Long-term antibacterial surface properties of composite resin incorporating polyethyleneimine nanoparticles

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Objective: The development of new longer-lasting composite resins is an urgent public health need. It has been shown that surface roughness of composite resins is increased by *Streptococcus mutans* biofilm in vitro and further that incorporation of small amounts of antibacterial nanoparticles (polyethyleneimine [PEI]) into composite resins renders a strong antibacterial effect against *S mutans* biofilm. The present study tested the hypotheses that incorporation of PEI nanoparticles into composite resins prevents the increase of surface roughness caused by *S mutans* biofilm and that PEI incorporation into composite resin has a long-lasting antibacterial effect. **Method and Materials:** Composite resin incorporating PEI nanoparticles was characterized using contact angle goniometry, X-ray photoelectron spectroscopy (XPS), and SEM. Six-month-aged samples were tested for antibacterial effect against *S mutans* using the direct contact test. Surface roughness following 1 month of bacterial challenge was depicted using atomic force microscopy (AFM). **Results:** Contact angle increased following PEI incorporation, and XPS revealed surface iodide and nitrogen elements. Direct contact test results showed that 6-month-aged composite resins incorporating PEI nanoparticles completely inhibited *S mutans* growth ($P < .05$). AFM analysis showed an increase in root mean square roughness following bacterial challenge in composite resin samples ($P < .05$); no effect was depicted in samples incorporating PEI. **Conclusion:** Changing the surface properties of composite resins by incorporating PEI antibacterial nanoparticles may improve their clinical performance both by inhibiting bacterial growth and by preventing changes in the surface roughness. *(Quintessence Int 2010;41:827–835)*

Key words: biofilm, composite resins, nanoparticles, polyethyleneimine

The key evaluation of composite resin restorations is clinical survival. Although these restorative materials have been reasonably successful for both anterior and posterior restorations, studies have indicated a wide range of failures. The main reason for replacement of dental restorations is secondary caries.[1] It was reported that mutans streptococci adhesion and colonization on restorative materials are essential steps in secondary caries evolvement, compromising the restoration's longevity.[2] This failure may be attributed to the tendency of composite resin restorations to accumulate more dental plaque than other restorations[3–5] and to their lack of antibacterial properties.[6] Indeed, composite resin materials used for restorations are subjected to a harsh chemical and mechanical environment in the oral cavity. Plaque-forming bacteria and ingested liquids contribute to the degradation of composite resin restorations. Moreover, it was suggested that nonpolymerized monomers, which can be extracted from composite resins, accelerate growth of cariogenic bacteria.[7]

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Table 1	Characteristics of the synthesized quaternized alkylated PEI-based nanoparticles
^{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, $-CH_2-$, PEI hydrogens and 2H, $-CH_2-$, 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

(C) Carbon, (H) hydrogen, (N) nitrogen, (I) iodine.

Previously, it was shown that S mutans growth on polymerized composite resins is accelerated and increases the composite resin's surface roughness.[8] This change in surface integrity may further accelerate dental plaque accumulation and, as a result, increase the risk for recurrent caries at the restoration's margins.

A potential solution to this cascade is to modify and improve the existing resin systems. This can be accomplished by changing the composite resin's antibacterial properties. Subsequently, two main methods were previously described to obtain antibacterial properties: One was the addition of soluble antimicrobial agents,[9–11] and the other was the immobilization of antibacterial components in the materials' substance,[12,13] the latter having the advantages of being nonvolatile and chemically stable, thus enabling a longer antibacterial activity.

It was reported that small amounts of quaternary ammonium polyethyleneimine (PEI) nanoparticles can be immobilized into composite resins during polymerization without leaching out and without compromising the mechanical properties of the composite resin.[14] Furthermore, it was shown that incorporation of small amounts of PEI antibacterial nanoparticles into composite resins renders a strong antibacterial effect against a wide range of bacteria for at least 1 month with no measured effect on biocompatibility.[15]

The present study further investigates the PEI nanoparticles when incorporated into a composite resin. Here, the hypotheses tested were that incorporation of PEI nanoparticles has an antibacterial effect that lasts at least 6 months and that the addition of these particles may prevent the increase of surface roughness caused by *S mutans* biofilm.

