Antibacterial potential of colloidal platinum nanoparticles against *Streptococcus mutans*

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This study evaluated the antibacterial activity of colloidal platinum nanoparticles (CPNs) toward *Streptococcus mutans* (S. mutans) viability. S. mutans 109c was treated with water and three CPN solutions at 37°C for 24 h (i.e., control, PAA-Pt, C-Pt, C-CyD-Pt). Dilution series (10⁻¹–10⁻⁵) were prepared using brain heart infusion (BHI) broth for all samples, and a 100 µL suspension of each dilution was spread onto a BHI agar plate. Colony-forming units (CFU/mL) were determined after 24 h. The effects of CPNs on S. mutans survival and biofilm formation were investigated using fluorescence and scanning electron microscopies. The antibacterial rate of S. mutans increased with increasing concentrations of all three CPNs, with PAA-Pt nanoparticles exhibiting the highest antibacterial efficacy. CPNs were found to reduce S. mutans growth and inhibit biofilm formation remarkably.

**Keywords:** Nanoparticle, *Streptococcus mutans*, Antibacterial agent, Biofilm, Scanning electron microscopy

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**INTRODUCTION**

Dental caries is a disease of the teeth’s hard tissue demineralization, caused by constant exposure of free sugar to the dental biofilm that alters the ecological balance towards caries ecological disorders¹⁻³. Carious lesions in adults mainly occur on proximal surfaces or are related to existing restorations (secondary caries) in permanent teeth⁴. In clinical situations, remaining bacteria in the cavity pose potential risks of secondary carious lesions⁵. Caries around restorations is usually caused by the accumulation of biofilm (dental plaque) above and at the restoration edge and the release of acids and enzymes produced by the cariogenic bacteria during fermentation⁶⁻⁷. New generations of restorative materials with antibacterial are being developed to prevent the development of secondary caries. For instance, in the last decades, there has been an increased interest in employing metallic nanoparticles as an antibacterial agent in restorative materials⁸⁻¹⁰.

Several metallic compounds have been used for centuries as antibacterial agents¹¹. These metallic agents demonstrate efficacies by damaging bacterial cell walls, producing reactive oxygen species, or inactivating intracellular proteins¹². The potential of such inorganic compounds depends on the extent of their contact surfaces, and therefore, a compound with a larger surface area exhibits superior performance¹³. Metallic nanoparticles have demonstrated substantial antimicrobial sensitivity because these nanoparticles inherently have larger surface areas, allowing a broader gamut of interactions with other organic and inorganic molecules¹⁴. Chwalibog et al. reported that 50 ppm hydrocolloid of platinum nanoparticles (PtNPs), produced from high purity platinum, manifested excellent antibacterial activity by successfully disintegrating the cell walls of *Staphylococcus aureus* and *Candida albicans*¹⁵. Tahir et al. showed that PtNPs exhibit potent antibacterial activity against antibiotic-resistant *Pseudomonas aeruginosa* and *Bacillus subtilis*¹⁶. Hashimoto et al. found that PtNPs adhere to the surfaces of *Streptococcus mutans* (S. mutans) and inhibit biofilm formation¹⁷. Platinum has been certified safe as a food additive, and several commercial products supplemented with platinum nanoparticles as antioxidants, including mineral water, yogurt, gum, candies, mouthwash, and skin lotion, are marketed in developed countries¹⁸.

Colloidal platinum nanoparticles (CPNs), which consist of metallic nanomaterials synthesized from the colloidal suspension of platinum particles, are effective in reducing inflammation¹⁹,²⁰ and preventing apoptosis¹⁹. CPNs have also been found to delay the aging process because CPNs can act as antioxidants by scavenging reactive oxygen species²¹. The successful outcome of CPNs in potentially treating diseases related to oxidative stress and aging has been studied²²⁻²⁴. In the field of dental biomaterials, CPNs were found to enhance the bonding performance of 4-META/MMA-TBB-based resin cement to sound dentin²²⁻²⁴. However, caries-affected dentin particularly proved to be a challenge to the bonding outcome of a restorative material as the complete removal of cariogenic bacteria can hardly be confirmed by a clinician²⁵.

Surprisingly, no literature has tested the efficacy of CPNs against one of the causative cariogenic microorganisms, *S. mutans*. Therefore, the objective of
this study is to assess the antibacterial effect of three types of CPNs on S. mutans. The null hypothesis was that the three types of CPNs would not affect the growth and biofilm formation of S. mutans.

**MATERIALS AND METHODS**

*Bacterial strain and culture conditions*

*Streptococcus* 109c (S. mutans 109c, wildtype, serotype c) was cultured overnight at 37°C in brain heart infusion (BHI) broth (Becton, Dickinson and Company, Sparks, MD, USA) under anaerobic conditions (80% N₂, 10% H₂, 10% CO₂). The resulting bacterial suspension was adjusted to an optical density at 600 nm (OD₆₀₀) of 1.0 using a spectrophotometer (Bio-Rad Smartspec™ plus, Hercules, CA, USA) for further use.

