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Supporting Information for

O^2-Protected Diazeniumdiolate-Modified Silica Nanoparticles for Extended Nitric Oxide Release from Dental Composites

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Experimental

Materials

Tetraethoxysilane, chloropropyltrimethoxysilane, bromopropyltrimethoxysilane, iodopropyltrimethoxysilane, *N*-(6-aminohexyl)aminopropyltrimethoxysilane, and tetramethoxysilane were purchased from Gelest, Inc. (Morrisville, PA). *N,N*-dimethylformamide (anhydrous), methanol (anhydrous), acetonitrile (anhydrous), tetrahydrofuran (anhydrous), ethanol, and ammonia solution (30%) were purchased from Fisher Scientific (Fair Lawn, NJ). 1-Ethoxycarbonylpiperazine, 5.4 M sodium methoxide in methanol, anhydrous sodium carbonate, chloromethyl methyl ether, sodium sulfate, magnesium sulfate and cetyltrimethylammonium bromide were purchased from Sigma Aldrich (St. Louis, MO). Nitrogen (N_2), argon (Ar), and nitric oxide calibration (26.81 PPM, balance N_2) gases were purchased from National Welders (Raleigh, NC). Pure nitric oxide gas (99.5%) used for *N*-diazeniumdiolate formation was purchased from Praxair (Sanford, NC). Reagents for the Griess assay (sulfanilamide, *N*-1-naphthylethylenediamine, and nitrite standard) were purchased from Sigma Aldrich (St. Louis, MO). Distilled water was purified

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using a Millipore Milli-Q UV Gradient A-10 system (Bedford, MA), resulting in a total organic content of ≤6 ppb and a final resistivity of 18.2 mΩ·cm. *Streptococcus mutans* (ATCC # 25175) was received from American Type Culture Collection (Manasses, VA). Estelite Σ Quick (Tokuyama Dental) resin-based dental composite was a gift from UNC Dental School.

Synthesis of mesoporous silica nanoparticles

Cetyltrimethylammonium bromide (290 mg) was added to 50 mL of a 5 mM solution of ammonium hydroxide in Milli-Q-purified water. The mixture was stirred for 1 h and heated to 40 °C to allow micelle formation. An initial 110 μL aliquot of tetraethoxysilane (TEOS) was added to the solution, with subsequent stirring for 5 h. A second 600 μL aliquot of TEOS was then added, and the solution was stirred for an additional 2 h. The particles were then allowed to age without stirring at 40 °C for 24 h. The resulting mesoporous silica nanoparticles were collected by centrifugation at 16,770×*g* for 20 mins, washed thrice with ethanol and dried in vacuo. The surfactant was removed by stirring the particles in 50 mL acidic ethanol (1:9 HCl:EtOH) at 60 °C overnight.

Synthesis of O^2-methoxymethyl 1-(4-(3-(trimethoxysilyl)propyl)piperazin-1-yl)diazen-1-ium-1,2-diolate

O^2-Methoxymethyl 1-(piperazin-1-yl)diazen-1-ium-1,2-diolate (MOM-Pip/NO) was prepared as previously described.(J. E. Saavedra, M. N. Booth, J. A. Hrabie, K. M. Davies and L. K. Keefer, *J. Org. Chem.* , 1999, **64**, 5124-5131) Briefly, 10 g of 1-ethoxycarbonylpiperazine was dissolved in 30 mL of methanol with 1.167 mL 5.4 M sodium