METHOD AND MATERIALS

PEI nanoparticle synthesis

The synthesis of quaternary ammonium PEI nanoparticle was previously described by Beyth et al.[14] Briefly, PEI (10 g, 0.23 mol monomer units) dissolved in 100 mL ethanol reacted with dibromopentane at a 1:0.04 mol ratio (monomer units of PEI/dibromopentane) under reflux for 24 hours. 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 hours followed by neutralization with sodium hydroxide (1.25 equimolar, 0.065 mol) for an additional 24 hours under the same conditions. N-methylation was conducted as follows: 43 mL of methyl iodide (0.68 mol) were added, and methylation was continued at 42°C for 48 hours, followed by neutralization with sodium bicarbonate (0.23 mol, 19 g) for an additional 24 hours. 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. Average yield was 70% (mol/mol). PEI particles were chemically characterized as shown in Table 1.

Table 2 Contact angle and XPS analysis

Sample	Contact angle*	Atomic concentration %/Mass concentration %†				
		O	C	Si	N	I
Composite resin	55.20 (0.45)	26.42/31.90	72.42/65.64	1.16/2.46	–	–
Composite resin+1%PEI	68.60 (0.55)	17.65/21.22	77.82/70.24	0.90/1.90	3.29/3.47	0.33/3.18

(O) Oxygen, (C) carbon, (Si) silicon, (N) nitrogen, (I) iodine.
*Contact angle in degrees as measured three times on each composite resin disk with and without 1% by weight PEI nanoparticles. n = 5 in each test group.
†XPS component peak assignments.

Sample preparation and characterization

The tested samples were prepared by adding 1% by weight of synthesized PEI nanoparticles to Filtek Flow (3M ESPE). An equal amount of material was pressed between two glass slides to form disks (5 ± 1 mm diameter, 1 mm thickness). The disks (n = 5 each test group) were light polymerized for 40 seconds on each side using a conventional light-curing unit (Elipar high light, 3M ESPE) with a light intensity of 600 ± 3 mW/cm^2. Then, each disk's surface was characterized by means of surface chemical analysis, surface contact angle depiction, and surface imaging.

Chemical surface analysis of the disks was performed using X-ray photoelectron spectroscopy (XPS). Measurements were performed using a Kratos Axis Ultra X-ray photoelectron spectrometer (Kratos Analytical). The spectra were acquired with a monochromatic aluminum K-alpha (1,486.7 eV) X-ray source with a 0-degree takeoff angle. The pressure in the test chamber was maintained at 1.5 ± 10^{-9} Torr during the acquisition process. The survey spectra were collected from 1,200 to –5 eV (binding energy) with pass energy 80 eV. High-resolution XPS scans were collected for carbon 1s, oxygen 1s, nitrogen 1s, silcon 2p, and iodine 3d peaks with pass energy 20 eV. The step size was 1.0 eV for the survey scans and 0.1 eV for the high-resolution spectra. The XPS binding energy was calibrated with respect to the C 1s peak position as 285.0 eV.[16] Data analysis and processing were performed with Vision processing data reduction software (Kratos Analytical) and CasaXPS (Casa Software) as summarized in Table 2. Signals attributed to the composite resin were 1s oxygen, 1s carbon, and 2p silicon (530.12 to 532.7, 285.0 to 289.1, 102.77, respectively). In addition, signals such as iodine (618.57 [$3d_{5/2}$] and 630.07 [$3d_{3/2}$] eV) and nitrogen (399.7 1s [I] and 402.5 1s [II] eV) appeared in the samples of the composite resin incorporated with 1% by weight PEI.

Furthermore, the surface contact angle was evaluated. The angle was measured by the tangent between a drop of deionized water (Mili-Q, Milipore) and the disk's surface. Contact angles were measured using a Ramé-Hart model 100 contact angle goniometer. All measurements were repeated three times for each disk. Increase in the contact angles was depicted in the disks incorporating 1% by weight PEI nanoparticles as shown in Table 2. In addition, the surface of each disk was imaged using a scanning electron microscope (SEM). The disks were coated with gold and scanned by an SEM (Philips 505 SEM [Philips] at accelerating voltage). SEM imaging depicted no difference in the surface views of Filtek Flow when compared to Filtek Flow incorporating 1% by weight PEI nanoparticles (data not shown).

