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Title: Doxycycline functionalized polymeric nanoparticles inhibit *Enterococcus faecalis* biofilm formation on dentine.

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Running title: Doxycycline NPs harm *E. faecalis*.

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Abstract

Reinfection and tooth fracture are the most common causes of failure after endodontical treatment. **Aim:** The purposes of this study were to evaluate the antimicrobial and inhibition of biofilm formation potential of novel remineralizing polymeric nanoparticles (NPs) when applied on the dentine surface; and to ascertain the effect of the functionalization of these NPs with zinc, calcium or doxycycline. **Methodology:** The antimicrobial activity and the inhibition of biofilm formation have been analyzed on human dentine blocks that were infected with *Enterococcus faecalis* before or after NPs application. LIVE/DEAD ® testing under the confocal laser scanning microscopy and bacterial culturing were employed to analyse the biofilm biovolume and the bacterial viability. Field Emission Scanning Electron Microscopy was also employed to assess the biofilm morphology. One-way ANOVA with Welch's correction and *post-hoc* comparison by the Games-Howell test were performed for comparisons between groups. **Results:** The un-functionalized NPs displayed the highest antimicrobial activity against *E. faecalis* biofilms as they provided the lowest biovolume (3,865.7 μm^3) and the highest dead/injured cells percentage (79.93%), followed by Dox-NPs (biovolume: 19,041.55 μm^3, dead/injured cells: 45.53%). Doxycycline loaded NPs showed the highest values of inhibition of biofilm formation with the lowest biofilm biovolume (8,517.65 μm3) and a high dead/injured bacterial percentage (68.68%). Un-functionalized NPs did not reduce biomass growth, but exerted the highest percentage of compromised cells (93%), being able to disrupt biofilm formation. It also produced occlusion of dentinal tubules, probably interfering with bacterial tubule penetration. **Conclusions:** A new generation of bioactive nano-fillers (doxycycline-functionalized polymeric NPs) which may be included as primers in endodontic sealers, promoting antibacterial activity and dentinal tubules occlusion is proposed.

Introduction

Root canal treatment aims to remove the bacterial biofilms, pulp tissue and infected dentine of the root canal system as well as to create a coronal and apical tight seal to avoid reinfection. It will prevent microorganisms to impair from periapical healing or contributing to the development of apical lesions (Wang et al. 2016). Instruments and irrigating solutions are used to clean and disinfect the root canals. However, they sometimes fail to achieve this goal, jeopardizing the outcome of the endodontic treatment. Up to 48% of the main root canal areas remain untouched and therefore infected after instrumentation (Paqué et al. 2011, Gergi et al. 2015, Peters et al. 2015). The most accepted irrigation protocol includes the use of sodium hypochlorite (NaOCl) during root canal instrumentation, followed by a final rinse with a chelating agent such as ethylenediaminetetraacetic acid (EDTA) (Zehnder 2006). Although this protocol is effective in reducing the bacterial load, it is still incapable of eradicating bacterial infection (Arias-Moliz et al. 2015, Ruiz-Linares et al. 2017, Busanello et al. 2019). However, the most crucial point is that EDTA and/or NaOCl can adversely alter the structure of the root canal dentine. It has been previously reported that using these solutions on root dentine, produced structural dentine damage (Gu et al. 2017), reduced dentine hardness and could lead to root fractures (Lynch et al. 2002, Saha et al. 2017, Gu et al. 2017). As a consequence, an endodontically-treated tooth is more likely to fracture (Baras et al., 2019; Toledano et al. 2020). To overcome these problems, it would be a powerful approach to incorporate substances into root canal sealers able to strengthen and prolong the antimicrobial effect, and also mechanically reinforce and remineralize the endodontically treated root dentine (Baras et al. 2019, Brezhnev *et al*. 2019). The search for reinforcement of the root dentine, after endodontic treatment, has gained prominence in the last years (Toledano et al. 2020, Toledano-Osorio et al. 2020a, Baras et al. 2019).

Recently, polymeric nanoparticles with anionic carboxylate (*i.e*., COO^-) groups placed along the backbone of the polymer, have been developed. Polymeric NPs can be loaded with calcium (Ca-NPs), doxycycline (Dox-NPs) or zinc (Zn-NPs) and have been shown to be non-toxic to fibroblasts (Osorio et al. 2016a). When zinc doped NPs are applied as a primer to endodontically treated root dentine, they are able to produce an efficient seal, dentine collagen remineralization and mechanical reinforcement of this treated radicular dentine (Toledano et al. 2020). Doxycycline and zinc loaded on NPs, may also potentiate hard tissues regeneration when locally administrated because of its anticollagenolytic effect by inhibiting dentine metalloproteinases (Osorio et al. 2014, Osorio et al. 2016b).

Calcium, zinc and doxycycline loaded-NPs were also shown to exert some antimicrobial effect against different oral bacteria in planktonic cultures (Toledano-Osorio et al. 2018a) and were able to reduce periodontal bacterial biofilm formation (Sánchez et al. 2019); moreover doxycycline loaded-NPs were also able to reduce cariogenic biofilm formation at the dentine-resin interface (Toledano-Osorio et al, 2020b). However, although root canal infections are mainly polymicrobial (Tzanetakis et al. 2015), *Enterococcus faecalis* seems to be involved in most of the endodontic refractory infections due to its strong resistance to extremely harsh environments (Ròças et al. 2004, Baras et al. 2019). This reason, along with its easy handling in the laboratory, makes *E. faecalis* the endodontic associated key specie for biofilm disease models (Kreth et al. 2019, Fan et al. 2018). So far, no information is available regarding the antimicrobial activity of zinc, calcium or doxycycline loaded polymeric NPs against *E. faecalis* infected dentine as well as their capacity to inhibit biofilm growth in order to avoid dentine recontamination.

The purposes of this study were to evaluate the antimicrobial and the inhibition of biofilm formation potential of the polymeric NPs when applied on the dentine surface and to ascertain the effect of loading these NPs with zinc, calcium or doxycycline. It was then hypothesized that incorporating NPs, Ca-NPs, Zn-NPs or Dox-NPs would not inhibit the growth and biofilm formation of *E. faecalis* on dentine and would not exert antimicrobial effect if NPs are applied on contaminated dentine.

