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Kinetic-dependent Killing of Oral Pathogens with Nitric Oxide

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Appendix

Materials and Methods

Materials

Tetramethyl orthosilicate (TMOS), trypsin, penicillin streptomycin (PS), phenazine methosulfate (PMS), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS), Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS) for cell culture, and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 3-Methylaminopropyltrimethoxysilane (MAP3), *N*-(6-aminohexyl)aminopropyltrimethoxysilane (AHAP3), and *N*-(2-aminoethyl)-3-aminopropyltrimethoxysilane (AEAP3) were obtained from Gelest (Morrisville, PA, USA). Anhydrous methanol (MeOH), *N,N*-dimethylformamide (DMF), and sodium methoxide (5.4 M in MeOH) were purchased from Acros Organics (Geel, Belgium). Ethanol (EtOH), ammonium hydroxide (30 wt%), Tris(hydroxymethyl) aminomethane (Tris), and Tris hydrochloride were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Pure nitric oxide (NO) gas (99.5%) was purchased from Praxair (Sanford, NC, USA). Nitrogen (N_2), argon (Ar), carbon dioxide (CO_2), biological atmosphere mixture (5% CO_2, 10% H_2, 85 % N_2), and NO calibration (26.81 ppm, balance N_2) gases were acquired from National Welders (Raleigh, NC, USA). 4,5-Diaminofluorescein diacetate (DAF-2 DA) was purchased from Calbiochem (San Diego, CA, USA). Brain heart infusion (BHI) broth, BHI agar, Wilkins-Chalgren agar, and anaerobe broth were obtained from Becton Dickinson (Sparks, MD, USA). *Streptococcus mutans* (ATCC 25715), *Aggregatibacter actinomycetemcomitans* (ATCC 43717), and human gingival fibroblasts (HGF-1) (ATCC CRL-2014) were purchased from the American Type Culture Collection (Manassas, VA, USA). *Porphyromonas gingivalis* (strain A7436) was provided by the UNC Dental School. Distilled water was purified to a resistivity of 18.2 MΩ and a total organic content of <6 ppb using a Millipore Milli-Q Gradient A-10 system (Bedford, MA, USA). All other solvents and reagents were analytical grade and used as received.

Synthesis and Characterization of NO-releasing Silica Particles

The Stöber co-condensation of aminosilanes with alkoxysilanes to create secondary amine-modified silica particles has been reported previously (Shin et al. 2007). Briefly, hybrid particles were synthesized by combining TMOS with an aminosilane (MAP3, AHAP3, AEAP3) and bolus injection of the silane mixture into a flask containing water (27.84 mL), ammonium hydroxide (9.8 mL), and EtOH as a cosolvent to a total volume of 100 mL. After stirring for 2 h under ambient conditions, the particles were collected via centrifugation (2,907 × *g*; 4 °C; 10 min) and decanting the supernatant. The pellet was resuspended in EtOH, centrifuged, and decanted twice more to remove unreacted silanes. To achieve different particle compositions (i.e., 50 and 70 mol% MAP3, 60 mol% AHAP3, and 80 mol% AEAP3), the total silane concentration (0.12 M for MAP3 and AHAP3; 0.16 M for AHAP3) for each particle system was held constant while varying the mol% aminosilane (balance TMOS). For example, 50 mol% MAP3 particles were prepared by mixing 1.20 mL MAP3 and 0.90 mL TMOS. To control particle size, the concentration of water and catalyst, as well as the cosolvent chain length, were kept constant while the identity and percent composition of the silanes were optimized to provide spherical, monodisperse particles.

The resulting secondary amine-functionalized silica particles were dried under vacuum. A portion (30 mg) of the particles was suspended in 6 mL 9:1 DMF/MeOH with 25 or 50 µL NaOMe. This solution was sealed in a Parr reaction bottle, flushed briefly with Ar 3 times, and followed by 3 longer (10 min) purges with Ar to remove oxygen before being pressurized to 10 bar NO. After 3 d of constant stirring, the Ar purging process was repeated to remove unreacted NO. Particles were collected via centrifugation (2,907 × *g*; 4 °C; 10 min) and decanting of the supernatant. The pellet was resuspended in EtOH, centrifuged, and decanted twice more to remove

