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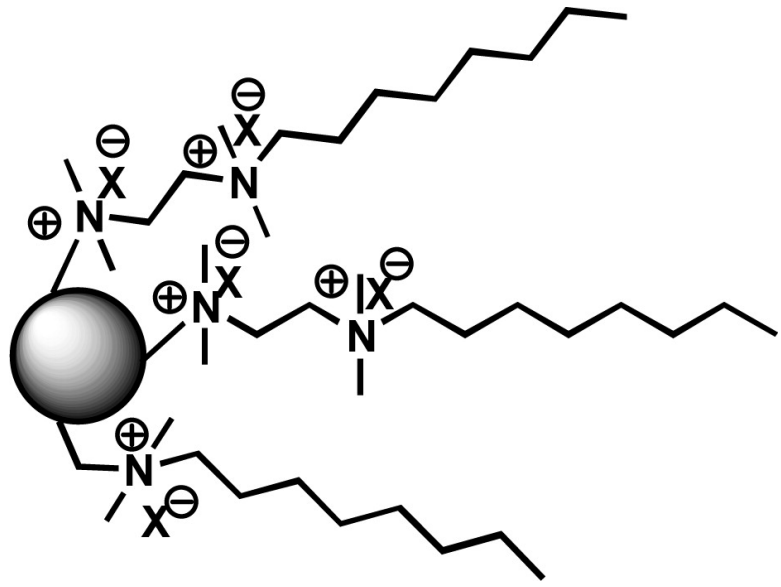

Surface Characterization and Biocompatibility of Restorative Resin Containing Nanoparticles

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Composite resins that are used to restore hard tissues have several drawbacks including the accumulation of biofilm on teeth and restorations. Recently, quaternary ammonium poly(ethylene imine) (QA-PEI) nanoparticles were developed for additional antibacterial activity of restorative composite resins. QA-PEI nanoparticles were synthesized from cross-linked poly(ethylene imine) that was N-alkylated with octyl halide, followed by quaternary methylation with methyl iodide. QA-PEI particles that were embedded in restorative composite resin at 1% w/w resulted in the complete growth inhibition of *Streptococcus mutans*. Moreover, the antibacterial activity was retained for at least 3 months. The active substances on the surface of the restorative composite resin that were incorporated with QA-PEI particles were detected by X-ray photoelectron spectroscopy (XPS) and confocal microscopy measurements. The in vitro cytotoxicity tests showed a similar effect on the viability of the cell line that was tested with composites including modified and unmodified dental composite resins. In vivo toxicity studies, which were assessed on Wistar rats by the implantation of modified composite specimens, revealed no inflammation response 1 week after the implantation of restorative composite resin that was embedded with up to 2% w/w QA-PEI.

Introduction

In the past 30 years, many studies have dealt with the antibacterial properties of various composite resins as well as their constituents.[1−9] In vitro studies have shown that the tooth-restoration interface that is created by the use of self-etching adhesive systems does not eliminate microleakage and bacterial penetration, which can lead to secondary caries, the most common reason for dental restoration failure. The lack of antibacterial properties of cured composites can lead to plaque accumulation on the surface, and bacteria such as *Streptococci mutans* can easily grow on composites.

Several studies present resin composites that release antimicrobial agents.[10−12] The conventional method for preparing antibacterial composite materials is to impregnate them with low-molecular-weight antibacterial agents, such as antibiotics, silver ions, iodine, and quaternary ammonium compounds that are gradually released over time. The low-molecular-weight antimicrobial agents have the shortcoming of the residual toxicity of the agents endangering the environment, and their effects are short lived because of the difficulty of controlling their rate of diffusion.[13,14] Another disadvantage of composites that release antimicrobial agents is an adverse influence on mechanical properties.[15] Another method of conferring an antibacterial effect to resin components is to immobilize the antibacterial components in the bulk of the composite material. For instance, Imazato et al. reported on a new monomer 12-methacryloyloxydodecylpyridinium bromide (MDPB) that was covalently bonded to the polymer network of resin composite.[16] MDPB demonstrated a long-lasting antibacterial effect without altering the mechanical properties and the curing behavior of the composite resins.[16,17] However, the antibacterial effect that was achieved with the MDPB-containing composites was less intensive in comparison with composite materials that release antibacterial agents. To overcome the disadvantages of antibacterial composite materials that release antiseptic or low-molecular-weight antimicrobial agents, a possible solution is the usage of polymeric macromolecules with antimicrobial properties. Compared with low-molecular-weight antimicrobial agents, macromolecular bactericides have some advantages; they are nonvolatile, chemically stable, have long-term antimicrobial activity, and do not permeate through the skin.[14,18,19] Distinctively, antimicrobial polymers that bear quaternary ammonium moieties have high charge densities and exhibit high antimicrobial activity.[13]

Recently, we reported on octyl alkylated quaternary ammonium poly(ethylene imine) (QA-PEI)-based nanoparticles that were embedded in restorative composite resin at 1% w/w, which completely inhibited *S. mutans* growth without leaching out and did not alter the original mechanical properties of the composite material.[20] We prepared more than 50 derivatives of polycationic particles starting from different polyamines and alkyl halides that had 4 to 16 methylene groups by using different synthetic methods. Although some of the tested nanoparticles were found to be effective in inhibiting bacterial growth, octyl alkylated QA-PEI nanoparticles that were embedded in dental composite resin at 1% w/w showed superior antibacterial effects against *S. mutans*. Octyl alkylated QA-PEI nanoparticles were prepared

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from cross-linked poly(ethylene imine) (PEI) that was N-alkylated with octyl halide, followed by quaternization of the amino groups with methyl iodide. Because the surface long-lasting antibacterial effect of the modified restorative composite resin is essential in the reduction of the bacterial biofilm, long-lasting studies were performed with modified resins, which indicated a strong antibacterial effect that lasted for at least 3 months[21] (unpublished results).

