

Nanoparticulates for Antibiofilm Treatment and Effect of Aging on Its Antibacterial Activity

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Abstract

Introduction: Issues pertaining to the effective elimination of bacterial biofilm and disruption of biofilm structure still remains in endodontic disinfection. Nanoparticulates such as chitosan (CS-np) and zinc oxide (ZnO-np) are known to possess significant antibacterial properties. This study aimed to test (1) the efficacy of CS-np and ZnO-np in disinfecting and disrupting biofilm bacteria and (2) the long-term efficacy of these nanoparticulates following aging. **Methods:** *Enterococcus faecalis* (ATCC & OG1RF) in planktonic and biofilm forms were treated with different concentrations of CS-np and ZnO-np. The treated bacteria were quantified by using microbiologic methods. The biofilm viability and structure after nanoparticulate treatment were assessed by using confocal laser scanning microscopy. The effect of aging by using sterile saliva and phosphate-buffered saline on the antibacterial properties of the nanoparticulates was also determined. **Results:** The rate of bacterial killing by the nanoparticulates depended on the concentration and time of interaction. Total elimination of planktonic bacteria was observed in contrast to the biofilm bacteria, which survived even after 72 hours. The confocal microscopy images showed predominantly dead bacterial cells and significant reduction in the thickness of biofilm ($P < .01$) after nanoparticulate treatment in both groups. Both CS-np and ZnO-np were found to retain their antibacterial properties after aging for 90 days. **Conclusions:** The present study highlighted the efficacy of the nanoparticulates to reduce biofilm bacteria, disrupt biofilm structure, and retain the antibacterial property even after aging. CS-np and ZnO-np present a potential approach in biofilm disinfection. (*J Endod* 2010;36:1030–1035)

Key Words

Antibacterial, antibiofilm, chitosan, *Enterococcus faecalis*, nanoparticulates, zinc oxide

Endodontic infection is a biofilm-mediated infection, and the success of an endodontic treatment will depend on the effective elimination of bacterial biofilm from the root canal system (1, 2). Resistance of bacterial biofilm to present disinfectants such as sodium-hypochlorite and chlorhexidine is due to inherent microbiologic factors and the complex anatomy of the root canal system (3, 4). It was reported that almost 35% of the root canal surfaces remain untouched by the root canal files, regardless of the filing method (5). The bacteria present within the dentinal tubules are inaccessible to the currently used irrigants (eg, sodium hypochlorite), medicaments (eg, calcium hydroxide), and sealers (Grossman sealer) as a result of their limited penetrability into the dentinal tubules (5–7). The obturating materials are also reported to have a limited antibacterial activity because most of the sealers lose their antibacterial property by 1 week (8–10). All these factors contribute to the persistence of bacterial biofilm within the root canal system (1, 4).

Biofilm mode of bacterial growth is one of the adaptive processes that allow bacteria to survive in nutrient-depleted environment similar to the treated root canals (11, 12). The apical portions of the root canal–treated mandibular molars revealed presence of bacteria in the form of biofilm mainly in the anatomical complexities (1). The biofilm bacteria show higher antimicrobial resistance as compared with their free-floating “planktonic” counterparts (13, 14). The resistance of biofilm bacteria has been attributed to the protective barrier provided by the extracellular polymeric matrix (EPM) (15). Therefore, agents that possess the ability to disrupt the EPM would allow better penetration of antibacterial agents into the biofilm structure and result in significant bacterial elimination. This approach would be advantageous in nonsurgical root canal treatment (NSRCT). Various nanoparticles have gained popularity as antimicrobial agents as a result of their broad spectrum of activity and biocompatibility (16). Recent studies have focused on using nanoparticulate materials to disinfect root canals (17, 18). Nanoparticulates exhibit higher antibacterial activity as a result of their polycationic/polyanionic nature with higher surface area and charge density, resulting in greater degree of interaction with the bacterial cell (17). It has been observed that the size of nanoparticulates plays an important role in their antibacterial activity, with smaller particles showing higher antibacterial activity than the macroscaled ones (18–20).