Materials and Methods

Nanoparticles fabrication

PolymP-*n* Active nanoparticles (NPs) (NanoMyP, Granada, Spain) were obtained through polymerization precipitation. In order to control the process of precipitation, a thermodynamic approach has been used: the Flory-Huggins model based on the Hansen solubility parameters. The model was developed based on the growth of polymeric chains and solvent molecules interactions by hydrogen-bonding, polar and dispersion forces (Medina-Castillo et al. 2010). The NPs are designed with 2-hydroxyethyl methacrylate as the backbone monomer, the methacrylic acid as the functional monomer and ethylene glycol dimethacrylate as the cross-linker. NPs functionalized with calcium (Ca-NPs) and zinc (Zn-NPs) were produced. The NPs complexation processes with zinc and calcium were realized via immersion of 30 mg of NPs in aqueous solutions of 15 mL of $ZnCl_2$ or $CaCl_2$ solutions (containing 40 mg mL^{-1} of $ZnCl_2$ or $CaCl_2$ at pH 6.5) for 2 h at room temperature under constant shaking, with the objective of reaching the metal ions adsorption equilibrium (Osorio et al. 2016a). The suspensions were

subsequently centrifuged (7800 rpm/G-force=6461) for 20 min and the particles were detached from the supernatant. The same centrifugation technique, with the addition of phosphate buffer solution (PBS) to wash the sample, was repeated twice. Achieved values of ion complexation were 0.96 ± 0.04 μg Ca/mg NPs and 2.15 ± 0.05 μg Zn/mg NPs (Osorio et al. 2016a). For doxycycline functionalization, 30 mg of NPs were immersed in 18 mL of a doxycycline hyclate aqueous solution of 500mgL^{-1} (Sigma Aldrich, Chemie Gmbh, Riedstr, Germany) for 30 min under constant shaking. After incubation the amount of doxycycline per gram of NPs was 70 μg (Toledano-Osorio et al. 2018a). The experiments were carried out using 10 mg of NPs in 10 ml of phosphate buffer solution (PBS). Four different nanoparticles were tested in this study: a) non-functionalized nanoparticles (NPs) developed as previously described by Osorio et al. (2016a); b) NPs functionalized with zinc (Zn-NPs); c) with calcium (Ca-NPs); and d) with doxycycline (Dox-NPs). NPs diameter is about 200 nm, these NPs do not agglomerate. The functionalization of NPs with zinc, calcium or doxycycline does not produce changes in NPs diameter (Toledano-Osorio et al. 2018b).

Dentine specimen preparation

Dentine blocks were obtained from 43 human non-carious freshly extracted teeth, after polishing (500–800-grit SiC papers) 2 × 2 × 1.2 mm specimens were obtained. Dentine smear layer was removed using 17% EDTA for 4 min. The dentine specimens were then sterilized in autoclave and kept in sterile saline solution at 4°C. A total of 170 dentine blocks were prepared, half of them were used for the antimicrobial activity test, and the other half to test for the inhibition of biofilm formation effect (Baca et al. 2011). The research protocol was approved by the institutional Ethics Committee (UGR #438).

Antimicrobial activity testing

The dentine blocks were incubated in 200 μl of a 1×10^5 colony forming units per millilitre (CFUs/ml) *E. faecalis* ATCC 29212 bacteria suspension in a 96 well microtiter plate. This bacterial suspension was done in Brain Heart Infusion (BHI) broth (Scharlau Chemie, Barcelona, Spain) with the aid of a densitometer (DensiCHEK plus, biomerieux, Lyon, France). The plate was placed on a rocking table (Swing Sw 8 10000-00015, OVAN, Badalona, Spain) and incubated at 37°C for 12 hours at 5 rocks per minute. Thereafter, all the specimens were washed by immersion in 200 μl of saline solution in order to eliminate the planktonic bacteria that are loosely adhered to the surface.The specimens were randomly divided into 4 experimental groups (n = 14) according to the type of NPs: NP, Dox-NP, Zn-NP and Ca-NP. A

positive control, in which only 2.5 µl of PBS were applied, without NPs treatment was also included. Moreover, 5 non-treated and non-infected dentine blocks were prepared and served for sterility verification. NPs were applied onto dentine surfaces using a micropipette and a sterile microbrush. 2.5 µl of the NPs suspension (10mg/ml) was applied on each specimen; it was left undisturbed for 1h, to accomplish solvent evaporation.

After the exposure, the specimens were rinsed with 0.9% saline solution. Five blocks were observed under the confocal laser scanning microscopy (CLSM) (Leica, TCS-SP5 II, Wetzlar, Germany) and 9 specimens were used for bacterial culturing. For the CLSM analysis the dentine blocks were stained with the LIVE/DEAD® BacLigth viability kit (Invitrogen, Eugene, OR, USA) which includes Syto-9 and propidium iodide. Both dies target the nucleic acids. Syto-9 is a green fluorescent stain labeling microorganisms with intact membranes (considered as live bacteria) whilst propidium iodide is a red fluorescent stain that penetrates only the cells with damaged membranes (considered as dead bacteria). After staining the samples with 100 µl of the dyes for 15 min, they were rinsed with saline solution and observed using a CLSM. Four microscopic confocal volumes from random areas were acquired from each sample using the 40× oil lens, 1 µm step-size and a format of 512 pixels. Each picture represented an area of 387.5 × 387.5 µm. *Bio*image_L software was used for quantification purposes (Chávez de Paz et al 2009). The parameters evaluated in each group were the total biovolume (μm^3) and the percentage of dead cells or cells with injured membranes.

For the bacterial culturing, each one of the rest 9 specimens were placed in Eppendorf tubes with 200 µL BHI, vortexed for 2 seconds, and then sonicated on a water-table sonicator (Model 5510E–MT; Branson, Danbury, CT) for 10 minutes to assure biofilm recovery. Disrupted biofilm cultures were diluted serially ($10\text{-}10^{-5}$) and plated for viable cell counting. Results of the CFUs/ml counts were expressed as Log_{10}.*Inhibition of biofilm formation testing*

Seventy five specimens were randomly divided into the same 4 experimental groups and a positive control without NPs treatment (n = 14). Once sterile, NPs were applied onto the dentine surfaces using a micropipette and a sterile microbrush. 2.5 µl of a PBS solution of the NPs (10mg/ml) was applied on each specimen. Specimens were left undisturbed for 1 h, until the solvent was evaporated. Five specimens that were not treated and not infected were used for sterility verification.

The dentine blocks were then incubated in 200 µl of the *E. faecalis* suspension in a 96 well microtiter plate at 37°C for 12 hours on a rocking table at 5 rocks per minute. Thereafter, all the specimens were washed by immersion in 200 µl of saline solution in order to eliminate

the planktonic bacteria that are loosely adhered to the surface, and after they were split: 5 of them were stained and observed under CLSM by using the same methodology described above, and the other 9 were sonicated to recover the biofilms and obtain the CFUs/ml counts as explained above.

Analysis of the Biofilms by Field Emission Scanning Electron Microscopy (FESEM)

Two additional samples from each experimental group were prepared for FESEM. The specimens were fixed in a solution at 4% paraformaldehyde and 2.5% glutaraldehyde solution in 0.1 mol/L sodium cacodylate buffer, rinsed three times in 0.1 mol/L sodium cacodylate buffer, then washed in PBS and in sterile water for 10 min. Samples were placed in an apparatus for critical point drying (Leica EM CPD 300, Wien, Austria). They were sputter-coated with carbon by means of a sputter-coating Nanotech Polaron-SEMPRE2 (Polaron Equipment Ltd., Watford, UK) and observed with a field emission scanning electron microscope (FESEM Gemini, Carl Zeiss, Oberkochen, Germany) at an accelerating voltage of 3 kV.

Statistical analysis

A Kolmogorov-Smirnov test was applied to confirm that the variables total biovolume and Log_{10} CFUs followed a normal distribution. Dead percentage was normalized by means of the Anscombe transformation before statistical analysis (Robert 2000). Global comparisons were performed by one-way ANOVA with Welch's correction, and *post-hoc* comparison by the Games-Howell test. Previously, the Levene test showed significant differences of variances among groups. The level of statistical significance was set at $p < 0.05$. Statistical analysis was performed using SPSS 24.0 (SPSS Inc., Chicago, IL) software package.