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methoxide in methanol. The solution was placed in a Parr stainless steel pressure vessel connected to an in-house NO reactor, purged thoroughly with Ar, then pressurized to 10 bar with NO that had been scrubbed with KOH. The pressure was maintained at 10 bar for 2 d, after which it was released and the solutions were again purged with Ar to remove unreacted NO. Cold ether was added, and the resulting white precipitate (**1**) was collected by filtration, washed with cold methanol followed by ether and dried in vacuo. To a slurry of **1** (2.2 g) and anhydrous sodium carbonate in tetrahydrofuran (100 mL) was added 0.75 mL chloromethyl methyl ether and 1 mL methanol simultaneously and dropwise under nitrogen at 0 °C. The mixture was then brought to room temperature and stirred overnight. The reaction was then filtered, evaporated to dryness, and taken up in dichloromethane. The product (**2**) was dried over sodium sulfate, filtered through magnesium sulfate, and evaporated to dryness. The ethoxycarbonyl protecting group was then removed by heating **2** to reflux in 10% ethanolic sodium hydroxide for 1 h. After cooling to room temperature, the ethanol was removed and the residue was extracted in dichloromethane, filtered, extracted with aqueous hydrochloric acid, washed with dichloromethane, and then made basic with aqueous sodium hydroxide. Lastly, the product was extracted with dichloromethane, dried over sodium sulfate and filtered through magnesium sulfate. Removal of dichloromethane yielded MOM-Pip/NO, which was further purified by Flash 40 chromatography using a 4x15 cm KP-Sil column and an eluent of 10:1 dichloromethane/methanol.

Hünig's base (0.79 mmol) was added to a solution of MOM-Pip/NO (0.53 mmol) in 1 mL anhydrous *N,N*-dimethylformamide. Chloro-, bromo-, or iodopropyltrimethoxysilane (0.58 mmol) was then added, and the reaction was allowed to proceed for 24 h at 60 °C to yield O^2-methoxymethyl 1-(4-(3-(trimethoxysilyl)propyl)piperazin-1-yl)diazen-1-ium-1,2-diolate

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(MOM-Pip/NO-TMS). *N,N*-dimethylformamide was removed by vacuum and the residue was taken up in THF. The insoluble HI salt of Hünig's base was removed by filtration, and the solvent was removed. The percent yields from chloro-, bromo- and iodotrimethoxysilane were 58, 81 and 94% as determined by ^{1}H NMR. ^{1}H NMR (CD_3CN) δ 0.61 (t, $SiCH_2$), 1.64 (m, $SiCH_2CH_2CH_2$), 2.57 (t, $Si(CH_2)_2CH_2N$), 2.83 (t, NCH_2CH_2N), 3.41 (s, OCH_2OCH_3), 3.51 (s, $Si(OCH_3)_3$), 3.55 (t, NCH_2CH_2N), 5.16 (s, OCH_2OCH_3) ppm. ^{13}C NMR (CD_3Cl) δ 6.46 ($SiCH_2CH_2$), 12.08 ($SiCH_2CH_2$), 50.56 ($SiOCH_3$), 50.97 ($CH_2NCHCHN$), 54.48 (OCH_2OCH_3), 57.07 ($CH_2NCH_2CH_2N$), 59.94 ($SiCH_2CH_2CH_2N$), 97.88 (OCH_2OCH_3) ppm. ESI/MS (in CH_3CN): 353.08 m/z.

Synthesis of O^2-protected diazeniumdiolate-modified silica nanoparticles

MOM-Pip/NO-TMS was dissolved in 1 mL tetrahydrofuran and added to a suspension of 10 mg of MSNs in 9 mL tetrahydrofuran. The mixture was refluxed overnight, and the resulting MOM-Pip/NO-modified MSNs were collected by centrifugation (3645×*g*, 10 min), washed twice with tetrahydrofuran, then twice with ethanol. The particles were then dried in vacuo overnight, and stored in a vacuum-sealed container protected from light at -20 °C until further use.

Synthesis of piperazine-modified silica nanoparticles

Iodopropyltrimethoxysilane (250 µL) was added to a suspension of 25 mg MSNs in 10 mL toluene and refluxed overnight. The resulting iodopropyl-modified silica particles were collected by centrifugation (3645×*g*, 10 min), washed twice with toluene, then twice with ethanol. The particles were then dried in vacuo. Energy dispersive spectroscopy (INCA

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PentaFET –x3, Oxford Instruments) indicated that the resulting particles contained 6.33 wt% iodine (0.5 μmol iodine/mg particle). To a suspension of 10 mg iodopropyl-modified particles in 5 mL DMF was added 8.55 mg piperazine dissolved in 5 mL DMF and 17.4 μL Hünig's base. After reacting at 60 °C for 24 h, the particles were collected by centrifugation (3645×*g*, 10 min), washed once with DMF, then twice with ethanol. The particles were then dried in vacuo overnight, and EDS was used to confirm the absence of iodine (<1 wt%) and successful addition of piperazine.

