Supporting Information

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SI Materials and Methods.

The protocol of the study was approved by the Helsinki Committee for Human Clinical Trials (http://clinicaltrials.gov/ Identifier: NCT00299598). Ten adults aged 25–55 yr volunteered to participate in the study. All participants were healthy and each signed an informed consent form. The general anamnesis for each subject was unremarkable. They were in healthy dental condition. Exclusion criteria were the use of (1) antibacterial mouthwashes and/or (2) antibiotics in the half year preceding the study.

PEI Nanoparticle Preparation.

The synthesis of quaternary ammonium polyethylenimine (QPEI) nanoparticle was previously described by Beyth et al. (1). Briefly, 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 (1.68 mol) were added and 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 (Scheme S1). The average yield was 70% (mol/mol). FTIR (QPEI nanoparticles, KBr): 3,440 cm^{-1} (N-H), 2,956, 2,926, and 2,853 cm^{-1} (C-H), 1,617 cm^{-1} (N-H, small band), 1,465 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, $-CH_2-$, PEI hydrogens, and 2H, $-CH_2-$, octyl hydrogens.

Preparation of Test Samples.

The test specimens were prepared by adding the synthesized polymers to a commercial resin composite FILTEK FLOW (47% zirconia/silica average particle size 0.01–6.0 μ; BIS-GMA, TEGDMA), 3M ESPE Dental. A 1% wt/wt polymer powder was added to 100 ± 20 mg of the commercial composite resin, which was homogeneously mixed in a dark room for 20 s with a spatula before polymerization. The mixed material was then pressed between two glass slides after being placed in a Teflon mold (6 mm diameter, 3 mm height), forming a disc, followed by light polymerization for 40 s using the Elipar Highlight (3M ESPE) light cure. Two test discs as described above and two similarly prepared control discs without the additional nanoparticles were prepared for each volunteer. The discs were inserted into a removable acrylic appliance prepared from heat-polymerized methyl methacrylate. Each participant wore the appliance on the upper jaw for 4 h and maintained regular behavior during the experiment. After 4 h the discs were disconnected from the appliance without touching the disc surface to be tested. The surface to be examined was turned toward the palate but was not in direct contact with it, so that the space between the disc and the tissue remained free. This allowed the biofilm to be washed with saliva and nutrients but avoided the possibility of disturbing the biofilm through contact with the tongue.

Confocal Laser Scanning Microscopy.

Confocal laser scanning microscopy (CLSM) allowed us to explore the vitality of the bacteria in the different layers of the biofilm following treatment. Biofilm was allowed to form on the discs. The removed discs ($n = 40$) were tested for biofilm formation and viability using a confocal laser microscope (2). After exposure, the samples were dyed using a live/dead kit (Live/Dead BacLight viability kit, Molecular Probes). The kit consists of two stains: propidium iodide (PI) and SYTO® 9, that both stain nucleic acids. Green fluorescing SYTO® 9 is able to enter all cells and is used for assessing total cell counts, whereas red fluorescing PI enters only cells with damaged cytoplasmic membranes. The emission properties of the stain mixture bound to DNA change due to the displacement of one stain by the other and quenching by fluorescence resonance energy transfer.

Stock solutions of the dyes were prepared as follows: PI and SYTO® 9 were used from the LIVE/DEAD BacLight kit according to the manufacturer's instructions, and SYBR green (concentrated $10^4\times$ in DMSO) was diluted 100-fold in 0.22-μm-filtered DMSO. All stock solutions were stored at −20 °C. The molar concentration of the SYBR green was not supplied by the manufacturer. The final SYBR green concentration used was diluted $10^4\times$ from the original stock (concentrated $10^4\times$ in DMSO).