Results

Antimicrobial activity testing

The NPs antimicrobial activity analysed by the CLSM is shown in the Table 1. Representative CLSM images of the different NPs groups are presented in Figure 1. The un-functionalized NPs displayed the highest antimicrobial activity against *E. faecalis* biofilms as they gave the lowest biovolume (3,865.7 μm^3) and the highest damaged cells percentage (79.93%), followed by Dox-NPs (biovolume: 19,041.55 μm^3, dead/injured cells: 45.53%)($p<0.05$). Zn-NPs and Ca-NPs did not exert antimicrobial activity, as their total biovolumes were similar to those of the control group ($p>0.05$). However, dead/injured cells

percentages of Zn-NPs and Ca-NPs groups were slightly higher than those of the control group (p<0.05). CFUs counts were similar in all groups (p>0.05), with logarithm values ranging from 5.45 in the control to 5.86 in the Ca-NPs group (Table 2A).

*Inhibition of biofilm formation testing*CLSM data on the inhibition of biofilm formation are shown in the Table 3. Representative CLSM images of the different NPs groups are presented in Figure 2. Dentine treatment with Dox-NPs exerted the highest values of inhibition of biofilm formation with the lowest biofilm biovolume (8,517.65 μm^3) and a high dead/injured bacterial percentage (68.68%). It is followed by Zn-NPs showing intermediate values of biovolume (23,716.05 μm^3) with no statistical differences with Dox-NPs neither with the positive control. Un-functionalized NPs attained the highest dead bacteria percentage (93%), followed by Dox-NPs (68.68%) and Zn-NPs (36.77%), being these values significantly different from the control group (9.71%). Data from CFUs counts of *E. faecalis* stated that Dox-NPs strongly inhibited the biofilm formation with a 4 Log reduction respect to the control group (p<0.001) (Table 2B).

Morphological Analysis of the Biofilms by Field Emission Scanning Electron Microscopy (FESEM)

Selected FESEM images showing antibacterial activity, where the *E. faecalis* biofilm was grown on the dentine surface, and then treated with the different NPs for 1 h, are presented in Figure 3. A structured *E. faecalis* biofilm is covering the dentine surface of the control group specimens. The observed exopolymeric substance (EPS) is preferentially located around the dentinal tubules (Figure 3a and 3b). In the group of un-functionalized NPs, bacteria and NPs are spread on the dentine surface (Figure 3c). At higher magnification, it is observable how NPs bind the EPS and completely occluded the dentinal tubules. Biofilm is disrupted and bacteria presented elongations trying to connect each other (Figure 3d). At grown *E. faecalis* biofilms treated with Dox-NPs, some bacteria were also shown, but they appeared partially covered by NPs (Figure 3e). It was also observed a tight contact between NPs and bacterial membranes which appeared extremely rough at their surface, showing small protrusive spherical shapes (Figure 3f). Zn-NPs were homogeneously distributed onto the dentine surface and did not interact with bacteria's membranes, however they facilitated biofilm disruption. EPS can be observed in between the bacteria (Figures g and h). Many bacteria could be observed at the biofilms that were treated with Ca-NPs, EPS was also evidenced (Figure 3i). Even when Ca-NPs seem to produce some biofilm disruption, Ca-NPs were not in contact with

bacteria which tried to reach each other by elongations emerging from their membranes (Figure 3j).

FESEM images showing inhibition of the *E. faecalis* growth on dentine surfaces that were previously covered with the different NPs are presented in the Figure 4. (a) *E. faecalis* biofilm development on the control dentine surface is shown in Figure 4a. First stages of biofilm formation and loose attachment of the bacteria on the surface were seen. Un-functionalized NPs were spread on the dentine surface, probably competing with *E. faecalis* for unmineralized collagen binding sites. As a result few bacteria were observed on the surface (Figure 4b). At a higher magnification, bacterial agglomerations with membranes alterations could be observed. Most of the bacteria showed visible changes in morphology (Figure 4c). Dox-NPs were completely covering the dentine surface and occluding dentinal tubules. Few bacteria were observed (Figure 4d). At higher magnification, tight contact of Dox-NPs with *E. faecalis* is shown at the image (Figure 4e). Dentine surfaces completely covered by Zn-NPs (Figures 4f, 4g) and Ca-NPs (Figure 4h) are shown with partially occluded dentinal tubules at dentine surfaces treated with Zn-NPs or Ca-NPs, before bacterial incubation.

Discussion

There are multiple *in vitro* testing models for the efficacy of antibacterial agents in endodontics, which may involve single or multispecies bacteria. The microcosm model is the most clinically relevant (Kreth et al. 2019), but the attained results are not easy to interpret due to difficulties in controlling the behavior of individual bacterial species (Ferracane et al. 2017). It is also a challenge to decide which species are appropriate in each experiment and their relative amounts (Ferracane et al. 2017). In the present study, as novel antibacterial agents are being analyzed, mono-culture tests were used in order to facilitate the interpretation of results and to obtain the basic understanding of the antimicrobial properties of these NPs. *E. faecalis* was selected because it is commonly found in root canal infections (Ròças et al. 2004) and it has been proposed as key specie in endodontics disease models (Kreth et al. 2019). It has been previously used to evaluate the antimicrobial properties of NPs and other different chemicals applied for endodontic therapy (Barbosa-Ribeiro et al. 2016, Bukhari et al. 2018). The ATCC 29212 strain was selected as it has been reported to be similar to a clinical isolate (Seneviratne et al. 2013). *E. faecalis* ATCC 29212 has been shown to form a biofilm after 12 h of incubation (Yang et al. 2018) and the EPS matrix has been demonstrated to cover the biofilm after 6 hours (Kristich et al. 2004, Ramos et al. 2019). At the present study,

a 12 h biofilm was grown trying to create a young biofilm, as it is the biofilm that the residual bacteria would form on dentine walls, after performing chemo-mechanical cleaning during endodontic treatment. A lightly infected dentine model with a bacterial suspension of 10^5 CFUs/ml (Hirose et al. 2016) was used. However, as the endodontic infections are known to be polymicrobial (Tzanetakis et al. 2015, Sánchez-Sanhueza et al. 2018), further studies need to be conducted on clinical isolates and multi-species biofilms, in order to corroborate these results. Both the persistence of bacteria after endodontic treatment and bacterial reinfection of the root canal can be the cause of the treatment failure (Siqueira & Rôças 2008). In an attempt to address these challenges, the antimicrobial effect and inhibition of biofilm formation potential of novel polymeric NPs have been tested.