Synthesis of unprotected diazeniumdiolate-modified silica nanoparticles

Silica nanoparticles composed of *N*-(6-aminohexyl)-aminopropyltrimethoxysilane (AHAP) and tetramethoxysilane (TMOS) were synthesized via a modified Stöber method as previously described.(Carpenter et al., *Biomacromolecules*, **2012**, *13*, 3334-3342) Briefly, AHAP (1.173 mL) and TMOS (0.708 mL) were mixed and added to a solution of ethanol (59.16 mL), water (27.84 mL), and ammonium hydroxide (9.8 mL). After 2 h reaction at room temperature, the resulting particles were collected by centrifugation (3645×*g*, 10 min, 4 °C) and washed three times with ethanol. After drying in vacuo, 20 mg of AHAP particles were suspended by sonication in 4 mL of a 1:9 mixture of methanol and *N,N*-dimethylformamide with 50 μL 5.4 M sodium methoxide in methanol. The particles suspensions were then purged with Ar and exposed to NO in the same manner as described above. After 3 d, the resulting *N*-diazeniumdiolate-modified silica nanoparticles (AHAP/NO) were collected by centrifugation (3645×*g*, 10 min, 4 °C), washed three times

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with ethanol, and dried in vacuo.

Characterization

Total amounts of NO released from the particles were evaluated using the Griess assay. MOM-Pip/NO-modified particles were suspended in oxygenated phosphate buffered saline (PBS, pH 7.4) and liberated NO was converted to nitrite (NO_2^-). Aliquots of the particle suspension were taken at the time points indicated in Figure 4. The particles were removed by centrifugation, and 50 μL aliquots of the supernatant were reacted with equal volumes of 1 wt% solutions of sulfanilamide and *N*-1-naphthylethylenediamine. The formation of an azo compound was detected by measuring the absorbance at 540 nm and comparing the results to a calibration curve constructed using nitrite standards to determine the total NO released from the particles. Real-time nitric oxide release was monitored using a Sievers NOA 280i Chemiluminescence NO Analyzer (Boulder, CO) connected to a customized reaction vessel. Nitric oxide-releasing materials were placed in deoxygenated media at 37 °C with NO carried to the analyzer by passing N_2 through the solution at a constant rate of 70 mL/min. The analyzer was calibrated with air passed through a NO zero filter (0 ppm NO) and a 26.39 ppm NO standard gas (balance N_2).

Particle size and morphology were characterized using a Hitachi S-4700 Scanning Electron Microscope (Pleasanton, CA). Covalent incorporation of MOM-Pip/NO-TMS onto the MSNs was confirmed using solid-state cross-polarization/magic angle spinning (CP/MAS) ^{29}Si NMR with a Bruker 360 MHz DMX spectrometer (Billerica, MA) equipped with wide-bore magnets (triple-axis pulsed field gradient double-resonance probes). Sampleswere packed into a 4 mm rotor (double-resonance frequency of 71.548 Hz) and spun at 10kHz. Chemical shifts were

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determined in parts per million relative to a tetramethylsilane external standard. Nitrogen adsorption/desorption isotherms were obtained using a Micromeritics Tristar II Porosimeter (Norcross, GA) after outgassing the particles at 110 °C for 18 h. The specific surface area was calculated using Brunauer-Emmet-Teller theory. Carbon, hydrogen and nitrogen content were determined on a Perkin Elmer CHN/S elemental analyzer operated in CHN mode.

Particle-doped dental composites

Particle-doped resins were prepared by mixing 1.4 mg particles into 138.6 mg resin with a spatula. Particle-doped resins were pressed between glass slides into uniform disks with a diameter of 1 cm and a thickness of 0.1 cm. The composites were cured using a Translux Power Blue dental curing light (440–480 nm) from Heraeus Kulzer (South Bend, IN).

