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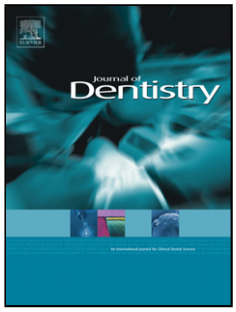

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Key words

dentine; sub-micron particles; surfactants; dentinal tubules; microscopy; surface coating

Abstract

(word count – 244)

Objectives: Sub-micron particles may assist in the delivery of compounds into dentine tubules. The surface interactions of the particles with dentine may prevent them from entering the tubules. The aim of this study is to investigate whether silica particles, treated with surfactants improves dentine tubules occlusion using both artificial and human tooth models

Methods: Spherical silica particles (size 130 to 810 nm) bearing an encapsulated ruthenium luminescent complex were coated with the following surfactants: Zonyl® FSA, Triton® X-100 and Tween20®. The particles were prepared as 0.004% w/v and 0.04%w/v solutions with deionized water and were applied to the surface of; (1) *in vitro* model of PET ThinCert cell culture inserts; (2) 0.1mm thick sections of human molar teeth.

Results: Scanning electron and confocal fluorescence microscopy images show that particles without any coating and with TritonX-100 coating had the highest aggregation. Particles with Tween-20 are less aggregated on the surface and show inclusion in the tubules. Particles coated with fluorosurfactant Zonyl show a preference for aggregation at the tubule. With the Thinsert membranes high aggregation within the artificial tubules was increased by particle concentration.

Conclusions: The use of silica sub-micron particles on hard dental tissues is dependent on the modification of the surface chemistry of both the particle and the dentine and the employment of the fluorosurfactant may improve tubule occlusion. The use of Thinserts membrane is useful *in vitro* model to mimic dentinal tubules and observe the ability of particles to occlude small channels.

Clinical Significance: The use of silica sub-micron particles on hard dentine tissues is dependent on the modification of the surface coating of the particles. This may influence how particles are incorporated in potential delivery vehicles applied to the dentine surface with the employment of a fluorosurfactant showing promise.

Introduction

Dentinal tubules are microscopic channels within the dentine structure, which traverse from the junction of the protective outer enamel to the internal pulp containing the soft tissues[1]. They are around 1 to 4 microns in width and formed via the odontoblast cells[2]. There is variation in both arrangement and width of the tubules depending on their position within the tooth[3]. Once the outer enamel structure is breached, exposure of dentine occurs and allows communication through the tubular space to the pulp underneath. This can lead to irreversible damage to the soft tissues of the tooth. Nano- and sub-micron sized particles offer the possibility of a multi-functional dental agent, by both delivering anti-pathogenic or analgesic drugs into the tubules combined with the ability to occlude of the internal space, thus helping to prevent infection or infiltration into the pulp[4-6]. Their size makes them an attractive vehicle for entering into the dentinal tubule and investigators have looked to different types of particles to undertake such a task. These include calcium fluoride[7], combinations of carbonate-hydroxyapatite nanocrystals[8-10] as well as bioactive glass[11-12]. The general aim of these studies is to increase mineral content of enamel and dentine, where the particle acts as a seed for further growth of crystalline structure leading to the closure of the tubules. One approach is that the sub-micron particles may be introduced into a scaffold of collagen and provide a structure for this growth and subsequent mineralisation[13]. Furthermore, antibacterial actives or compounds may be attached to the particles to inhibit or break up bacterial growth.

A problem with the use of such particles is that they are liable to aggregate[14], thus preventing them from entering a dentinal tubule. Methods of preventing this from occurring include adding surfactants to the particles. However, it is not known how the addition of surfactants influences the movement of particles on the dentine surface and into the tubules. Researchers have used mechanical forces such as cavitation form imploding bubbles to push particles into the dentinal tubules[15]. Observing such activity of the sub-micron particles may be problematical due to the complex surface of dentine and a simple *in vitro* model system may assist in observing how particles behave on a dentine like

surface.

We have chosen silica particles for their distinct advantages: availability in sizes ranging for nanometer to submicron without altering the particle surface and their porous structure, which can act as a container for molecules. Most studies involving silica particles in dental applications have concentrated in their property as a composite in biomaterials. We have previously introduced the influence of surfactants of metal complexes attached on the outside of the silica surface[16].

