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Antibacterial Efficacy of Exogenous Nitric Oxide on Periodontal Pathogens

ABSTRACT

Current treatments for periodontitis (*e.g.*, scaling/root planing and chlorhexidine) have limited efficacy since they fail to suppress microbial biofilms satisfactorily over time, and the use of adjunctive antimicrobials can promote the emergence of antibiotic-resistant organisms. Herein, we report the novel application of nitric oxide (NO)-releasing scaffolds (*i.e.*, dendrimers and silica particles) as anti-periodontopathogenic agents. The effectiveness of macromolecular NO release was demonstrated by a 3-log reduction in periodontopathogenic *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* viability. In contrast, *Streptococcus mutans* and *Streptococcus sanguinis*, caries-associated organisms, were substantially less sensitive to NO treatment. Both dendrimer- and silica-based NO release exhibited substantially less toxicity to human gingival fibroblasts at concentrations necessary to eradicate periodontopathogens than did clinical concentrations of chlorhexidine. These results suggest the potential utility of macromolecular NO-release scaffolds as a novel platform for the development of periodontal disease therapeutics.

INTRODUCTION

Oral care comprises a significant fraction of health care costs in the United States, with $104.8 billion devoted to dental services in 2010 (Ebersole *et al.*, 2012). A considerable portion of these expenses is attributed to periodontal disease caused by dental plaque biofilms. Approximately 30% of the US population is afflicted by periodontitis (Kandelman *et al.*, 2012). Left unmanaged, periodontitis can result in tooth loss and has been associated with increased risk for systemic conditions such as cardiovascular disease and stroke (Cobb, 2008; Sen *et al.*, 2013).

The current "gold standard" non-surgical periodontal treatment is scaling and root planing (SRP). However, even successful treatment *via* SRP is accompanied by a high probability of reinfection, with periodontal surgery to reduce periodontal pockets as a likely treatment outcome (Cobb, 2008). Other non-surgical treatments for periodontal diseases, such as the use of chlorhexidine (CHX), have undesirable side effects, including altered taste, discoloration of the mouth, and mucosal irritation (Charbonneau and Snider, 1997; Gürgan *et al.*, 2006). In combination, CHX (0.2% w/w) with SRP does not significantly affect microbial composition compared with SRP alone. Quiryen *et al.* suggested that CHX treatment should be re-evaluated as an adjunct to SRP periodontal therapy (Quiryen *et al.*, 2000). While the use of antibiotics (*e.g.*, minocycline and tetracycline) represents a possible adjunct to SRP, and local drug delivery systems can improve clinical and microbial outcomes (Williams *et al.*, 2001), their systemic use has undesirable side effects (*e.g.*, antibiotic resistance, pseudomembranous colitis) (Radvar *et al.*, 1996; Kinane and Radvar, 1999). The development of alternative treatments for periodontitis thus remains an important area of current oral health care research.

Nitric oxide (NO) is a reactive, gaseous radical produced endogenously during the immune response to invading organisms (DeGroote and Fang, 1999). For example, oral mucosal epithelial cells generate NO in response to bacteria and pro-inflammatory stimuli initiated upon the deposition of dental plaque (Carossa *et al.*, 2001). While exogenous NO has been utilized as an antimicrobial agent with no previously observed resistance (Privett *et al.*, 2012; Schairer *et al.*, 2012), the application of NO-releasing scaffolds for the treatment of periodontitis represents an unexplored opportunity.

The design of macromolecular scaffolds (*e.g.*, dendrimers, silica, polymers) capable of storing and delivering biocidal levels of NO is crucial to the application of gaseous NO as an antimicrobial therapy (Carpenter and Schoenfisch, 2012; Riccio and Schoenfisch, 2012). Previously, we have synthesized *N*-diazeniumdiolated silica and dendrimer scaffolds capable of spontaneous NO release under biological conditions as potential *in vivo* antimicrobial agents (Shin *et al.*, 2007; Sun *et al.*, 2012). The antimicrobial activity of such scaffolds was demonstrated against several Gram-positive,

Gram-negative, and antibiotic-resistant bacteria (Hetrick *et al.*, 2008; Sun *et al.*, 2012) as well as biofilms (Hetrick *et al.*, 2009; Lu *et al.*, 2013). Collectively, this prior work suggests exogenous NO delivered *via* macromolecular NO-release scaffolds may prove useful against dental microbes, including those organized within supragingival and subgingival biofilms.