Chitosan (poly (1, 4), b-d glucopyranosamine) is obtained from deacetylation of the second most abundant polymer, chitin. Chitosan is an excellent bioadhesive that possesses broad spectrum of antimicrobial properties. Nanoparticulates are those particles with diameter of 100 nm or less. Previous studies have reported significant antibacterial efficacy of chitosan nanoparticulates (CS-np) and zinc oxide nanoparticulates (ZnO-np) against planktonic *Enterococcus faecalis* (17, 21).

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Antibacterial activity of polycationic CS-np and ZnO-np could be attributed to the electrostatic attraction with the negatively charged bacterial cell, which might lead to the altered cell wall permeability, resulting in leakage of the proteinaceous and other intracellular components and death of the cell (22, 23). Certain metallic nanoparticulates (ZnO, MgO) are also known to cause membrane damage as a result of lipid peroxidation by the reactive oxygen species (ROS) such as superoxide (O_2^-) and hydroxyl radicals (OH^\cdot) (24). Direct or close contact between the nanoparticulates and the bacterial membrane appeared to be essential for the ROS toxicity to be effective in peroxidation (16, 25). In addition, CS-np and ZnO-np possess several important properties for clinical application such as biocompatibility, color (white), cost-effectiveness (as compared with bioactive glass), availability, and ease of modification.

E. faecalis is one of the primary organisms in patients with post-treatment endodontic infection. It has been shown to exist as biofilm in the anatomical complexities and possess ability to penetrate dentinal tubules (26, 27). Even though nanoforms of bioactive glass have demonstrated significant antibacterial properties, effects on biofilm structure and effects of aging have not been established (18, 28). The objective of this study was 2-fold: (1) to investigate the efficiency of CS-np and ZnO-np to eliminate and disrupt the structure of a 7-day-old *E. faecalis* biofilm *in vitro* and (2) to evaluate the long-term antibacterial activity of CS-np and ZnO-np following aging process.

Materials and Methods

All chemicals and bacteriologic media used in this study were purchased from Sigma-Aldrich Inc (St Louis, MO), unless otherwise stated. CS-np was synthesized according to the method reported in an earlier work (29). In brief, chitosan obtained from Sigma was dissolved

in 1 v/v % acetic acid solution at a concentration of 0.1 w/v %, and the pH was raised to 5 with 1 mol/L NaOH. CS-np was formed by adding 0.1% sodium tripolyphosphate in water to chitosan solution in a ratio of 3:1 under stirring at a speed of 1000 rpm. CS-np was separated by centrifugation at 15,000 rpm for 30 minutes. The supernatant was discarded, and CS-np was extensively rinsed with deionized water to remove any residual NaOH and then freeze-dried before further use. ZnO-np with a particle size of <100 nm was purchased and tested in this study.

Assessment of Antibacterial Efficacy of Nanoparticulates

Two strains of *E. faecalis* with biofilm-forming capacity were tested in both planktonic and biofilm state. OG1RF strain is known to form aggressive biofilm compared with ATCC 29212 strain (30). *E. faecalis* was incubated overnight at 37°C under agitation in brain-heart infusion (BHI) broth. The cultures were centrifuged (6000 rpm, 10 minutes, 4°C), supernatants were discarded, and the cells were washed twice in sterile deionized water. The cells were resuspended in deionized water and adjusted to 10^7 cells/mL (optical density ≈ 0.1) at 600 nm (UV-VISIBLE Spectrophotometer; Shimadzu, Kyoto, Japan). One milliliter of the planktonic bacterial cells was inoculated into each well of the multiwell plate. The bacterial cells were divided into 2 treatment groups (to test the effects of CS-np and ZnO-np) and 1 control group. Three concentrations, 2, 5, and 10 mg/mL, of both CS-np and ZnO-np were tested. At different time intervals, 100 μ L of the bacterial inoculum was withdrawn and plated onto freshly poured BHI-agar plates after serial dilutions.