The aim of this research is to investigate the interaction of silica particles with human molar teeth sections, with a particular focus on entering the opening of dentine tubules. We have also used different surfactants as coatings for the particles to address their influence upon the surface interactions of the particle with the dentine surfaces. An alternative *in vitro* models has also been evaluated against dentine slices of human teeth to determine its suitability as a research tool.

Materials and methods

Tooth preparation

Human decay-free lower first permanent molar teeth, which were covered by the United Kingdom Human Tissue Act (HTA), were chosen for the purpose of assessing the entry of sub-micron particles into the tubules. The crown removed and the root sliced in half along the vertical axis using the bone saw. The sectioned roots were ground down using a grinder/polisher with 400/800 papers, to approximately 0.1mm thick sections measured by a micrometer. This method was used to produce sections with perpendicular tubule openings. Before imaging/nanoparticle application, the sections were etched by a 60 second immersion in a 10% citric acid solution. The sections were then ultrasonicated for 180 seconds. The sections were dehydrated using successive ethanol baths for ten-minute periods of 50%, 75% then 100% be-fore drying for 1 hour in a 60 degree oven.

Particle preparation

The particles used in this investigation consisted of spherical silica encapsulated with a luminescent complex, tris-(2,2'-bipyridyl) ruthenium(II) dichloride, represented as SiO_2-Ru[17], with a size of 640nm were designated as SiO_2-Ru-640 (640±90 nm). The effect of polymer coating did not change the size within the error limit of the particle size analysed. The sizes were determined by Dynamic Light Scattering measurement using a Zetasizer Nano ZS, (Malvern Instruments Ltd, Malvern, UK). Visualization of the particles was performed using Nanosight tracking Analysis instrument (Malvern Instruments Ltd, Malvern, UK). For confocal fluorescence imaging studies, three different sizes were used (particle concentration 0.004 % w/v). SiO_2-Ru-130 (130 ± 50 nm), SiO_2-Ru-430 (430 ± 50 nm), SiO_2-Ru-810 (810 ± 160nm) estimated by the confocal reflectance images. They were prepared as 0.004 % w/v solutions by addition of 50 ml deionised water to 2 mg particles (dry weight), followed by ultrasonication for 5 minutes. For surfactant coated nanoparticle preparations Zonyl® FSA, Triton® X-100 or Tween20® (purchased by Sigma Aldrich) were added to 0.04 % w/v solutions of the SiO_2-Ru-640. The 0.04 % w/v solution required addition of 10 ml deionized water to 4 mg

particles (dry weight), followed by ultrasonication for 5 minutes. Additions of the various surfactants were done on 10 ml aliquots of the 0.04 % w/v SiO_2-Ru-640. Preparations are therefore as follows:

1.SiO_2-Ru-640: Control sample – (no surfactant)

2. Zonyl- SiO_2-Ru-640: (a) 10ml - SiO_2-Ru + 10µl Zonyl FSA (1.3 g/mL) (b) 10ml - SiO_2-Ru + 100µl Zonyl FSA (1.3 g/mL) . The excess of Zonyl FSA in (b) was used to examine if this affected the particle interaction with dentine.

3. Tween20- SiO_2-Ru-640:10ml - SiO_2-Ru + 10µl Tween20 (1.095 g/mL)

4.Triton- SiO_2-Ru-640:10ml - SiO_2-Ru + 10µl TritonX – 100 (1.7 M)

After addition of the surfactants, 1 ml aliquots were transferred to Eppendorfs centrifuge tubes and centrifuged at 6000 rpm for 6 minutes, the supernatant discarded and the particles re-suspended in deionized water. This wash step was done to remove excess surfactant.

The photophysical properties of the luminescent particles were analyzed using an Edinburgh Instruments FLS920 Series fluorescence spectrometer.

Application of particles to dentine

The particles were dropped on to the tooth surface with no mechanical agitation taking place. For the SiO_2-Ru-640, Zonyl- SiO_2-Ru-640, Tween20-SiO_2-Ru-640 and the Triton-SiO_2-Ru-640 10µL of the supplied NP solution, was pipetted on to the dentine surface and uniformly spread using the pipette tip. The surface was left to dry completely and then subsequently washed three-times with 10 µL of de-ionised water by pipetting it on to the surface and aspirating it off. Dentine samples were then prepared for imaging.