Bacterial adhesion assay

Streptococcus mutans was grown to 10^8 CFU/mL in BHI broth, centrifuged (3645×*g*, 10 min), resuspended in PBS, and diluted to 10^6 CFU/mL in 10% (v/v) BHI in PBS. Particle-doped composite resins were placed in 1.0 mL of 10^6 CFU/mL and incubated at 37 °C with gentle shaking. After 24 h, the composites were removed and rinsed with distilled water to remove loosely adhered bacteria. The composites were then placed in 1.0 mL of fresh PBS. Adhered bacteria were removed by sonication (60 kHz, 10 min). Complete removal of adhered bacteria was confirmed by imaging the composites with atomic forcemicroscopy (AFM). Aliquots (100 µL) were taken from the resulting bacteria suspensions,plated on BHI agar nutrient plates, and enumerated after incubating for 48 h at 37 °C.

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Atomic force microscopy imaging of composites

Prior to imaging, substrates were gently rinsed in sterile water and allowed to dry under ambient conditions. AFM images were collected in contact mode using an Asylum MFP-3D AFM (Santa Barbara California) and Olympus TR400PSA silicon nitride probes. At least three 10 μm^2 images of each substrate were taken at a resolution of 1024x1024 pixels and scan speed of 1 Hz. Root-mean-squared (rms) roughness of substrates was determined using MFP-3D software over four 2-μm^2 scan regions.

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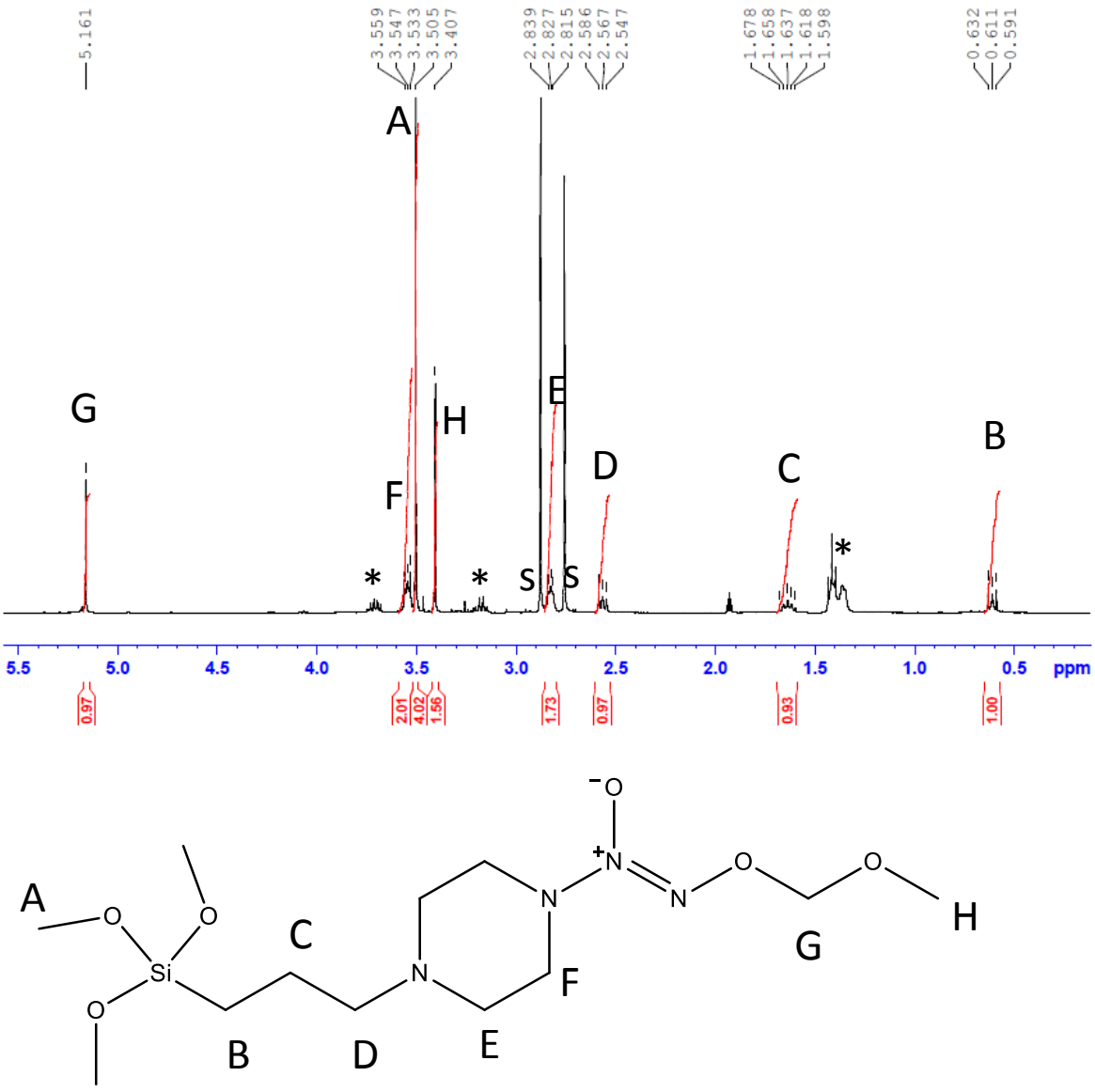

