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Characterisation of the antibacterial effect of polyethyleneimine nanoparticles in relation to particle distribution in resin composite

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ABSTRACT

Objectives: To characterise the antibacterial effect of resin composite incorporating cross-linked quaternised polyethyleneimine (QPEI) nanoparticles in relation to their distribution in the bulk material.

Methods: The antibacterial effect of resin composite incorporating QPEI nanoparticle was tested against various oral pathogens, including *Enterococcus faecalis*, *Streptococcus mutans*, *Actinomyces viscousus*, *Lactobacilus casei* and whole saliva. Nanoparticle distribution in the modified resin composite was assessed using X-ray photoelectron spectroscopy (XPS). Additionally, the degree of conversion was recorded.

Results: Total bacterial inhibition was detected against all the tested pathogens following direct contact with the outer surface of the modified resin composite. Similarly, the inner surface of the modified resin composite caused total inhibition. Electron microscope images showed bacterial death. XPS revealed surface I^- ions on both the outer and the inner surfaces of the modified composite. No I^- ions were detected in the unmodified composite. Nanoparticle distribution was higher on the inner surface of the modified composite. The composite's degree of conversion was unaffected by nanoparticle addition.

Clinical significance: QPEI nanoparticles represent a new generation of antibacterial nanoparticles which are highly promising in preventing bacterial recontamination when restoring teeth.

1. Introduction

In dentistry, surface coating with antibacterial materials is challenging, as the oral environment offers harsh conditions that may result in detachment or wear of the coating. Thus, enhancement owing to antibacterial properties through full material modification may be more durable.

Studies have shown higher bacterial accumulation on resin composites relative to other materials such as amalgam and glass ionomer.[1,2] Resin composite materials are widely used in tooth restoration, core buildup and in cementation due to their benefits, including aesthetics and adhesion to tooth structure.[3] However, the main drawback of resin composite-based materials is marginal leakage, which may result in secondary caries formation.[4]

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In previous studies it was shown that incorporation of antibacterial quaternised polyethyleneimine (QPEI) nanoparticles in resin composites results in a potent and long-lasting antibacterial effect. The antibacterial compound is stable and does not leach out from the material into the surrounding environment.[5–7] To provide antibacterial properties, QPEI nanoparticles were incorporated into resin composites. These particles most probably disrupt the passage of ions through the bacterial membranes, leading to membrane destruction and death.[5,8,9]

Particle surface area size is an essential component in nanoscale materials.[10] Specifically, particle surface area has a critical role when nanoparticles are used as antibacterial agents. As particle size is reduced, the proportion of the atoms found on the surface interface is enhanced relative to the proportion of the particle volume. This results in nanoscale particles, which are likely to be more reactive than microscale particles, thus generating a more efficient antibacterial effect upon application.[11,12] Unfortunately, alteration of the material surface properties using nanoparticles may be compromised because of the high tendency of the nanoparticles to aggregate,[5] resulting in a less effective surface antibacterial effect.[13] However, to generate an efficient effect, their mode of distribution in the bulk material is critical.[2–6,12]

The purpose of the present study was to characterise the antibacterial activity of QPEI against oral pathogens when incorporated in a resin composite material, in relation to the distribution of the nanoparticles in the bulk material.

2. Materials and methods

2.1. Test materials

Synthesis was as previously described.[7] Polyethylenimine dissolved in ethanol was reacted with dibromopentane under reflux for 24 h. N-alkylation was conducted using octyl. Alkylation was carried out under reflux for 48 h, followed by neutralisation with sodium bicarbonate for an additional 24 h under the same conditions. N-methylation was conducted using methyl iodide. Methylation was continued at 42 °C for 48 h, followed by neutralisation with sodium bicarbonate for an additional 24 h. The supernatant obtained was decanted and precipitated in double distilled water (DDW), washed with hexane and DDW and then freeze-dried. The average yield was ≥85% (mol/mol). Then the particles were washed with a 2% solution of N-lauryl-sarcosine surfactant (NLS). A total 20 g of prepared QPEI nanoparticles was placed in a Buchner funnel, using a paper filter and a vacuum source. A 200 ml volume of NLS solution was allowed to pass through the nanoparticles under vacuum conditions. Treated nanoparticles were freeze-dried overnight and a fine powder was obtained.