Herein, we investigated the bactericidal efficacy of NO-releasing 3-methylaminopropyltrimethoxysilane (MAP3) silica particles and propylene oxide (PO)-modified generation 1 (G1) poly(amidoamine) (PAMAM) dendrimers against cariogenic bacteria (*Streptococcus mutans* and *Streptococcus sanguinis*) and periodontopathogens (*Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*). The cytotoxicity of the NO-release scaffolds to human gingival fibroblasts (HGF-1) was also evaluated and compared with CHX to ascertain the therapeutic potential of these materials for oral care.

MATERIALS & METHODS

Synthesis of Nitric Oxide Release Scaffolds

We prepared *N*-diazeniumdiolate NO donor-modified proline by dissolving 2.05 g proline and 2.00 g of sodium methoxide in 25 mL methanol. After brief mixing, this solution was placed in a Parr reaction bottle (Saavedra *et al.*, 1996). The bottle was purged with argon (Ar) and subsequently pressurized with NO gas (10 bar). After 3 days of constant stirring, the bottle was purged again with Ar, and the solution was treated with cold ether to precipitate the *N*-diazeniumdiolate-modified product. The NO donor ("PROLI/NO") was collected by vacuum filtration, washed with cold ether, and stored at -20°C.

Hybrid tetramethyl orthosilicate (TMOS) (Sigma, St. Louis, MO, USA) MAP3 (Gelest, Morrisville, PA, USA) particles were synthesized by the injection of a solution containing 0.538 mL TMOS and 1.68 mL MAP3 into a flask containing 60.1 mL ethanol, 27.8 mL water, and 9.8 mL ammonium hydroxide (30 wt%) (Shin *et al.*, 2007). This solution was stirred for 2 hr under ambient conditions and then centrifuged at 2900 x *g* at 4°C for 10 min. Particles were collected by decanting the supernatant. The pellet was re-suspended in ethanol, centrifuged, and decanted twice more to remove unreacted silanes and residual solvent. The resulting amine-functionalized MAP3 particles (70 mol% aminosilane) were dried under vacuum, and a portion (30 mg) was suspended in 6 mL 9:1 *N,N*-dimethylformamide (DMF) and methanol. This solution was sealed in a Parr bottle containing 50 μL sodium methoxide (5.4 M in methanol). The bottle was flushed with Ar to remove oxygen and pressurized to 10 bar NO. After 3 days of constant stirring, the bottle was again flushed with Ar to remove unreacted NO. The particles were re-collected by centrifugation and decanting of supernatant. The pellet was re-suspended in ethanol, centrifuged, and decanted twice more to remove residual solvent and sodium methoxide. Particles were dried under vacuum, vacuum-sealed, and stored at –20°C to yield *N*-diazeniumdiolate NO donor-modified MAP3 silica.

Secondary amine-functionalized G1 PAMAM dendrimers were synthesized by the dissolution of primary amine-functionalized G1 dendrimers (100 mg) in methanol (1 mL) with 39.2 μL propylene oxide (PO) (Lu *et al.*, 2013). After 3 days of constant stirring, the solvent was removed under vacuum to yield PO-modified PAMAM dendrimers. Sodium methoxide (51.8 μL; 5.4 M in methanol) was then added to 45 mg of dendrimers in methanol (1 mL), and the resulting solution was placed in a Parr bottle, sealed, flushed with Ar, and filled with NO (10 bar). After 3 days of constant stirring, the bottle was purged with Ar again to remove unreacted NO. The NO-releasing G1-PAMAM-PO dendrimers were then stored at -20°C.

Characterization of Nitric Oxide Release

A Sievers 280i Chemiluminescence Nitric Oxide Analyzer (Boulder, CO, USA) was used to quantify NO release at 37°C (Coneski and Schoenfisch, 2012). The NO-releasing material (~1 mg) was added to a flask containing 30 mL deoxygenated phosphate-buffered saline (PBS; pH = 7.4). Nitrogen was used to purge this solution continuously and thus carry liberated NO to the analyzer (flow rate, 80 mL/min). The NO analysis was in real time and continued until the NO levels fell below 10 ppb.