Figure SI.1 ^{1}H NMR O^{2}-methoxymethyl 1-(4-(3-(trimethoxysilyl)propyl)piperazin-1-yl)diazen-1-ium-1,2-diolate. * = Hunig's base, s = solvent (DMF)

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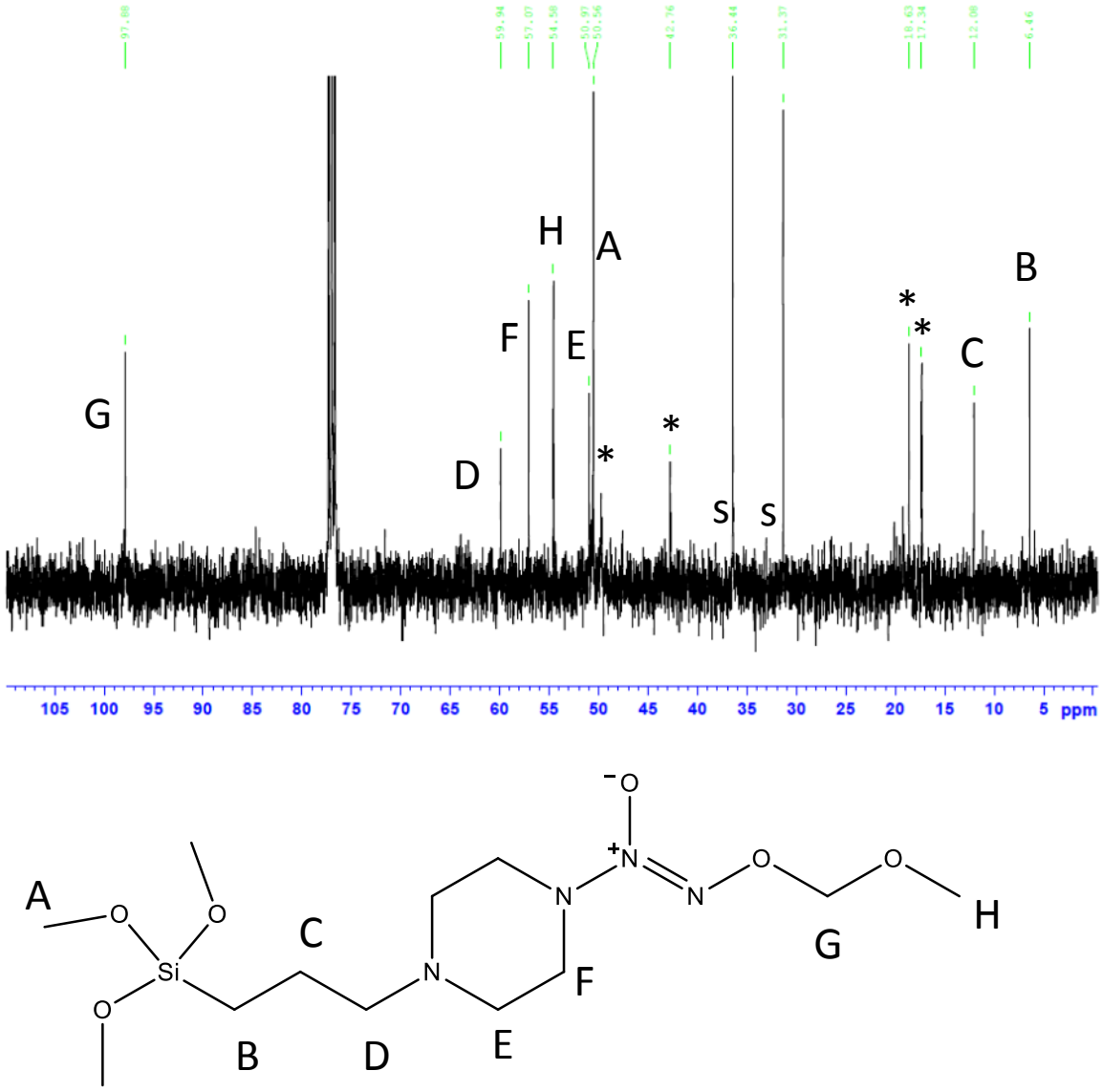