QPEI nanoparticles were incorporated in resin composite (3M™ ESPE™ Filtek™ Supreme XTE Flowable Restorative, St. Paul, MN, USA). Material curing was preformed according to the manufacturer's instructions.

2.2. Direct contact test (DCT)

The antibacterial effect was evaluated against various oral pathogens (the bacterial species are summarised in Table 1) and against whole saliva bacteria (as approved by the Helsinki Committee for Human Clinical Trials-HMO052511). Bacteria were grown to 10^6 colony forming units (CFU)/ml in brain heart infusion (BHI). The antibacterial effect of modified resin composites incorporating QPEI nanoparticles was tested using the direct contact test.[14] Briefly, triplicate wells in a 96-well microtiter plate were coated with resin composite incorporating QPEI nanoparticles (0%, 1% and 2% wt/wt). The plate was then aged by adding to each well 250 μl PBS, which were replaced every 48 h for 1 month at 37 °C. At the end of the ageing period the plate was dried under sterile conditions and 10 μl of tested bacterial suspension were placed on the surface of each test group to allow direct contact. After an hour, growth medium was added to each well and the plate was placed in a spectrophotometer for 24 h. Optical density readings in each well were recorded continuously every 20 min, with a 5 s mix before each reading. Data were analysed using Kruskal-Wallis One Way Analysis of Variance on Ranks.[15]

Table 1 – Bacterial strains used in the direct contact test.

Microorganisms	Source	Comments
Enterococcus faecalis	Clinically isolated at the Maurice and Gabriela Goldschleger School of Dental Medicine, Tel Aviv University, Israel	Streptomycin-resistant OD_{650} = 1 CFU/ml = 10^6
Streptococcus mutans	ATCC 700610	OD_{650} = ~1 CFU/ml = 10^6
Actinomyces viscousus	ATCC43146	
Lactobacillus casei	ATCC334	
From saliva	Clinical isolate	OD_{650} = 0.3 CFU/ml = 10^6

2.3. Inner and outer surface antibacterial effect

Discs (4.5 mm diameter × 4 mm height) incorporating 0% or 2% wt/wt QPEI nanoparticles were prepared using a silicone template. The discs were then cut in the middle, using a sterile scalpel. The test groups included: #1 – no added nanoparticles (0%, outer surface); #2 – no added nanoparticles (0%, inner surface); #3 – added nanoparticles (2%, outer surface); #4 – added nanoparticles (2%, inner surface).

The discs were inserted in a 24-well microtiter plate (flat bottom plate, Nunclon, Nunc, Denmark) and an *Enterococcous faecalis* (*E. faecalis*) suspension (10 μl; $OD_{650\ nm}$ of 0.5 ≈ 10^5 CFU/ml) was placed on the surface of each disc to test the antibacterial effect of the inner or outer surface of the material. The plate was incubated for 1 h at 37 °C. During this period the suspension liquid evaporated and a thin layer of bacteria was obtained, ensuring direct contact between all the bacteria and the tested surface. *E. faecalis* placed on the surface of the microtiter plate served as control.

After incubation, 1 ml of BHI was added to each well and the microtiter plate was placed on a titermix for 5 min (450 RPM). A 660 μl volume was transferred from each well to a fresh 96-microtiter plate and divided between three wells (220 μl in each well). The plate was then placed in a temperature-controlled microplate spectrophotometer

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(VERSAmax, Molecular Devices Corporation, CA, USA), at 37 °C with 5 s vortex mixing before each reading. Bacterial growth was estimated by following changes in $OD_{650\ nm}$ in each well every 20 min for 14 h. Each point on the curve is the average absorbance measured simultaneously in eight wells in the same microtiter plate.

