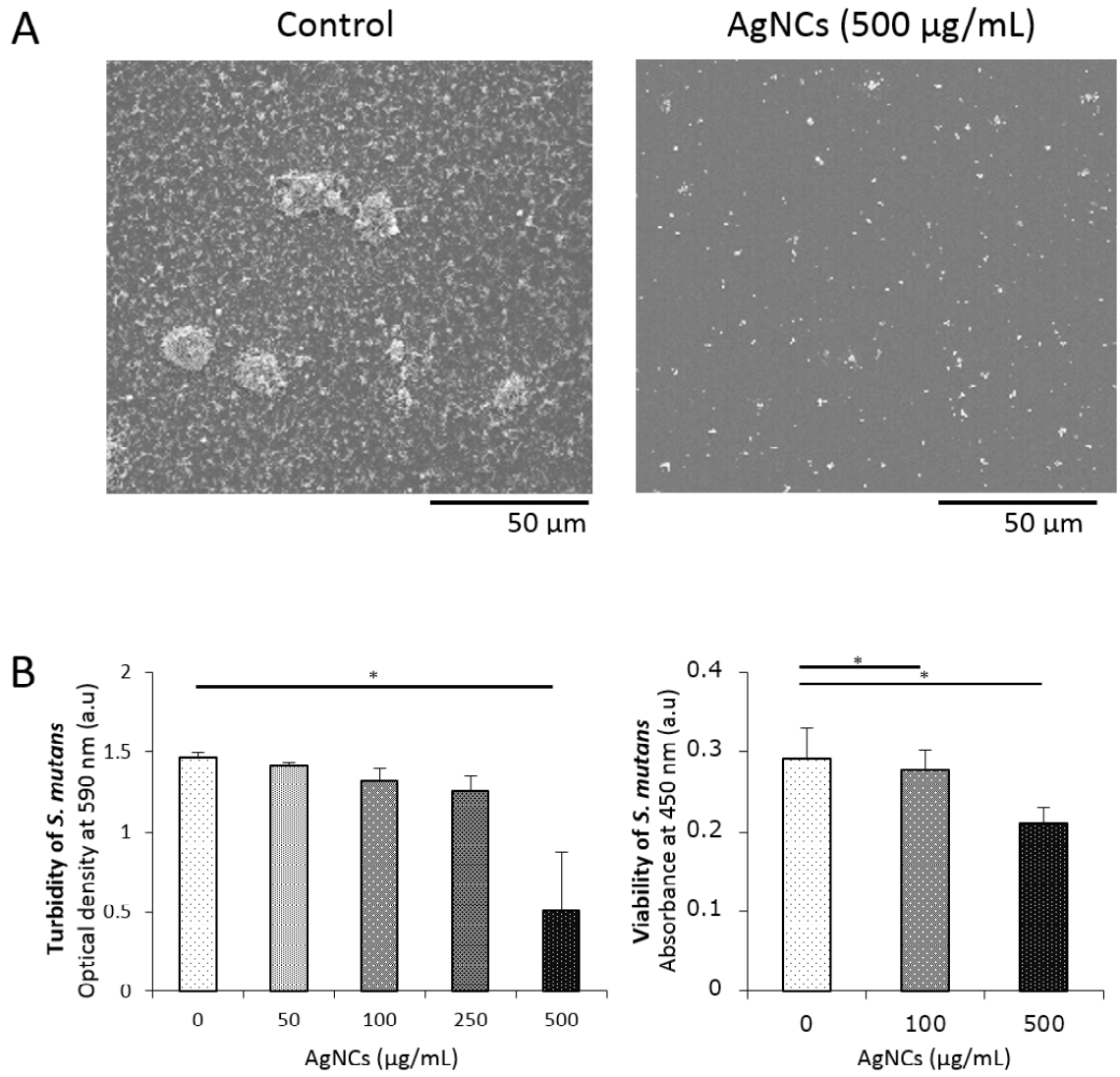


**Fig. 3 Antibacterial characterization of AgNCs**
(A) SEM observation of *S. mutans* after 24 h incubation in control and AgNCs groups. (B) Turbidity (n=5, mean ± standard deviation) and viability (n=4, mean ± standard deviation) of *S. mutans.* *, $P<0.05$.
Abbreviations: AgNCs, silver nanoclusters; SEM, scanning electron microscopy.

Free fluoride ions are available to remineralize dentin and enamel; however, silver precipitate is black [14]. In contrast, the present results show that the dentin surface is not discolored after AgNC application, similarly to the PBS group. AgNCs are stabilized by glutathione protection, and thus the silver precipitate is suppressed [15]. AgNCs may therefore be a beneficial material for esthetic dental treatment and to provide Ag to the tooth surface.

