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Surface antimicrobial activity and biocompatibility of incorporated polyethylenimine nanoparticles

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ABSTRACT

The antimicrobial effect and biocompatibility of insoluble cross-linked quaternary ammonium polyethylenimine (PEI) nanoparticles incorporated at 1 or 2% w/w in a resin composite were assayed. The antimicrobial effect against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Escherichia coli* was tested using the direct contact test (DCT), agar diffusion test (ADT) and scanning electron microscopy (SEM). Biocompatibility was tested by assessing macrophage viability, and TNFα secretion. Samples incorporating 2% w/w nanoparticles inhibited the growth of all bacterial strains tested. Reducing the amount of the added nanoparticles to 1% w/w resulted in complete inhibition of *S. aureus* and *E. faecalis*, and decreased growth of *S. epidermidis*, *P. aeruginosa* and *E. coli* ($p < 0.0001$). The DCT results were confirmed by SEM. However, ADT showed no inhibition halo in all test bacteria, indicating the antimicrobial nanoparticles are not diffusing into the agar *milieu*. Biocompatibility tests revealed macrophage viability, and TNFα secretion was not altered by the presence of the nanoparticles in the resin. Incorporation of PEI nanoparticles in a resin composite had a long lasing antimicrobial effect against a wide range of bacteria with no measured effect on biocompatibility.

1. Introduction

In the clinical environment there is a common call for prevention of microbial adhesion and biofilm formation, for they may have crucial implications. The conventional approach to the use of antibacterial materials is based on the release of antibacterial agents over time. However, leaching of these agents from the material has several disadvantages: a decrease in the mechanical properties of the carrier material with time, short-term effectiveness, and possible toxic effect if the release is not properly controlled. As compared with conventional antibacterial agents of low molecular weight, the advantage of polymeric antibacterial agents is that they are nonvolatile, chemically stable, and can be chemically bound within the polymer carrier via active groups for improved integration [1–3].

Recently [4] we described the synthesis of nanoparticles made from cross-linked polyethyleneimine (PEI) that underwent quaternarization with octyl groups, followed by methylation to form quaternary amines. Incorporation of these nanoparticles at a low concentration (1% w/w) into dental resin composites during polymerization resulted in modified resin composites possessing a potent antibacterial effect against *Streptococcus mutans*, the principal bacterium causing dental caries. The modified resin composite materials (incorporated with PEI nanoparticles) maintained their full antibacterial properties over 1 month without leaching out and without alteration of mechanical properties [4]. This demonstrated the possibility of obtaining solid materials with stable and long lasting antibacterial surface properties.

To consider the use of the modified resin composite *in vivo*, biocompatibility has to be established by comparing the modified resin composite with the native resin composite. Monocytes and macrophages have been shown to be critical cells in the biological response to materials [5]. Due to the fact that monocytes direct much of the chronic inflammatory response, the ability of a material to alter a cell's viability or secretory function may have significant consequences on the overall biological response to a given material [6].

In the present study we further characterize biological properties of the insoluble cross-linked quaternary ammonium polyethylenimine (PEI) nanoparticles incorporated into the resin

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matrix. We tested (i) the antibacterial spectrum and (ii) the biocompatibility of this novel class of modified dental resin composites.

2. Materials and methods

2.1. Quaternary ammonium PEI nanoparticle synthesis

The synthesis of quaternary ammonium PEI nanoparticle was previously described by Beyth et al. [4]. In brief, PEI (10 g, 0.23 mol monomer units) dissolved in 100 ml ethanol was reacted with dibromopentane at a 1:0.04 mol ratio (monomer units of PEI/dibromopentane) under reflux for 24 h. *N*-alkylation was conducted as follows: octyl halide was added at a 1:1 mol ratio (monomer units PEI/octyl halide). Alkylation was carried out under reflux for 24 h followed by neutralization with sodium hydroxide (1.25 equimolar, 0.065 mol) for an additional 24 h under the same conditions. *N*-methylation was conducted as follows: 43 ml of methyl iodide (0.68 mol) were added. Methylation was continued at 42 °C for 48 h followed by neutralization with sodium bicarbonate (0.23 mol, 19 g) for an additional 24 h. The supernatant obtained was decanted and precipitated in 300 ml of double distilled water (DDW), washed with hexane and DDW and then freeze-dried. The purification step was repeated using additional amounts of hexane and DDW. The average yield was 70% (mol/mol). Characteristics of the synthesized quaternized alkylated PEI-based nanoparticles are shown in Table 1.