The objective of this study was to examine the surface chemical composition of the restorative composite resin that was embedded with 1% w/w QA-PEI by the use of X-ray photoelectron spectroscopy (XPS) along with confocal microscopy. Also, the biocompatibility of the restorative composite resins that were embedded with QA-PEI was evaluated relative to the corresponding untreated resin by the use of in vitro cytotoxicity tests of the eluted supernatants that were obtained from modified restorative composite resins and in vivo local toxicity studies.

Materials and Methods

Materials. PEI of an average molecular weight of 750 kDa (50% w/w aqueous solution) that was purchased from Fluka (Rehovot, Israel) was freeze dried before use. Bromooctane, iodooctane, dibromopentane, diiodopentane, iodomethane, and sodium bicarbonate were purchased from Sigma-Aldrich (Rehovot, Israel). All solvents and reagents were of analytical grade.

IR spectra were recorded on a Perkin-Elmer System 2000 FT-IR spectrometer. ^{1}H NMR spectra (DMSO-d_6 or $CDCl_3$) were obtained on a Varian 300 MHz spectrometer in 5 mm o.d. tubes. $CDCl_3$/DMSO-d_6 that contained tetramethylsilane served as the solvent and shift reference. We determined the particle size by the dynamic light scattering method by using a high-performance particle sizer (ALV-NIBS/HPPS, Langen, Germany). Measurements were done in triplicate in ethyl alcohol at 25 °C by the use of an unweighted method. The degree of alkylation and methylation was estimated by nitrogen (% N), carbon (% C) and iodine (% I) microanalysis by the use of a Perkin-Elmer 2400/II CHN analyzer. We measure the zeta potential by using a Zetasizer 2000 (Malvern, UK) at 25 °C in DDW, and measurements were done in triplicate. XPS measurements were performed on a Kratos Axis Ultra X-ray photoelectron spectrometer. The distribution of labeled QA-PEI nanoparticles that were embedded in restorative composite resin at 1% w/w was detected by the use of the Zeiss LSM 410 confocal laser scanning system. The system was equipped with an argon laser (488 nm excitation time with 510 nm long-pass barrier filter). We collected the fluorescence by employing a 100/1.35 Plan-Apochromat oil immersion lens (Zeiss). In each experiment, the exciting laser intensity, background level, PMT gain, aperture, contrast, and electronic zoom size were maintained at the same level.

We made OD measurements by using a spectrophotometer (VERSAmax, Molecular Devices, Menlo Oaks Corporate Center, Menlo Park, CA). Multiwell plates were purchased from Nunclon (Nonc, Copenhagen, Denmark). Filtek Flow was kindly supplied by 3M Dental (St. Paul, MN). Brain heart infusion broth (BHI) was purchased from Difco (Detroit, MI). Phosphate-buffered saline (PBS) and bacitracin were purchased from Sigma (St. Louis, MO).

Methods. *Synthesis of the Quaternary Ammonium Poly(ethylenene imine) Nanoparticles.* An aqueous solution of PEI was lyophilized to dryness before use. PEI (10 g, 0.23 mol monomer units) was dissolved in 100 mL of absolute ethanol. Dibromopentane was added at a 1:0.04 mol ratio (monomer units of PEI/dibromopentane). The cross-linking reaction was carried out under reflux conditions for 24 h.

We conducted N-alkylation as follows: Octyl halide was added at a 1:1 mol ratio (monomer units of PEI /octyl halide) to the same flask. The alkylation step was carried out under reflux conditions for 24 h. Excess $NaHCO_3$ (1.25 equimolar of octyl halide, 0.065 mol, 5.5 g) was added to neutralize the released acid. The neutralization reaction continued for 24 h under the same conditions.