Imaging of particles

All of the samples were prepared by mounting onto a carbon coated aluminum stub before splutter coating with gold using a Quorum Emitech K550X splutter coater. A scanning electron microscope,

SEM (Zeiss EVO MA 10) was used under high vacuum and at a range of electron acceleration voltages between 5 and 15 eV.

The confocal microscopy studies were performed using a Leica confocal microscope in reflectance and fluorescence modes. Excitation was at 488nm at approximately 20% power and at 458, 476 and 488nm at 100% power, with emission collection at 478-498nm and 580-800 nm, respectively. The samples were mounted on 12 mm diameter carbon stubs (used in SEM) and were attached to a glass slide.

Thinsert preparation and imaging

Polyethylene terephthalate (PET) ThinCert cell culture inserts, normally used for cell tissue work, were selected with a pore size of approximately 3 micrometers (3μm) in diameter. They consisted of a polystyrene housing containing a PET membrane. Prior to application of the particles, one batch of control membranes was not splutter coated; the second batch had one coat of gold applied and the third batch had two coats of gold applied. The gold splutter coating deposition was for 2 minutes delivering a thickness of 15nm. Sub-micron particles were applied to the surface of each batch of membranes with no mechanical agitation taking place. The membranes and particles were splutter coated before SEM imaging in a similar manner to the dentine surfaces.

Results

When applied directly to the molar root dentine surfaces that had been acid etched with 10% citric acid for 60 seconds to simulate hypersensitive dentine, SEM examination revealed unique interactions of the particles with the dentine surface. The 0.04 % w/v SiO_2-Ru -640 particles formed large aggregates of several micrometers in diameter clustered on top of each other (Figure 1A). Although these clusters are large enough to potentially occlude a tubule opening it seemed that most of the particles attached to the area surrounding the tubule periphery (Figure 1A*). Partial occlusion is thus only achieved in a small minority of the tubules where small clusters or single particles have been able to enter.

The Triton® X-100 coated particles, TritonSiO_2-Ru -640, show similar interactions with the dentine surface section as the SiO2-Ru -640 (no surfactant); however particle aggregation occurred to a lesser degree (Figure 1B). Nevertheless, the particle clusters that formed were still too large to occlude the tubule openings and instead were restricted to the periphery of the tubule opening. The few tubules that were partially occluded to a small degree, were blocked with small clusters or single s (Figure 1B*, 1B**).

Tween20-SiO_2-Ru-640 particles were markedly less aggregated on the acid etched dentine surface with clusters qualitatively smaller than the SiO_2-Ru -640 or the Triton100-SiO_2-Ru -640 preparations (Figure 1C). With these smaller clusters it is perhaps easier for the particles/clusters to at least partially occlude the tubules (Figure 1C*). This is particular relevant when compared to the SiO_2-Ru-640 particles (Figure 1A), where large clusters are potentially preventing tubule occlusion by particles/small clusters by attaching to and blocking the area surrounding the tubule periphery.

With regards to the Zonyl-SiO_2-Ru-640 preparation, Zonyl is a relatively hydrophobic surfactant, bearing polar fluorine groups. This may explain why Zonyl-SiO_2-Ru-640 particles were observed as

fairly large aggregated clusters that seem to have an affinity for the tubule openings (Figure 2). The particles were placed on dry dentine and further research on how moisture affects the clustering is required. The physico-chemical properties of the particles, in this instance, may be such that these clusters of particles prefer attachment within the tubule as opposed to the tubule periphery, as in the case of the SiO_2-Ru -640 and the Triton-SiO_2-Ru -600. Furthermore, the clusters seem to be of a suitable size to occlude the majority of the tubule openings.

The silica particles exhibited red luminescence based on the properties of the encapsulated ruthenium bipyridyl complex (Figure 3). We carried out spectroscopic studies to quantify the effects of the surfactants on the luminescence signal. The experiment was carried out by aliquoting silica particles into 1 ml samples, followed by taking emission scans before and after the addition of the surfactants. This approach accounts for any small but significant variations in the concentration of the particles in the stock solution. The luminescence spectra are accompanied by excitation spectra (Figure 3).