Immediately after the discs were removed from the oral cavity, they were washed and then incubated in solution containing propidium iodide and SYTO® 9 (final concentration, 5 μM) and PI (30 μM). The samples were incubated in the dark at room temperature for 20 min and then were washed with PBS. A 50-μL volume of glycerol 50% was added to ensure that the samples remained hydrated. The stained bacteria were examined using a confocal microscope; the fluorescence emission of the discs was detected using a Zeiss LSM 410 confocal laser microscope (Carl Zeiss Microscopy). Red fluorescence was measured at 630 nm and green fluorescence was measured at 520 nm; objective lenses: ×60/oil, 1.4 numerical aperture. Live and dead bacteria can be viewed separately or simultaneously by fluorescence microscopy. Horizontal plane (*x-y* axes) optical sections were made at 5-μm intervals from the surface outward, and images were displayed individually. Biofilm was quantified by measuring the area occupied by the microorganisms in each individual layer in relation to the tested area. The bacterial index was determined with the aid of Image Pro 4.5 software (Media Cybernetics).

Statistical Analysis. Statistical analysis of biofilm viability and biofilm thickness in the CLSM experiments was performed using the T-paired test and the Wilcoxon nonparametric paired rank test.

Scanning Electron Microscopy (SEM).

Following examination of the 40 discs using the CLSM, one test disc and one control disc—from each volunteer—were fixed in 2% glutaraldehyde, washed in cacodylate buffer (0.1M, pH 7.2), and postfixed in 2% osmium tetra oxide for 1 h. Samples were then dehydrated through a graded series of ethanol and dried in a graded series of freon-113 in absolute ethanol. Samples were gold-coated using a Sputter coater (Polaron ES100) and examined with the aid of a Philips 505 SEM at accelerating voltage. To compare the surface views of resin composite and resin composite discs incorporating 1% QPEI, an additional set of control discs was examined without the biofilm (Fig S1 *A* and *B*, respectively).

Distribution Analysis.

The distribution of QPEI nanoparticles incorporated in resin composite were further examined to investigate whether the nanoparticles are present on the surface of the modified material and can be used to achieve antibacterial surface properties. Surface distribution analysis of QPEI incorporated at 0, 0.25, 0.5, or 1% wt/wt labeled with dansyl chloride was performed using CLSM. QPEI nanoparticles preparation steps were repeated as described above. QPEI nanoparticles (2 g, max 4.00 mmol of ε-NH_2) were dissolved in 10 mL of anhydrous dichloromethane. A volume of 1 mL (0.04 mmol equivalent to 1% mol/mol to ε-NH_2) of dansyl chloride dissolved in anhydrous dichloromethane was added to the QPEI solution. The mixture was stirred at room temperature for 3 h. After stirring, labeled QPEI nanoparticles were precipitated, filtered, and washed several times with dichoromethane to remove unbound dansyl chloride. The product was dried overnight on silica gel. Samples were examined using confocal microscopy; the fluorescence emission of the discs was detected using Olympus 300 confocal laser scanning microscope (Carl Zeiss Microscopy). Fluorescence was measured at 405 nm; objective lenses: ×63/oil, 2.0 numerical aperture. The fluorescence microscopy images confirmed the homogenous distribution of QPEI particles, with some tendency to aggregate (see Fig S2 *B–D*). The distribution of QPEI particles in relation to the tested area was quantified using Image Pro 4.5 software (Media Cybernetics) depicting 9.5%, 20%, or 31% area coverage in the 0.25, 0.5, or 1% wt/wt samples, respectively.

In Vitro Antibacterial Tests.

To distinguish between the antibacterial effect of QPEI nanoparticles and the effect of the biofilm cells on their neighboring cells, we used the extract of a biofilm grown on the surface of a resin composite incorporating QPEI. To this end, a 10-μL volume of fresh whole saliva collected from a volunteer was inoculated on the surface of each of the resin composite discs incorporating QPEI nanoparticles prepared as described previously (3). Then the discs were placed in liquid medium to allow biofilm growth for 24 h. Each disc was carefully transferred into a new test tube containing medium, and the biofilm cells were detached from the disc into the medium by sonication for 15 min (Tuttnauar-Ultrasonic cleaner, model U1424 43 KHz, (Tuttnauer). The medium collected was then incubated for 3 h and centrifuged. To isolate the factors secreted from the biofilm cells, the supernatant was then filtered through a 22-μm vacuum-driven filtration system. The extract obtained was lyophilized and the powder obtained was absorbed on the resin composite surface, which was then photopolymerized.