We conducted N-methylation as follows: The methylation step was carried out with 43 mL of methyl iodide (0.69 mol), which was added at a 1:3 mol ratio (monomer units of PEI /methyl iodide) to the same flask. Methylation continued at 42 °C for 48 h. An equivalent amount of sodium bicarbonate (0.23 mol, 19 g) was added to collect the released HI during the methylation step. Neutralization was continued under the same conditions for an additional 24 h. The formed NaI salt and excess unreacted $NaHCO_3$ were discarded by decantation, and the obtained product was cooled to room temperature, was precipitated in 300 mL of double-deionized water (DDW), was washed with hexane and DDW to remove traces of the unreacted octyl halide, methyl iodide, and inorganic salts, and was freeze dried. The purification step was repeated with additional amounts of hexane and DDW, and the purified product was freeze dried. The average yield was 70% (mol/mol). FT-IR (alkylated PEI nanoparticles, NaCl, cm^{-1}): 3480 (N−H), 2950, 2930 and 2850 (C−H), 1635 (N−H, small band), 1454 (C−H). FT-IR (QA-PEI nanoparticles, KBr, cm^{-1}): 3440 (N−H), 2956, 2926 and 2853 (C−H), 1617 (N−H, small band), 1465 (C−H), 967 (quaternary nitrogen) ^{1}H NMR (cross-linked PEI nanoparticles, $CDCl_3$, δ): 1.43 (m, 2H, alkyl protons), 1.58 (m, 4H, alkyl protons), 2.1−3 (m, 4H of PEI protons and 4H of alkyl protons). ^{1}H NMR (cross-linked PEI octyl alkylated nanoparticles, $CDCl_3$, δ): 0.86 (t, 3H, CH_3, octyl protons), 1.24 (m, 10H, $-CH_2-$, octyl protons), 1.39 (m, 2H, $-CH_2-$, octyl protons), 2.36−2.7 (m, 4H, $-CH_2-$, PEI protons and 2H, $-CH_2-$, octyl protons). ^{1}H NMR (QA-PEI nanoparticles, DMSO, δ): 0.845 (t, 3H, CH_3, octyl protons), 1.24 (m, 10H, $-CH_2-$, octyl protons), 1.65 (m, 2H, $-CH_2-$, octyl protons), 3.2−3.6 (m, methyl of quaternary amine, 4H, $-CH_2-$, PEI protons and 2H, $-CH_2-$, octyl protons).

Dansyl-Labeled Quaternary Ammonium Poly(ethylenene imine)-Based Particles. PEI (2 g, 12.2 mmol ε-NH_2) was dissolved in 10 mL of anhydrous dichloromethane. Dansyl chloride (1 mL, 0.12 mmol equivalent to 1% mol/mol to ε-NH_2) dissolved in anhydrous dichloromethane was added to the PEI solution. The mixture was stirred at room temperature for 3 h. Dibromopentane (265 μL) at a 1:0.04 mol ratio (monomer units of PEI/dibromopentane) was added to the flask. Cross-linking was continued under reflux conditions for 24 h. During the cross-linking step, labeled PEI was precipitated, filtered and washed with several amounts of dichloromethane to remove unbound dansyl chloride. The product was dried overnight on NaOH pellets. Further steps including alkylation and methylation were repeated as described in the Materials and Methods section.

Bacteria and Growth Conditions. Streptococcus mutans (ATCC no. 27351), which was originally isolated from human dental plaque, was used in this study. Bacteria were cultured aerobically overnight at 37 °C in 5 mL of BHI supplemented with 0.0625 g/mL bacitracin to minimize contamination. The top 4 mL of the undisturbed overnight bacterial cultures were transferred to a new test tube and were centrifuged for 10 min at 3175*g*. The supernatant was discarded, and the bacteria were resuspended in 5 mL of PBS supplemented with 0.0625 g/mL bacitracin. The suspensions were adjusted to an OD of 0.9 at 650 nm, were 10-fold serially diluted, and were plated on BHI agar so that colony-forming units (CFU) could be determined.

Preparation of Modified Composite Resins. We prepared the experimental samples by adding QA-PEI to Filtek Flow (47% zirconia/silica, average particle size 0.01−6.0 μm; BIS-GMA, TEGDMA; 3M Dental). A 1% w/w QA-PEI powder was added to the composite resins and was homogeneously hand mixed with a spatula in a dark room for 20 s.

Direct Contact Test. A 96-well flat-bottomed microtiter plate was vertically positioned. Eight wells were coated with tested material samples (30 ± 4 mg in each well). The samples were applied on the sidewall by the use of a flat-ended dental instrument (spatula), which ensured uniform surface area. The composite resin samples were polymerized according to the manufacturers' instructions.

The bacterial suspension (10 μL, 10^6 CFU) was placed on each sample in a set of eight wells, and the plate was incubated in the vertical

position for 1 h at 37 °C. During that time, most of the suspension liquid was evaporated, which ensured direct contact between the bacteria and the tested surface.[22,23] Then, 220 μL of growth medium was added to each well, and the plate was incubated in a temperature-controlled microplate spectrophotometer that was set at 37 °C with 5 s of mixing before each reading. We estimated the growth of bacteria that shed from the biofilm by following the changes in OD_{650} every 20 min for 24 h.

Aging of Samples. Test materials that were similarly prepared and placed in microtiter plates, as above, were aged for 1 week. During this period, each well was filled with 250 μL of PBS, which was replaced every 48 h. Before we started the direct contact test, the PBS was aspirated and the plate was dried under sterile conditions.

Statistical Analysis. The absorbance measurements were plotted, which provided bacterial growth curves for each well in the microtiter plate. The linear portion of the logarithmic growth curve was used for statistical analysis. The data are expressed as the slope of the regression line that is plotted from the logarithmic bacterial growth phase. The slope values that correlated with the growth rate were expressed as percent of controls and were normalized to 100%. The data were analyzed by one-way ANOVA and the Tukey multiple comparison test. The level of significance was determined to be $p < 0.05$.

In Vitro Toxicity of Restorative Composite Resin Supplemented with Quaternary Ammonium Poly(ethylenene imine) (1 and 2% w/w). A 24-well flat-bottomed microtiter plate was vertically positioned. Four wells were coated with tested material samples (30 ± 4 mg in each well). The samples were applied on the sidewall by the use of a flat-ended dental instrument (spatula), which ensured a uniform surface area. The modified composite resin samples were polymerized according to the manufacturers' instructions. Test materials that were similarly prepared and placed in 24-well plates, as above, were aged for 1 week. Each well was filled with 1 mL of RPMI. The test restorative composite resin samples were stored in the dark at 37 °C. The supernatants of the first through seventh days and of the seventh day were individually collected in test tubes and were stored at −20 °C until further use. After every removal of the supernatants at the end of each collection, the composite samples were again immersed in 1 mL of fresh RPMI medium until the next scheduled sampling day.