The samples were excited with visible light at 450 nm, where the characteristic absorption of the ruthenium complex occurs based on a metal to ligand charge transfer transition. They luminesce in the red region of the spectrum with a peak around 615 nm. The luminescence is sensitive to the environment, and it clearly showed a blue shift upon coating of the particles with the Zonyl surfactant. The emission studies showed that for all samples of SiO_2-Ru particles, the effect of the surfactant was most pronounced for the Zonyl coating leading to particles with the highest luminescence and a 5 nm blue shift on the emission maximum. The effect of Zonyl is highest when excess of Zonyl was added (sample2b). The excitation spectra confirmed that the emission originates from the ruthenium core.

The red luminescent particles SiO_2-Ru -130, SiO_2-Ru -430and SiO_2-Ru -810 (0.004 % w/v) can also be observed interacting with molar root sections with confocal luminescence microscopy, utilising the luminescence of the encapsulated ruthenium. Overlays of the red luminescence channel and the

reflectance channel ($\lambda_{exc/em}$ = 488 nm) of the dentine surface, demonstrated nanoparticle association with the surface (Figure 4). However, due to the resolution limit of confocal microscopy, only the larger SiO_2-Ru -810 were clearly resolved, and were readily observed as clusters occluding multiple tubules (Figure 4).

To further characterize the particle-surface interactions, Zonyl coated particles were applied to plain and gold splutter-coated polyethylene terephthalate (PET) micropore sheets (Figure 5).

A sample of Zonyl-SiO_2-Ru (sample 2b) was applied to "thinserts" in a similar manner. Three thinsert samples were used. The control sample was an unaltered PET thinsert, the second sample had one splutter coating of gold, and the third sample had two consecutive splutter-coatings of gold before the particles were applied. The most interesting and noticeable result of this experiment is that nanoparticle aggregation is clearly surface-dependent. Aggregation between particles is a well-documented phenomenon that is thought to be mainly driven by Van der Vaals forces, and exaggerated by a large surface-to-volume ratio. The repulsive forces between polar surfactant-coated particles and charged ions are overcome in favor of the attractive Van der Vaals forces on the PET surface, resulting in obvious aggregation. It is also possible, given the chemical structure of PET, that delta negative charges on the surface repel the delta negative oxygen atoms of the silica shell. On the gold coated polymer surface, surfactant-coated particles adsorb more evenly onto the surface, presumably driven by electrostatic forces. The same particles used in all three gold coated PET samples, were also used on dentine. It is notable that the gold-coated PET membranes bound the particles similar to how the particles bound to dentine. This could be useful for future experiments, but could also be an indicator that similar strength forces are involved. Overall, the particles showed distinct aggregation on the plain PET micropores, and strong surface interaction on the gold-coated version.

Discussion

There is much interest in the use of sub-micron particles in dentistry and generally these particles

may be used as a method to remineralise dentine. There have been nanosized particles of different materials introduced into the dentinal tubules and these include calcium fluoride, hydroxyapatite and bioactive glass (8, 20, 21). All these approaches use the particles to initiate a scaffold for the remineralisaiton of the dentine. The silica particles used in this project have a porous structure and therefore have the potential to be a carrier for a range of molecules including calcium based compounds. These particles may also serve as a carrier for antimicrobial compounds and therefore offer a novel approach to treating contaminated hard tissues (16,17).

Previous work has shown the ability of particles to infiltrate dentinal tubules although the difficulty of preventing the particles from aggregating due to interaction with each other is a challenge (11). Previous work has suggested that surfactants may prevent this from occurring (16) although this was not conducted in a biological situation.

In this study, when the particles with surfactant coatings were applied to the flat dentine surfaces, differences in the aggregation were observed with SEM. The no surfactant and Triton X-100 treated preparations resulted in the greatest aggregation of the particles with little surface spread or occlusion of the tubules. The dentinal tubule occlusion was greater in the Tween20 preparation, but was most readily observed with the Zonyl preparation, possibly due to the physico-chemical properties of the sub-micron particles when coated with the relatively hydrophobic surfactant containing polar fluorine groups. The properties of this preparation are such that the particles achieve suitable interaction with the surface and clustered to achieve optimum tubule occlusion, compared to the other preparations investigated. The use of luminescent particles also provides an avenue for the use of luminescence imaging to investigate their use on dental surfaces (17).