To test the antibacterial efficacy of the nanoparticulates on bacterial biofilm, 7-day-old biofilm of *E. faecalis* (ATCC 29212 and OG1RF)

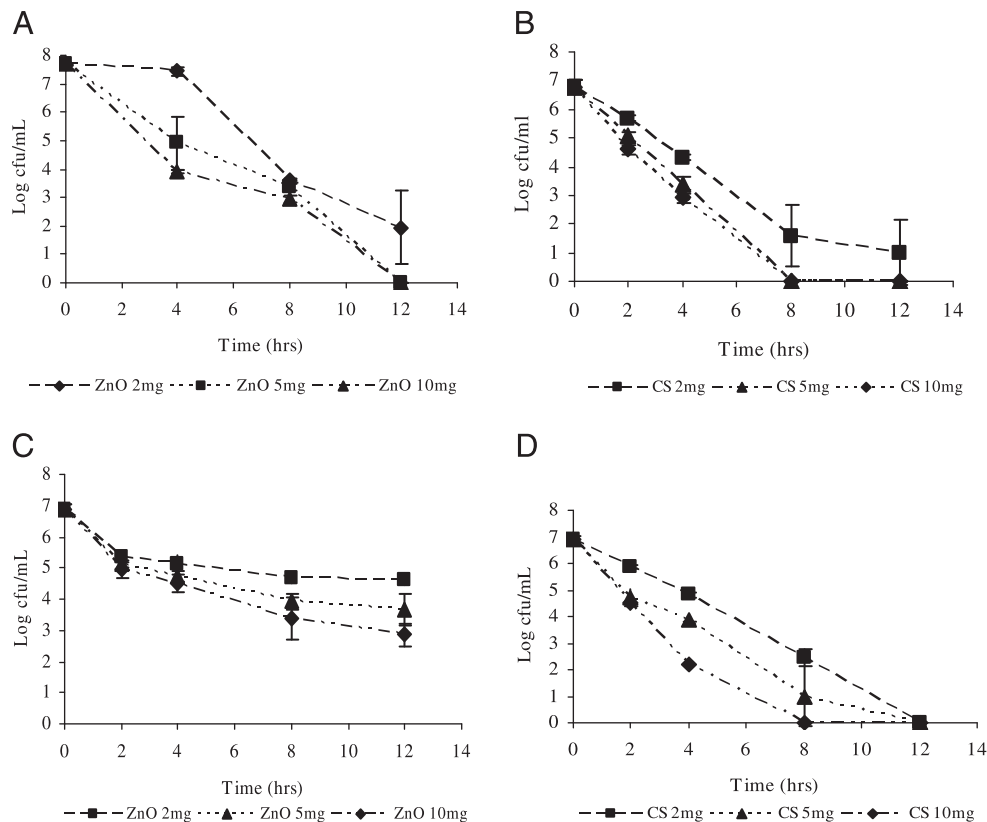


Figure 1. Antibacterial efficacy of CS-np and ZnO-np by using 3 different concentrations on planktonic *E. faecalis* strains ATCC 29212 (A, B) and OG1RF (C, D).

was grown in multiwell plates. Single colony from the agar plate was inoculated into 50 mL of BHI broth (Sigma) and cultured overnight in an orbital incubator at 37°C at 100 rpm. Fresh culture adjusted to an optical density of 1 at 600 nm, 1 mL of *E. faecalis* culture was added into each well of the multiwell plates preconditioned overnight with sterile saliva and incubated in an orbital incubator at 37°C, 100 rpm. Fresh medium was replenished every 48 hours to provide a constant supply of nutrients and to remove dead bacterial cells. On the eighth day, the medium was removed from the wells, and the biofilm was carefully washed twice with sterile deionized water. The washed biofilm was maintained in 1 mL of sterile deionized water. The bacterial cells were divided into 2 treatment groups (to test the effects of CS-np and ZnO-np) and 1 control group. Three concentrations of 5, 10, and 20 mg/mL of both the nanoparticulates were tested. Nanoparticulates were added to the bacterial culture and incubated at 37°C, 100 rpm. The wells in the control group were maintained in sterile deionized water. At different time intervals the nanoparticulates were removed from the wells, and the biofilms were washed gently by using deionized water. One milliliter of deionized water was added, the biofilm was then disrupted, and 100 μ L of the biofilm bacterial inoculum was plated onto freshly poured BHI-agar plates after serial dilutions. Colonies were counted after 24 hours of incubation at 37°C and expressed as log colony-forming units (CFU) per milliliter. The experiments were carried out in triplicates, and the mean values were calculated.