Serial dilutions of the collected supernatants were prepared in RPMI 1640 growth medium. The cytotoxicity of supernatants was evaluated in murine bladder tumor (MBT) cells. Growth inhibition was estimated by the [^{3}H]-thymidine incorporation method. Cells were cultured in flat-bottomed flasks at 37 °C. Before each experiment, the cells were washed and removed by a trypsin treatment or were scraped from the bottom of the flask, and an appropriate volume was centrifuged, resuspended, and diluted in growth medium to the desired cell concentration (4000 cells/200 μL). The growth medium consisted of RPMI 1640 and 10% fetal calf serum (FCS). After an overnight incubation, supernatant dilutions of 50 μL were added in triplicate to the test wells. Supernatant-free medium was used as the control. [^{3}H]-thymidine (0.5 μCi) in 20 μL of medium was added the next day. The plate was harvested and read by a liquid scintillation counter (LKB, Finland) after an additional 24 h. The percent growth inhibition of the cells by the supernatants was calculated to be [100 − (count with supernatant/control count) × 100].

In Vivo Toxicity of the Restorative Composite Implants Containing Quaternary Ammonium Poly(ethylenene imine). Male Wistar rats that were 10 weeks old with starting body weights of 243−256 g (Harlan Laboratories, Israel) were given free access to irradiated sterile food and acidified water throughout the experiment. All experiments were carried out in accordance with the guidelines of the local Animal Welfare Committee. The Ethical Committee of the Hebrew University of Jerusalem (reg. no. 10581-4) approved the study protocol.

Restorative composite resin specimens (5 mm diameter and 1.2 mm width) that contained various concentrations of QA-PEI (i.e., E) ranging from 1 to 100% w/w were subcutaneously implanted into groups of two male Wistar rats. One group was implanted with two blank samples on the left side (control) and two restorative composite resin samples that were embedded with 1 and 2% w/w QA-PEI on the right side. A second group was implanted with restorative composite resin that was embedded with 4 and 5% w/w QA-PEI on the left side and 20 and 100% w/w QA-PEI on the right side.

Anesthetized rats were sacrificed by cervical dislocation 8 days after implantation, and the animals' skin was elevated to expose the polymer implant. The implants were taken for chemical analysis, and the surrounding tissue was fixed in 4% neutrally buffered formaldehyde solution for histopathological examination.

Histopathological Analysis. The tissue samples were processed in paraffin, and 5 μm sections were prepared and stained with hematoxylin and eosin for histological evaluation. Each sample was evaluated and graded for histopathological changes. The reactive and inflammatory changes were assigned severity grades of 0−4, which represent unremarkable, minimal, mild, moderate, and marked changes, respectively. Evaluated parameters included the presence of the capsule and histological components of the capsule such as necrosis and inflammatory cells including giant cells, fibroblasts, and mature collagen.

Chemical Surface Analysis by X-ray Photoelectron Spectroscopy. The XPS measurements were performed on a Kratos Axis Ultra X-ray photoelectron spectrometer. Spectra were acquired with a monochromated Al Kα (1486.7 eV) X-ray source with a 0° 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 1200 to −5 eV (binding energy) with 80 eV pass energy. High-resolution XPS scans were collected for C 1s, O 1s, N 1s, Si 2p, and I 3d peaks with 20 eV pass energy. 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 to be 285.0 eV.[24] Data analysis and processing were performed with Vision processing data reduction software (Kratos Analytical) and CasaXPS (Casa Software).

Results

Antibacterial Activity. QA-PEI-based nanoparticles were prepared from cross-linked poly(ethylene imine) that was N-alkylated with octyl halide, followed by quaternization with methyl iodide. Five QA-PEI-based derivatives (A, B, C, D, and E) were prepared under similar conditions and were characterized, as shown in Table 1.

The degree of alkylation was determined from the carbon/nitrogen (C/N) content following the alkylation step, and it ranged from 67 to 74%, whereas the degree of quaternization was estimated from the iodine content after the methylation step. The purpose of this experiment was to evaluate the reproducibility of the antibacterial activity of the synthetic polycation-based particles that are incorporated within the matrix of restorative composite resins. QA-PEI particles were physically embedded within restorative composite resin at 1% w/w, and their antibacterial activity against *S. mutans* was quantified by the direct contact test. We demonstrated the antibacterial effect by measuring the average OD changes that correlated with the bacterial growth of *S. mutans* that was exposed to samples that were aged for 1 week. Figure 1 shows the antibacterial activity of the modified restorative composite resins in which representative QA-PEI batches were applied as antibacterial additives. All samples showed similar potencies in complete bacterial decay.

Surface Analysis. XPS was used for surface chemical analysis of the restorative composite resin that was embedded with 1% w/w QA-PEI (i.e., E). Figure 2 shows the XPS survey spectra of the modified and unmodified resins. In addition, atomic percentage values of the analyzed samples that we measured by collecting data at the high-resolution mode are presented in Table 2.

