Assessment of bactericidal effects of quaternary ammonium-based antibacterial monomers in combination with colloidal platinum nanoparticles

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Pretreatment of dentin using colloidal platinum nanoparticles (CPtN) can enhance the bond strength of dentin adhesives. However, the combination of CPtN, which is negatively charged, with cationic monomer-containing adhesive may reduce the antibacterial activity of the original material. Thus, the purpose of this study was to assess the effect of CPtN on the bactericidal activity of two cationic antibacterial monomers, 12-methacryloyloxydodecylpyridinium bromide (MDPB) and methacryloxylethyl cetyl dimethyl ammonium chloride (DMAE-CB). The rapid killing effects of the two monomers against planktonic or attached Streptococcus mutans were achieved by the two cationic monomers. Combination with 0.1 mM CPtN did not reduce the bactericidal effects of the two monomers, indicating that CPtN may be used as a pretreatment with antibacterial adhesives.

Keywords: Antibacterial monomer, Quaternary ammonium, Platinum nanoparticles, Bactericidal effects

INTRODUCTION

The fields of medicine and dentistry use nanotechnology in an increasing number of applications. Taking advantage of the unique properties of nanosized particles, various types of nanomaterials have been designed to aid in the transport of diagnostic or therapeutic agents through biologic barriers, target specific molecules, mediate molecular interactions, and detect molecular changes in a sensitive, high-throughput manner. Colloidal platinum nanoparticles (CPtN) were recently developed and are currently being evaluated for the ability to reduce inflammation, prevent apoptosis, and delay the aging process. As for dental applications, it has been reported that usage of CPtN in combination with a 4-methacryloyloxyethyl trimellitic anhydride (4-META)/methyl methacrylate (MMA) adhesive increases the dentin bond strength to twice that of the parent material, probably due to enhanced polymerization. Since CPtN has been demonstrated to be a potent antioxidant in vitro, the addition of CPtN may also improve the biocompatibility of resin-based materials.

Several studies have reported the use of quaternary ammonium-based antibacterial monomers in dental resins. In particular, 12-methacryloyloxydodecylpyridinium bromide (MDPB) has been incorporated into dental adhesives, resulting in the successful commercialization of the world’s first antibacterial dental adhesive system. The combined use of CPtN with antibacterial adhesives containing quaternary ammonium-based monomers may enhance their clinical usefulness by improving bonding properties and biocompatibility, thereby contributing to durable restoration. However, for the colloidal system of platinum nanoparticles currently under investigation for dental applications, sodium citrate is used as a stabilizer because it can adsorb to the surface of the nano-sized particles, providing negative charges that prevent aggregation. Like conventional quaternary ammonium compounds, cationic antibacterial monomers based on quaternary ammonium interact electrostatically with negatively charged bacterial membranes to damage them, resulting in the leakage of intracellular components. Thus, sodium citrate-protected CPtN may electrostatically neutralize cationic antibacterial monomers, reducing their antibacterial activity. In this study, we tested the hypothesis that CPtN reduces the bactericidal activity of cationic antibacterial monomers by evaluating the rapid killing effects of two antibacterial monomers against planktonic or attached Streptococcus mutans in the presence or absence of platinum nanoparticles.

MATERIALS AND METHODS

Monomers and colloidal platinum nanoparticles

The quaternary ammonium-based antibacterial monomers MDPB and methacryloxylethyl cetyl dimethyl ammonium chloride (DMAE-CB) were used. These monomers were synthesized as previously described and dissolves in sterile distilled water. The CPtN solution obtained from the manufacturer contained 0.02% platinum, 0.29% sodium citric acid, and 99.69% water. The average diameter of the particles was approximately 2 nm, and the particles did not exhibit a second phase of...
Bacterial species

*S. mutans* UA159 was cultured in brain heart infusion (BHI) broth (Becton Dickinson, Sparks, MD, USA) supplemented with 0.5% yeast extract (Becton Dickinson, Sparks, MD, USA).