There may be other factors involved in nanoparticle aggregation, mainly local concentration and surface morphology. However these observations merit further research to establish the extent of

influence the surfactant has with respect to the chemistry, bonding and other interactions. More research should be directed at optimizing the entry of these particles for occluding dentine tubules. If these particles are able to occlude the tubule opening, their novel structure will allow active compounds to be bound onto the particle. Such an approach may be useful in the topical treatment of dentine sensitivity or the treatment of carious dentine.

The particles also showed distinct aggregation on the plain PET micropores and strong surface interaction on the gold-coated PET membranes, again revealing the importance of the surface chemistry of both the nanoparticle, and the surface to which they are binding. Therefore, the occlusion of the dentinal tubules may be assisted by the use of surface chemistry modification prior to delivery of the sub-micron particles.

For the use of particles to occlude for example hypersensitive dentine, the interactions of the particles with the dentine surface present a paradox. Electrostatic interactions of the particles with dentine surfaces are a requirement to keep the particles on the tooth surface, but they may prove an obstacle when attempting to deliver the particles deeper into the tubules. It may be that other techniques are required to overcome the surface attractive forces and move the particles to a deeper position in the tubules.

These studies also demonstrated the use of PET thinserts as potential *in vitro* dentine models for observing the activity of sub-micron particles. Human teeth are difficult to procure and require a Human Tissue Act (HTA) license for handling, as well as inherent variations between individuals. Part of this project involved preliminary examinations of PET thinserts as a possible model for dentine. The thinserts are semi-transparent capillary pore membranes produced from polyethylene terephthalate (PET) from which they derive their name. The average tubule diameter for the openings of the dentinal tubules was measured as 2.3μm (Standard deviation: ± 0.50μm). For the Thinserts the

average diameter of the pore was calculated at 2.17μm (Standard deviation: ± 0.23μm), and the percentage of the total area covered by the pores was 3.0% which is similar to that of superficial dentine[18]. In Pashley (1996), the area of dentine occupied by the tubules is 2.85-3.96% in mid-coronal dentine[18]. It is only 0.96% in superficial dentine. So the tubules, which are in dentine vs. PET membrane, are similar. These values are unsurprising, given the gross appearance of the dentine in comparison to the thinserts. Summing up the similarities and differences, the channel openings are very similar in diameter although the thinserts are less variable as shown by the standard deviations. The area in the thinserts occupied by pores is similar to the area of superficial dentine occupied by the dentinal tubules[18]. The other difference is the pattern of arrangement of the pores which is more random than the dentine. However, these images do suggest that the thinserts may be useful as a dentine model.

This study showed distinct aggregation of the sub-micron particles on plain PET micropore membranes and strong surface interaction on the gold-coated version, revealing the importance of the surface chemistry of both the nanoparticle and the surface to which they are binding. Silica particles offer the possibility of different sizes, coating and inclusion of therapeutic agents and their use on hard dental tissues will be dependent on the modification of the surface chemistry of the dentine prior to drug delivery.

There are several limitations with this work. The deposition of the submicron particles may affect subsequent bonding systems ability to attach to the dentine. The potential effect on bond strength to dentine is an area of future investigation. Furthermore silica absorbs water from the dentine and this in itself could potential cause post-operative discomfort if the particles are to be used as an anti-hypersensitivity agent. Again this will be investigated in further studies.

Conclusion

The use of silica sub-micron particles on hard dentine tissues is dependent on the modification of the surface coating of the particles. The hydrophobic Zonyl surfactant coating appeared to allow the most penetration of particles into the tubules. The use of a Thinsert membrane provides a useful *in vitro* model to mimic dentinal tubules and observe the ability of particles to occlude small channels.

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Figure 1. SEM images of SiO_2-Ru-640 (A) particles suspended in: Triton-SiO_2-Ru -640 (B), Tween20- SiO_2-Ru -640 (C) applied to acid etched root surface sections. (Scale bar 10 µM).

Concentrations:

Triton- SiO2-Ru-640:10ml - SiO2-Ru + 10□l TritonX – 100 (1.7 M),

Tween20- SiO2-Ru-640:10ml - SiO2-Ru + 10µl Tween20 (1.095 g/mL)

Figure 2. SEM image of Zonyl-SiO_2-Ru -640 (and corresponding magnifications) applied to molar root surface sections. (Scale bar 20 µM).