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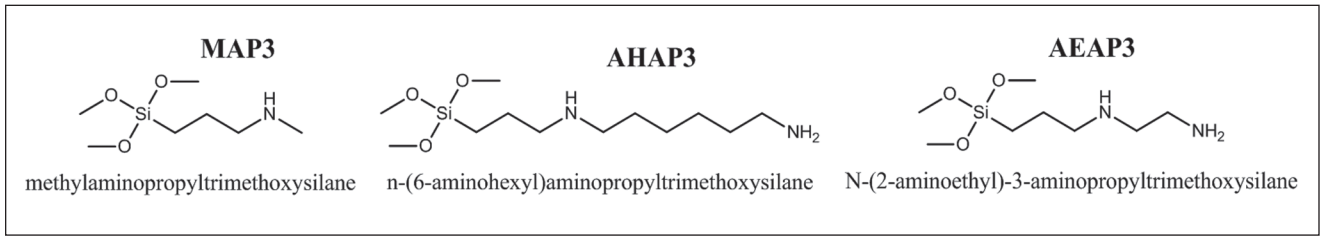
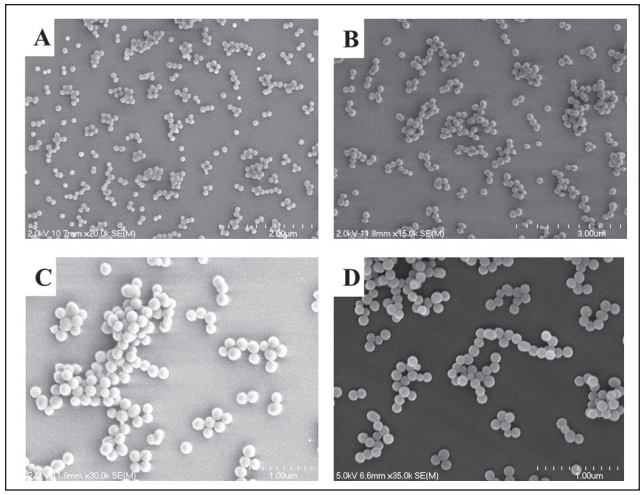
Appendix Table. Silica Nanoparticle Characterization.[a]

Aminosilane	Diameter (nm)[b]	Diameter (nm)[c]	PDI[c]	Zeta Potential (mV)[d]	Nitrogen wt%[e]
MAP3	166 ± 14	197 ± 7	0.04 ± 0.02	20.9 ± 1.8	3.42 ± 0.17
AHAP3	174 ± 15	239 ± 14	0.05 ± 0.02	31.4 ± 2.5	4.28 ± 0.07
AEAP3	125 ± 13	148 ± 9	0.06 ± 0.02	18.3 ± 1.1	5.83 ± 0.17

AEAP3, *N*-(2-aminoethyl)-3-aminopropyltrimethoxysilane; AHAP3, *N*-(6-aminohexyl)aminopropyltrimethoxysilane; MAP3, 3-methylaminopropyltrimethoxysilane.
[a]Results presented as mean ± standard deviation for $n = 3$ or more pooled experiments.
[b]Geometric diameter estimated using scanning electron microscopy and ImageJ software.
[c]Hydrodynamic diameter and particle polydispersity index measured in water using dynamic light scattering.
[d]Zeta potential measured using DLS in Tris–phosphate-buffered saline (pH 7.4).
[e]Quantified using carbon, hydrogen, and nitrogen (CHN) elemental analysis.



Appendix Figure 1. Chemical structures of MAP3, AHAP3, and AEAP3 aminosilanes. AEAP3, *N*-(2-aminoethyl)-3-aminopropyltrimethoxysilane; AHAP3, *N*-(6-aminohexyl)aminopropyltrimethoxysilane; MAP3, 3-methylaminopropyltrimethoxysilane.



Appendix Figure 2. Scanning electron micrographs of (**A**) 80% AEAP3, (**B**) 60% AHAP3, (**C**) 50% MAP3, and (**D**) 70% MAP3 silica nanoparticles. AEAP3, *N*-(2-aminoethyl)-3-aminopropyltrimethoxysilane; AHAP3, *N*-(6-aminohexyl)aminopropyltrimethoxysilane; MAP3, 3-methylaminopropyltrimethoxysilane.