MIC and MBC measurement

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of MDPB, DMAE-CB, and CPtN against *S. mutans* UA159 were determined by microdilution assay. Serial two-fold dilutions of MDPB, DMAE-CB, and CPtN were prepared, and 50-µL aliquots were used for analysis. The concentrations tested for MDPB and DMAE-CB ranged from 0.12 to 250 µg/mL, and the concentration of CPtN ranged from 0.001 to 2.5 mM. A bacterial suspension was prepared from the stock culture, incubated for 12 h, and adjusted to about $2 \times 10^6$ colony-forming units (CFU)/mL; 50 µL of this suspension was added to each well of the 96-well culture plate containing an antibacterial monomer or CPtN solution. The plate was then incubated anaerobically at 37°C for 24 h. The MIC value was determined as the lowest concentration of the tested agent that resulted in no detectable turbidity upon visual examination. Aliquots of bacterial suspension were obtained from wells that showed no visible growth and inoculated on BHI agar plates. After anaerobic subculture for 24 h, the MBC value was determined as the lowest concentration of the tested agent that resulted in no colony formation on the plates.

Influence of CPtN on the rapid killing effects against planktonic bacteria

*S. mutans* prepared from the stock culture was adjusted to approximately $1 \times 10^6$ CFU/mL in 0.01 M phosphate buffered saline (PBS; pH 7.4). To 80 µL bacterial suspension, 10 µL of 1 mM CPtN solution (for the CPtN-monomer combination group) or distilled water (for the monomer-only group) was added. This mixture was gently mixed for 60 s, and then 10 µL antibacterial monomer solution was added (final concentration, 250 µg/mL MDPB or 10 µg/mL DMAE-CB). As a negative control, 10 µL distilled water was used. After 20, 40, or 60 s of gentle agitation, the bacterial suspension was diluted 100 times by adding 9.9 mL PBS to inactivate the antibacterial monomers. Aliquots of the diluted suspension (100 µL) were inoculated onto BHI agar plates, and the viable bacteria (CFUs) were counted after a 48-h anaerobic incubation at 37°C.

Influence of CPtN on the rapid killing effects against attached bacteria

Human saliva was collected from a healthy volunteer (S.M) and sterilized. Then, wells of a tissue culture chamber (Lab-Tek™ Chambered Coverglass, Thermo Fisher Scientific Inc. Waltham, MA, USA) were coated with 200 µL sterilized saliva for 1 h. After the saliva was removed, a 200-µL aliquot of *S. mutans* suspension (adjusted to about $1 \times 10^7$ CFU/mL with PBS) was added to each well. The chambers were anaerobically incubated for 24 h to allow the bacteria to attach to the bottom of the wells. To confirm attachment, live/dead staining (LIVE/DEAD BacLight Bacterial Viability kit L7012; Molecular Probes Inc., Eugene, OR, USA) was performed, and the chambers were observed by confocal laser scanning microscopy (CLSM, LSM510, Carl Zeiss, Oberkochen, Germany, FITC/Cy3, LP 543 nm, BP 505–530 nm). For CLSM analysis, the bacteria in each well were stained with 200 µL of the viability staining solution for 15 min and rinsed with distilled water. CPtN solution (0.1 mM) was added to each well containing attached bacteria. After 60 s, the CPtN solution was removed with a pipette, and the cells were treated with MDPB (250 or 1,000 µg/mL) or DMAE-CB (10 or 100 µg/mL) for another 60 s. The bacteria were then carefully detached from the bottom of the wells by pipetting, and an evenly distributed bacterial suspension was prepared. A 100-µL aliquot of this suspension was inoculated onto BHI agar plates and incubated anaerobically for 48 h at 37°C to enumerate CFUs.
Statistical analysis
Results were compared by one-way analysis of variance followed by the post hoc Student-Newman-Keuls test using SPSS Statistics 13.0 (SPSS Inc. Chicago, IL, USA). A $p$-value lower than 0.05 was considered significant.