To gain some insight into the observed antibacterial effect, we quantified the growth inhibition of bacteria obtained from the saliva of the same volunteer using the direct contact test as described previously by Beyth et al. (4). Briefly, using the direct contact test (DCT) we studied the kinetics of bacterial growth; the bacteria were allowed to come in direct contact, under controlled conditions, with the resin composite without added QPEI but covered with the previously obtained bacterial extract in a set of 8 wells in a 96-well microtiter plate. Bacterial growth was also measured in additional wells after direct contact with the resin composite ($n = 8$) and with resin composite incorporating 1% QPEI ($n = 8$), as shown in Scheme S2. In an additional set of experiments, the effect of nanoparticle concentration on the antibacterial nonreleased efficacy of this system was tested on 2-wk-aged samples as previously described (5). Briefly, the antimicrobial effect of QPEI nanoparticles incorporated at 0, 0.25, 0.5, or 1% wt/wt in resin composite was assayed against bacteria obtained from the saliva of the same volunteer on 2-wk-aged samples using the DCT. No bacterial growth was detected in samples incorporating 1% wt/wt nanoparticles. This effect was reduced in accordance with the added concentration of nanoparticles (Fig. S3*A*). The growth of bacteria shed from the biofilm was estimated by recording the changes in optical density during 16 h. The absorbance measurements were plotted, providing bacterial growth curves. The linear portion of the logarithmic growth curve, derived from the ascending part of the curve, was expressed by two variables: the slope (a) and the constant (b) of the linear function $ax + b = y$. These variables correlate with growth rate ($a =$ slope) and the initial number of viable bacteria ($b =$ constant) as deduced from the calibration curve.

Calibration. To allow interexperimental comparison, each micotiter plate also included a set of wells for calibration of bacterial outgrowth. Ten microliters of the bacterial suspension were placed on the sidewalls of two uncoated wells, 275 μL of brain heart infusion broth were added, and the plates were gently mixed for 30 s. A 55-μL sample from each well was transferred to an adjacent set of wells containing 220 μL of medium and the fivefold dilution was repeated six consecutive times. An example of the gradual and reproducible decrease in optical density correlated with serial dilution, as can be seen in Fig. S3*B* (the calibration curves shown were obtained for the in vitro antibacterial test described in the main text). This also indicates that the initial number of viable bacteria had no effect on growth rate or on the final optical density in the stationary phase.

Statistical Analysis. The data were analyzed by one way ANOVA and the Tukey multiple comparison test. The level of significance was determined as $P < 0.05$.

1. Beyth N, Yudovin-Farber I, Bahir R, Domb AJ, Weiss EI (2006) Antibacterial activity of dental composites containing quaternary ammonium polyethylenimine nanoparticles against Streptococcus mutans *Biomaterials* 27:3995–4002.
2. Zaura-Arite E, van Marle J, ten Cate JM (2001) Conofocal microscopy study of undisturbed and chlorhexidine-treated dental biofilm *J Dent Res* 80:1436–1440.
3. Beyth N, Bahir R, Matalon S, Domb AJ, Weiss EI (2008) Streptococcus mutans biofilm changes surface-topography of resin composites *Dent Mater* 24:732–736.
4. Beyth N, Domb AJ, Weiss EI (2007) An in vitro quantitative antibacterial analysis of amalgam and composite resins *J Dent* 35:201–206.
5. Beyth N, et al. (2008) Surface antimicrobial activity and biocompatibility of incorporated polyethylenimine nanoparticles *Biomaterials* 29:4157–4163.