Figure SI.2 ^{13}C NMR *O*^{2}-methoxymethyl 1-(4-(3-(trimethoxysilyl)propyl)piperazin-1-yl)diazen-1-ium-1,2-diolate. * = Hunig's base, s = solvent (DMF)

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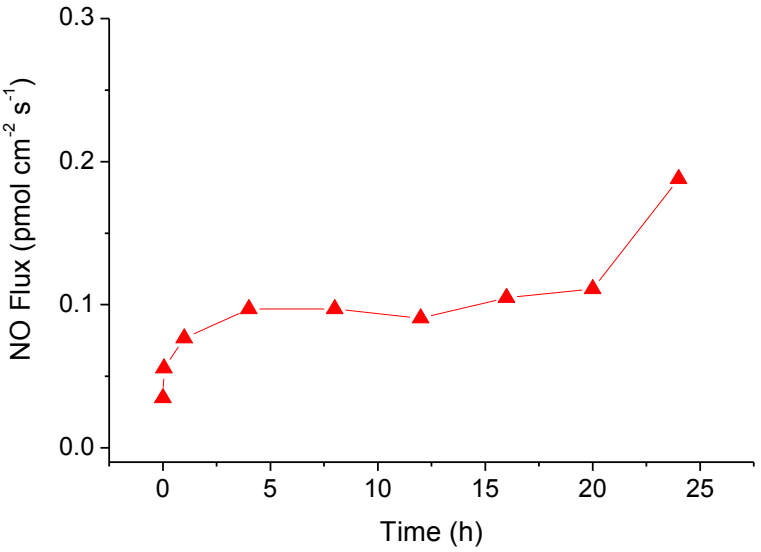

Figure SI.3 Nitric oxide release in 10 vol% BHI in PBS from dental composites doped with MOM-Pip/NO (triangle) particles.

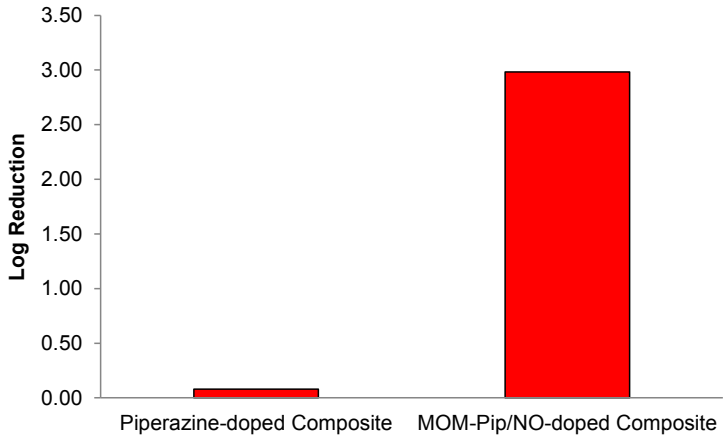

Figure SI.4 Log reduction in the number of viable *S. mutans* (CFU/mL) adhered to composites doped with piperazine-modified particles and MOM-Pip/NO-modified particles compared to control (undoped) composites. The insignificant difference between undoped and piperazine-doped composites indicates that the piperazine moiety does not contribute to the resistance to adhesion exhibited by the MOM-Pip/NO-doped composites.