Concentration: Zonyl- SiO2-Ru-640: 10ml - SiO2-Ru + 100µl Zonyl FSA (1.3 g/mL)

Figure 3. (a) Schematic diagram of silica particles with encapsulated luminescent ruthenium probe SiO_2-Ru and surfactant coating.

(b) Excitation (left) and emission (right) spectra of the silica particles coated with different surfactants, λ_{exc} = 450 nm, λ_{em} = 620 nm.

Figure 4. Confocal luminescence and reflection microscopy image overlays (and corresponding magnifications) of SiO2-Ru-130 (A), SiO2-Ru-640 (B) and SiO2-Ru-810 (C) on the molar root surface. (Magnified image, scale bar 2 µM).

Figure 5. SEM images of Zonyl-SiO2-Ru-640 applied to PET Thinserts (A) uncoated, (B) with a single gold splutter Coat, (C) with two gold splutter coats. (Scale bar 20 µM).

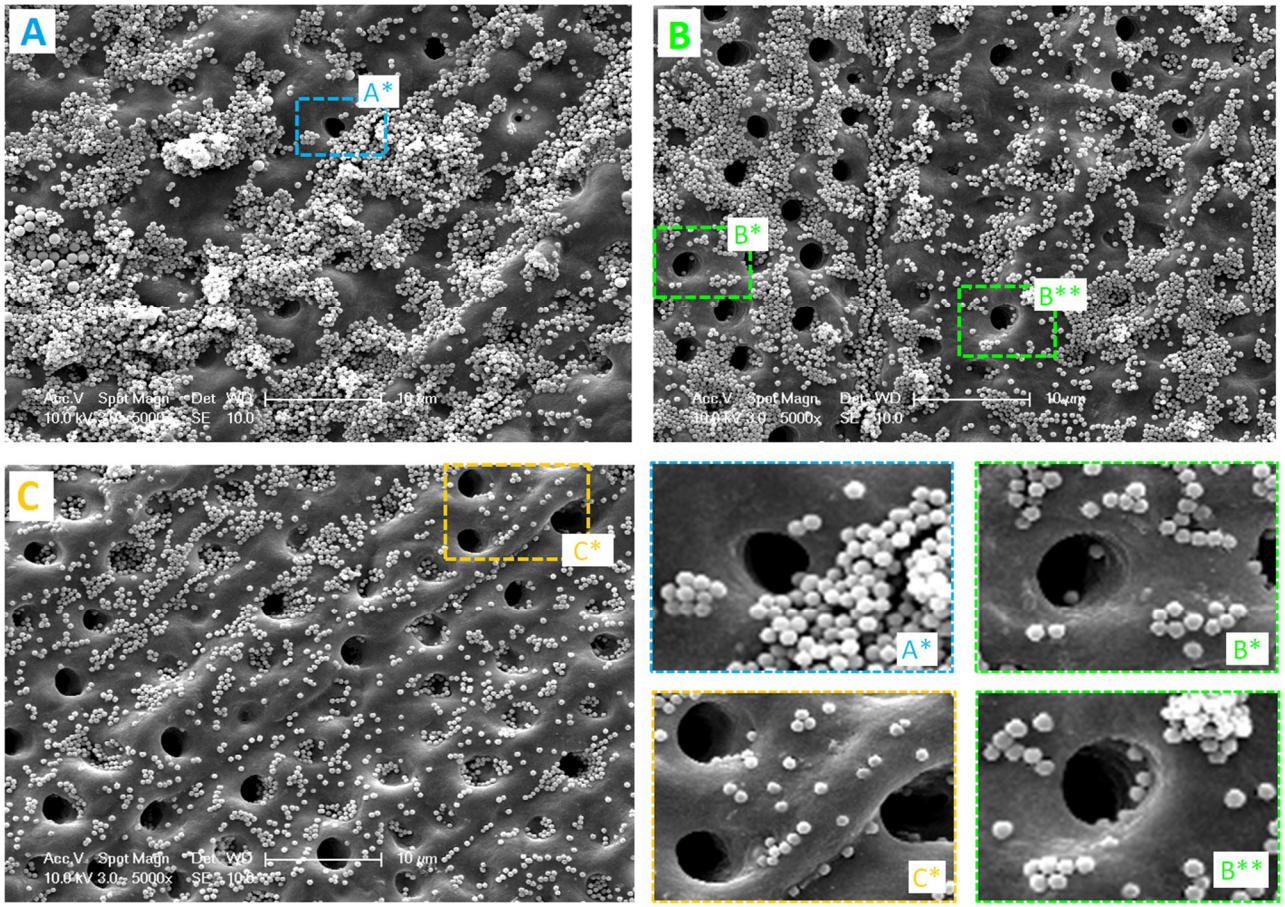

Fig. 1

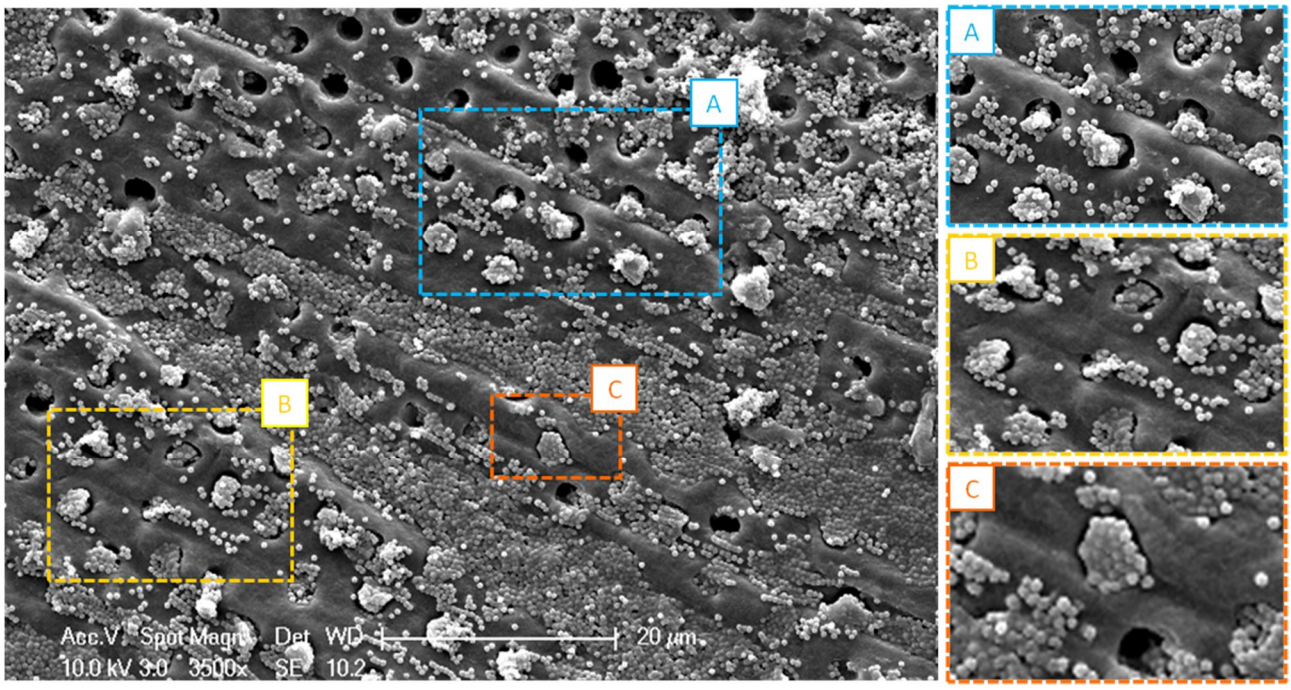

Fig. 2

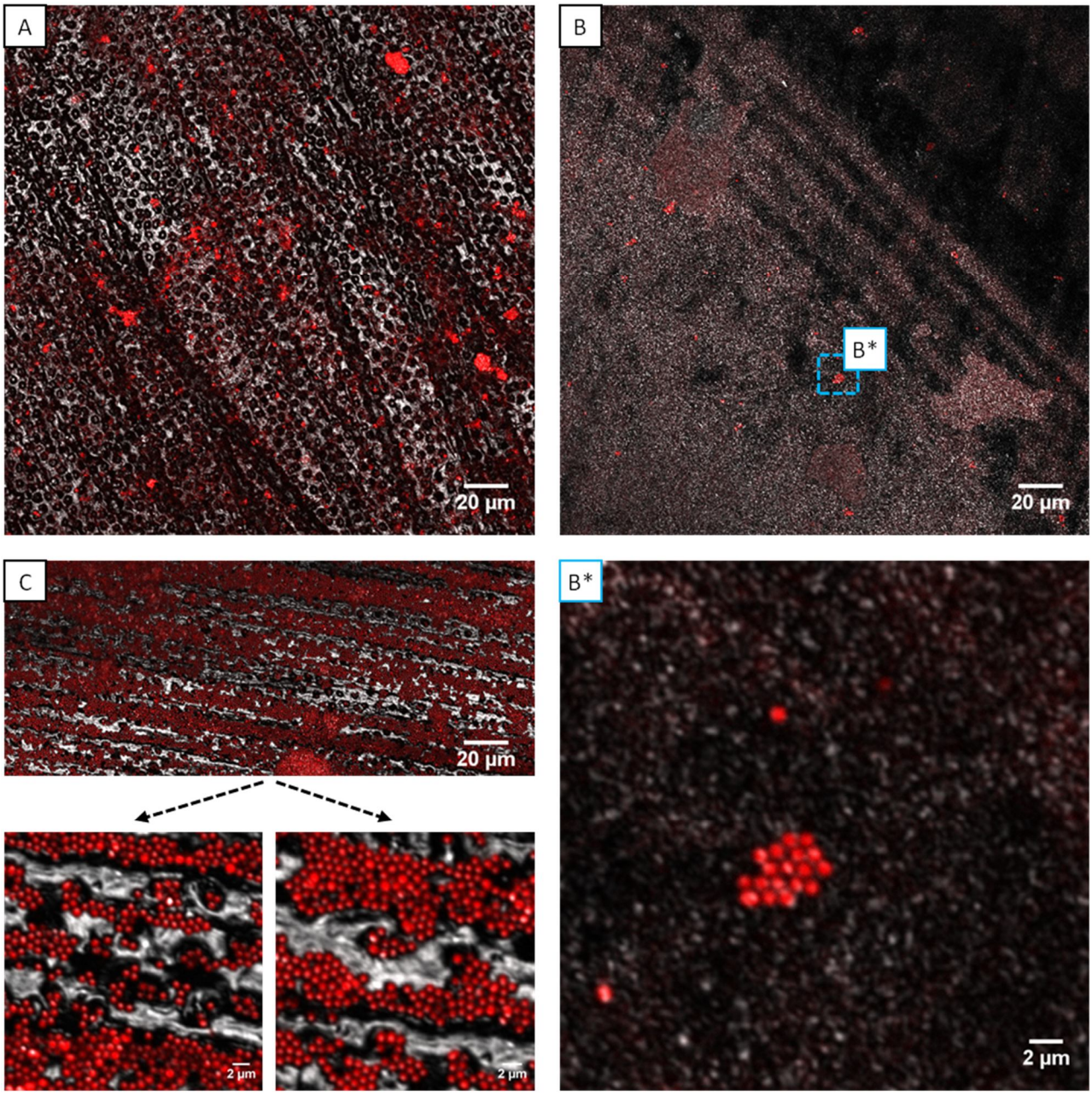

Fig. 4

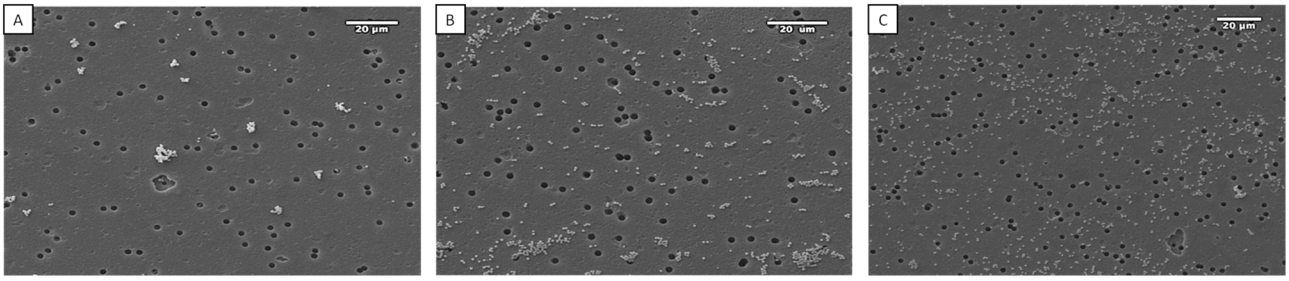

Fig. 5