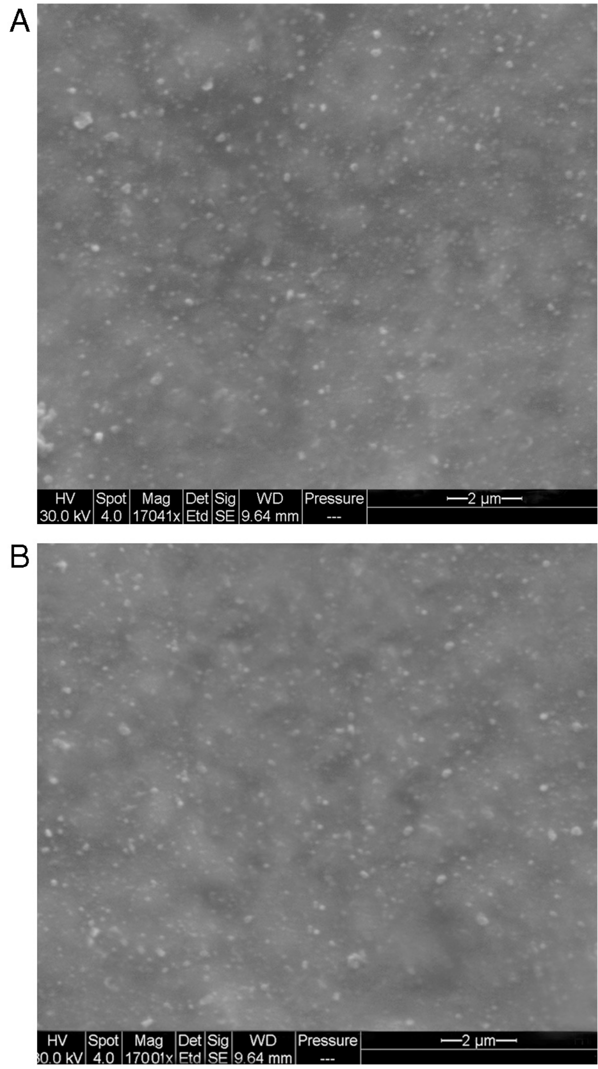


Fig. S1. SEM micrographs of resin composite and resin composite incorporating QPEI nanoparticles. Surface views of resin composite (*A*) and resin composite incorporating 1% wt/wt QPEI nanoparticles (*B*) without biofilm cells (mag. 17,000×).