NPs application on contaminated dentine during 1h has been shown to possess different antibacterial activity depending on the applied NPs, as encountered after LIVE/DEAD® BacLigth viability results (Table 1) and CFUs counts testing (Table 2A). From CLSM, it was demonstrated that un-functionalized NPs were able to significantly reduce total biovolume of grown biofilm (20-times reduced respect to the control group) and to exert damage to *E. faecalis* (up to 80% of existent cells were damaged or injured) (Table 1). A limited but significant effect in reducing biovolume (4-fold reduction) and compromising *E. faecalis* viability was also exerted by Dox-NPs. After CFUs counts, all groups were similar in total cells number. Differences between the two employed testing procedures are somehow expected. It has been previously reported (Kreth et al. 2019) that LIVE/DEAD® dyes may not be used as an exact quantitative measurement of cell death. Propidium iodide stained cells (red cells) may be falsely identified as dead cells, and represent cells that are injured, dead or starving viable cells. Therefore, red cells percentages should be taken as injured or dead cells. Bacteria were exposed to NPs just for 1 h, which may account for the scarce encountered effect of NPs on bacterial viability. However, when longer exposure times of bacteria to NPs were attempted,, the complete dentine surface was covered by a layer of mineral deposition, and even when images were obtained (data not shown); it was not possible to execute CLSM or CFUs measurements. This mineral deposition is exerted by NPs, due to their chelating efficacy and has been previously described on dentine surfaces (Toledano-Osorio et al. 2018b). FESEM images do also facilitate some information about NPs antibacterial effect. Three main effects are observed: i) EPS is preferentially placed around dentinal tubules (Figure 3), probably facilitating bacterial adhesion and tubules penetration. *E. faecalis* has the ability to invade dentinal tubules and withstand prolonged nutritional deprivation, and thus is difficult to eradicate (Wang et al. 2016). NPs bind to bacterial EPS. This interaction between NPs and EPS has produced biofilm disruption, probably altering the bacterial penetration trough dentinal

tubules. ii) Un-functionalized NPs and Dox-NPs occluded dentinal tubules (Figure 3) competing with bacteria for this location. iii) Only Dox-NPs have a close interaction with bacterial membranes and altered the *E. faecalis* appearance (Figure 3). Even when EPS may be observed at FESEM images, it should be taken into account that probably some part of it may be altered or diminished due to polymers collapsing trough desiccation procedures, during sample preparation. All this information obtained from FESEM images is only qualitatively assessed and may be taken with caution.

When dentine was pre-treated with NPs, it was found that Dox-NPs exerted a high anti-biofilm formation activity (Table 3, Figure 2) inhibiting biomass growth (4.5-fold times reduction if compared to the control group) and producing damage in most of the grown cells (69%). A high inhibition activity was also corroborated by the CFUs counts testing with a 4 Log reduction with respect to the control group. It is important to stress that even when it exists a disagreement between CFU and CLSM results regarding the unfunctionalized NPs; the results of the inhibitory effect of biofilm formation using Dox-NPs were in accordance. These results are also consistent with previous studies, where Dox-NPs produced a significant lowering in viable bacterial cells from a periodontal biofilm attached to hydroxyapatite discs (Sánchez et al. 2019), and Dox-NPs were also able to reduce cariogenic biofilm formation at the dentine-resin bonded interface (Toledano-Osorio et al, 2020b). Doxycycline may have a dual mechanism of action: i) a direct one by inhibiting the microbial protein synthesis (Kim et al. 2002) and ii) an indirect effect as it binds to the calcium ions of dentine, causing a chelation reaction (Porter et al. 2016). Furthermore, doxycycline can be absorbed onto root canal dentine (Rasimick et al. 2010), forming complex agglomerates that may serve as an antibiotic reservoir (Porter et al. 2016) impeding the bacterial adherence and exerting its antimicrobial direct effect. These effects of Dox-NPs were also observed at FESEM images, where dentine was covered by a Dox-NPs film and Dox-NPs were in tight contact with bacteria. The bacteria's damage was shown as membranes alteration (Figure 4). However, the antimicrobial activity of Dox-NPs observed in this study may be considered low if compared with previous reports that demonstrated 97% (Toledano-Osorio et al. 2018a) and 99% (Sánchez et al. 2019) bacterial viability reduction after 24 h exposition to Dox-NPs. However, comparisons between different studies using distinct biofilms models are difficult, as microbes' susceptibility to doxycycline is different for bacterial species. It should also been taken into account, that high doses are needed to act in a biofilm if compared to planktonic cultures, with reported concentrations of approximately 250 times greater than what may be required to destroy the same strains grown planktonically (Hojo et al. 2009). If the concentration range of doxycycline antimicrobial

activity for *E. faecalis* is about 0.02 to 6 μg/ml (Barbosa-Ribeiro et al. 2016), then biofilms may require from 5 to 1500 μg/ml of doxycycline. Tested Dox-NPs have been seen to release about 1200 μg/ml at 12 h time-point (Toledano-Osorio et al. 2018a).

Important differences are found between antibacterial activity results when Dox-NPs are applied on grown biofilms (Tables 1 and 2A; Figure 1 and 3) or on the dentine surface previous to the biofilm formation (Tables 3 and 2B; Figure 2 and 4). These results are due to difficulties of Dox-NPs and liberated doxycycline in penetrating the formed biofilm from the top. A continuous concentration decrease of the antimicrobial will be produced through the biofilm thickness, and may lead to infectivity in the deeper regions of the biofilm (Chambles et al. 2006). When NPs are placed at the bottom of the biofilm, early colonizers responsible for the biofilm anchorage are mostly affected, and biofilm survival is highly compromised (Kim et al. 2002, Sanchez et al. 2019).

Un-functionalized NPs did not inhibit biomass growth but exerted the highest percentage dead/injured cells (93%) after LIVE/DEAD® BacLigth viability testing. This antimicrobial effect could be explained by the interaction between the COO^- groups exposed at the NPs' surfaces and the bacterial proteins containing amino groups that will form covalent peptide bonds (Sanchez et al. 2019), consequently inhibiting bacterial functions and/or producing bacterial membranes alterations. Moreover, the negatively charged NPs may have a certain level of antibacterial activity due to molecular crowding, which leads to interactions between the NPs and the bacterial surface (Figure 4) (Arakha et al. 2015). On the other hand, the high biovolume within this group can be a consequence of different factors. First, *E. faecalis* presents heterogeneity in the zeta potential which offers the organism an advantage in adhesion to a negatively charged surface (van Merode et al. 2006). It is speculated that in this case, *E. faecalis* would have not avoided its interaction with the anionic charge of the un-functionalized-NPs spread onto the dentine surface. Biofilm formation observed at CLSM images (Figure 2) could not be evidenced after the FESEM analysis (Figure 4), because sample preparation procedures for FESEM produce the detachment of dead or injured cells that are not firmly attached to the dentine surface. Moreover, the antimicrobial activity obtained in the CLSM test contrasts with the high log of CFU counts. This discrepancy is explained by the associated uncertainty to the LIVE/DEAD® BacLigth viability testing, where bacteria stained with the propidium iodide (red cells), as mentioned above, may be damaged or injured bacteria although being viable for CFU counts analysis. On the other hand, in other studies, when comparing these two bacterial viability testing methods, CLSM has been shown to reflect better the true viability of the biofilm than culturing (Shen et al. 2010). It may be due to the

fact that this effect was observed during the starvation phase of the bacteria as they can enter in a viable but non-culturable state, and could return to the normal physiological state. These discrepancies are then, a consequence of differences in the methodologies, bacteria, biofilm treatment, period of evaluation, etc… It is clear that there is not a gold standard antimicrobial test to study the properties of a material. Thus, a combination of several methods is recommended when investigating adherent microorganisms (Hannig et al. 2010).

The action mechanism of Zn is related to the electrostatic attraction with the negatively charged bacterial cell membranes (Vargas-Reus et al. 2012), compromising the membrane permeability, and producing cytoplasmic fluid leakage that leads to cell death (Tamura et al. 2009). The results of the present study show that Zn-NPs did not exert antibacterial activity against previously grown biofilms, although it showed a moderate capacity to inhibit the biofilm formation and to obtain dead/injured cells. Gram-positive bacteria like *E. faecalis*, seem to be less sensitive to Zn ions than gram-negative cells (Pal et al. 2007). Probably, the entrapment of Zn ions into thick negatively charged peptidoglycan layers causes less toxicity than that of thinner peptidoglycan layered gram-negative bacteria.