Assessment of Biofilm Structure after Nanoparticulate Treatment

The structure of the 7-day-old biofilm after treatment with nanoparticles was assessed by using confocal laser scanning micros-

copy (CLSM). *E. faecalis* (ATCC 29212) biofilm was grown on a glass coverslip that was fixed covering a circular hole (6 mm diameter) made in the base of a Petri dish (3 cm diameter). One milliliter of fresh culture of *E. faecalis* grown in BHI broth was adjusted to optical density of 1 at 600 nm and added into the Petri dish with the glass coverslip, which was preconditioned overnight with 0.5 mL of sterile saliva and incubated in an orbital incubator at 37°C at 100 rpm. Fresh medium was replenished every 48 hours to provide a constant supply of nutrients and to remove nonadherent bacterial cells. Three Petri dishes containing the biofilm were tested under each group. On the day of experiment, medium was removed from the wells; biofilm was washed gently twice with sterile deionized water and was maintained in 1 mL of sterile deionized water. CS-np and ZnO-np (20 mg/mL) were added into the wells and incubated at 37°C at 100 rpm for 24 hours. After this period of nanoparticulates-biofilm interaction, the suspension was gently removed, and the biofilms were washed with sterile deionized water to remove the remaining nanoparticulates. The biofilms were then stained with 20 μ L of Live/Dead *BacLight* stain (Molecular Probes, Eugene, OR) and incubated in the dark for 10 minutes. The biofilm structures were then viewed under a confocal laser scanning microscope (Olympus, Tokyo, Japan). Kr/Ar laser was the source of illumination, with 488 nm excitation and long-pass 500–523 nm and 622–722 nm emission filter settings for green and red signals, respectively. Nine different areas were imaged from each sample by using a 60 \times water objective. The optical sections of the biofilm structure were recorded and analyzed by using FluoView software (Olympus). Student *t* test was used to compare the thickness of the biofilm before and after nanoparticulate treatment.