ANOVA was used for statistical analysis, followed by the Tukey test as non-parametric tests showed similar results.[15]

2.4. Electronic microscopy

An *E. faecalis* suspension (1 ml; $OD_{650\ nm}$ of $1 \approx 10^8$ CFU/ml) was placed in a glass tube containing 9 ml DDW or in a tube containing 9 ml DDW with 5 mg/ml QPEI nanoparticles. The tubes were placed on a titermix in a 37 °C incubator for 1 h. The specimens were then centrifuged and the pellet was fixed with formaldehyde, gluteraldehyde, and osmium tetraoxide in cacodylate buffer, followed by dehydration with a graded ethanol and Freon series, and coated with gold. Specimens were observed using a high-resolution scanning electron microscope (SEM) at magnifications of 20,000× and 40,000×. Similar samples were observed using Transmission electron microscopy (TEM). The samples were fixed in Karnovsky's Fixative after 30 min incubation in a 37 °C incubator. Post-fixation was carried out in osmium tetraoxide. Specimens were dehydrated in graded ethanol and embedded in epoxy resin. Then 70 nm thin sections were contrasted with uranyl acetate and lead citrate. The slices were examined at magnifications of 40,000× and 15,000×.

2.5. Nanoparticles distribution in modified resin composite

Resin composite discs were prepared as described above and analysed using X-ray photoelectron spectra (XPS). The XPS were recorded using a Kratos Axis Ultra spectrometer (Kratos Analytical Ltd., Manchester, UK), with an Al Kα monochromatic radiation X-ray source (1486.7 eV). The emission current was set at 15 mA and the anode high voltage at 15 kV.

All XPS spectra were collected with a take-off angle of 90° (normal to analyser), the vacuum condition in the chamber was 1.9×10^{-9} Torr. The survey XPS spectra were acquired with pass energy of 160 eV and 1 eV step size. High-resolution spectra were collected for C 1s, O 1s, Si 2p, Zr 3d and I 3d levels, with a pass energy of 20 eV and 0.1 ev step size. The binding energies were calibrated using C 1s peak energy as 285.0 eV.[16] The collected data were analysed with a Casa XPS (Casa Software Ltd., Center for Nanoscience and Nanotechnology, The Hebrew University of Jerusalem, Israel) and a Vision Data processing program (Kratos Analytical Ltd., Center for Nanoscience and Nanotechnology, The Hebrew University of Jerusalem, Israel).

I^- ions were selected as indicators of the presence of the QPEI nanoparticles due to the fact that I^- ions are found solely in the QPEI nanoparticles, and not in the resin composite.

2.6. Degree of conversion (%DC) of modified resin composite

The number of double carbon links present in the monomers, which are converted into single links to form the polymeric chain during the polymerisation process, is called degree of conversion. Monomer conversion of the double carbon–carbon bonds was tested using a Fourier Transform Infra-Red (FTIR) spectrometer with an Attenuated Total Refraction (ATR) device. Resin composite samples with QPEI concentrations of 0%, and 2% w/w were prepared as above. A thin layer of approximately 1.5 mm was placed on top of the ATR device of the FTIR spectrometer (Nicolet™ iS™10 FT-IR Spectrometer, Thermo Electron Scientific LLC.,