Antibacterial effect

The antibacterial effect of Filtek Flow incorporating 1% by weight PEI nanoparticles was tested after 6 months of aging using a direct contact test.[6] In brief, a microtiter plate (96-well flat-bottom plate, Nunclon, Nunc) 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 light polymerized for 40 seconds. Then, the microtiter plate was aged for 6 months. During this time, each well in the microtiter plate was filled with 250 μL phosphate-buffered saline (PBS) (Sigma), and the plate was incubated at 37°C. PBS was replaced every 48 hours. At the end of the aging period, the PBS was aspirated, and the plates were dried under sterile conditions.

S mutans (ATCC#27351) was cultured overnight at 37°C in brain-heart infusion (BHI) broth (Difco) supplemented with bacitracin (Sigma) (0.0625 g/mL). The top 4 mL of the suspension were harvested into a fresh test tube and centrifuged for 10 minutes at 3,175 × g to obtain single- and short-chain bacterial cells. The supernatant was discarded, and the bacteria were resuspended in 5 mL of PBS containing 0.0625 g/mL of bacitracin to obtain 10^9 cells/mL. A 10-μL volume of the bacterial suspension 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 hour 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 tested surface. A 10-μL volume of the bacterial suspension was placed on the uncoated walls of eight wells that 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.

The microtiter plates were placed in a temperature-controlled microplate spectrophotometer at 37°C (VERSAmax, Molecular Devices), with 5 seconds' vortex mixing before each reading. Bacterial growth was estimated by following changes in optical density absorbance (A_{650}) in each well every 20 minutes for 18 hours.

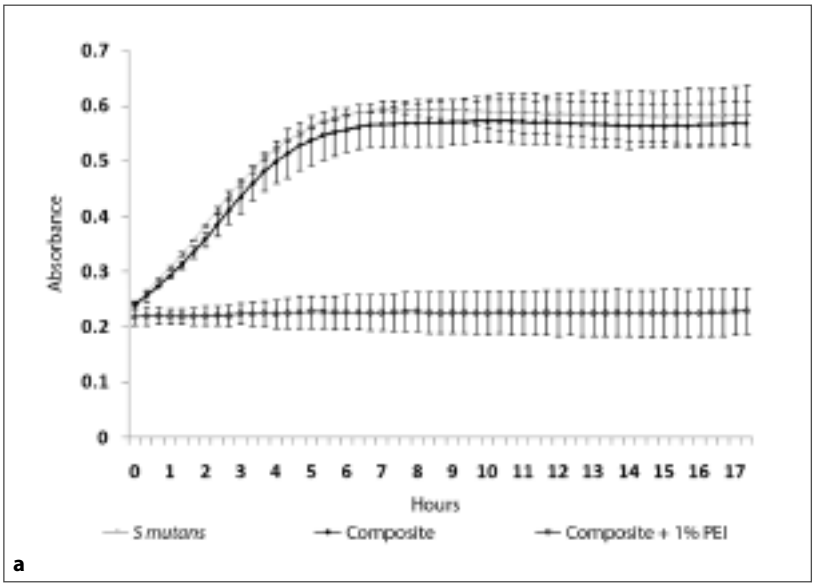
The absorbance measurements were plotted, providing bacterial growth curves for each well in the microtiter plate. The linear portion of the logarithmic growth phase was subjected to statistical analysis. The results are expressed according to two variables: the slope (a) and the constant (b) of the 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.

Surface roughness

Surface roughness of Filtek Flow incorporating 1% by weight PEI nanoparticles was tested after 1 month of bacterial challenge using atomic force microscopy (AFM) as previously described by Beyth et al.[8] Disks for each test group (n = 5) were prepared as described above. Direct contact between the bacteria and the composite resins was achieved by placing 10 μL of bacterial suspension on each disk. The disks were then incubated at 37°C for 1 hour to evaporate excess water and placed in tubes containing 5 mL of BHI broth supplemented with 0.0625 g/mL of bacitracin. The tubes were incubated at 37°C for 1 month, during which time the broth was replaced every 48 hours. Before the broth was replaced, the disks were vortexed for 10 seconds and the broth decanted without disturbing the biofilm. Lack of contamination was verified by microscopic examination.