Table 1. Characterization of QA-PEI Nanoparticles[a]

code	bacterial growth (%)[c]	elemental analysis[b] % N	% C	% I	C/N[d]	particle size (nm)[e]	zeta potential (mV)[f]
A	2.08	5.65	42.97	42.61	6.3	340	74.1 ± 17
B	2.35	5.94	40.53	39.79	5.9	370	72.1 ± 19.1
C	0.44	5.92	42.89	36.29	6.2	380	45 ± 12
D	0.123	5.72	44.56	35.65	6.8	380	73.4 ± 6.3
E	0.021	6.23	40.42	39.62	6.5	250	60.3 ± 17.3

[a] Octyl halide was reacted with cross-linked PEI nanoparticles at a 1:1 mole ratio (monomer units of PEI/octyl halide), followed by N-methylation. [b] Found nitrogen, carbon, and iodine content of QA-PEI (elemental analysis). [c] Bacterial growth (%) onto restorative composite resin containing 1% w/w QA-PEI particles. Restorative composite resin was used as a negative inhibitory control and resulted in 100% bacterial growth. [d] C/N was determined by elemental analysis of the alkylated samples before the methylation step, whereas C/N of the nonalkylated PEI was found to be 2.26. [e] Mean particle size of QA-PEI nanoparticles (diameter). [f] Zeta potential of QA-PEI nanoparticles.

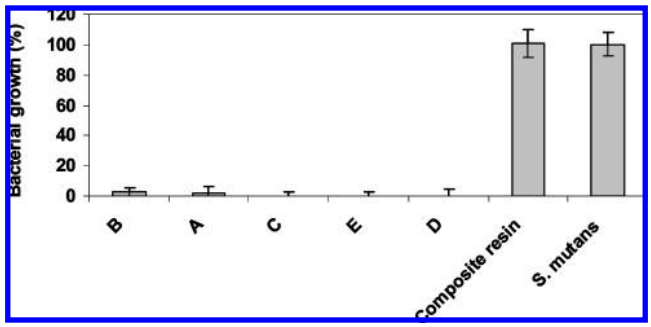

Figure 1. Analysis of *S. mutans* growth following the direct contact test with restorative composite materials that were aged for 1 week and were supplemented with 1% w/w QA-PEI particles. The linear portion of the logarithmic growth curve was used for statistical analysis. The results are expressed by the slope that was derived from the ascending part of the bacterial growth curve, correlating with the growth rate. The values are normalized to percent controls.

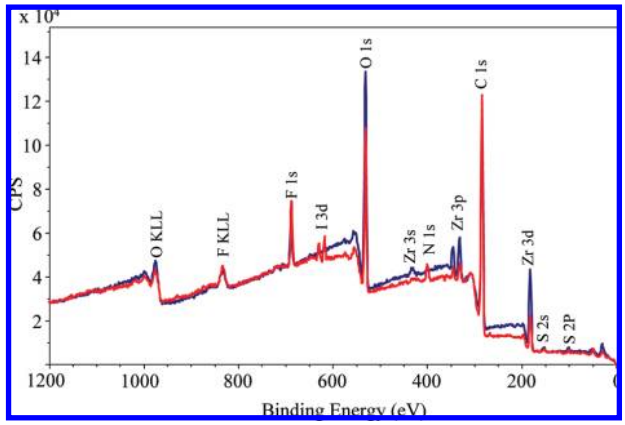

Figure 2. A wide survey scan, low-resolution XPS (0−1200 eV) of restorative composite resin (blue line) and restorative composite resin that was embedded with 1% w/w QA-PEI (red line).

Figure 3 shows the C 1s high-resolution spectrum curve-fitting analysis of modified and unmodified restorative composite resins. The major component of C 1s is based on C 1s (I) at 285.0 eV, which resulted from C−C/C−H bonds. The further deformation of the C 1s peak is attributed to C 1s (II) at 286.5 eV, which was obtained from C−COOR, and C 1s (III) at 289.1 eV, which was related to the O═C−O− bond. The C 1s peak form is attributed to the poly(methyl methacrylate) (PMMA) structure, the main component of the restorative composite resin. In addition, atomic percentage values of modified resins represent 54.83% of C 1s (I) compared with those of unmodified restorative resin, which represent only 50.58% of C 1s (I). This indicates QA-PEI contribution to the C 1s (I) content.

Atomic percentage values of O of modified and unmodified restorative composite resins are demonstrated in Table 2. The same oxygen species were detected in each spectrum of both samples. In particular, a signal that was assigned at 530.12 eV corresponded to C═O, whereas a major signal at a higher energy (532.7 eV) directed to an organic oxygen neighborhood that was received from the ester groups (−CO−O−) in PMMA resin.

Table 2. Atomic Percentage Values of Elements Determined by XPS Analysis

peak	atomic concentration (%)	mass concentration (%)
Restorative Composite Resin		
O 1s	26.42	31.90
C 1s	72.42	65.64
Si 2p	1.16	2.46
Restorative Composite Resin Embedded with 1% w/w QA-PEI		
I 3d	0.33	3.18
O 1s	17.65	21.22
N 1s	3.29	3.47
C 1s	77.82	70.24
Si 2p	0.90	1.90

The semimetal signal that resulted from Si 2p appears in both samples at a binding energy of 102.77 eV, which can be attributed to the Si restorative composite resin component.