**Determination of antibacterial activity**

Three different formulations of the CPNs (APt company, Tokyo, Japan) were used in this study (Table 1). The pH values of these three CPNs were measured by pH meter (392R, AS ONE, Osaka, Japan). The CPNs were diluted with BHI broth to various concentrations (0.4–0.9 mM). The concentration of platinum in the solution ranged from 80 to 180 ppm (manufacturer’s information). Sterile water and suspended bacteria were used as the control group.

The antibacterial activity of six different concentrations (0.4, 0.5, 0.6, 0.7, 0.8, and 0.9 mM) of each of the CPNs on S. mutans was initially tested. For the assessment, 20 µL S. mutans suspension was mixed with various concentrations of innovative solutions (980 µL) and incubated at 37°C for 24 h. Following CPNs treatment of each group, dilution series (10⁻¹–10⁻⁵) were prepared using a sterilized saline solution, and 100 µL of each dilution was spread onto BHI agar plates and the plates incubated at 37°C for 48 h. The number of bacterial colonies on each BHI agar plate was obtained using a bacterial colony counter (Count™, Heathrow Scientific, Vernon Hills, IL, USA). The bacterial concentration in samples after co-culturing (N, CFU/mL) was calculated by using Eq. (1), whereas the antibacterial rate was determined by using Eq. (2):

\[
N = (C \times d \times 1000)/l \tag{1}
\]

\[
\text{Antibacterial rate} = (N_{\text{control}} - N_{\text{material}})/N_{\text{control}} \times 100\% \tag{2}
\]

In Eq. (1), \(C\) represents the average colony count on the plates, \(d\) is the dilution factor with \(d=50\) in this study, and \(l\) is the volume of the bacterial suspension used in the samples with a value of \(l=100\) in this study. In Eq. (2), \(N_{\text{control}}\) and \(N_{\text{material}}\) are the average colony counts for the control (water) and CPNs groups, respectively. According to the National Standard of China (GBT 4789.2-2010), an antibacterial rate≥99% means that the sample has substantial antibacterial activity, and an antibacterial rate≥90% indicates that the sample has antibacterial activity. Experiments were performed in triplicate.

**Growth inhibition study**

Growth inhibition analysis was performed to determine the inhibition kinetics of CPNs against S. mutans. The three CPNs were added to bacterial suspensions to a final concentration of 0.7 mM (the value was determined previously, as described before). The suspensions were incubated at 37°C under anaerobic conditions. After 1, 2, 3, 4, 5, and 6 h incubation, 100 µL of each suspension was serially ten-fold diluted and spread onto BHI agar plates. The number of viable bacterial colonies was counted after 48 h. The experiment was performed in triplicate.

**Live/dead staining for visualization of S. mutans viability**

S. mutans was incubated at 37°C for 24 h. Bacteria in the logarithmic growth phase (concentration OD₆₀₀=1.0) were treated with 0.7 mM CPNs for 24 h. Sterile water was used as the control group. Bacterial suspensions were stained using the Live/Dead BacLight Bacterial Viability kit L7012 (Molecular Probes, Eugene, OR, USA) for 15 min in the dark at room temperature. The kit consists of Syto 9, which stains all cells green, and

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**Table 1** The composition of the three types of CPNs

| Product   | Lot No.   | Composition                                      | pH value |
|-----------|-----------|--------------------------------------------------|----------|
| PAA-Pt    | 111170908 | Pt (platinum): 0.02%, 1 mM                       | 6.40     |
|           |           | PAA (polyacrylic acid): 1.16%                    |          |
|           |           | Water: 98.82%                                    |          |
| C-Pt      | 331180316 | Pt (platinum): 0.02%, 1 mM                       | 6.12     |
|           |           | Sodium citric acid: 0.29%                        |          |
|           |           | Water: 99.69%                                    |          |
| C-CyD-Pt  | 251180316 | Pt (platinum): 0.02%, 1 mM                       | 6.10     |
|           |           | Sodium citric acid: 0.29%, Cyclodextrin: 0.3%    |          |
|           |           | Water: 99.39%                                    |          |

Three types of CPNs are provided by Apt company (Tokyo, Japan).
propidium iodide, which stains dead cells red because these cells have a damaged cell membrane. Samples were observed using a fluorescence microscope (BZ-X800, Keyence, Osaka, Japan). The experiment was performed in triplicate. Images were analyzed using Image-Pro Plus 5.0.