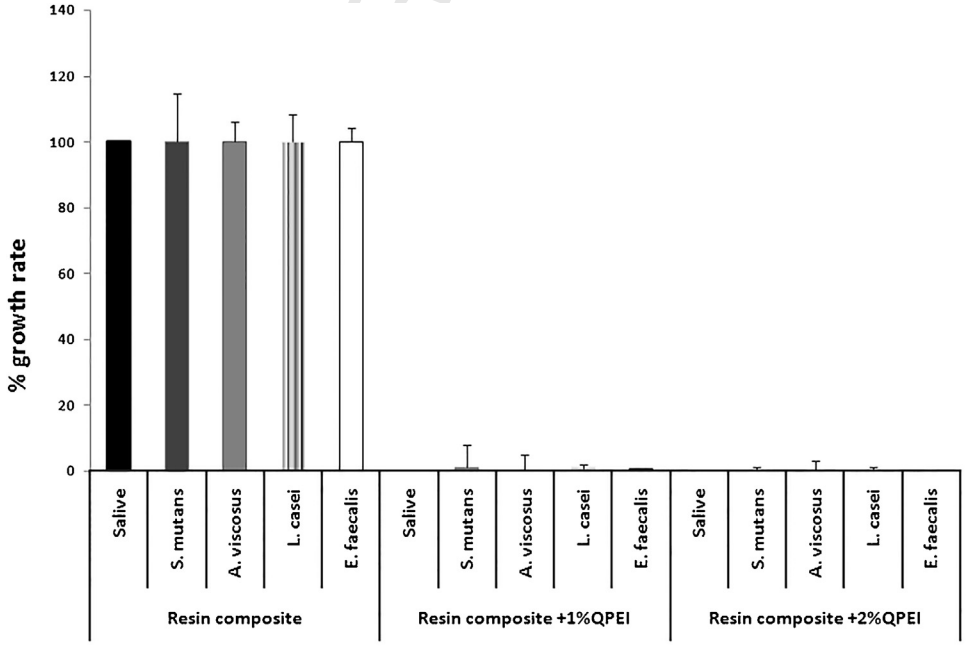

Fig. 1 – Antibacterial activity of modified resin composites incorporating QPEI nanoparticles. Microorganism growth was evaluated in the presence of resin composite incorporating 1% or 2% wt/wt QPEI nanoparticles after 1 month using DCT.

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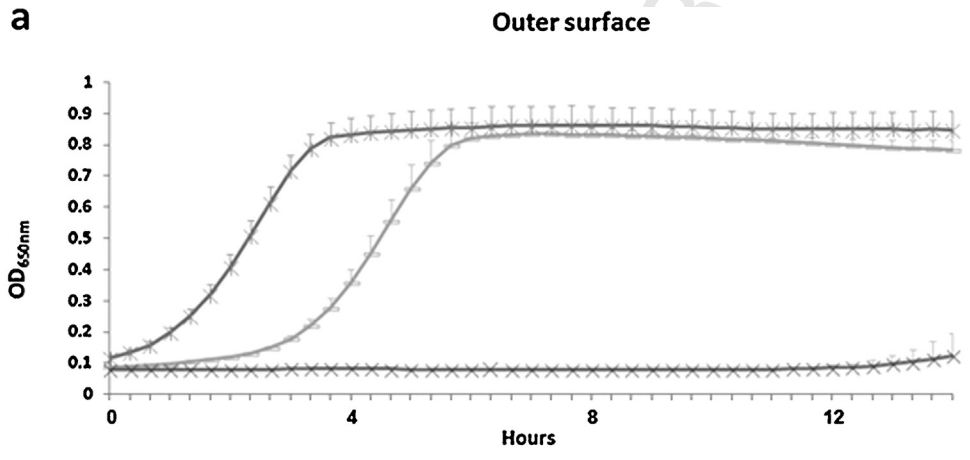
Madison, WI USA). First, spectra of un- polymerised material were taken as a baseline for further DC calculations. The material sample was irradiated, with a dental light curing LED unit for 20 s according to the manufacturer's instructions and then the spectra were collected. The material sample was allowed to polymerise for an additional 10 min. Spectra were taken every 60 s. The DC was calculated by dividing the peak heights of the double carbon–carbon bonds at wavenumber 1637 cm^{-1} of the final polymerised state by similar peaks in the baseline spectra. The peaks were normalised by aromatic double carbon–carbon peaks at wavenumber 1608 cm^{-1}. This is to eliminate other factors that may affect peak height variability, such as liquid to solid state transformation and shrinkage that is likely to occur during polymerisation of acrylates and methacrylates. The formula used for the DC calculation is:

$$\%DC = 100 \times \left(1 - \frac{A(1638\ cm^{-1})\,final / A(1608\ cm^{-1})\,final}{A(1638\ cm^{-1})\,initial / A(1608\ cm^{-1})\,initial}\right)$$