RESULTS

MIC and MBC measurement
The MIC/MBC values for MDPB, DMAE-CB, and CPtN against \textit{S. mutans} UA159 are shown in Table 1. The MIC and MBC values of DMAE-CB were lower than those of MDPB, indicating a stronger antibacterial activity. CPtN alone did not inhibit the growth of \textit{S. mutans}, even at 2.5 mM, the highest concentration tested.

Influence of CPtN on the rapid killing effects of antibiotic monomers against planktonic bacteria
The number of viable cells after treatment with CPtN and the two antibacterial monomers is shown in Figs. 2 and 3. The reduction in cell numbers, calculated as a percentage of the control, is presented in Table 2. Both antibacterial monomers significantly reduced the number of viable cells ($p<0.05$). MDPB (250 µg/mL) exhibited a time-dependent killing effect against planktonic \textit{S. mutans}, and all bacteria were killed after 60 s (Fig. 2). The monomer DMAE-CB (10 µg/mL) killed 100% of the cells after 40 s (Fig. 3). CPtN alone did not show any killing effects against planktonic bacteria. Furthermore, the number of viable bacteria after contact with the cationic monomer did not differ in the presence or absence of CPtN ($p>0.05$), indicating that adding CPtN did not reduce the antibacterial effects of either

<table>
<thead>
<tr>
<th>MIC</th>
<th>MBC</th>
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<tr>
<td>MDPB</td>
<td>7.81 µg/mL</td>
</tr>
<tr>
<td>DMAE-CB</td>
<td>3.91 µg/mL</td>
</tr>
<tr>
<td>CPtN</td>
<td>&gt;2.5 mM</td>
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Table 1 Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) against \textit{Streptococcus mutans} UA159 for 12-methacryloyloxydodecylpyridinium bromide (MDPB), methacryloxylethyl cetyl dimethyl ammonium chloride (DMAE-CB), and colloidal platinum nanoparticles (CPtN)

Table 2 Reduction in planktonic \textit{Streptococcus mutans} after contact with 12-methacryloyloxydodecylpyridinium bromide (MDPB) or methacryloxylethyl cetyl dimethyl ammonium chloride (DMAE-CB) in the presence or absence of colloidal platinum nanoparticles (CPtN)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Reduction in the number viable bacteria (%)</th>
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<tbody>
<tr>
<td></td>
<td>20 s</td>
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<tr>
<td>250 µg/mL MDPB</td>
<td>32.80 (13.12)</td>
</tr>
<tr>
<td>250 µg/mL MDPB+CPtN</td>
<td>15.03 (5.42)</td>
</tr>
<tr>
<td>10 µg/mL DMAE-CB</td>
<td>99.45 (0.59)</td>
</tr>
<tr>
<td>10 µg/mL DMAE-CB+CPtN</td>
<td>99.42 (0.52)</td>
</tr>
</tbody>
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Results are expressed as mean (standard deviation); $n=3$. 

Fig. 2 Effect of colloidal platinum nanoparticles (CPtN) on the viability of \textit{Streptococcus mutans} after contact with 12-methacryloyloxydodecylpyridinium bromide (MDPB). The presence of 0.1 mM CPtN did not significantly reduce the rapid killing effect of MDPB against planktonic \textit{S. mutans}. *No viable cells.
Influence of CPtN on the rapid killing effects of antibiotic monomers against attached bacteria

The CLSM image of attached bacterial cells on the bottom of the chamber is shown in Fig. 4. Bacterial cells were sparsely attached to the bottom, and almost all the cells were viable, as shown by green staining.