Bacteria-killing Assays

Streptococcus mutans (ATCC #25715), *Streptococcus sanguinis* (ATCC #49297), and *Aggregatibacter actinomycetemcomitans* (ATCC #43717) were purchased from the American Type Tissue Culture Collection (Manassas, VA, USA). *Porphyromonas gingivalis* strain A7436 was provided by the UNC School of Dentistry, Chapel Hill, NC. Stock cultures, initially stored in 15% (v/v) glycerol in PBS at -80°C, were grown overnight in brain heart infusion (BHI) broth at 37°C or Difco anaerobic broth for *P. gingivalis*. A 500-μL aliquot of this solution was re-inoculated into 50 mL fresh broth, incubated at 37°C, and grown to 10^8 colony-forming units *per* milliliter (CFU/mL). *S. mutans* and *S. sanguinis* were cultured aerobically. *A. actinomycetemcomitans* was cultured in a microaerophilic environment (6% -16% O_2 and 2%-10% CO_2) in a GasPak EZ Campy Container System (Becton, Dickinson and Company; Franklin Lakes, NJ, USA). *P. gingivalis* was cultured anaerobically in an atmosphere of 5% CO_2, 10% H_2, and 85% N_2.

We quantified the antimicrobial activity of the NO-releasing materials against planktonic cultures of bacteria by determining the minimum bactericidal concentration (MBC_{2h}) required to achieve a 3-log reduction in viability after 2 hr. Bacteria (10^8 CFU/mL) were re-suspended in tris(hydroxymethyl)aminomethane phosphate-buffered saline solution (Tris-PBS; pH = 7.4) and diluted to 10^6 CFU/mL. This bacteria solution was added to vials containing NO-releasing material and respective controls, sonicated or vortexed briefly, and incubated at 37°C with shaking. After 2 hr, these solutions were diluted and spiral-plated on BHI agar or Wilkins-Chalgren agar for *P. gingivalis*. Agar plates containing *S. mutans* and *S. sanguinis* were incubated aerobically for 72 hr. *A. actinomycetemcomitans* plates were incubated under microaerophilic conditions for 48 hr. *P. gingivalis* plates were incubated for 96 hr under anaerobic conditions.

Cytotoxicity Evaluation

Human gingival fibroblasts (HGF-1) (ATCC #CRL-2014) were grown in Dulbecco's modified Eagle's medium (DMEM)

Table. Characterization of NO-releasing Materials by Means of a Chemiluminescent Nitric Oxide Analyzer

NO Release Scaffold	t[NO] (μmol/mg)	[NO]$_m$ (ppb/mg)	t$_{1/2}$ (min)	t[NO]$_{2h}$ (μmol/mg)
70 mol% MAP3	0.84 ± 0.14	9200 ± 4300	19.8 ± 3.3	0.81 ± 0.15
G1-PAMAM-PO	0.96 ± 0.11	2900 ± 800	43.8 ± 4.3	0.77 ± 0.11
Proline	6.29 ± 0.24	370000 ± 11000	2.3 ± 0.1	6.29 ± 0.24

Results shown for particles (70 mol% MAP3), dendrimers (G1-PAMAM-PO), and small-molecule NO donor (NO-releasing proline). Values presented as means ± standard deviations. For all measurements, n = 3 or more pooled experiments.

containing 10% v/v fetal bovine serum and 1 wt% penicillin/streptomycin under 5% CO_2 and humidified conditions at 37°C. After reaching confluence, cells were trypsinized and seeded onto a 96-well plate. The NO-release scaffolds or controls were added to the wells. Following incubation for 2 hr at 37°C, the supernatant was aspirated, and DMEM, phenazine methosulfate (PMS) (100/20/1, v/v/v), and [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) were added to the wells. After incubation for an additional 2 hr at 37°C, solutions were transferred to a microtiter plate for absorbance measurement at 490 nm. Cell viability was quantified with respect to untreated cells and subtraction of blanks. The cytotoxicity of chlorhexidine was evaluated similarly.