**Effects of CPNs on S. mutans morphology**

*S. mutans* were incubated in the presence of 0.7 mM CPNs for 24 h. The samples were harvested by centrifugation, washed twice with sterilized phosphate-buffered saline (PBS), and fixed in 2.5% glutaraldehyde overnight. The attached bacteria were adhered to glass coverslips, dehydrated serially in ethanol (50% for 10 min, 80% for 10 min, and 100% for 20 min), and air-dried overnight. Samples were then sputter-coated with gold-palladium and examined with a scanning electron microscope (SEM; S-4800, Hitachi, Tokyo, Japan).

**Biofilm analysis**

CPNs were diluted with BHI broth containing 1% sucrose to various concentrations (0–0.9 mM). *S. mutans* strains (OD_{600}=1.0) were then cultured in sterile 96-well microtiter plates in BHI broth (containing CPNs and 1% sucrose) at 37°C for 24 h. Sterile water was used as the control group. After incubation, the planktonic bacteria were removed, and the wells were washed three times with PBS. The biofilm was fixed with 100 µL 99.99% methanol per well, and after 15 min, microplates were aspirated and air-dried. Crystal violet (CV) staining was used to measure bacterial biofilm formation\(^{29}\). One hundred microliters CV was added to stain the biofilm and incubated at 37°C for 5 min. The excess stain was removed by placing the microplate under running tap water. After air-drying the microplates, dye bound to the adherent cells was resolubilized with 100 µL 33% (v/v) glacial acetic acid per well. The optical density of each well was measured at 570 nm using a microplate reader (Model 3550, Bio-Rad). Biofilm formation by bacteria after co-culturing with CPNs was calculated by Eq. (3):

$$ P = \frac{O_{\text{material}}}{O_{\text{control}}} \times 100\% \quad (3) $$

In Eq. (3), \(O_{\text{control}}\) and \(O_{\text{material}}\) are the optical densities for the control (water) and CPNs groups, respectively.

**Live and dead staining (S. mutans biofilm viability test)**

For the biofilm viability test, *S. mutans* were grown in sterile 24-well microtiter plates with glass cover slides (Matsunami, Chiba, Japan) for 24 h with BHI broth containing 1% sucrose. Sterile water was used as the control group. After exposure to 0.5 mM CPNs for 24 h, biofilms were stained with Live/Dead BacLight Bacterial Viability kit L7012 (Molecular Probes). Sample fluorescence was observed using the fluorescence microscope (BZ-X800, Keyence) at 488 and 543 nm excitation wavelengths. The experiment was performed in triplicate.

**Effect of CPNs on S. mutans biofilm morphology**

SEM was used to observe biofilms formed overnight in 24-well plates with glass cover slides at the bottom of each well. Before SEM imaging, the biofilm was treated with 0.5 mM CPNs for 24 h at 37°C. The CPNs-containing medium was removed, and the wells were gently washed twice with sterilized distilled water and fixed in 2.5% glutaraldehyde overnight. The fixed samples adhered to glass coverslips were dehydrated serially in ethanol (50% for 10 min, 80% for 10 min, and 100% for 20 min) and finally air-dried in a desiccator overnight. The samples were sputter-coated with gold-palladium and examined by SEM (S-4800, Hitachi).

**Statistical analysis**

Statistical analyses were carried out with SPSS 19.0 (SPSS, Chicago, IL, USA). The normality of the data was checked with the Shapiro-Wilk test and the homogeneity with Levene’s test. Since the data were normal and homogeneous, the one-way ANOVA with Dunnett’s multiple comparison test was used to determine the statistical significance\((p<0.05)\) between different groups.

**RESULTS**

**Determination of antibacterial activity**

Figure 1 shows the changes in the antibacterial rate of CPNs on *S. mutans*. The antibacterial rate of 0.7 mM PAA-Pt was about 91.5% (>90%) while the antibacterial rate of 0.7 mM C-Pt and 0.7 mM C-CyD-Pt were only about 69.0% (<90%). This result suggests that 0.7 mM PAA-Pt is antibacterial material, whereas 0.7 mM C-Pt and 0.7 mM C-CyD-Pt are not according to the National Standard of China GB4789.2.

**Growth inhibition study**

Figure 2 shows the growth-inhibitory curves of the three types of CPNs at 0.7 mM against *S. mutans*. The three types of CPNs showed potent antibacterial activity up to 6 h. The PAA-Pt group demonstrated the most potent antibacterial activity with no significant increase in the concentration of each type of CPNs.
Fig. 2 Growth inhibition assay of bacteria. The number of bacteria at different treatment times (0–6 h) for the four groups were examined. The concentration of each CPNs group was 0.7 mM.

Fig. 3 Representative live/dead staining images of S. mutans treated with CPNs (concentration of CPNs: 0.7 mM; treatment period: 24 h). Live bacteria are stained green, whereas dead bacteria are stained red. Magnification: ×200.