However, at FESEM images Zn-NPs and Ca-NPs have shown disrupted biofilms (Figure 4), which is mainly produced through EPS binding and alterations (Kumar et al. 2019). A similar event was previously observed using ZnO NPs (Shrestha et al. 2010).

The attained limited antibacterial activity of NPs on grown *E. faecalis* biofilm may be explained by structural resistance mechanisms of biofilms to impede antibacterials penetration (Chambles et al. 2006). This effect will be potentiated by the shown fact that NPs may be prone to bond to EPS produced by *E. faecalis,* being even more difficult for NPs to penetrate the biofilm from the top of it and interact with cells. Same rationale will also help to explain that NPs may probably require more time to act if they are applied onto a previously grown biofilm. However, the antibiofilm activity is favored by NPs competition for binding sites (unmineralized collagen segments) (Love et al. 1997, Osorio et al. 2014) and it will be potentiated by the antibacterial effect of Dox-NPs, which is acting from the bottom of the biofilm. It will mainly affect to first bacterial colonizers (Sanchez et al. 2019). Lastly, the efficacy of NPs for occluding dentinal tubules may also be a crucial point affecting *E. faecalis* survival and their dentine invading capacity, as *E. faecalis* is able to persist and infect dentine from this location (Wang et al. 2016). This point should be further ascertained.

Some other NPs with antimicrobial activity have been previously tested. Silver and chitosan-based NPs were reported to be antimicrobial, but they require a prolonged contact time to effectively kill the bacteria. Besides, the toxicity of silver NPs possessed an

inconvenience in clinical use (Wu et al. 2014, Shrestha & Kishen 2016, Baras et al. 2019). Moreover, silver has been shown to hamper dentine remineralization, diminishing mechanical properties of dentine (Baras et al. 2019, Toledano et al. 2019).

The results of the present study, even when limited, should be considered as highly valuable. Taken into account that *E. faecalis,* when grown in biofilms, is one of the most difficult species to eliminate (Wang et al. 2016). Previous studies using tetracycline HCl, erythromycin, clindamycin, and metronidazole revealed poor *in vitro* activity against *E. faecalis* isolates and, therefore, were claimed to be ineffective therapeutic agents in failed root canals (Wang et al. 2016). Moreover, tested NPs in the present study were able to disrupt the external EPS. It may be a crucial point as EPS has been shown to protect pathogens from antibacterial agents and to contribute to the virulence of *E. faecalis* (Wang et al. 2016).

This article presents the possibility of using a new generation of bioactive nano-fillers to be included as primers in endodontic sealers promoting antibacterial, dentinal tubules occlusion and tooth mineral-regeneration effects. However, results from the present *ex vivo* study should be considered with certain caution as a different behaviour might be expected against a polymicrobial biofilm and under the parameters that simulate the clinical conditions. Therefore, clinically relevant *in vivo* methodologies need to be implemented in future studies to assess the potential applicability of these novel NPs with antimicrobial, MMPs inhibiting and remineralizing effects.

Conclusions

Doxycycline functionalized NPs are proposed to be included as primers in endodontic sealers to promote antibacterial activity against *E. faecalis* dentine biofilm, mainly through: i) direct cells injuring; ii) dentinal tubular occlusion and iii) probably, by disruption of the protective exopolymeric substance produced by these bacteria.

Acknowledgements

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Table 1. CLSM results attained at the antimicrobial activity testing: mean (standard deviation) of the total biovolume (μm^3) and dead/injured cells percentage of the *Enterococcus faecalis* biofilms after being grown on dentine and then exposed to NPs for 1 h.

	Total biovolume	**Dead/Injured cells percentage***
Un-functionalized-NPs	3,865.7 (2,926.97)[1]	79.93 (18.40)[1]
Dox-NPs	19,041.55 (17,638.23)[2]	45.53 (26.50)[2]
Zn-NPs	72,180.85 (50,218.14)[3]	16.59 (12.37)[3]
Ca-NPs	74,417.05 (45,716.19)[3]	16.55 (11.08)[3]
Positive control	84,534.75 (62,078.81)[3]	4.76 (2.28)[4]
***p*-value**	<0.001	<0.001

* Before statistical analysis, data were previously subjected to the Anscombe transformation.
Global comparison by ANOVA with Welch's correction.
Different superscript numbers indicate significant differences by the Games-Howell test ($p<0.05$).

Table 2. Results from CFUs counts: mean (standard deviation) of the *Enterococcus faecalis* counts expressed as Log_{10} (CFUs). **A)** Antimicrobial activity testing: dentine was first contaminated with bacteria and after treated with NPs during 1 h. **B)** Inhibition of biofilm formation testing: dentine was covered with NPs and after contaminated with bacteria.

	A	**B**
	Log_{10} (CFUs)	**Log_{10} (CFUs)**
Un-functionalized-NPs	5.50 (0.18)	8.02 (0.54) [1]
Dox-NPs	5.51 (0.22)	3.59 (0.14) [2]
Zn-NPs	5.53 (0.41)	8.03 (0.54) [1]
Ca-NPs	5.85 (0.89)	8.06 (0.52) [1]
Positive control	5.45 (0.38)	7.63 (0.88) [1]
***p*-value**	0.823	<0.001

Global comparison by ANOVA with Welch's correction. Read vertically, different superscript numbers indicate significant differences by the Games-Howell test ($p<0.05$).

Table 3. CLSM results attained at the inhibition of biofilm formation testing: mean (standard deviation) of the total biovolume (μm^3) and dead/injured cells percentage of the *Enterococcus faecalis* biofilms after being grown on dentine surfaces that were previously covered by NPs.

	Total biovolume	Dead/Injured cells percentage*
Un-functionalized-NPs	71,099.70 (72,388.01) [1,3]	93.00 (8.23)[1]
Dox-NPs	8,517.65 (7,055.81)[2]	68.68 (12.50)[2]
Zn-NPs	23,716.05 (24,435.16)[1,2]	36.77 (24.57)[3]
Ca-NPs	49,838.70 (20,966.46)[3]	5.33 (6.85)[4]
Positive control	37,149.25 (35,615.57)[1,3]	9.71 (10.65)[4]
***p*-value**	<0.001	<0.001

* Before statistical analysis, data were previously subjected to the Anscombe transformation.
Global comparison by ANOVA with Welch's correction.
Different superscript numbers indicate significant differences by the Games-Howell test ($p<0.05$).

Figure 1. Representative LIVE/DEAD® staining images of biofilms created for antibacterial activity testing. The *E. faecalis* biofilm was grown on the dentine surfaces, and after was treated with PBS -control group- (a), Un-functionalized NPs (b), Dox-NPs (c), Zn-NPs (d) and Ca-NPs (e) for 1 h. Biofilms on control group were mainly alive. Biofilms on Un-functionalized NPs group were mostly compromised and a strong reduction in the biovolume is observed. Dox-NPs produced a high percentage of injured/dead cells. However, biofilms on Ca and Zn-NPs groups presented a high number of live cells. Compromised bacteria were stained red and live bacteria were stained green. All images had the same magnification, each image represents an area of 387.5 × 387.5 µm.