RESULTS

Nitric oxide-release half-life (t$_{1/2}$), maximum NO flux ([NO]$_m$), and total NO release over 2 hr (t[NO]$_{2h}$) were determined for the 3 NO donor systems (Table). Of note, t[NO]$_{2h}$ represents the NO dose delivered during the MBC$_{2h}$ bacterial killing assay. Of the 3 NO donors, *N*-diazeniumdiolate-modified proline (PROLI/NO) had the largest NO payload (6.29 ± 0.24 μmol/mg) and fastest NO release (t$_{1/2}$ of 2.3 ± 0.1 min). The t[NO]$_{2h}$ and t[NO] for PROLI/NO were equal, since the NO release is exhausted within 10 min. Compared with PROLI/NO, the silica particles released less NO (t[NO]$_{2h}$ = 0.81 ± 0.15 μmol/mg) and did so more slowly (t$_{1/2}$ = 19.8 ± 3.3 min). Despite releasing an equivalent level of NO (t[NO]$_{2h}$ = 0.77 ± 0.11 μmol/mg) *vs.* the silica, the dendrimer system exhibited the slowest NO release (t$_{1/2}$ = 43.8 ± 4.3 min).

With respect to bactericidal efficacy (MBC$_{2h}$), a 3-log reduction in viability was observed for *A. actinomycetemcomitans* at 4 mg/mL in NO-releasing silica or PROLI/NO (Fig. 1). These scaffold concentrations corresponded to NO doses of 3.2 and 25.2 μmol/mL, respectively (Fig. 1). Less NO-releasing dendrimer was required for analogous killing (MBC$_{2h}$ = 2 mg/mL; NO dose = 1.5 μmol/mL). *P. gingivalis* proved even more sensitive to NO treatment, with a 3-log reduction in viability observed at 0.5, 1, and 2 mg/mL for NO-releasing proline, dendrimers, and silica, corresponding to NO doses of 3.1, 0.8, and 1.6 μmol/mL, respectively. In contrast, cariogenic *Streptococcus* species were less susceptible to NO-releasing silica, with no observed 3-log killing up to 64 mg/mL. As shown in Fig. 2, similarly large concentrations of NO-releasing proline and dendrimers (48 mg/mL) were required to kill *S. mutans* (NO doses of 301.9 and 37.0 μmol/mL, respectively). Likewise,

S. sanguinis required 32 and 48 mg/mL PROLI/NO and NO-releasing dendrimers, respectively (NO doses of 201.3 and 37.0 μmol/mL).

Both NO-releasing dendrimers and silica showed reductions in HGF-1 viability (75 ± 3 and 61 ± 12% compared with untreated cells) at the maximum concentrations required to kill periodontopathogens (2 and 4 mg/mL, respectively), but only minimally when compared with chlorhexidine (Fig. 3). In contrast, PROLI/NO was more toxic to HGF-1 at the maximum concentration required to kill periodontopathogens (18 ± 2% compared with untreated cells for 4 mg/mL). Likewise, 0.12 and 0.20% (w/w) chlorhexidine (CHX) negatively affected HGF-1 viability (17 ± 2 and 22 ± 3% compared with untreated cells, respectively).