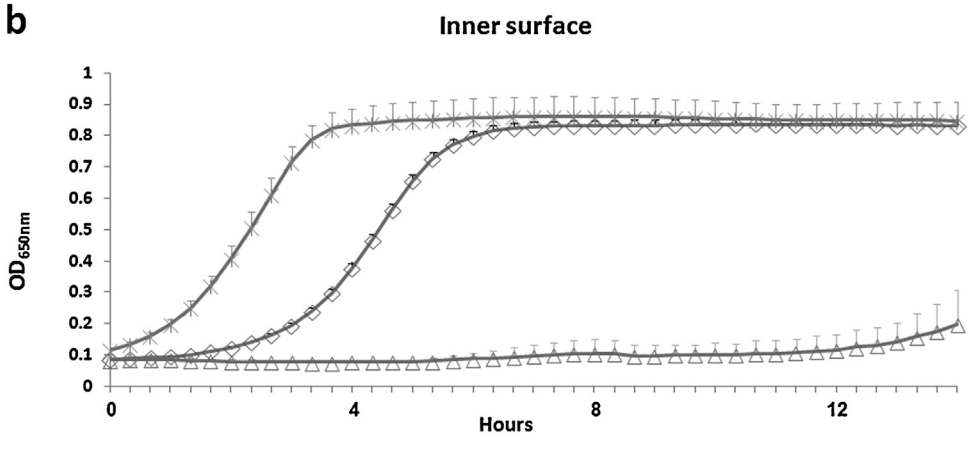
3. Results

The antibacterial properties of the resin composite were evaluated and compared *in vitro* with those of modified composite incorporating QPEI nanoparticles.

Incorporation of QPEI nanoparticles resulted in a broad spectrum ($p < 0.05$) antibacterial effect against all the tested



	Outer surface	
QPEI nanoparticles concentration (%)	0	2
Bacterial growth (%)	100	~0



	Inner surface	
QPEI nanoparticles concentration (%)	0	2
Bacterial growth (%)	100	~0

Fig. 2 – Antibacterial activity of the inner portion of modified resin composites incorporating QPEI nanoparticles. *E. faecalis* growth following direct contact with the resin composite discs' inner portion and outer surface was tested. The resin composite was modified by incorporating 2% wt/wt QPEI nanoparticles.

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microorganisms at both tested concentrations (1% and 2% wt/wt) (Fig. 1).

The properties were further examined following direct contact with the inner and outer portions of the prefabricated composites which were modified using QPEI nanoparticles. Resin composite incorporating 2% wt/wt QPEI nanoparticles exhibited antibacterial activity against *E. faecalis* (Fig. 2). The addition of 2% wt/wt nanoparticles resulted in total bacterial inhibition. The antibacterial effect was evident ($p < 0.05$) in both test groups of the modified resin composites, *i.e.* following direct contact both with the outer and inner disc surfaces (Fig. 2a and b respectively). SEM and TEM micrographs of *E. faecalis* showed normally dividing cells with intact membranes prior to exposure to the QPEI nanoparticles (Fig. 3a and c, respectively). Bacteria exposed to the nanoparticles showed distinct morphologic changes, without visible signs of cell division. Bacterial aggregation, syncytium-like cell wall fusion and membrane disruption were also evident (Fig. 3b and d).

XPS analysis of QPEI nanoparticle distribution in the resin composites showed the presence of I^- ions only in the test group with added 2% wt/wt QPEI nanoparticles (Fig. 4). Nanoparticle distribution was more evident in the inner surface of the modified resin composite incorporating the QPEI nanoparticles. Changes in the materials' degree of conversion (DC) were recorded using FTIR. QPEI incorporation in resin composites did not affect the DC: modified and unmodified resin composites with QPEI nanoparticles showed similar % DC (Fig. 5).

4. Discussion

In the present study antibacterial properties of resin composite incorporating QPEI nanoparticles were characterised in relation to nanoparticle distribution. The QPEI nanoparticles caused bacterial lysis and produced total bacterial inhibition when incorporated in the resin composite. These properties were further tested following direct contact with the inner and outer surfaces of the composites. Interestingly, total bacterial inhibition was recorded following direct contact with both the inner and the outer surface of the modified composite,