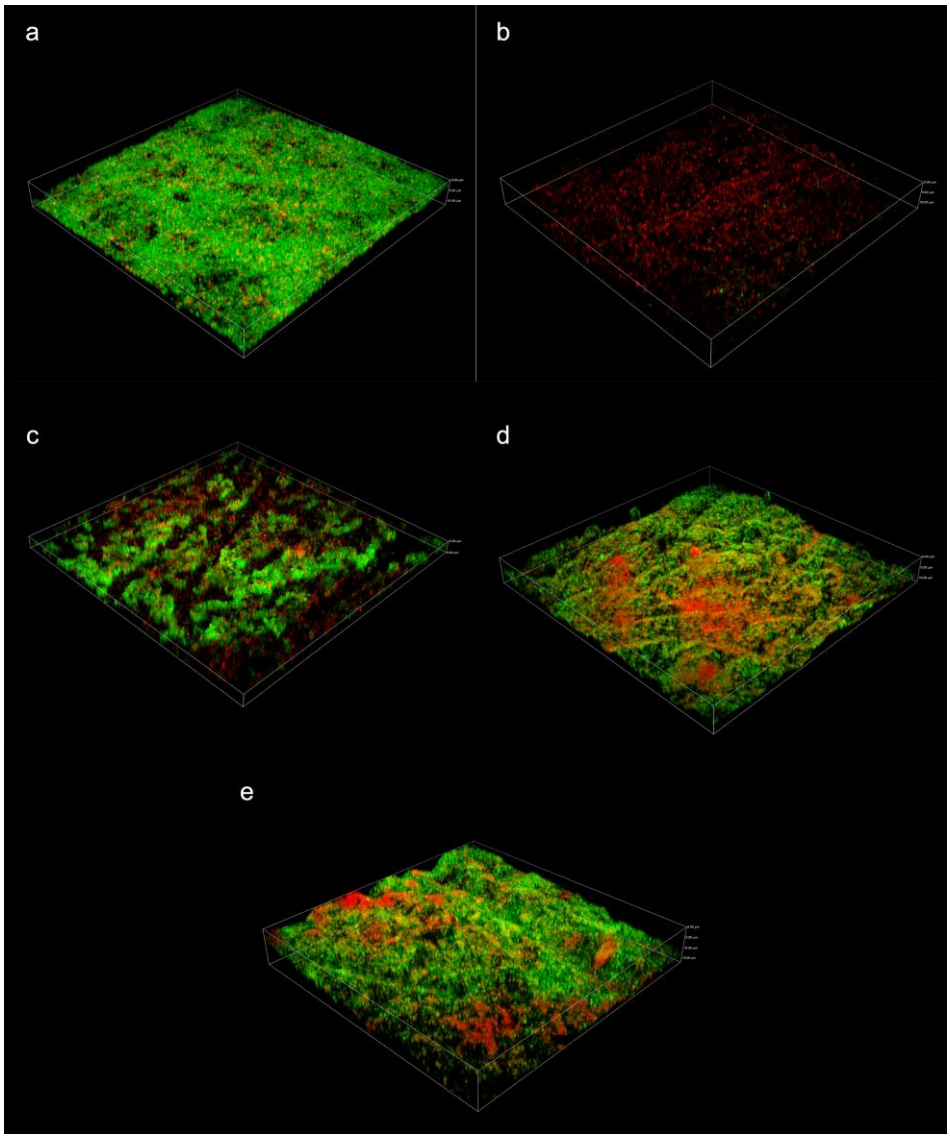


Figure 2. Representative LIVE/DEAD® staining images of biofilms created for testing the inhibition of the *E. faecalis* biofilm formation on the dentine surfaces, which were previously treated with PBS -control group- (a), Un-functionalized NPs (b), Dox-NPs (c), Zn-NPs (d) and Ca-NPs (e). Biofilms on control and Ca-NPs groups were mostly alive. Some damaged bacteria may be observed at biofilms on Zn-NPs groups. For Dox-NPs groups most of the bacteria in biofilms appeared injured and a low biovolume is observed. For Un-functionalized NPs group it was difficult to observe live cells in grown biofilms. Compromised bacteria were stained red and live bacteria were stained green. All images had the same magnification, each image represents an area of 387.5 × 387.5 μm.