DISCUSSION

This study examined the antimicrobial efficacy of macromolecular NO-release scaffolds against 4 microbes affecting oral health (*S. mutans*, *S. sanguinis*, *A. actinomycetemcomitans*, and *P. gingivalis*). While NO release from macromolecular scaffolds has proven effective against *Candida*, *Escherichia*, *Pseudomonas*, and *Staphylococcus* species (Hetrick *et al.*, 2008; Sun *et al.*, 2012), its efficacy against dental micro-organisms and the impact of NO-release scaffold on antibacterial activity are unexplored topics. Although prior research has indicated salivary nitrite-derived NO as a means to inhibit cariogenic and periodontopathogenic growth (Silva Mendez *et al.*, 1999; Allaker *et al.*, 2001), our work is the first to evaluate the effects of exogenous NO delivered *via* macromolecular scaffolds against periodontopathogens. Similar to an earlier study showing enhanced action against Gram-negative species (Hetrick *et al.*, 2008), NO treatment was highly effective against *A. actinomycetemcomitans* and *P. gingivalis*. The NO-releasing dendrimers possessed superior bactericidal activity compared with silica, even though they released similar amounts of NO (t[NO]$_{2h}$). Indeed, less NO was required to eradicate *A. actinomycetemcomitans* (1.5 *vs.* 3.2 μmol/mL for dendrimer and silica, respectively) and *P. gingivalis* (0.8 *vs.* 1.6 μmol/mL for dendrimer and silica, respectively) because of enhanced bacterial association of the dendritic scaffold (Sun *et al.*, 2012). Despite storing and releasing more NO, similar concentrations of PROLI/NO were required to kill periodontopathogens, albeit at larger NO levels (3.1 and 25.2 μmol/mg for *P. gingivalis* and *A. actinomycetemcomitans*, respectively). This result would be expected, since the PROLI/NO produces NO without bacteria association—and thus
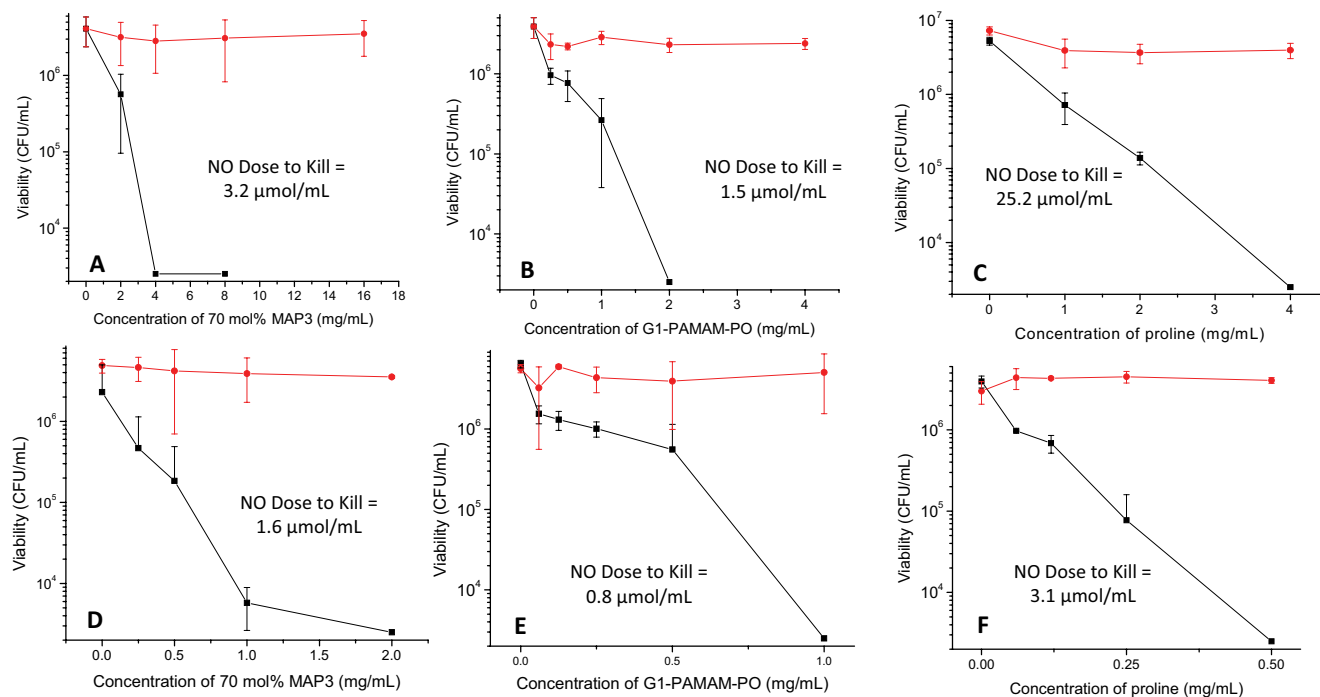
Figure 1. Bactericidal efficacy of **(A)** 70 mol% MAP3 particles, **(B)** G1-PAMAM-PO dendrimers, and **(C)** proline against *A. actinomycetemcomitans* in Tris-PBS after 2 hr. Bactericidal efficacy of **(D)** 70 mol% MAP3 particles, **(E)** G1-PAMAM-PO dendrimers, and **(F)** proline against *P. gingivalis* in Tris-PBS after 2 hr. NO-releasing material denoted by rectangles (-■-) and non-NO-releasing controls denoted by circles (-●-). Error bars signify standard deviation of the mean viability (CFU/mL). For all measurements, n = 3 or more pooled experiments.
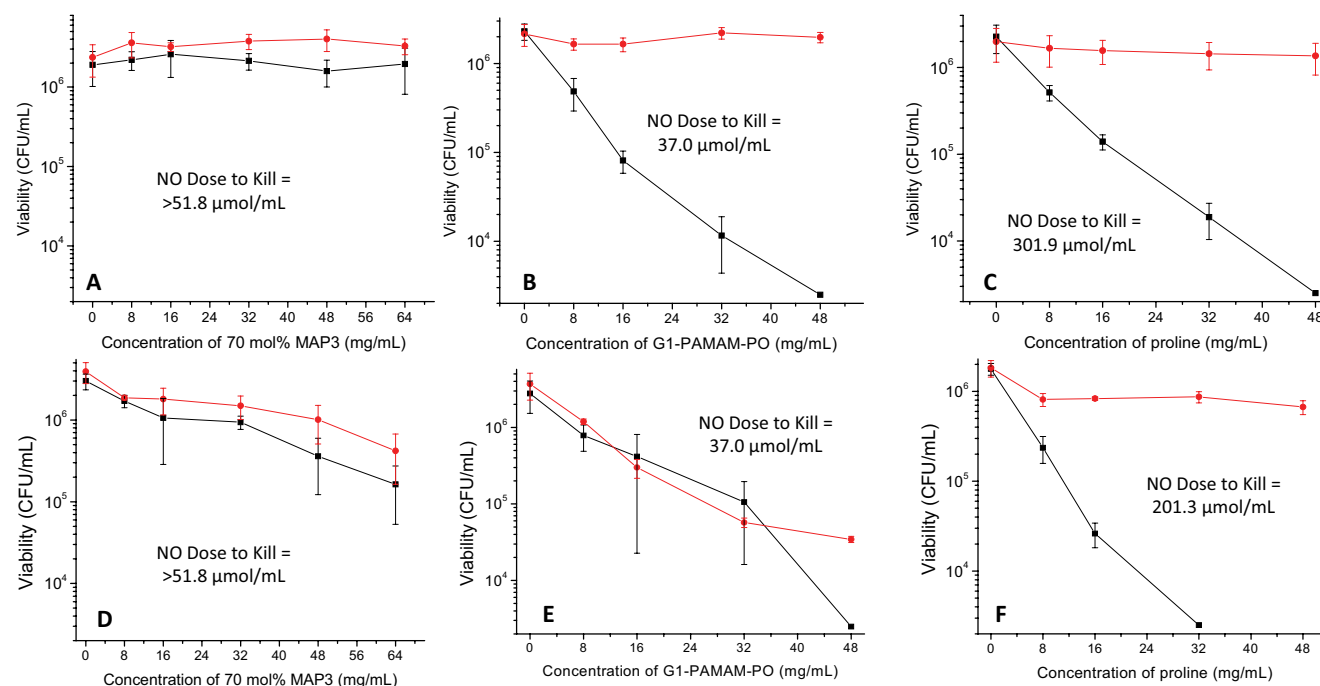