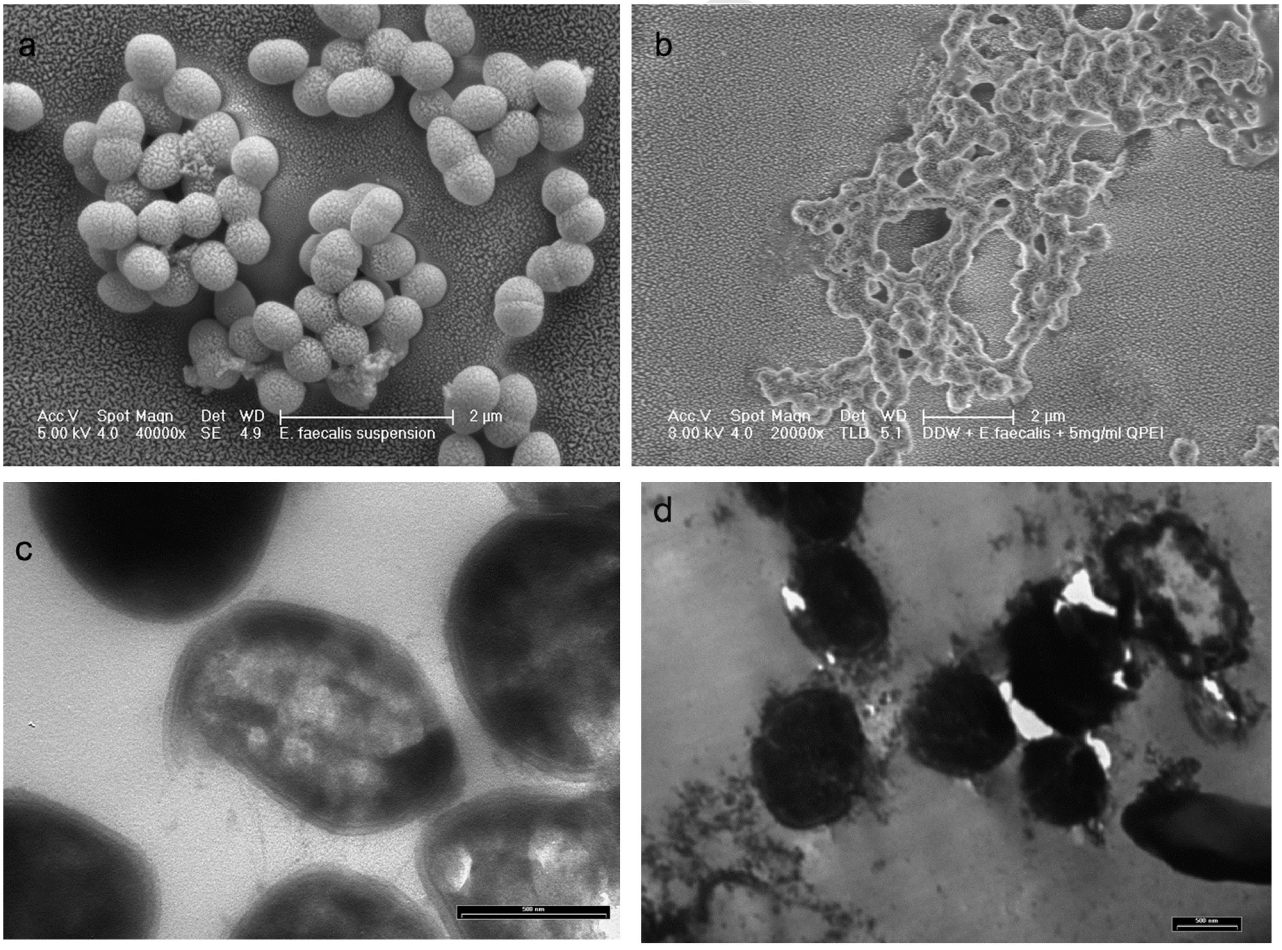

Fig. 3 – Bacterial morphologic changes following exposure to QPEI nanoparticles. Images were observed using high resolution SEM at magnifications of 40,000× and 20,000×) (a and b respectively), and TEM at magnifications of 40,000× and 15,000× (c and d). Control *E. faecalis* cells (panels a and c with no added QPEI nanoparticles), show dividing bacterial cells with intact membranes. Images taken following exposure to QPEI show no visible signs of cell division, with bacterial aggregation and syncytium-like cell wall fusion (b). Adherence of QPEI nanoparticles to bacterial membrane and morphological changes are also observed (d).