At the end of the test period, the disks were washed three times for 30 seconds with DDW and placed in test tubes containing 5 mL of DDW supplemented with 0.02% azide at 4°C. To obtain a clean surface without bacteria or bacterial products, the disks were sonicated for 15 minutes (Tuttnauer ultrasonic cleaner, model U1424 43 KHz, Tuttnauer). The disks were then dried at room temperature (20°C). Bradford protein staining (Bio-Rad Protein Assay, BIO-RAD Laboratories) of each disk was used to ensure that all biofilm residues had been removed.[17]

To obtain measurements of root mean square (RMS) roughness, each disk was scanned by a Dimension 3100 Scanning Probe Microscope (D3100 Nanoscope Dimension, Nanoscope IV-A control station) in three different areas. AFM was performed using nanoprobe-etched silicon cantilevers with a spring constant of k = 0.06 N/m in TappingMode by oscillating the cantilever in free air at its resonant frequency.



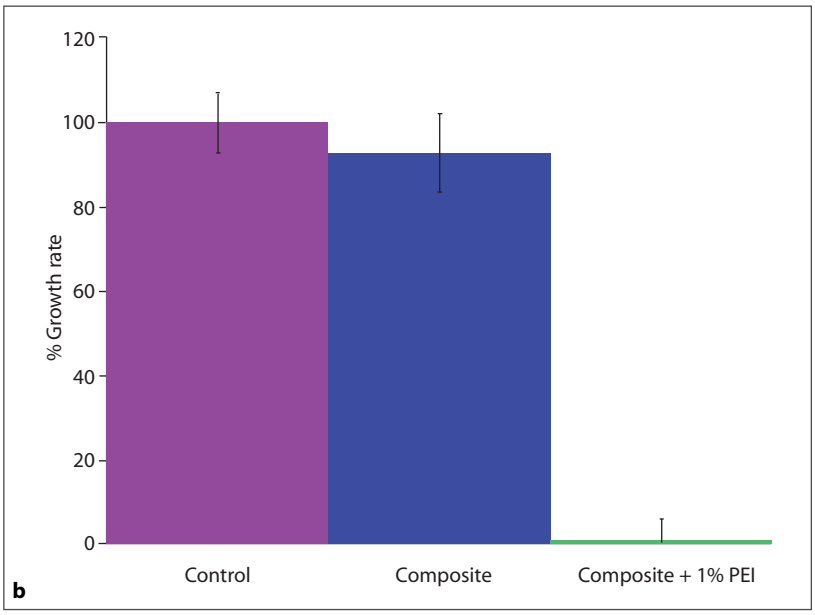


Fig 1 Antibacterial effect. *(a) S mutans* growth determined by optical density following direct contact with 6-month-aged resin composite with 1% by weight incorporated PEI nanoparticles and composite resin without nanoparticles. Each point on the curve is the mean (± SD) absorbance (A_{650}) measured in eight replica wells; eight uncoated wells served as control in the same microtiter plate. *(b)* Analysis of the logarithmic portion of *S mutans* growth phase. Results are expressed as the mean percentage (± SD).

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and the Tukey multiple comparison test. The level of significance was determined as $P < .05$.

RESULTS

Antibacterial effect

S mutans growth determined by optical density (A_{650}) in a 96-well microtiter plate aged for 6 months is shown in Fig 1a. Analysis of the growth rate showed an evident antibacterial effect ($P < .05$) of the composite resin samples incorporating PEI nanoparticles, as seen in Fig 1b, whereas the composite resin