Figure 2. Bactericidal efficacy of **(A)** 70 mol% MAP3 particles, **(B)** G1-PAMAM-PO dendrimers, and **(C)** proline against *S. mutans* in Tris-PBS after 2 hr. Bactericidal efficacy of **(D)** 70 mol% MAP3 particles, **(E)** G1-PAMAM-PO dendrimers, and **(F)** proline against *S. sanguinis* in Tris-PBS after 2 hr. NO-releasing material denoted by rectangles (-■-) and non-NO-releasing controls denoted by circles (-●-). Error bars signify standard deviation of the mean viability (CFU/mL). For all measurements, n = 3 or more pooled experiments.

indiscriminately—whereas the macromolecular scaffolds associate with bacteria and localize (*i.e.*, target) the NO (Hetrick *et al.*, 2008). The efficient killing of *A. actinomycetemcomitans* and *P. gingivalis* with dendrimers or silica illustrates the advantage of macromolecular NO-release scaffolds over small-molecule NO donors for delivering bactericidal levels of NO.

As evidenced by the larger NO-donor scaffold concentrations and NO doses required for equivalent killing, *S. mutans* and *S. sanguinis* were less sensitive to treatment with NO. While these bacteria are Gram-positive species with a thicker peptidoglycan layer, the substantial disparity in bactericidal efficacy relative to periodontopathogens was surprising and has not been previously observed. Of note, *S. mutans* makes use of nitrite reductase, an enzyme that converts nitrite to NO, to facilitate survival in nitrite-rich, acidic environments (Choudhury *et al.*, 2007). Gusarov and Nudler reported that NO facilitates protection against oxidative stress ("NO-mediated cytoprotection") in *Bacillus subtilis* (Gusarov and Nudler, 2005), and this may be observed for other Gram-positive species. In this respect, *S. mutans* and *S. sanguinis* may tolerate NO because of its role as a metabolite rather than a biocidal agent.