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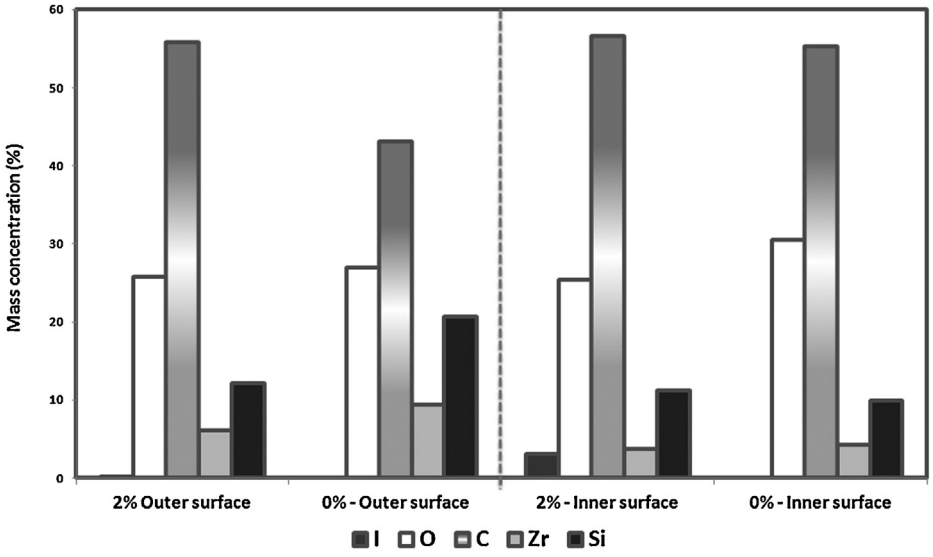

	0% - Outer surface	0% - Inner surface	2% - Outer surface	2% - Inner surface
I	0.00	0.00	0.23	3.05
Raw Area	0	0	92.0	1448.7
O	26.93	30.57	25.72	25.40
C	43.14	55.37	55.77	56.63
Zr	9.32	4.22	6.14	3.73
Si	20.60	9.84	12.14	11.20

Fig. 4 – QPEI nanoparticles distribution in resin composite. Flowable resin composite discs incorporating 0% or 2% wt/wt QPEI nanoparticles were prepared and cut. The inner and outer surfaces were tested for nanoparticle distribution using XPS. I^- ions were selected as indicators of the QPEI nanoparticles. Analysis show mass concentrations of I^- ions and raw area distribution only in the 2% wt/wt nanoparticle group. No I^- ions were detected in the unmodified composite.

although the nanoparticles were distributed to a lesser extent in the outer surface.

The conventional method for preparing antibacterial materials is to impregnate them with antibacterial agents that are gradually released over time. However, leaching of the agents from the bulk material, and more specifically from dental materials, has several disadvantages.[17–19] An alternative approach is the development of macromolecular materials possessing antimicrobial properties without releasing an agent into the solution. Insoluble polymeric contact disinfectants may inactivate or remove target microorganisms by contact without releasing biocide into the bulk phase.[5,20]

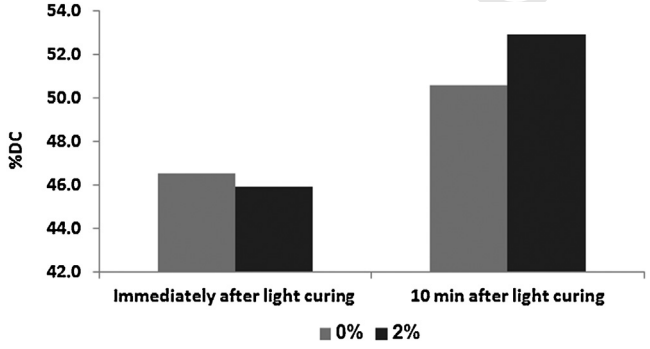

Fig. 5 – Degree of conversion (%DC). Monomer conversion of the double carbon–carbon bonds was tested in a composite resin (3M™ ESPE™ Filtek™ Supreme XTE Flowable Restorative) disc incorporating 0% and 2% wt/wt QPEI nanoparticles.