2.2. Bacterial strains and growth conditions

Clinically isolated *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Staphylococcus epidermidis* (the listed bacteria were clinically isolated at the Maurice and Gabriela Goldschleger School of Dental Medicine at Tel-Aviv University, Israel), and *Staphylococcus aureus* ATCC 8325-4 were used in this study. The bacteria were cultured aerobically overnight in 5 ml of brain–heart infusion (BHI) broth (Difco, Detroit, MI), at 37 °C.

2.3. Preparation of test samples

The tested materials were prepared by adding the synthesized PEI nanoparticles to the dental restorative resin composite Filtek Flow (47% Zirconia/silica, average particle size 0.01–6.0 μ; BIS-GMA, TEGDMA (3 M ESPE Dental St Paul, MN, USA)). The nanoparticle powder was added at 0, 1 or 2% w/w to the resin composite and homogeneously mixed in a dark room for 30 s with a spatula.

2.4. Preparation of the microtiter plate

A microtiter plate (96-well flat bottom plate, Nunclon, Nunc, Copenhagen, Denmark) was vertically positioned. Using a flat-ended dental instrument (dental spatula) the sidewalls of eight wells were coated evenly with an equal amount of the material tested (30 ± 5 mg in each well). Special care was taken not to touch the bottom of the well to avoid false readings during incubation in the spectrophotometer. The materials were polymerized according to the manufacturer's instructions.

2.5. Direct contact test

The direct contact (DCT) test was used to determine the antibacterial properties of the tested materials, as previously described [7,8]. A 10 μl volume of bacterial suspension ($\sim 10^6$ bacteria) was placed on the surface of each tested material in a set of eight wells, and the plate was incubated in a vertical position for 1 h at 37 °C. During the incubation period, the suspension liquid evaporated and a thin layer of bacteria was obtained, ensuring direct contact between all the bacteria and the tested surface. A 10 μl volume of the bacterial suspension was placed on the uncoated walls of eight wells, which served as control in the same microtiter plate. The plate was then positioned horizontally and 220 μl of BHI broth were added to each well containing the material.

Table 1
Characteristics of the synthesized quaternized alkylated PEI-based nanoparticles

FT-IR (KBr)	3440 cm^{-1} (N–H), 2956 cm^{-1}, 2926 cm^{-1} and 2853 cm^{-1} (C–H), 1617 cm^{-1} (N–H, small band), 1465 cm^{-1} (C–H), 967 cm^{-1} quaternary nitrogen
^{1}H NMR (DMSO)	0.845 ppm (t, 3H, CH_3, octane hydrogens), 1.24 ppm (m, 10H, –CH_2–, octyl hydrogens) 1.65 ppm (m, 2H, CH, octyl hydrogens), 3.2–3.6 ppm (m, CH_3 of quaternary amine, 4H, –CH2–, PEI hydrogens and 2H, –CH2–, octyl hydrogens)
Particle size (R, nm)	7.5 ± 2:49%, 140 ± 37:51%
Elemental analysis	%C = 40.93, %H = 7.84, %N = 6.23, %I = 38.26

2.6. Kinetic measurement of bacterial growth

The microtiter plates were placed in a temperature-controlled microplate spectrophotometer at 37 °C (VERSAmax, Molecular Devices Corporation, Menlo Oaks Corporate Center, Menlo Park, CA), with 5 s vortex mixing before each reading. Bacterial growth was estimated by following changes in optical density absorbance (A_{650}) in each well every 20 min for 24 h.