Figures 5 and 6 show the number of viable cells after contact with each monomer with/without CPtN pretreatment. Both monomers significantly reduced cell viability ($p<0.05$). Treating attached bacteria with CPtN before adding antibacterial monomers did not significantly alter their bactericidal effects ($p>0.05$). MDPB (250 µg/mL) reduced the number of viable cells by 99.20% (without CPtN) or 99.23% (with CPtN) (Fig. 5). When the concentration of MDPB was increased to 1,000 µg/mL, more than 99.80% of the bacteria were killed, regardless of whether they were pretreated with CPtN. DMAE-CB (10 µg/mL) reduced the number of viable cells by 79.48% (without CPtN) or 80.63% (with CPtN). Increasing the concentration of DMAE-CB to 100 µg/mL killed 99.90% of the adherent bacteria regardless of CPtN pretreatment (Fig. 6).
DISCUSSION

Platinum nanoparticles tend to aggregate because of their large specific surface area. According to the Derjaguin-Landau-Verwey-Overbeek theory\(^24\), nanoparticle aggregation can be prevented by providing the particles with charges to generate an electrostatic repulsion force\(^25,26\). In the CPtN system, negatively charged citrate ions are used for this purpose. Since quaternary ammonium monomers depend mainly on the positively charged head group for their bactericidal effects, it is logical that electrostatic interactions with negatively charged platinum nanoparticles may reduce the antibacterial activity of those cationic monomers. However, our results did not support this hypothesis. We found no reduction in the bactericidal effects of MDPB or DMAE-CB against S. mutans (planktonic or attached) by pretreating with CPtN.

Before investigating the possible interaction between CPtN and cationic monomers, we evaluated the potential antibacterial activity of CPtN itself. A previous study reported that the bond strength of a 4-META/MMA-based adhesive was greatly improved by using 0.1 mM CPtN as a primer solution\(^9\). Therefore, we first examined CPtN at 0.1 mM, and found no antibacterial activity (data not shown). The further MIC/MBC measurement indicated that CPtN does not inhibit the viability or growth of S. mutans even at 2.5 mM, the highest concentration not to cause aggregation. Chwalibog et al. reported that hydrocolloid of platinum nanoparticles produced from high purity Pt and demineralized water disintegrated the cytoplasmic membrane and cell wall of Staphylococcus aureus and Candida albicans at the concentration of 50 ppm\(^27\). Different results obtained in the present study may be due to the composition of CPtN used, which is stabilized with sodium citrate.

Since quaternary ammonium compounds have a positive charge, they interact with the negatively charged bacterial cell surface, rapidly inducing the cell wall to burst. Izutani et al. investigated the killing effects of MDPB against S. mutans NCTC10449 after short period contact, demonstrating that the complete killing of planktonic cells was obtained in 60 s with 1,000 µg/mL MDPB\(^28\). Similarly, DMAE-CB achieved 99.44% killing of S. mutans ATCC25175 after a 60-s contact, at a concentration four times that of MBC (19.2 µg/mL)\(^23\). To examine effects on planktonic S. mutans, the concentration of 250 µg/mL was chosen for MDPB based on the study by Izutani et al. For DMAE-CB, we used a concentration of 10 µg/mL, which is slightly higher than MBC value. When combined with 0.1 mM CPtN, complete killing of the bacteria was achieved with 250 µg/mL MDPB after 60 s, and with 10 µg/mL DMAE-CB after 40 s, demonstrating that CPtN does not significantly reduce the antibacterial activity of the two monomers at the concentration used. Bacterial pretreatment conditions (0.1 mM CPtN for 60 s) before exposure to antibacterial monomers was based on previous studies reporting that high bond strength was achieved by priming the dentin with 0.1 mM CPtN for 60 s\(^8\). When using 250 µg/mL MDPB (0.61 mM), it is possible that a sufficient number of free molecules remain to kill or inactivate bacteria even after some monomers were...
electrostatically neutralized by the negatively charged platinum nanoparticles. However, with 10 µg/mL DMAE-CB (0.02 mM), electrostatic interaction with bacteria would be expected to be greatly reduced by the nanoparticles, as its molar concentration was lower than that of CptN (0.1 mM). There are two possible reasons for our finding that adding CptN did not reduce the efficacy of the antibacterial monomers. First, the binding of cationic monomers with the bacterial cell surface may occur more quickly than with CptN. The bacterial cell is much larger than CptN; therefore, the antibacterial monomers may come into contact with bacteria more frequently. Second, antibacterial monomers may be able to inactivate bacteria even after its positively charged portion is bound to CptN. The alkyl group plays an important role in the antibacterial activity of alkyl pyridinium compounds. The alkyl group length is related to the molecule’s hydrophobicity, which plays a role in adsorption to the cell surface. Therefore, the cationic monomers combined with CptN may still be able to disrupt the cell surface.