In the present study a wide range antibacterial effect against oral pathogens was achieved by incorporating a small percentage of QPEI nanoparticles in resin composites. Moreover, this effect was long-lasting and caused total bacterial inhibition of whole saliva bacteria. These results coincide with previous findings demonstrating QPEI's excellent antibacterial activity and long-term durability,[21] which is attributable to the high cationic density on its backbone.[8,22]

An important parameter that researches on developing antibacterial dental materials should bear in mind is the effect of the salivary components on the materials' surface properties. Saliva in the oral cavity is adsorbed onto dental surfaces, and may change the surface–biofilm interaction. Although this adsorption may potentially mask the functional groups in the positively charged QPEI nanoparticles, in an *in vivo* study nanoparticles incorporated in resin composite have been shown to cause bacterial death.[5] Consequently it may be suggested that saliva is not a limiting factor for in the antibacterial effect of the incorporated nanoparticles.

Cationic polymers bearing quaternary ammonium groups exhibit high antibacterial potency by interacting with and disrupting the bacterial cell membrane.[23] Moreover, when

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these polymers are synthesised as nanosized particles, their use becomes advantageous, as their surface area is exceedingly outsized relative to their size and scope.[13] Thus, only small amounts of nanoparticles may provide high activity. The particles are preferably homogeneously distributed in the dental material in which they are incorporated, generating an active antibacterial outer surface. Their surface concentration should be close to 1 particle/sq μm for effective activity against bacteria, the average size of which is 1 μm^3.

Nonetheless, surface modification for production of an antibacterial effect is not sufficient in the dental environment, as dental materials are often subjected to wear or degradation as a result of the harsh oral conditions. Thus, incorporation of antibacterial nanoparticles into resin composite materials can be useful in preventing recontamination when the base material is modified, preserving the antibacterial potency in the inner portion of material. To test the antibacterial potency of the inner portion of the modified resin composite incorporating QPEI nanoparticles, we used *E. faecalis*, which is considered a highly resistant oral pathogen.[24–26] Although only a small percentage of nanoparticles was incorporated in the resin composite, direct contact of *E. faecalis* with the inner portion of the material resulted in total bacterial inhibition, similarly to the outer surface's effect. Consequently, it is conceivable that even if the outer surface of modified resin composite is worn out the antibacterial effect is likely to be conserved. Further investigation of the effect of QPEI on this persistent bacterium showed damaged membranes and various stages of lysis as demonstrated by SEM and TEM. These findings support the assumption that antimicrobial polycations are adsorbed onto the bacterial cell surface, diffuse through the cell membrane, bind to the cytoplasmatic membrane, release cytoplasmatic constituents such as K^+ ions, DNA and RNA and, finally, cause cell death.[8,27]

Although XPS analysis demonstrated that the QPEI nanoparticles are distributed unevenly, and are more evident in the inner portion of the material, the antibacterial compound added here to the resin composite was sufficient to inhibit bacterial growth. The antibacterial effect was not compromised in the outer surface. The uneven distribution could be attributed to the manual mixing of the nanoparticles into the resin composite or to the presence of a polymerisation inhibition layer often found in resin composites.[28] As dental materials tend to wear out during prolonged usage, the presence of the antibacterial nanoparticles in the inner layers is important. One of the most critical aspects of a composite resin restoration is the polymerisation stage. The %DC has an important effect on the physical and mechanical properties of composite resins.[29] Here, the %DC was unaffected following QPEI incorporation. These results lead us to the assumption that incorporation of a small percentage of QPEI nanoparticles does not decrease the monomer conversion of the material and thus will not affect its physical properties.

5. Conclusions

In our investigation, the extent of the antibacterial activity of modified resin composites incorporating QPEI nanoparticles was found to be comprehensive, and was evident against all the tested oral pathogens, indicating a nonspecific mode of action. The effect was long-lasting and was conserved in the inner portion of the modified materials. Thus, even when the outer surface of the material may be subjected to wear, the antibacterial effect will be preserved. Quaternary ammonium polyethyleneimine nanoparticles may be suitable candidates as additives to resin composite materials in order to endow their surface with antibacterial activity.

Acknowledgments

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