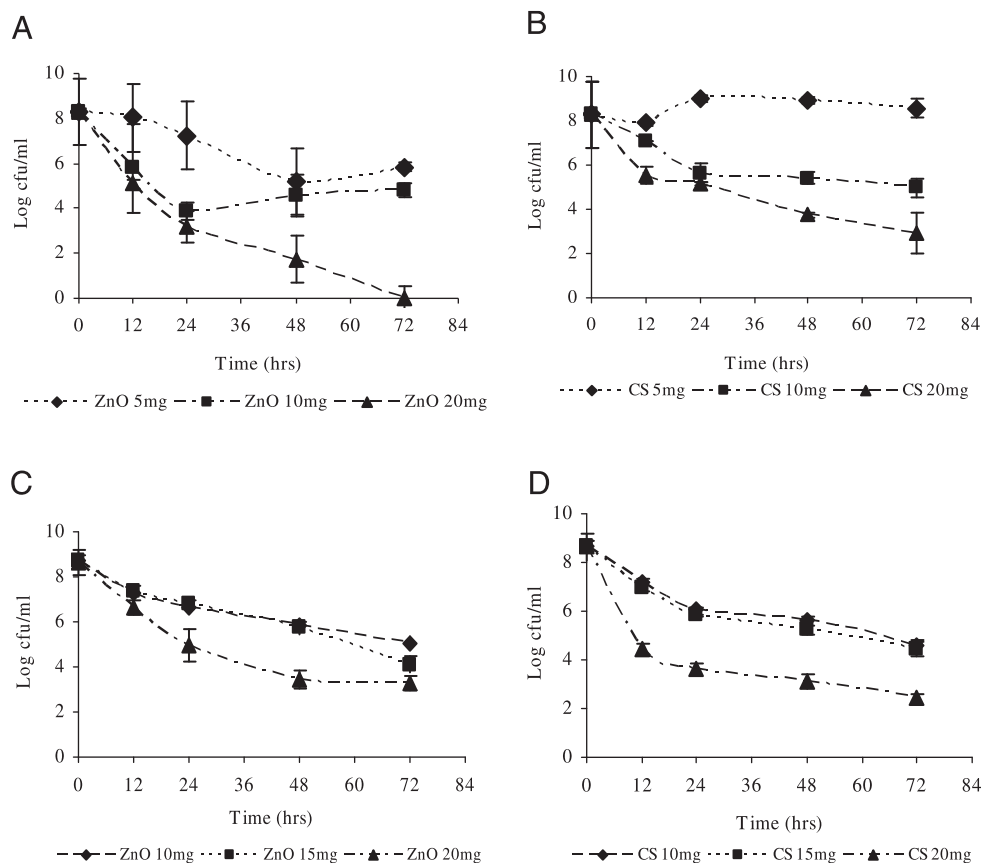


Figure 2. Antibacterial efficacy of CS-np and ZnO-np by using 3 different concentrations on biofilm of *E. faecalis* strains ATCC 29212 (A, B) and OG1RF (C, D).

Assessment of Aging Effect on Antibacterial Properties of Nanoparticulates

Thin coatings of ZnO-np and CS-np were prepared on the base of a 24-well plate by dehydrating the suspension (10 mg per well) in a hot air oven (70°C) for 6 hours. The wells were divided into 3 groups: group 1, nanoparticulates aged by using sterile filtered saliva (1 mL/well); group 2, nanoparticulates aged by using sterile phosphate-buffered saline (PBS) (1X) (1 mL/well); and group 3, nanoparticulates without aging, control group. All the groups were incubated in a CO₂ incubator at 37°C, simulating the root canal environment for different time intervals of 1, 7, 30, and 90 days. After different time intervals, *E. faecalis* (ATCC 29212) was incubated overnight at 37°C under agitation in the BHI broth. The culture was centrifuged (6000 rpm, 10 minutes, 4°C); supernatants were discarded and washed twice in sterile deionized water. The cells were resuspended in deionized water and adjusted to 10⁷ cells/mL (optical density ≈ 0.1) at 600 nm. One milliliter of the culture was inoculated into each well of the multiwell plate and incubated at 37°C, 100 rpm. The bacterial reduction was assessed after 24 hours of incubation by plating the cells onto freshly poured BHI-agar plates after serial dilutions. Colonies were counted after 24 hours of incubation at 37°C, and cell survival fraction was expressed as the ratio of the CFU of bacteria treated with nanoparticulates to the CFU of bacteria that did not receive any treatment.

Results

Assessment of Antibacterial Efficacy of Nanoparticulates

Figs. 1 and 2 show that the rate of reduction of the bacterial CFU on treatment with the nanoparticles depends on the state of the bacteria (planktonic or biofilm), duration, and concentration of the nanoparticulates used. CS-np showed total bacterial elimination within 8–12 hours of interaction with both *E. faecalis* strains. However, ZnO-np showed total elimination of only ATCC 29212 strain after 12 hours of interaction. The highest concentration (10 mg/mL) of ZnO-np showed maximum reduction up to 4 log of OG1RF strain (Fig. 1C). The antibacterial efficacy of both CS-np and ZnO-np decreased in the case of biofilm bacteria when compared with the planktonic bacteria (Fig. 2). At lower concentrations CS-np and ZnO-np (5 and 10 mg/mL) showed a maximum reduction of 3–4 log after 72 hours. However, in the case of ATCC 29212 strain, after the initial decrease, there was a slight increase in bacterial numbers (Fig. 2A, B). Significant reductions of biofilm bacteria of both strains were observed with the highest concentration used (20 mg/mL). In the case of the OG1RF strain, a maximum reduction of 3–4 log was observed (Fig. 2C, D). However, complete elimination of ATCC 29212 strain was observed only with ZnO-np after 72 hours of interaction (Fig. 2A). The biofilm cells in the control group maintained in sterile deionized water showed approximately 1 log reduction bacterial count after 72 hours (data not shown).