Similar to its effect on planktonic cells, CptN did not reduce the rapid killing effects of the cationic monomers on attached S. mutans. Although 250 µg/mL MDPB and 10 µg/mL DMAE-CB completely killed planktonic cells after 60 s, higher concentrations of the cationic monomers were required to inactivate all viable cells attached to the bottom of the chamber, because biofilm bacteria are more resistant to bactericides. However, at all monomer concentrations tested, CptN pretreatment did not reduce their antibacterial effects. We used a previously reported protocol for using CptN as a primer for dentin bonding, first treating the adherent cells with 0.1 mM CptN solution for 60 s, and then removing the CptN and adding various concentrations of the cationic monomers, allowing contact with the bacteria for another 60 s. X-ray photoelectron spectroscopy has demonstrated that some of the platinum nanoparticles remain adsorbed to the dentin surface even after rinsing with water. Contact with adherent bacteria may be less frequent than with planktonic bacteria because of limited movement of molecules and entrapment by collagen fibers; however, the CptN concentration appears to be too low to reduce the antibacterial effects of cationic monomers.

We found that DMAE-CB has a stronger intrinsic antibacterial activity than MDPB. This difference may be attributed to their chemical structures. For quaternary ammonium compounds, the length of the alkyl chain is an important determinant of their antibacterial activity. A comparative study revealed that both MDPB and its precursor hydroxydodecylpyridinium bromide exhibit lower bactericidal activity than cetylpyridinium chloride, which has a 16-carbon alkyl chain. Similarly, DMAE-CB exhibits higher antibacterial activity than its homologue, methacryloxyethyl tetradecyl ammonium chloride, which has similar structure except for a shorter alkyl chain (14 carbons). The cationic head group may be another possible reason for the different antibacterial activities of MDPB and DMAE-CB. Cetyltrimethylammonium bromide, which has a head group of trimethylammonium, displays stronger antibacterial activity against cariogenic bacteria than cetylpyridinium chloride, despite the same alkyl chain length (16 carbons). It is well known that strong base shows antibacterial activity. A slightly higher basic nature of trimethylammonium structure may be in part helpful to give greater antibacterial activity to DMAE-CB than pyridinium-based MDPB.

In summary, CptN did not reduce the antibacterial activity of cationic monomers under the experimental conditions of this study, indicating that it may be useful as a pretreatment solution in combination with an antibacterial adhesive system. Further study investigating the effect of CptN on the biocompatibility of the antibacterial adhesive is important to determine the appropriate role of CptN in dental applications.

CONCLUSION

The present study investigated the effect of colloidal platinum nanoparticles (CptN) on the bactericidal activity of two quaternary ammonium-based antibacterial monomers. CptN alone did not exhibit antibacterial activity under the experimental conditions used in this study. Both MDPB and DMAE-CB demonstrated rapid killing effects against planktonic and attached S. mutans, with DMAE-CB exhibiting stronger antibacterial activity than MDPB. Combining CptN with quaternary ammonium-based monomers did not reduce the bactericidal effects of the monomers against S. mutans (planktonic or attached). Based on these results, we conclude that CptN has no negative effects on the antibacterial activity of quaternary ammonium based antibacterial monomers. In the future, the bond strength and cytotoxicity of antibacterial adhesives in combination with CptN will be studied to investigate the potential of using CptN as a pretreatment solution to improve the bonding capacity and biocompatibility of antibacterial adhesive systems.

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