In contrast with the unmodified restorative composite resin, resins embedded with 1% w/w QA-PEI demonstrated a similar appearance of the N 1s peak with a similar contribution of N 1s (I) and (II) (Figure 4A, Table 2). The first component of N 1s was based on N 1s (I) at a binding energy of 399.7 eV, which is attributed to the tertiary amine bonds, whereas N 1s (II) at a higher binding energy of 402.5 eV originated from quaternary ammonium groups.[25] The relative concentration of N 1s is

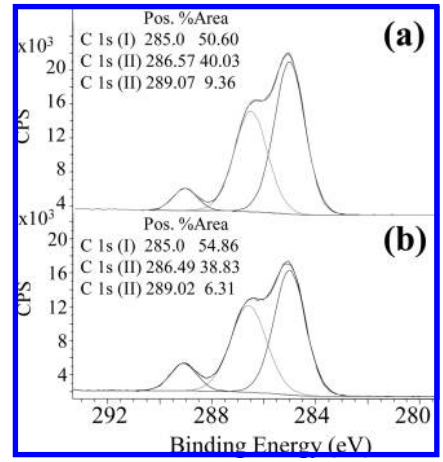

Figure 3. High-resolution XPS spectra of C 1s of (a) restorative composite resin and (B) restorative composite resin embedded with 1% w/w QA-PEI.

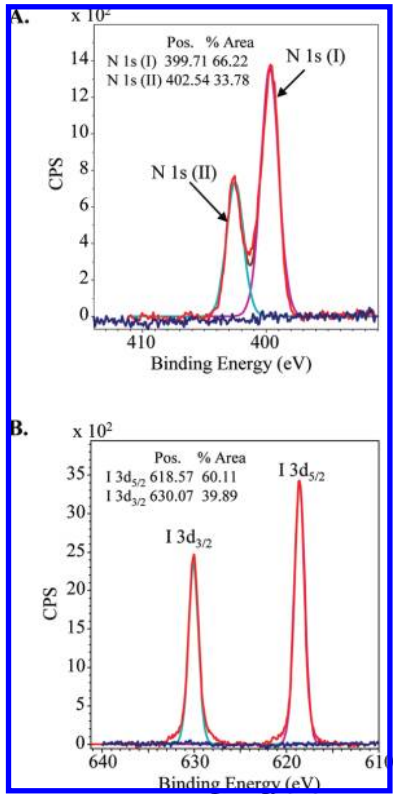

Figure 4. High-resolution XPS spectra of (A) N 1s and (B) I 3d of restorative composite resin embedded with 1% w/w QA-PEI compared with the corresponding untreated resin.

composed of 66.22% N 1s (I) and 33.78% N 1s (II), which indicates 1/3 of the quaternary ammonium groups of the overall amine content. Furthermore, two signals that were related to I 3d peaks were detected on the surface of the modified dental composite (Figure 4B, Table 2). Iodide peaks are composed of two components including I $3d_{5/2}$ at a binding energy of 618.57 eV and I $3d_{3/2}$ at 630.07 eV. There is no evidence of N 1s and I 3d peaks in unmodified resins in this area of binding energy, which indicates QA-PEI's contribution to the chemical composition of the modified restorative composite resin surface.

The dispersion state of QA-PEI in a polymerized matrix is of practical importance in the field of polymeric restorative composite resins. Therefore, for the evaluation of the qualitative distribution rate of QA-PEI in restorative composite resin, we used dansyl-labeled QA-PEI. The microstructure of the modified restorative composite resin is studied by the use of confocal microscopy. The fluorescence microscopy image (Figure 5) confirmed the homogeneous distribution of QA-PEI particles in restorative composite resin with some tendency of aggregation. QA-PEI nanoparticles that were <200 nm were out of the resolution rate, so larger particles or their aggregates were detected by the use of confocal microscopy.

In Vitro Cytotoxicity of the Restorative Composite Resins Embedded with QA-PEI. To examine the possible effect of leachable materials from the polymerized restorative composites incorporating QA-PEI (i.e., E), we tested the cytotoxicity properties of the eluted supernatants that were obtained from these materials on the MBT cell line.

After exposing the MBT cell line to the 1-day supernatants, we observed a minor reduction in cell viability that was caused by all tested samples including restorative composite resin and resins that were supplemented with 1 and 2% w/w QA-PEI (Figure 6A). The viability of MBT cells that were exposed to the materials decreased ~30% for all tested groups. There was no significant difference in the viability rates between the restorative composite resin and the resins that incorporated QA-PEI particles.

For sampling days 2−7, no significant difference in cell viability was measured for any of the tested samples. The cell viability rates of the restorative composite resin and resins that were supplemented with 1 and 2% w/w QA-PEI reached similar values during the experiment (ranging from 60 to 98%). The effects of both modified and unmodified restorative materials could be attributed to the unpolymerized monomers of methyl methacrylate, which is known to be a main component that is responsible for the cytotoxic effect of resin-containing materials.[26,27] Generally, there is no difference between the modified and the control resins throughout the experiment. Figure 6B depicts the effect of supernatants that were collected after 7 days of resin immersion in RPMI. The week old supernatants, which were obtained from both composites including modified and unmodified resins, inhibited cell growth in a similar manner. Therefore, the incorporation of QA-PEI within the matrix of the resins did not significantly increase their cytotoxicities compared with the unmodified dental composite resins.