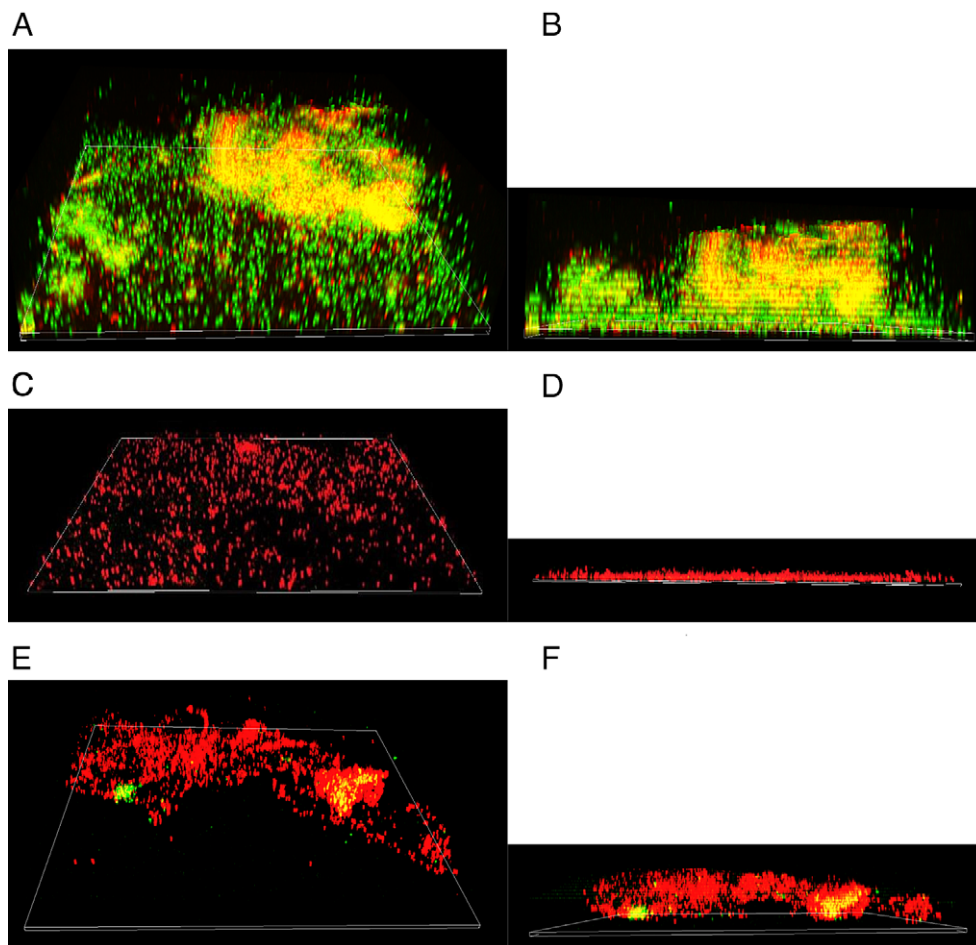


Figure 3. The 3-dimensional CLSM reconstruction of *E. faecalis* (ATCC 29212) biofilm (A, B) and after treatment with antibacterial ZnO-np (C, D) and CS-np (E, F). The number of live bacterial cells was reduced significantly, and the 3-dimensional structure was also disrupted. (B, D, and F show the sagittal sections of the biofilm structure) (original magnification, 60×).