2.7. Material aging

Similar microtiter plates were prepared with the tested materials and aged for 48 h, 1 and 4 weeks. During this time each well was filled with 250 μl PBS, which was replaced every 48 h, and the plates were incubated at 37 °C. Before the DCT the PBS was aspirated and the plates were dried under sterile conditions.

2.8. Data analysis

The absorbance measurements were plotted, providing bacterial growth curves for each well in the microtiter plate. The linear portion of the logarithmic growth phase was subjected to statistical analysis. The results are expressed according to two variables: the slope (a) and the constant (b) of the linear function $ax+b=y$ derived from the ascending portion of the bacterial growth curve. The slope (a) and the constant (b) correlate with growth rate and initial bacterial number, respectively. The data were analyzed by one-way ANOVA, and the Tukey multiple comparison test. The level of significance was determined as $p < 0.05$.

2.9. Agar diffusion test

A 200 μl volume of each bacterial suspension was spread on Tryptic Soy Blood Agar (TSBA) (Difco, MI, USA) and defibrinated sheep blood. Uniform test-disks 1 mm thick and 5 mm in diameter, weighing 10 ± 2 mg, were prepared by application of standard pressure to the resin composite samples, followed by light polymerization. Test-disks of the resin composite material Filtek Flow with 0, 1 or 2% w/w incorporated PEI nanoparticles were placed on the surface of each agar plate. The plates were incubated for 24 h at 37 °C and the inhibition zone around each specimen was measured.

2.10. Scanning electron microscopy

Filtek Flow resin composite with 1% w/w incorporated PEI nanoparticles, as described above, was scanned after an incubation of 1 or 24 h with each of the test bacteria. Uniform test-disks 1 mm thick and 5 mm in diameter, weighing 10 ± 2 mg, were prepared by application of standard pressure to the resin composite samples, followed by light polymerization.

The samples were pressed between two glass slides to obtain standardized smooth surfaces. The disks were light polymerized for 40 s, using a light curing unit (Elipar highlight, 3 M ESPE Dental). A 10 μl volume of bacterial suspension ($\sim 10^6$ bacteria) was placed on the surface of each tested sample and incubated for 1 h at 37 °C. Evaporation of the suspension liquid resulted in a thin layer of bacteria, ensuring direct contact between all the bacteria and the tested surface. The samples were fixed with glutaraldehyde and osmium oxide solutions, dehydrated in a graded ethanol series, and then coated with gold. An additional set of disks was processed as above and incubated for 24 h in 5 ml of BHI broth. The tested samples were examined by scanning electron microscopy (Philips 505 SEM at accelerating voltage). For each time point, additional disks without added polymer served as control.

2.11. Cell culture

The raw 264.7 macrophage cell line was cultured in Petri dishes in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS), 1% penicillin/streptomycin and 1% glutamine. Before the assay, the cells were seeded at a density of 60,000 cells per well in 96-well tissue culture plates (NUNC). At 24 h after plating, the cells were activated by heat killed *Porphyromonas gingivalis* 33277 ATCC (which was grown at 37 °C under anaerobic conditions and heat killed at 80 °C for 10 min), and exposed to the resin composites (which were prepared as described above and polymerized over a special insert that was placed in the wells). Following 24 h of incubation at 37 °C in a humidified atmosphere of 5% CO_2, the plates were analyzed for cell viability and the secreted levels of TNFα.

2.11.1. Cell viability

The viability of the cells was evaluated using a colorimetric XTT assay as described by Scudiero et al. [9]. The assay is based on the ability of metabolically active cells to reduce the tetrazolium salt XTT to orange colored compounds of formazan. In brief, following 24 h of incubation, 50 μl of XTT labeling mixture were added to each well and the microplate was incubated for a further 4 h. A Vmax microplate reader (Molecular Devices, Palo Alto, CA) with a 450 nm optical filter and a 650 nm reference wavelength was used to measure the absorbance of each well. The fraction of viable cells in the treatment groups was normalized according to control viability = 100%.