Local Toxicity of the Restorative Composite Implants Containing QA-PEI. Maximal activity against bacteria with no effect on cell viability is required for clinical applications of a dental composite resin. The toxicity of restorative composite implants was assessed in rats. A subcutaneous implantation enables an accurate examination of the tissue response. All rats survived the entire period of the experiment. The histopathological evaluation indicated that there was a good tolerance to implants that were embedded with 1 and 2% w/w QA-PEI (i.e., E). The degree of the formed capsular tissue reaction was comparable to that formed when the blank implant was present, which suggests that there was no adverse reaction upon the

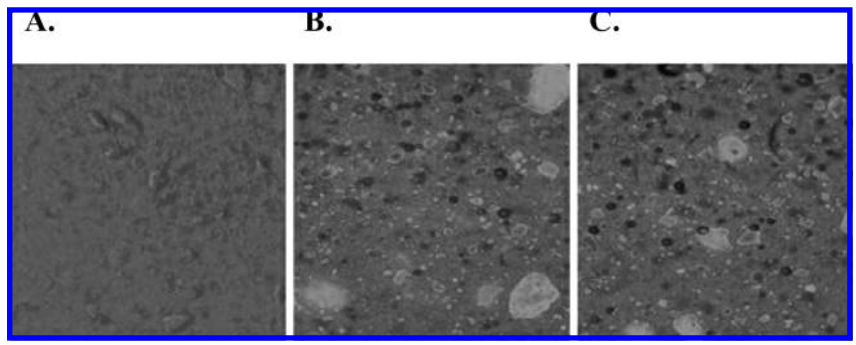

Figure 5. Confocal microscopy of dansyl-labeled QA-PEI embedded in restorative composite resin at 1% w/w. Magnification: lens ×100, zoom ×4. (A) Control restorative composite resin. (B, C) Restorative composite resin supplemented with 1% w/w QA-PEI.

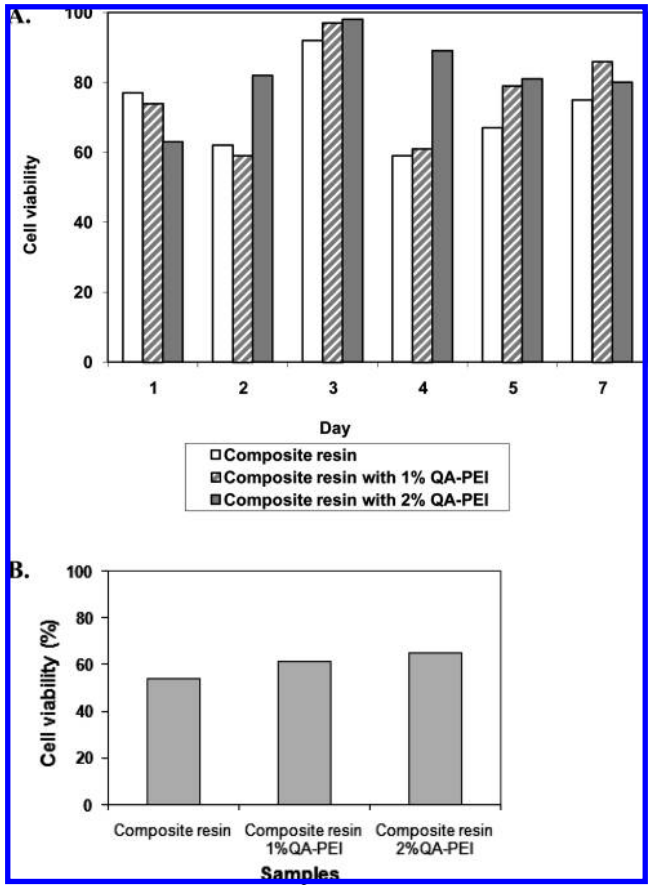

Figure 6. (A) MBT cell viability after incubation with eluted supernatants that were obtained from restorative composite resin and resins that were embedded with 1 and 2% w/w QA-PEI over the entire observation period. (B) MBT cell viability after incubation with eluted supernatants that were obtained from restorative composite resin and resins that were embedded with 1 and 2% w/w QA-PEI after 7 days of incubation.

application of the modified restorative composite resins. The formed capsule was predominantly composed of a mature collagen deposition that was associated with the presence of proliferating fibroblasts, sparse histiocytes, or other mononuclear cells. Within the capsule, there was a presence of sporadic foci that were composed of histiocytes and multinucleated giant cells that surrounded amorphous red-stained foreign material. No evidence of an active inflammatory reaction or tissue irritation was present within the capsule or extending beyond the local capsule. A photographic representation of the tissue reaction (e.g., capsule) that was observed in these groups is shown in Figure 7A. The implantation of restorative composite resins that were embedded with a higher concentration of QA-PEI (i.e., 5% w/w) resulted in a moderate tolerance, as evidenced by areas of necrosis and acute inflammation that were mainly located close to the internal surface of the capsule (Figure 7B). Furthermore, the implantation of restorative composites that were embedded with 20 and 100% w/w QA-PEI demonstrated poor tolerance, which was evidenced by a relatively thick layer of chronic active inflammation and necrosis, respectively, that surrounded the cavity (Figure 7C).