### 3. Antibacterial effects of AgNCs on *S. mutans*

We observed the sterilization effects of AgNCs by LIVE/DEAD staining. AgNC application increased the number of dead cells stained in red (Figure 2B). SEM images of controls (no application of AgNCs) revealed that colonies of *S. mutans* were frequently formed on the surface of the culture dish. In contrast, the AgNCs application group rarely showed any colonies of *S. mutans*, suggesting that AgNCs suppressed growth

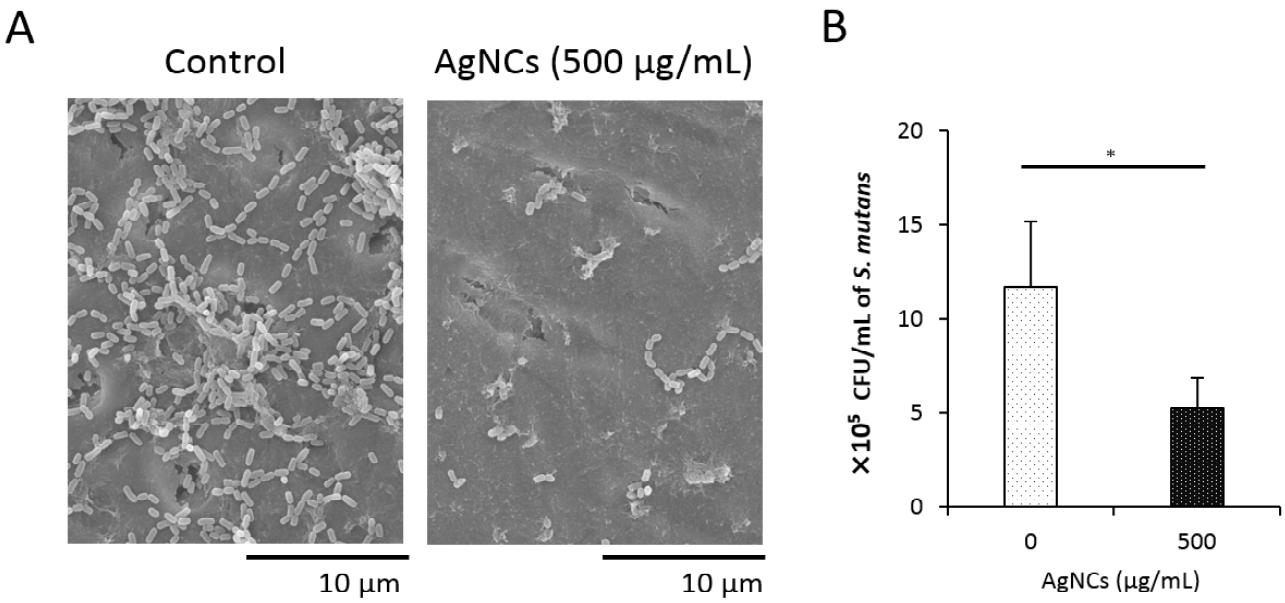


**Fig. 4 Antibacterial assessment of human dentin treated with AgNCs**
(A) SEM observation of dentin seeded with *S. mutans* after 24-h incubation in control and AgNCs groups. (B) CFU of *S. mutans* (n=4, mean ± standard deviation). *, $P<0.05$.

Abbreviations: AgNCs, silver nanoclusters; CFU, colony forming units; SEM, scanning electron microscopy.

of *S. mutans* (Figure 3A). In addition, the turbidity and viability of *S. mutans* were significantly reduced by application of 500 μg/mL AgNCs when compared to controls ($P<0.05$) (Figure 3B). These observations suggest that AgNCs exert bactericidal activity against *S. mutans*. AgNCs may inhibit bacterial growth through Ag ion release or generation of reactive oxygen species (ROS). Ag ions reportedly inhibit DNA replication to destroy bacterial cells [8]. Furthermore, Ag nanoparticles adhere to bacterial cell membranes to promote damage via generation of ROS [8, 16]. Nanoparticles penetrate cell membranes via interaction forces, such as electrostatic, van der Waals, hydrophobic forces and ligand-receptor binding [17]. Thus, AgNCs may invade the cell body to directly release ions and kill bacteria. Further study is necessary to elucidate the antibacterial mechanisms of AgNCs.

## 4. Antibacterial assessment of human dentin

Based on the screening assessments of AgNCs, 500 μg/mL AgNCs was used to assess the antibacterial coating of dentin. We frequently found *S. mutans* growth on normal dentin. In contrast, dentin immersed into the AgNC dispersion reduced *S. mutans* colony formation (Figure 4A). In addition, CFU tests revealed that the number of S. *mutans* on dentin was significantly lower than in controls ($P<0.05$) (Figure 4B). Therefore, AgNCs may remain on the surface of dentin to exert antibacterial effects. Nanoscale particles tend to adhere by attractive forces [18]. Nano-sized materials reportedly showed bioadhesive capability via electrostatic potential [19, 20]. As GSH has some carboxylic groups, it is thought that the electric potential of AgNCs in bodily fluids is negative. Tooth substrates, such as enamel, dentin and cementum, show anionic negative charge [21, 22]; however, pretreatment of dentin blocks was performed using cationic EDTA in this study. Thus, AgNCs may adhere to the dentin surface via electrostatic force. Tooth coating using AgNCs would therefore be clinically useful to obtain antibacterial and esthetically pleasing tooth surfaces via simple application procedures.

## Conclusion

We synthesized glutathione-protected AgNCs and assessed their esthetic and antibacterial effects. AgNCs did not discolor the dentin when compared to SDF and exerted marked antibacterial effects against the caries pathogen, *S mutans*.

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## Competing interests

The authors declare that they have no competing interests to disclose.

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