Compared with chlorhexidine at 0.12 and 0.20% (w/w)—concentrations used clinically—the toxicities of the NO-releasing dendrimers and silica to human gingival fibroblasts were minimal (Fig. 3) at the maximum concentrations required to kill periodontopathogens (2 and 4 mg/mL). In contrast, substantial toxicity was observed for PROLI/NO at the maximum concentration to kill periodontopathogens (4 mg/mL). This result is attributed to both the magnitude (25.2 μmol/mL) and the rapid nature of the NO release from this small-molecule NO donor. Hetrick *et al.* noted similar toxicity to L929 murine fibroblasts for PROLI/NO concentrations ≥ 5 mg/mL (Hetrick *et al.*, 2008). As expected, the relative toxicity for all of the NO donor systems was greater at concentrations required for complete eradication of the cariogenic bacteria (*S. mutans* and *S. sanguinis*).

The sharp disparity in NO susceptibility between the 2 classes of oral microbes investigated herein suggests the utility of NO-releasing macromolecular scaffolds as potential therapies for periodontal disease. While *S. mutans* and *S. sanguinis* are cariogenic, caries-associated bacteria, *A. actinomycetemcomitans* and *P. gingivalis* are periodontopathogens linked to periodontal disease (Silva *et al.*, 2012). In this manner, NO-releasing macromolecular scaffolds may prove useful in killing periodontopathogenic Gram-negative bacteria over Gram-positive cariogenic micro-organisms. Although the concept of therapeutics to kill periodontopathogens is appealing, methods for delivering antimicrobials locally without fostering antibiotic resistance and substantial toxicity to mammalian cells remain elusive. Macromolecular NO-release scaffolds represent a potential strategy for achieving this goal. Future work must evaluate the antibacterial activity of these NO-release scaffolds against other dental microbes to validate the proposed enhanced sensitivity of periodontopathogens over cariogenic oral microbes to NO treatment. The efficacy of these NO-release scaffolds against bacterial biofilms of both periodontopathogens and cariogenic microflora must also be examined, since plaque is a complex biofilm. Last, *in vivo* models of periodontal disease must be
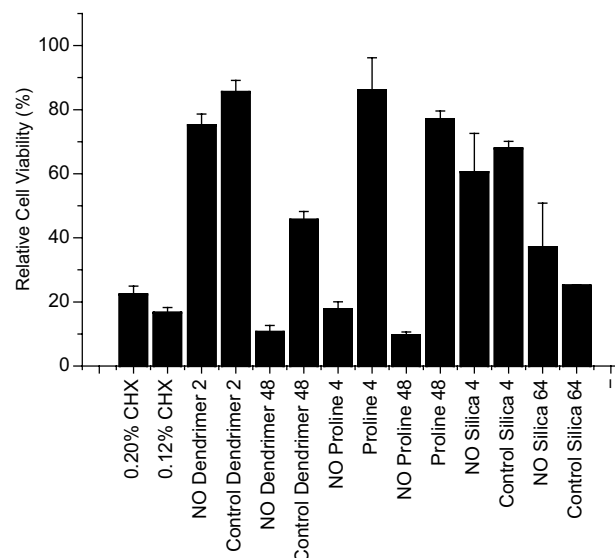

Figure 3. Cytotoxicity of NO-releasing materials and non-NO-releasing controls at minimum bactericidal concentration (MBC) two-hour concentrations (or maximum concentration tested). Viability measured as metabolically active fibroblasts with MTS and presented as normalized percentage relative to untreated cells. Chlorhexidine toxicity shown for clinical doses [0.12 and 0.20% (w/w)]. Of note, the numbers after the materials (*e.g.*, 2, 4, 48, and 64) correspond to the MBC data (in mg/mL). Error bars represent standard deviation of the mean. For all values, n = 4 or more replicate measurements.

used to elucidate the full potential of NO release as an effective adjuvant therapy.

ACKNOWLEDGMENTS

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