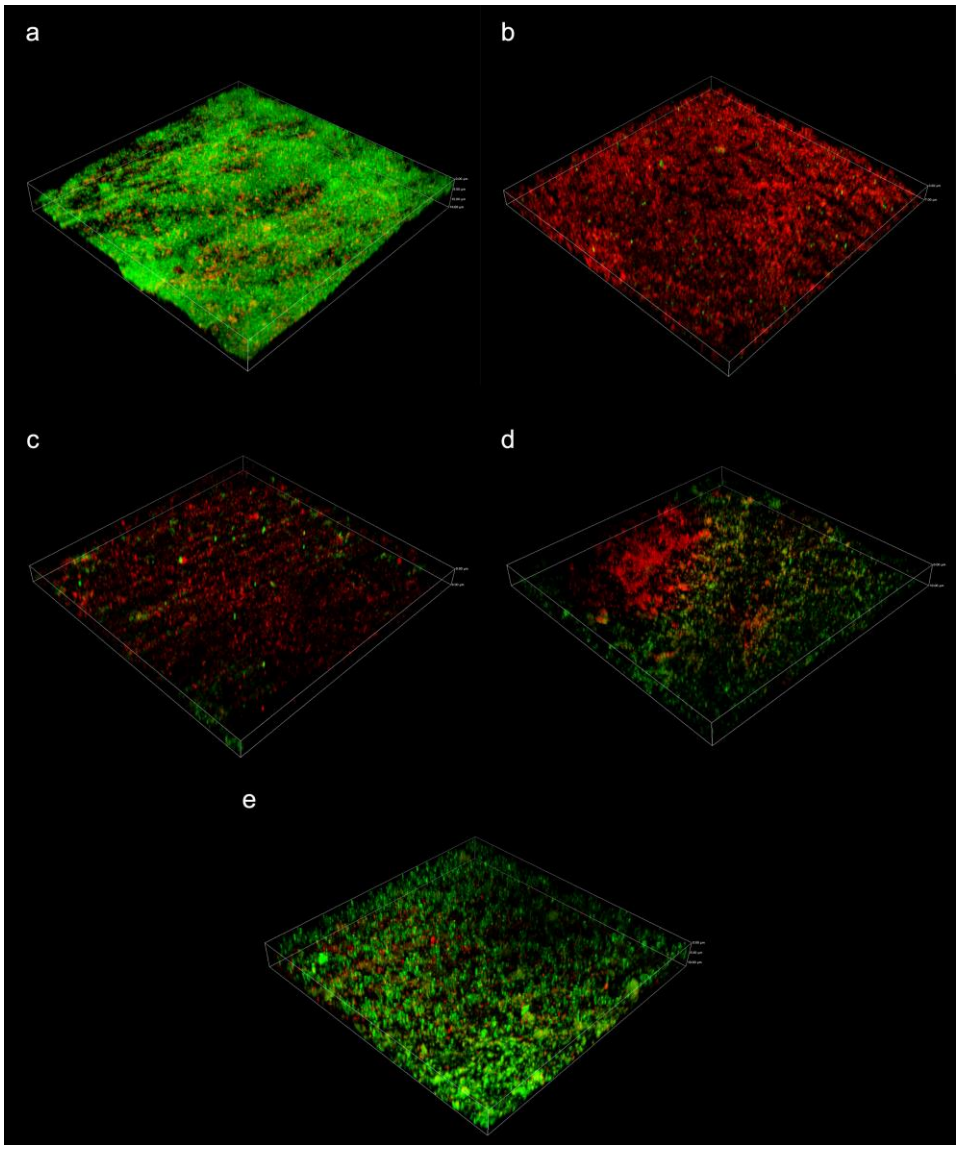
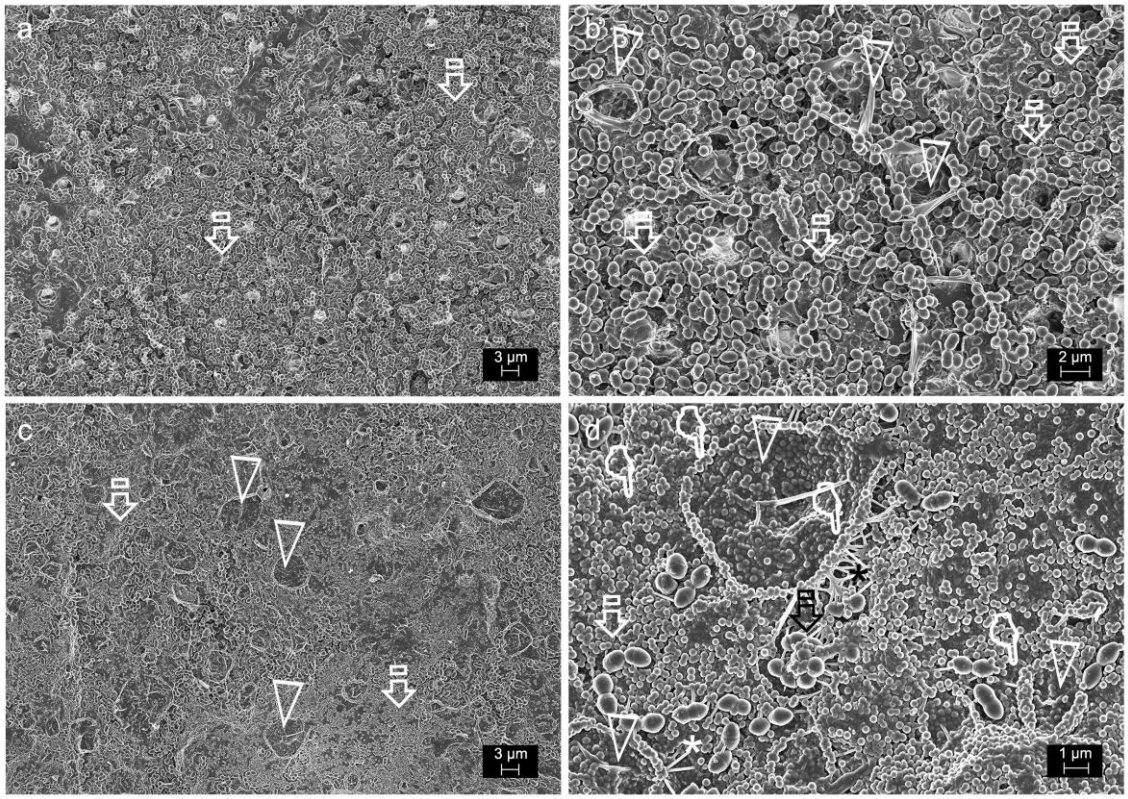

a
b
c
d
e

Figure 3. FESEM images showing antibacterial activity. The *E. faecalis* biofilm was grown on the dentine surface, and after was treated with the different NPs for 1 h. (a and b) A dense *E. faecalis* (arrow) biofilm was covering the dentine surface of the control group -PBS treated-specimens. EPS produced by bacteria was observable. At higher magnification (b), it may be seen how the EPS substance was preferentially placed around the dentinal tubules (open arrow), probably facilitating bacteria adherence and penetration through tubules. (c and d) Un-functionalized NPs were spread on the dentine surface and bacteria (arrow) may also be found. At higher magnification, it was observable how NPs (pointer) bind the produced EPS,NPs completely occluded the dentinal tubules (open arrows). Aggregation of bacteria within the biofilm seems to be interrupted, and bacteria presented elongations (asterisk) trying to connect each other. (e and f) At grown *E. faecalis* biofilms treated with Dox-NPs, some bacteria may also be seen (arrow), but they were partially covered by NPs (pointer), that were homogeneously distributed onto the surface. At higher magnification (f), it may be observed the intimate contact between NPs and bacteria membranes. Membranes alterations showing a rough appearance was also shown at the image. (g and h) correspond to *E. faecalis* biofilms treated with Zn-NPs. NPs (pointer) were homogeneously distributed onto the dentine surface and did not interact with bacteria`s membranes (arrow), however they facilitated biofilm disruption and at higher magnification, it was observable how bacteria connected each other by cytoplasmic elongations (asterisk). Some membranes alterations were present, conferring to bacteria a rough surface. Bacteria were mostly separated from Zn-NPs. (i and j) corresponded to biofilm treated with Ca-NPs, many bacteria were presented on the dentine surface (arrow), EPS was also evidenced at the image (open arrow). At higher magnification (j) Ca-NPs may be seen producing biofilm disruption. Ca-NPs (pointer) were not in contact to bacteria, which try to reach each other by elongations (asterisk) emerging from their membranes.