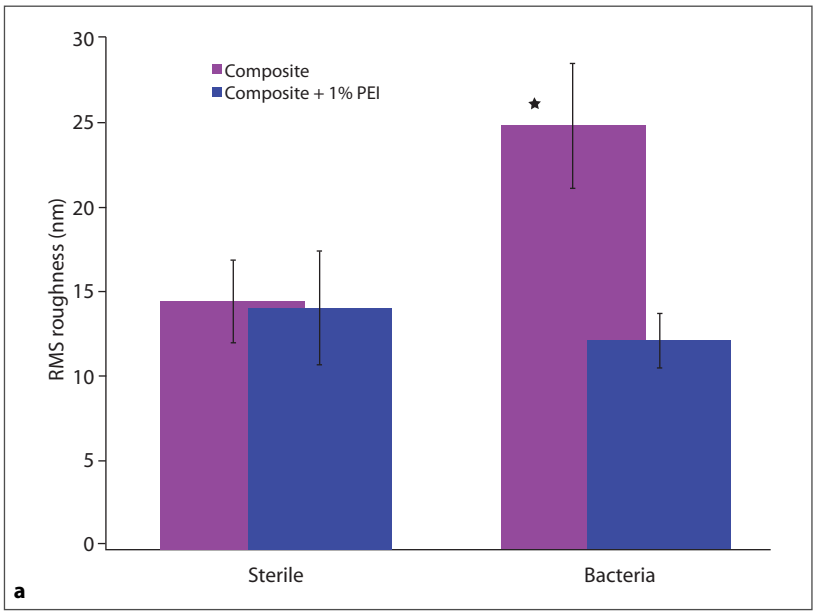
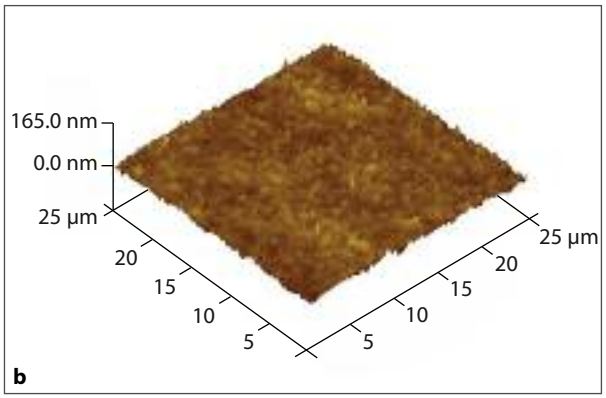
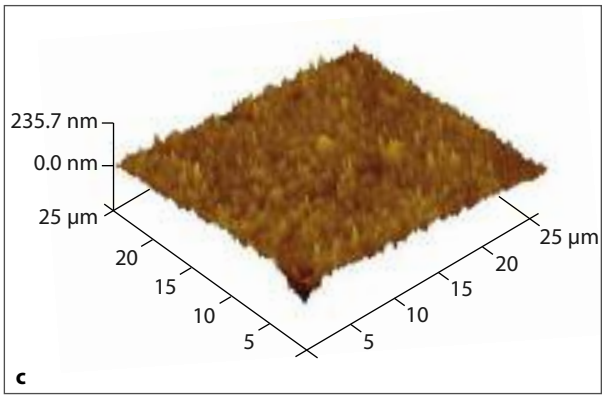


Fig 2a Root mean square (RMS) roughness analysis following 1 month of bacterial challenge compared with sterile samples depicted using AFM. *Star* denotes significant increase in RMS roughness ($P < .05$).

Figs 2b and 2c Surface views (25 × 25 μm) of composite resin incorporating 1% by weight PEI *(b)* and composite resin without the nanoparticles *(c)* following 1 month of bacterial challenge.





samples with no nanoparticles showed no antibacterial effect. The data are expressed as the slope of the regression line plotted from the logarithmic growth phase of each bacterium. Slope values correlating with the growth rate are expressed as the percentage of the controls, normalized to 100%.

Surface roughness

Surface roughness analysis showed increase in RMS roughness following 1 month of bacterial challenge ($P < .05$) in only the composite resin group without the nanoparticles, whereas no increase in RMS roughness was depicted in composite resin incorporating 1% by weight PEI nanoparticles before and after bacterial challenge (Fig 2a). Surface views following 1-month incubation with *S mutans* biofilm of composite resin with 1% by weight PEI nanoparticles compared with the composite resin without the nanoparticles are shown in Figs 2b and 2c, respectively.

DISCUSSION

Composite resin restorations are increasingly used in dental practice. However, longevity and survival studies show that composite resin restorations have an average replacement time of about 6 years. These failures are mainly due to secondary caries and fracture of the restoration. Thus, biomaterial research focusing on increasing the service life of dental composite resins is essential, and the development of new longer-lasting restorative composite resins is an urgent public health need. The present study further investigated the antibacterial PEI nanoparticles when incorporated in dental composite resin restorative material. The effect of the particles was studied after a long-term aging process and bacterial challenge. It was found, using the direct contact test, that contact of *S mutans* bacteria with the surface of a composite resin incorporating PEI nanoparticles resulted in total growth inhibition. The present findings match the reported antibacterial activity of quaternized PEI in itself[18] and when incorporated in various materials.[19] Moreover, this antibacterial effect was found not to diminish after 6 months of material aging. In addition, the PEI nanoparticles, when incorporated in the composite resins, prevented the increase in surface roughness that may be caused by cariogenic bacteria. Theoretically, the PEI nanoparticles establish a "protective" effect, maintaining the roughness of the composite resin but challenged 1 month with *S mutans* biofilm. These results coincide with the previous report that surface topographical changes in cured composite resins are caused by *S mutans*, and confirm previous investigations that reported of bacterial biofilm that promotes a negative effect on the surface morphology.[20]