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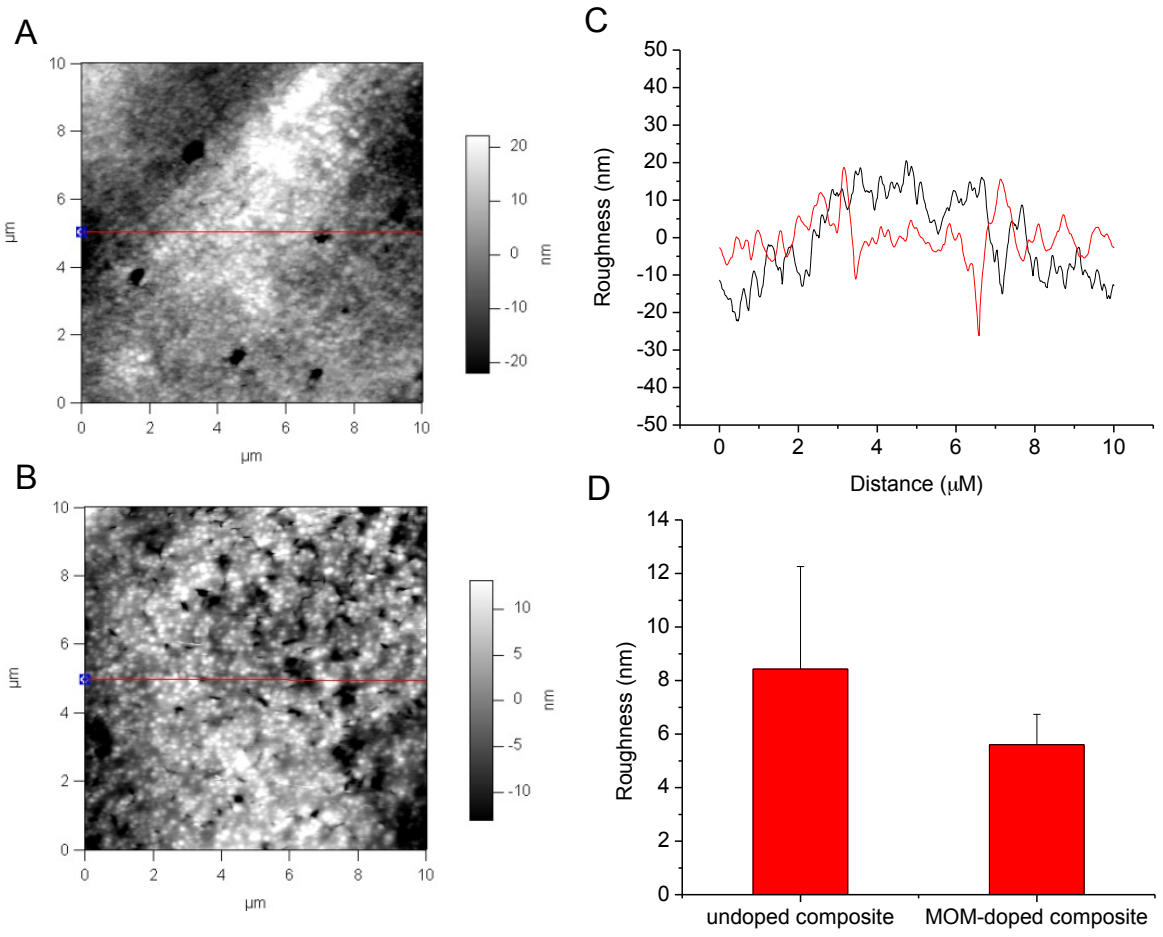

Figure SI.5 Atomic force micrographs of (A) control, (B) MOM-Pip/NO-doped composites, (C) representative height trace from center section of each image (indicated by horizontal red line), and (D) rms surface roughness determined from four 2 μm^2 areas.