Discussion

Numerous studies have reported on composite resin materials, including the restorative composite resins that were investigated in the current study, that enable bacterial growth and adhesion compared with other restorative materials or natural tooth surfaces.[28−34] A possible explanation of this phenomenon is based on the chemical surface characteristics of the substrate. The incorporation of polycations that possess antibacterial activity in the composite resins is a strategy for the prevention of bacterial growth.

QA-PEI-based nanoparticles, which are prepared from cross-linked poly(ethylene imine) that is N-alkylated with octyl halide, followed by quaternization of the amino groups with methyl iodide, demonstrated a long-lasting antibacterial effect of the modified restorative composite resins that was prolonged for at least 3 months[21] (unpublished results). The synthetic route of QA-PEI-based nanoparticles was found to be reproducible in terms of the degree of alkylation and iodine content (Table 1). In addition, the antibacterial activity of the synthetic particles that were incorporated in the restorative composite resin was also found to be reproducible in terms of *S. mutans* growth inhibition (Figure 1). The proposed mechanism of action of QA-PEI is suggested to be by transfusing and irreparably damaging the bacterial cellular membrane/wall,[35] whereas the hydrophobic nature and positive charge of QA-PEI elevate its antibacterial activity.[21] Surface chemical analysis of the restorative composite resin that was embedded with 1% w/w QA-PEI depicted surface modification of higher hydrophobicity and presence of quaternary amines in the modified composite resins compared with the corresponding unmodified commercial material. Furthermore, the water contact angles were increased following the

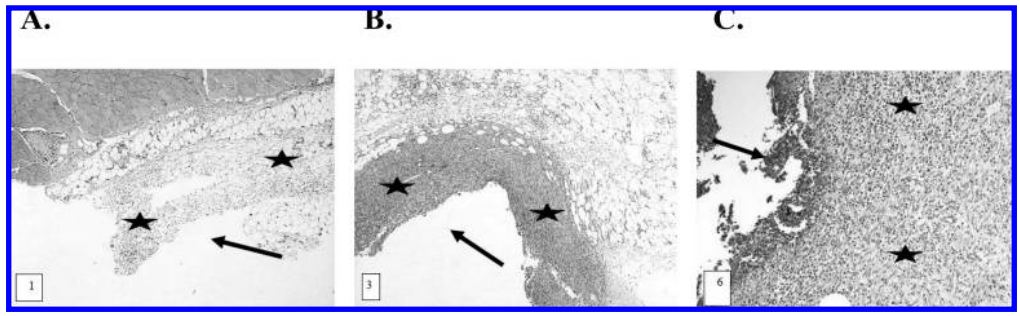

Figure 7. In all images (200×, hematoxylin and eosin), the arrow indicates the central cavity of implantation and the stars indicate the capsule. (A) Photographic presentation of a typical capsular reaction in the implanted restorative composite resin that was embedded with 1% w/w QA-PEI. Note: In all samples, the capsule had a comparable thickness and was composed of mature connective tissue and a minimal presence of dispersed mononuclear cells (graded as excellent tolerability). (B) Presentation of the typical capsular reaction of the restorative composite resin that was embedded with 5% w/w QA-PEI. Note: The capsule was composed of proliferating fibroblasts, mononuclear cells, and maturing collagen (graded as moderate tolerability). (C) Presentation of the typical capsular reaction of the implanted sample that was composed of 100% QA-PEI. Note: The cavity is surrounded by a relatively thick layer of chronic active inflammation and necrosis.

addition of the QA-PEI nanoparticles, which raised the material surface hydrophobicity[36] (unpublished results). The presence of active antibacterial groups on the surface of the modified restorative composite materials may offer an explanation of the long-lasting antibacterial properties that were achieved following the incorporation of QA-PEI.[20] In addition, in our previous studies, no appearance of surface topographical changes was detected following long-term exposure to biofilm, as presented by the use of AFM analysis[36] (unpublished results). Furthermore, the surface roughness of the composite resins that incorporated a low concentration of QA-PEI nanoparticles did not increase following 1 month of exposure to cariogenic bacteria.

The incorporation of QA-PEI at low concentrations in restorative composite resin did not change its biocompatibility when compared with the commercial composite resin in vitro and in vivo. No distinct difference in the cell viability of the eluted supernatants of the restorative composite and the composites that were supplemented with 1 or 2% w/w QA-PEI was detected in cytotoxicity studies. We note that we studied the release of QA-PEI from the restorative composite resins that contained 1% w/w QA-PEI by using various biological and chemical methods. In these tests, no residual particles or polymers were detected in the eluted supernatants. In addition, the bacterial outgrowth was similar to the outgrowth that was obtained from the eluted supernatants of the commercial composites.[20] The results of the dissolution behavior along with cytotoxicity studies indicated that the bioactivity of the composites is not through the release of compounds to the medium and that the activity is associated with surface contact.

Local in vivo studies presented by the implantation of modified dental composite materials into subcutaneous tissues of the rats indicate a normal response compared with the unmodified restorative composite resins, which suggests a possible application of the tested implants that were embedded with up to 2% w/w QA-PEI. In addition, no evidence of any active inflammatory reaction or necrosis was noted.

Conclusions

The incorporation of QA-PEI-based nanoparticles in dental composite resin at 1% w/w was biocompatible and resulted in a strong antibacterial effect against *S. mutans*. Therefore, QA-PEI nanoparticles have the potential to be incorporated into restorative composite materials to provide them with antibacterial properties without causing adverse effects.

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