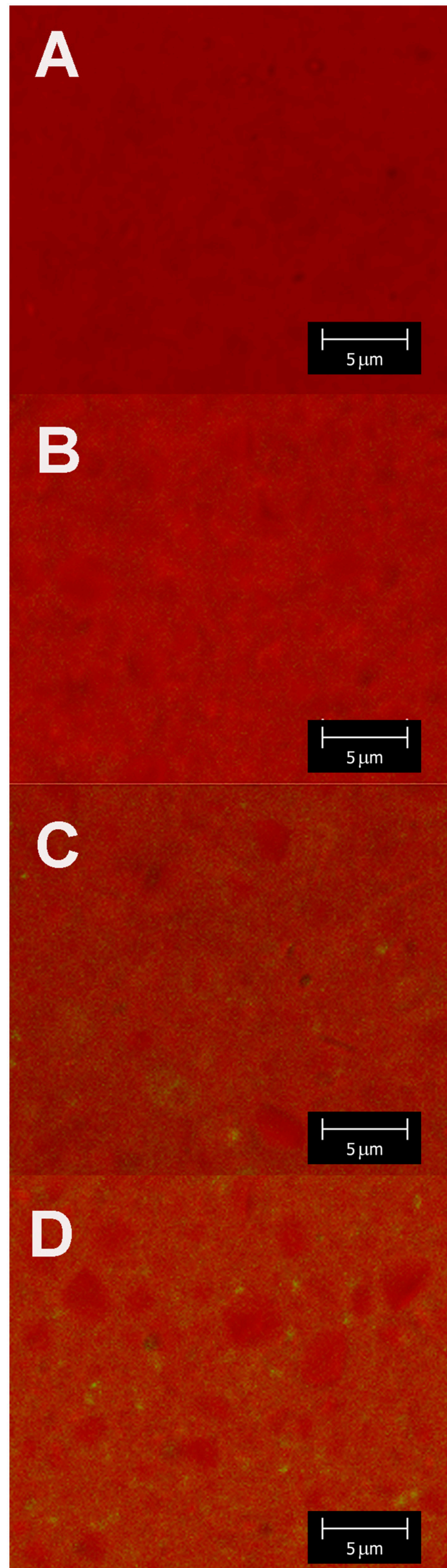
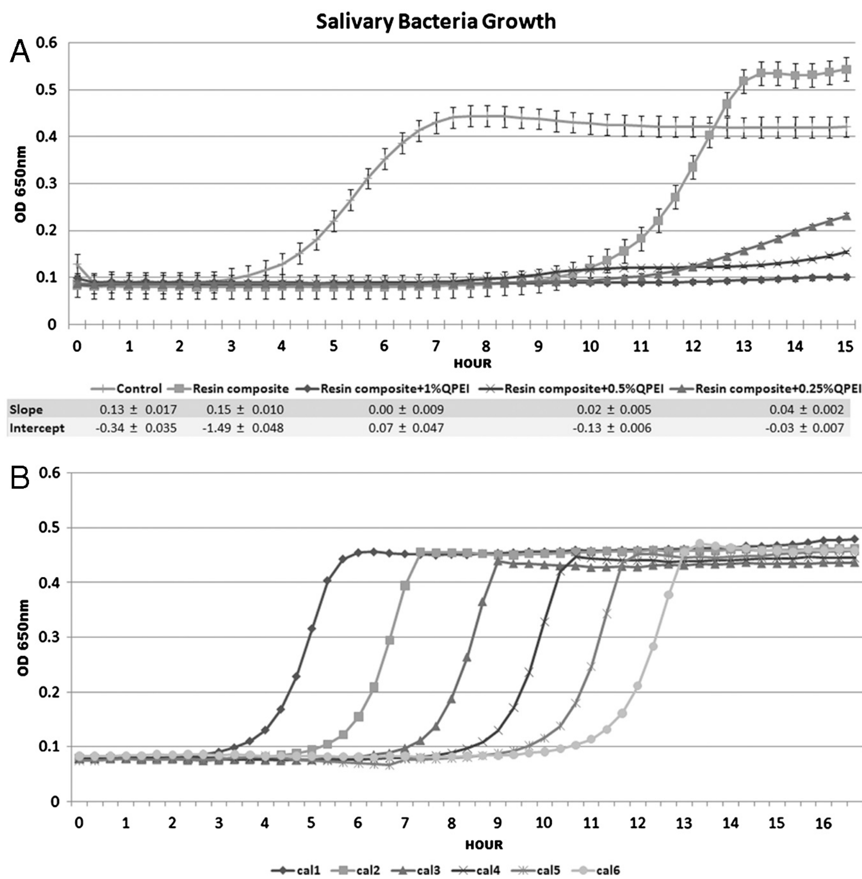
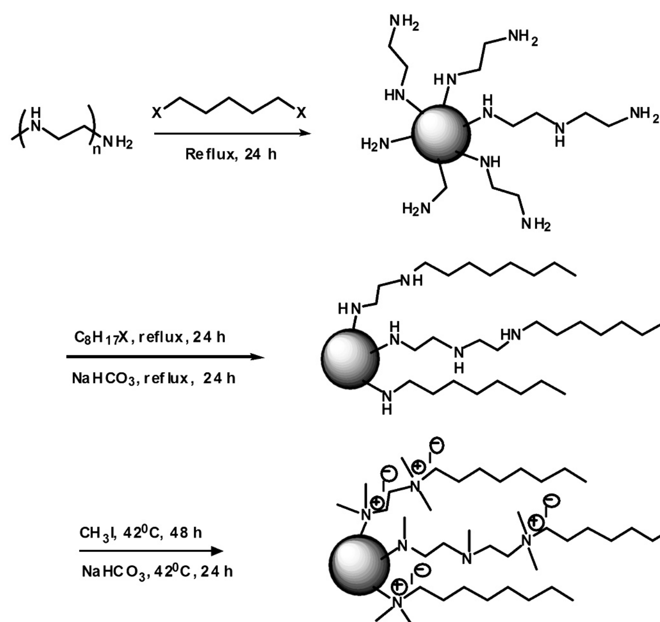