Restoration margins can provide a potential pathway to leakage of cariogenic microorganisms present in the normal human flora. Therefore, the antibacterial ability of materials themselves logically becomes an important factor in preventing infection and consequently preventing secondary caries. Many studies have tested the antibacterial activity of dental composite resins and demonstrated that most composite resin materials failed to display any inhibition after being cured. With the understanding of the pathogenesis and the adverse symptoms following placement of restorations that are attributed to the leakage of cariogenic microorganisms at the restoration margins, the authors introduced a potential solution using antibacterial polymer nanoparticles. Furthermore, although current dentin adhesive systems may show high bond strength, they do not have the ability to prevent the occurrence of microgaps between the tooth and the restoration. Therefore, composite resin restorations that possess antibacterial activity even after being placed in the cavity would be beneficial for eliminating the detrimental effect caused by bacterial microleakage.

The proposed mechanism of action of PEI nanoparticles is transfusing and irreparably damaging the bacterial cellular membrane/wall.[19] This antibacterial activity is dependent on the N-alkylated hydrophobic chain and its positive charge, which cause leakage of intracellular contents and lead to cell death. This cascade can occur provided that the active components are present on the composite resins' surface. Interestingly, when the sample surfaces were characterized, it was found that although only a small percentage of PEI nanoparticles were added, the active substances were detected on the surface using XPS spectroscopy and contact angle measurements. Additionally, we were interested in testing possible changes in the surface roughness. The roughness of intraoral hard surfaces is of clinical importance in the process of bacterial retention. Changes in this variable might, therefore, promote caries development. Interestingly, SEM micrographs and AFM images of the control composite resin surfaces and those of the composite resin incorporating PEI nanoparticles were found to be indistinguishable. Accordingly, it can be speculated that PEI nanoparticle incorporation affects mainly the chemical surface properties of the composite resin and not its surface topography.

This study focused on nanoparticle synthesis of cross-linked quaternized PEI for several reasons. First, use of nanoparticles is as advantageous as active antibacterial groups

since their surface area is exceedingly outsized relative to their size and scope. Thus, nanoparticles may provide high activity even though only a small amount of the particles is added. Second, cross-linking is required to form insoluble nanoparticles needed when materials such as composite resins are anticipated to withstand exposure to an aqueous environment. Furthermore, incorporation of nanoparticles is considered to be beneficial in durability over use of composite resins containing leachable antibacterial agents. It is recognized that the physical properties of resin-based materials containing a water-soluble antimicrobial deteriorate as the agent leaches out, resulting in poor clinical performance functionally and esthetically. On the contrary, quaternized PEI antibacterial nanoparticles when incorporated are not released and consequently have additional stability in a wet environment. The presence of active antibacterial elements on the material's surface as shown here may explain the long-lasting antibacterial properties achieved following incorporation of PEI nanoparticles. This new knowledge may help to devise better materials for longer-lasting composite resins and reduce the need for repeat restorative replacements.

This antibacterial effect may be of importance in the inhibition of bacterial growth on the surface of the material by preventing infection of the neighboring soft and hard tissues. In addition, this effect is significant in inhibiting bacterial growth in the restoration-tooth interface where microleakage occurs, resulting in secondary caries. However, the incorporation of PEI nanoparticles should be further investigated by in vivo usage tests to give substantial long-term effectiveness evidence. Hence, the practical value of a composite resin restoration incorporating PEI nanoparticles is not clear purely from the results of the in vitro antibacterial activity tests.

The future for restorative materials development should be bioactive materials that inhibit biofilm formation, inhibit caries-producing bacteria, and mitigate the effects of decreased pH during cariogenic activity.

CONCLUSIONS

The incorporation of hydrophobic nonleachable quaternized polyethyleneimine nanoparticles in composite resins is one strategy for prevention of bacterial infection.

Incorporation of PEI antibacterial nanoparticles in composite resins results in surface modification that may improve clinical performance both by inhibiting bacterial growth and by preventing changes in the surface roughness caused by bacterial presence. The long-term goal of future biomaterial research should be increasing the service life of composite resin restorations by managing the hazardous effect of biofilm bacteria.

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