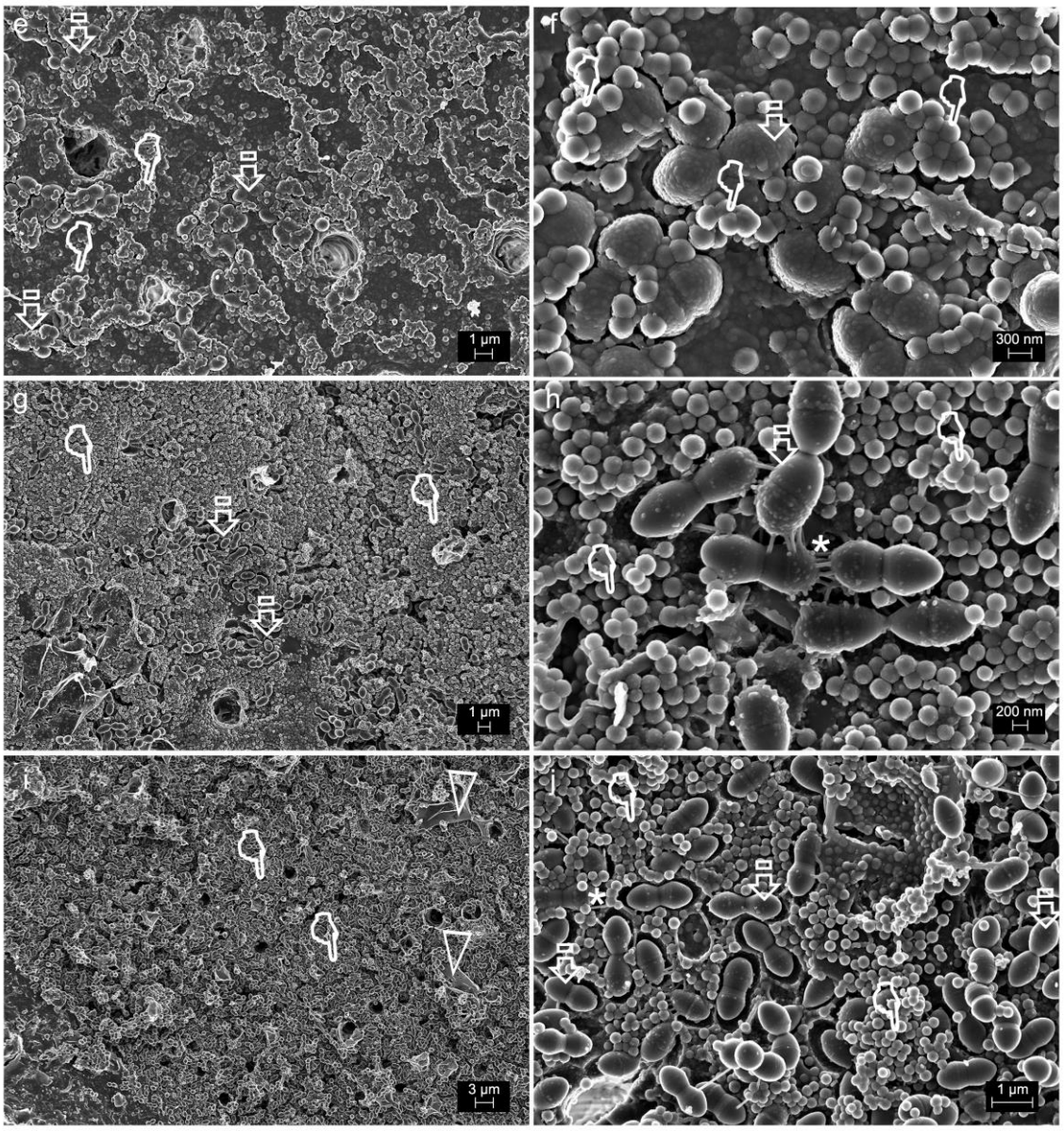

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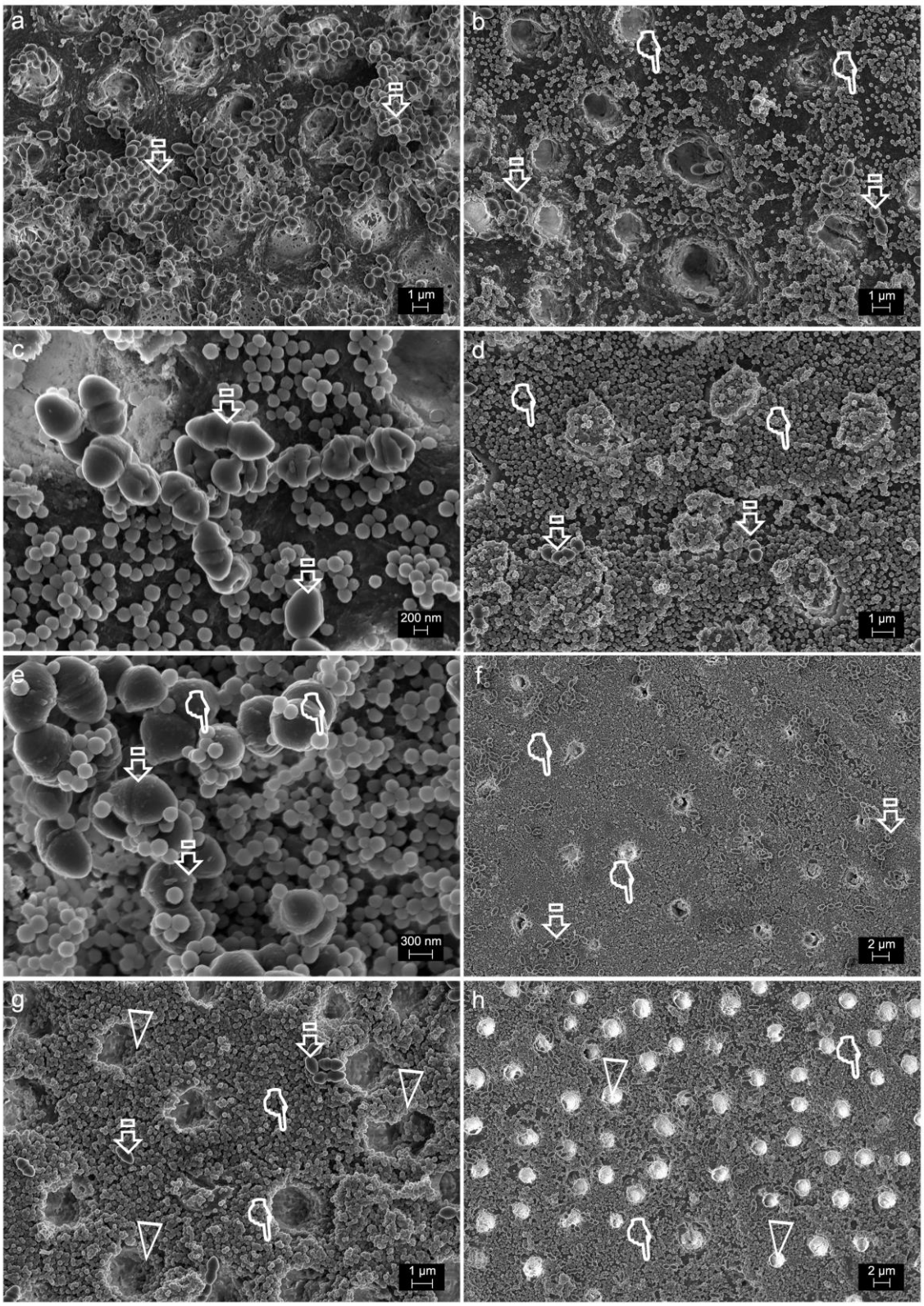
Figure 4. FESEM images showing inhibition of the *E. faecalis* biofilm formation on dentine surfaces that were previously covered with the different NPs (pointer). (a) *E. faecalis* biofilm development on the control dentine surface -PBS treated dentine- at the first stages loose physical association of the bacteria (arrow) with the surface was produced and binding activities of microbial cell surface adhesins to complementary receptors on demineralized collagen at the host surface may also occurred. (b) Un-functionalized NPs (pointer) were spread on the dentine surface, probably competing with *E. faecalis* for unmineralized collagen binding sites. As a result few bacteria (arrow) were observed on the surface. At a higher magnification (c), Aggregated cells (arrow) with deep membranes alterations can be observed. Most of the bacteria showed visible changes in morphology. (d) Dox-NPs (pointer) were completely covering the dentine surface and occluding dentinal tubules. Few bacteria (arrow) were observed. At higher magnification (e), tight contact of Dox-NPs (pointer) with *E. faecalis* (arrow) is shown at the image. (f and g) dentine surfaces completely covered by Zn-NPs (pointer) are shown, with non-aggregated bacteria (arrow). At higher magnification (g) Zn-NPs may be detected partially occluding dentinal tubules (open arrow). (h) Similar features were observed for dentine surfaces previously treated with Ca-NPs.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