Fig. S2. Distribution of QPEI nanoparticles incorporated in resin composite. Confocal laser scanning microscopy images showing nanoparticle distribution in resin composite incorporating 0, 0.25, 0.5, or 1% wt/wt QPEI dansyl chloride (green stain). Magnification: lens ×63, zoom ×2.0. (*A*) Control resin composite. Resin composite incorporating 0.25, 0.5, or 1% wt/wt (*B*, *C*, and *D*, respectively).

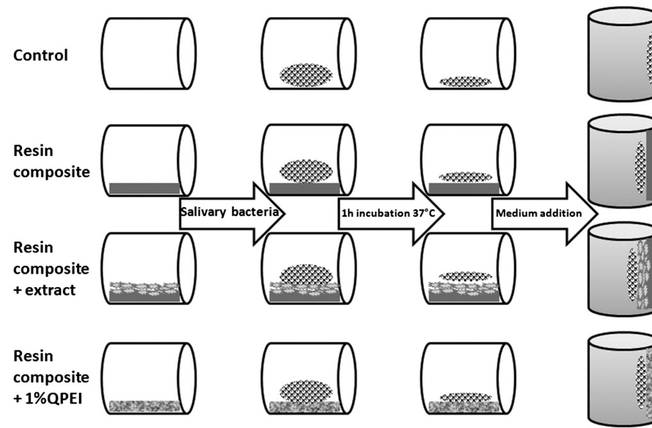


	Control	Resin composite	Resin composite+1%QPEI	Resin composite+0.5%QPEI	Resin composite+0.25%QPEI
Slope	0.13 ± 0.017	0.15 ± 0.010	0.00 ± 0.009	0.02 ± 0.005	0.04 ± 0.002
Intercept	-0.34 ± 0.035	-1.49 ± 0.048	0.07 ± 0.047	-0.13 ± 0.006	-0.03 ± 0.007

Fig. S3. In vitro antibacterial effect of resin composite incorporating 0, 0.25, 0.5, or 1% wt/wt QPEI nanoparticles, and microtiter plate surface on salivary bacteria. (*A*) Kinetic measurements depicting the growth of bacteria collected from saliva following direct contact with resin composite incorporating 0, 0.25, 0.5, or 1% wt/wt QPEI nanoparticles. Growth of the shed bacteria from the biofilm was measured every 20 min for 15 h. Each point on the curve is the average (±SD) optical density (650 nm) measured in eight replica wells similarly prepared in the same microtiter plate. The linear portion of the logarithmic growth phase was analyzed and expressed according to two variables: the slope (*a*) and the constant (*b*) of the linear function $ax + b = y$. The slope (*a*) and the constant (*b*) correlate with growth rate and initial bacterial number, respectively. (*B*) Calibration of bacterial outgrowth. An example of the gradual and reproducible decrease in optical density correlated with serial dilution (the calibration curves shown were obtained for the in vitro antibacterial test described in the main text).



Scheme S1. Quaternary ammonium polyethyleneimine nanoparticles synthesis.



Scheme S2. Direct contact test setup in a 96-well microtiter plate. The sidewall of the well was coated with the tested material. To assess the effect on early stage biofilm, bacteria were placed on the surface of the material. Incubation at 37 °C ensured bacterial interaction with the material. The kinetics of the bacterial growth was monitored.