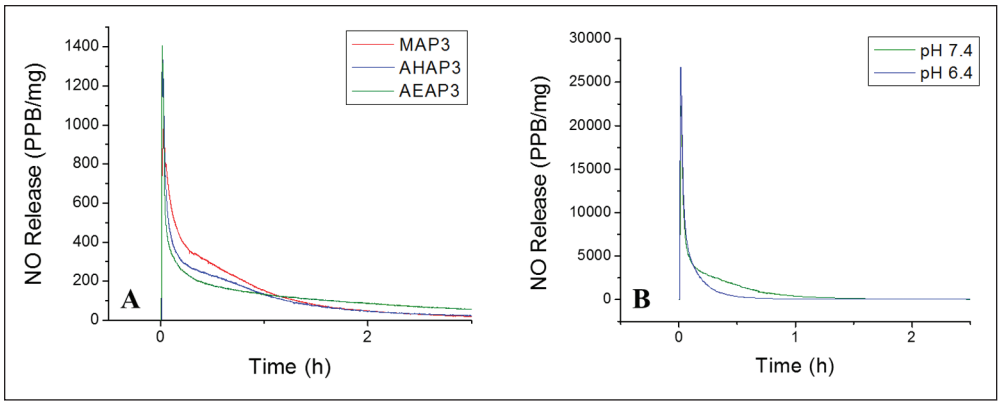
residual solvent and NaOMe. The resulting *N*-diazeniumdiolate NO donor-modified silica particles were dried under vacuum prior to storage in a vacuum-sealed freezer bag at −20 °C.

Particle size (i.e., hydrated diameter) and monodispersity were measured by dynamic light scattering (DLS) using a Malvern Instruments Zetasizer Nano (Worcestershire, UK). Samples were suspended in water at either 0.1 or 0.2 mg/mL and sonicated for 20 min prior to analysis at room temperature. Zeta potential measurements were performed at the same concentrations but using Tris-PBS (pH 7.4) or PBS (pH 6.4) as the dispersant. Particle morphologies and sizes were evaluated using a Hitachi S-4700 Scanning Electron Microscope (SEM; Chapel Hill Analytical Nanofabrication Laboratory) and ImageJ software. The concentration of aminosilane was quantified as nitrogen wt% using a PerkinElmer 2400 Series II CHNS/O Elemental Analyzer operated in CHN mode (Perkin Elmer, Waltham, MA, USA).

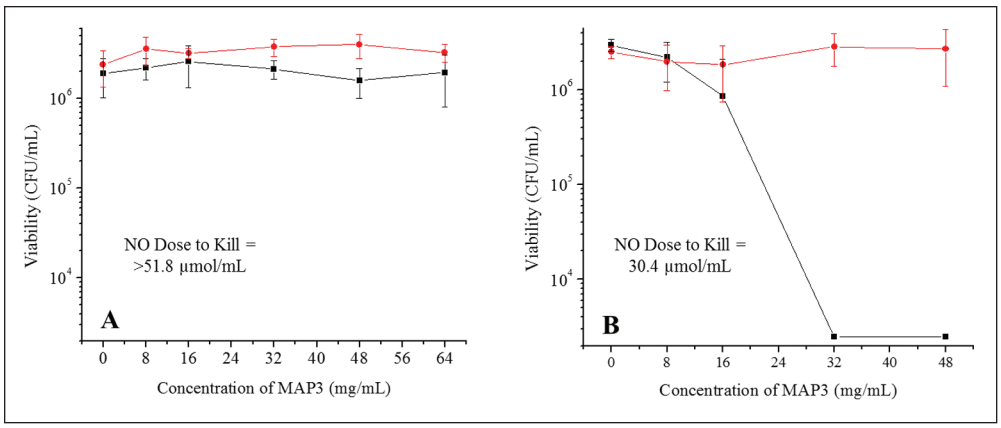
Real-time NO release was measured at 37 °C using a Sievers 280i Chemiluminescence Nitric Oxide Analyzer (NOA; Sievers, Boulder, CO, USA) (Coneski and Schoenfisch 2012). Briefly, NO-releasing particles (~1 mg) were added to a flask containing 30 mL deoxygenated PBS (pH 7.4 or 6.4). The flask was purged continuously with N_2 at 80 mL/min to carry liberated NO to the analyzer. Analysis was terminated when NO levels decreased to <10 ppb/mg.