Assessment of Biofilm Structure after Nanoparticulate Treatment

Fig. 3 shows the CLSM images of the bacterial biofilms before and after nanoparticulate treatment. In the untreated control, the biofilm structure consisted of both live (green) and dead (red) bacterial cells in a multilayered architecture. The number of live bacterial cells was observed to be higher as compared with the dead cells. The thickness of biofilm structure was found to be variable at different locations ($30.4 \pm 10.8 \mu\text{m}$). After treatment with CS-np and ZnO-np for 24 hours the biofilm architecture was altered. Distribution of viable bacteria reduced significantly, and the multilayered structure as observed in the control biofilm was disrupted. Bacterial biofilms exposed to ZnO-np were completely disrupted, with conspicuous loss of the intricate 3-dimensional form. In case of CS-np, the biofilm bacteria were found to be aggregated in clusters of dead cells, with few live bacterial cells in the deeper layers of the biofilm. The thickness of the biofilm reduced significantly to $9.33 \pm 2.42 \mu\text{m}$ ($P < .001$) and $16.88 \pm 11.7 \mu\text{m}$ ($P < .01$) after 24-hour treatment with ZnO-np and CS-np respectively.

Assessment of Aging Effect on Antibacterial Properties of Nanoparticulates

The findings from this experiment showed that CS-np and ZnO-np demonstrated antibacterial activity even after aging with PBS and saliva (Fig. 4). Total bacterial inhibition by both CS-np and ZnO-np was observed until 30 days without aging. CS-np showed bacterial inhibition after 30 days of aging in PBS. However, the antibacterial effect of CS-np aged in saliva showed pronounced reduction in antibacterial efficacy after 90 days. The survival fraction of CS-np aged in saliva was 0.6 as compared with <0.1 in the CS-np aged in PBS and control groups. ZnO-np showed faster reduction in antibacterial efficacy after aging in PBS than in saliva. The survival fraction of bacteria was 0.4 in case of ZnO-np aged in PBS and <0.1 in case of ZnO-np aged in saliva on day 90. After 90 days, the nanoparticulates without aging from the control group resulted in minimal increase in the survival fraction of <0.1 .

Discussion

This study demonstrated that CS-np and ZnO-np possessed significant antibiofilm properties and were able to disrupt the multilayered, 3-dimensional biofilm architecture. The antibacterial property was retained even after aging in saliva and PBS for 90 days. The planktonic bacterial cells were eliminated more rapidly and at lower concentrations of CS-np and ZnO-np when compared with the biofilm bacteria. Direct contact-dependent inhibition of planktonic bacteria might be the main killing mechanism by these nanoparticulates, whereas resistance to penetration of the nanoparticulates as a result of negatively charged biofilm EPM could be the cause of higher concentrations and a longer duration of contact required for elimination of biofilm bacteria (15, 31). In addition, the EPM might also serve as a chemical barrier by adsorbing the harmful ROS from reaching the cell surface, thereby decreasing the effect of ROS (32). The higher reduction of biofilm bacteria by ZnO-np compared with CS-np could be due to ROS production by ZnO-np, which was able to diffuse into the biofilm structure. The presence of moist or aqueous environment of the biofilm might augment the production of ROS by ZnO-np (21).

The bacterial biofilm structures demonstrated antimicrobial resistance even with higher concentrations of antimicrobials (15). The disruption of biofilm structure after treatment with the nanoparticulates was evident from confocal microscopy. The confocal images based on the *BacLight* staining indicated predominantly dead cells with disruption of the biofilm structures after 24 hours of treatment with both CS-np and ZnO-np. However, the microbiologic quantification showed