Fig. 4 Representative SEM images of S. mutans treated with CPNs (concentration: 0.7 mM; treatment period: 24 h).

number of bacteria observed up to 6 h.

Live/dead staining for visualization of S. mutans viability
Typical live/dead images of S. mutans exposed to the three CPNs at 0.7 mM for 24 h are shown in Fig. 3. The number of bacteria detected in the control group (i.e., water-only sample) was the largest. In contrast, the groups containing CPNs showed fewer live bacteria than the control group. In particular, the PAA-Pt group showed the lowest number of live bacteria among these groups.

Morphology changes to S. mutans
As shown in Fig. 4, S. mutans cells in the control group exhibited smooth surfaces, whereas cells exposed to 0.7

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mM CPNs for 24 h had a rough appearance. Additionally, CPNs-exposed S. mutans displayed an elongated morphology when compared with bacteria from the control group. Moreover, SEM images of S. mutans exposed to the three types of CPNs showed two or more ring-like structures along their circumferences.

Concentration-dependent inhibition of S. mutans biofilm formation by CPNs
Formation of S. mutans biofilm was reduced by PAA-Pt group at concentrations >0.5 mM, whereas C-Pt and C-CyD-Pt groups showed a biofilm inhibitory effect at concentrations higher than 0.8 mM (Fig. 5).

Live/dead staining for visualization of S. mutans biofilm formation
Figure 6 shows the live/dead staining results at 0.5 mM CPNs on S. mutans biofilm formation. PAA-Pt group showed the most substantial inhibitory effect on biofilm formation, whereas C-Pt and C-CyD-Pt groups generated a weaker inhibitory effect. Exposure of S. mutans biofilms to PAA-Pt, C-Pt, and C-CyD-Pt groups resulted in more dead bacteria when compared with that of the water (control) sample.

![Live/dead staining images of S. mutans biofilm formation treated with CPNs](image)

Fig. 6 Representative live/dead staining images of S. mutans biofilm formation treated with CPNs (concentration: 0.5 mM, treatment period: 24 h). Live bacteria are stained green and dead bacteria are stained red. Magnification: ×200.

![SEM images of S. mutans biofilm formation treated with CPNs](image)

Fig. 7 Representative SEM images of S. mutans biofilm formation treated with CPNs (concentration: 0.5 mM; treatment period: 24 h).

Fig. 5 Inhibition of bacterial biofilm formation by CPNs. * indicates statistically significant difference.
**Morphology changes to S. mutans biofilm**

As shown in Figs. 7A–D, reduction in biofilm formation by S. mutans mainly occurred when exposed to 0.5 mM PAA-Pt group. The anti-biofilm activity of C-Pt and C-CyD-Pt was greater than that of the PAA-Pt group but more robust than the water sample (control). As shown in Figs. 7F–H, bacteria treated with PAA-Pt, C-Pt and C-CyD-Pt demonstrated granular features on their surfaces. S. mutans exposed to CPNs also showed an elongated morphology compared with bacteria from the control sample (Fig. 4). These observations corroborate the observed morphological changes to S. mutans observed in Fig. 4.

**DISCUSSION**

This study showed that three different CPNs inhibited the growth of S. mutans. Therefore, the tested null hypothesis had to be rejected. According to our knowledge, the findings of this study are the first to demonstrate the inhibitory effect of CPNs on S. mutans biofilm formation. PAA-Pt exhibited the most significant inhibitory effect ($p<0.05$) on the biofilm formation of S. mutans among the tested groups. In the present study, 0.7 mM PAA-Pt showed the highest antibacterial activity among these three 0.7 mM CPNs, with an antibacterial rate of 91.5% (Fig. 1). This result was supported by the live/dead cell staining images presented in Fig. 3, where the PAA-Pt concentration of 0.7 mM showed the lowest fluorescence of live and dead cells.

The three types of CPNs have the same concentration of platinum (1 mM); however, the other components present in the nanoparticles differed: PAA-Pt contains 1.16% polyacrylic acid, C-Pt contains 0.29% sodium citric acid, and C-CyD-Pt contains 0.29% sodium citric acid and 0.3% cyclodextrin. Studies have shown that sodium citric acid is an antibacterial agent. Yamaguchi et al. report that a citric acid solution showed antibacterial activity among these three 0.7 mM CPNs, with an antibacterial rate of 91.5% (Fig. 1). This result was supported by the live/dead cell staining images presented in Fig. 3, where the PAA-Pt concentration of 0.7 mM showed the lowest fluorescence of live and dead cells.

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**CONCLUSION**

All three types of CPNs prepared were found to inhibit S. mutans growth and biofilm formation. These results indicate that CPNs represent a promising antibacterial agent to use in conjunction with adhesive materials for dental caries prevention around restorations.

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