Bacterial Growth

S. mutans was cultured aerobically. *A. actinomycetemcomitans* was cultured in a microaerophilic environment (6%−16% oxygen and 2%−10% carbon dioxide) produced by a GasPak EZ Campy Container System (Becton Dickinson, Franklin Lakes, NJ, USA). *P. gingivalis* was cultured anaerobically in 5% CO_2, 10% H_2, and 85% N_2 using a Coy Laboratory Products anaerobic chamber (Grass Lake, MI, USA). Bacterial cultures were grown overnight in BHI broth at 37 °C or Difco anaerobe broth (*P. gingivalis*). A 500-µL aliquot was reinoculated into 50 mL of fresh broth, incubated at 37 °C, and grown to 10^8 colony-forming units per milliliter (CFU/mL) as determined by optical density (OD) at 600 nm. This bacteria solution was pelleted via



Appendix Figure 3. Representative NO-release curves over 2 h for (**A**) NO-releasing 50% MAP3, 60% AHAP3, and 80% AEAP3 at pH 7.4 and (**B**) NO-releasing 70% MAP3 at pH 7.4 and 6.4. AEAP3, *N*-(2-aminoethyl)-3-aminopropyltrimethoxysilane; AHAP3, *N*-(6-aminohexyl)aminopropyltrimethoxysilane; MAP3, 3-methylaminopropyltrimethoxysilane; NO, nitric oxide.



Appendix Figure 4. Bactericidal efficacy of 70 mol% MAP3 particles against *Streptococcus mutans* in (**A**) Tris-PBS (pH 7.4) and (**B**) PBS (pH 6.4) after a 2 h exposure. NO-releasing material denoted by rectangles (■) and non-NO-releasing controls denoted by circles (●). Error bars signify standard deviation of the mean bacterial viability (CFU/mL). For all measurements, $n = 3$ or more pooled experiments. CFU, colony-forming units; MAP3, 3-methylaminopropyltrimethoxysilane; NO, nitric oxide; PBS, phosphate-buffered saline.

centrifugation (2,355 × *g*; 10 min), resuspended in distilled water, and diluted to 10^6 CFU/mL in Tris-PBS (pH 7.4) with 5% or 1% (v/v) broth for periodontopathogens and *S. mutans*, respectively. *S. mutans* was also tested in PBS (pH 6.4), with 5% BHI (v/v).

Confocal Microscopy for Intracellular NO Detection

A. actinomycetemcomitans and *S. mutans* were cultured as described above and diluted to 10^6 CFU/mL in Tris-PBS (pH 7.4) containing 5% BHI broth and 10 µM DAF-2 DA. *S. mutans* was also diluted to 10^6 CFU/mL in PBS (pH 6.4) containing 5% BHI broth and 10 µM DAF-2 DA. Aliquots of the bacteria solutions were incubated in a glass-bottom confocal dish (MatTek Corporation, Ashland, MA, USA) for 45 min at 37 °C. A Zeiss 510 Meta inverted laser scanning confocal microscope with a 488-nm Ar excitation laser (30.0 mW, 2.0% intensity) and a 505- to 530-nm band-pass filter was used to obtain DAF-2 (green) fluorescence images. Bright-field and fluorescence images were collected using an N.A. 1.2 C-Apochromat water immersion lens (Carl Zeiss Microscopy, LLC., Thornwood, NY, USA) with a 40× objective. Solutions of MAP3, AHAP3, and AEAP3 NO-releasing particles (2.0 mg/mL) in 1.5 mL Tris-PBS (10 µM DAF-2 DA) were added to 1.5 mL of the *A. actinomycetemcomitans* bacterial suspension in the confocal dish for a final concentration of 1.0 mg/mL. Solutions of 70 mol% MAP3 NO-releasing particles (0.75 mg/mL) in 1.5 mL pH 7.4 Tris-PBS or 1.5 mL pH 6.4 PBS (10 µM DAF-2 DA) were added to 1.5 mL of the *S. mutans* bacterial suspension in the confocal dish for a final concentration of 0.25 mg/mL. Images were collected every 5 min to observe the changes in intracellular NO temporally. The fluorescence signal was color inverted for clarity. Confocal micrographs of *S. mutans* were noise filtered with MATLAB (Natick, MA, USA) using a background subtraction threshold value equal to the average background pixel intensity. The resulting total fluorescence signal density was then summed.

Appendix References

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