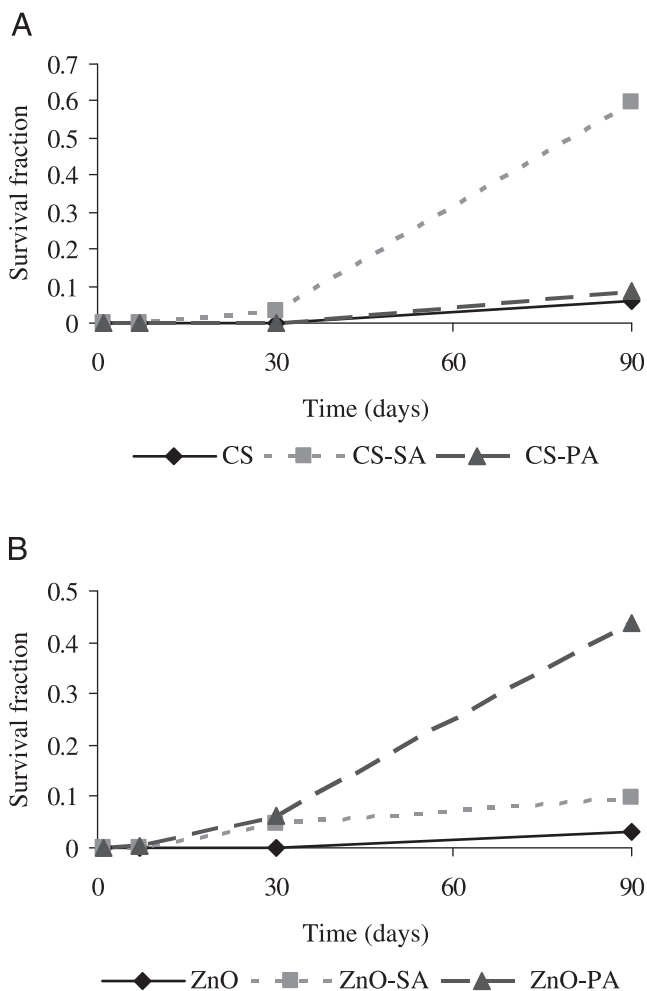


Figure 4. Effect of aging by using saliva and PBS in the antibacterial efficacy of (A) CS-np and (B) ZnO-np as a function of time. Graphs show the survival fraction of bacteria in contact with the nanoparticulates at different time intervals after aging. Groups: aging of CS-np with saliva (CS-SA), with PBS (CS-PA); and aging of ZnO-np with saliva (ZnO-SA), with PBS (ZnO-PA).

4–5 log of cells surviving after 24 hours. As discussed earlier, EPM acting as a physical barrier might result in a gradient of nanoparticulates affecting the biofilm bacterial cells (15, 33). In addition, the green fluorescence of the fewer number of surviving bacterial cells within the biofilm structure will be overwhelmed by the red fluorescence of the larger number of dead cells. Even though the number of live bacterial cells is reduced after treatment with CS-np and ZnO-np, they might possess the ability to grow and multiply once the exposure is eliminated and nutrients are provided. Therefore, the findings from this study highlight the importance of comparing the CLSM information with direct cell viability assay for better picture of biofilm disinfection.

The retention of the antibacterial activity of CS-np and ZnO-np for up to 90 days presents a promising advantage for biofilm elimination. To mimic interfacial fluid leakage in root-filled teeth, we have used saliva in addition to the routinely used PBS to induce aging of CS-np and ZnO-np. CS-np and ZnO-np showed good antibacterial property until 30 days of aging. Because the charge of nanoparticulates plays an important role in bacterial elimination, aging with saliva and PBS could have resulted in reduced efficacy as a result of interaction of ions present in these media with the nanoparticulates. However, CS-np aged in PBS showed better antibacterial efficacy than CS-np aged

in saliva. The use of saliva would help to evaluate the antibacterial efficacy of these nanoparticulates even in worst case scenario of leakage in a root-filled tooth *in vivo*. Saliva is known to consist of different types of ions (calcium, phosphates, potassium, etc), and these might be responsible for the earlier loss of activity in the aging group by using saliva (34). In NSRCT, these nanoparticulates could be delivered into the anatomical complexities and dentinal tubules by using high intensity focused ultrasound where conventional disinfectants are unable to reach (35). This could be highly beneficial because studies have shown that a significant portion of the root canal surfaces remain untouched by instrumentation, and bacteria survive in these favorable niches (1, 5). Use of liquids with higher diffusibility might further enhance diffusion of nanoparticulates into the biofilm structure and anatomical complexities within the root canals.

In summary, the present study highlighted the efficacy of CS-np and ZnO-np to reduce biofilm bacteria and disrupt biofilm structure. The antibacterial property of these nanoparticles was retained even after aging for 90 days. Therefore, CS-np and ZnO-np possess a potential anti-biofilm capability, and further studies with *ex vivo* or *in vivo* models are required to validate its potential application in NSRCT.

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