NITRIC OXIDE-RELEASE VEHICLES AS ORAL DISEASE THERAPEUTICS

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ABSTRACT

Christopher James Backlund: Nitric Oxide-Release Vehicles as Oral Disease Therapeutics
(Under the direction of Mark Schoenfisch)

Oral diseases (e.g., dental caries and periodontal disease) are caused by dental plaque biofilms. Due to the central role of nitric oxide (NO) in the immune response to bacterial infection, the delivery of exogenous NO as an antibacterial to combat dental biofilms is of interest. Herein, the synthesis of NO-release vehicles, followed by the evaluation of their chemical and physical properties in relation to their *in vitro* microbiological performance is described.

Hybrid silica nanoparticles exhibiting a range of NO-release totals and kinetics were synthesized by varying the aminosilane precursor identity and composition. The greatest NO storage obtained was ~1 μmol/mg silica nanoparticle. To study the effects of NO-release kinetics on bacterial killing, NO-release half-lives were also tuned (~30 and ~120 min), independent of both particle size (~150 nm) and NO-release totals (~0.2 μmol/mg).

Macromolecular nitric oxide-release vehicles (i.e., silica and dendrimers) proved more efficient than a small molecule NO donor (i.e., PROLI/NO) at delivering NO to oral pathogens, requiring lower NO doses to kill bacteria. Periodontopathogens were significantly more susceptible to NO treatment than cariogenic bacteria, suggesting a differential sensitivity to NO. At the concentrations required to kill periodontopathogens, NO-releasing silica and dendrimers were mildly toxic (>60% cell viabilities) to human gingival fibroblasts (HGF-1), and were significantly less toxic than clinical concentrations of chlorhexidine (0.12 and 0.20% w/w).

The kinetic-dependent killing of cariogenic and periodontopathogenic bacteria with NO-releasing silica nanoparticles was evaluated. Particles with slower NO-release kinetics (half-life ~120 min) demonstrated the greatest efficacy against periodontopathogens at pH 7.4. They were also least cytotoxic to HGF-1 cells, supporting the use of extended NO-release from silica-based materials for killing periodontopathogens. In contrast, cariogenic *Streptococcus mutans* was more susceptible to rapid NO release at lower pH (6.4).

The anti-biofilm activity of NO-releasing alkyl chain-modified poly(amidoamine) dendrimers was evaluated against *S. mutans* as a function of alkyl chain length, pH, and NO-release kinetics. Longer alkyl chains (i.e., hexyl, octyl, and dodecyl) were more bactericidal than shorter alkyl chains (i.e., propyl and butyl) at pH 7.4 due to greater membrane disruption. At lower pH, bactericidal efficacy was enhanced due to greater dendrimer-bacteria association, increased membrane damage (longer chains), and faster NO-release kinetics (shorter chains). The long alkyl chain dendrimers were toxic to mammalian cells, but less toxic with NO release. The efficient anti-biofilm activity and reduced cytotoxicity for NO-releasing long alkyl chain-modified dendrimers supports their future development as dental caries therapeutics.

This work is dedicated to my family and friends, who have always encouraged me to work hard, were there to pick me up when I fell, and celebrated my successes with me.

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LIST OF ABBREVIATIONS AND SYMBOLS

~	approximately
^{o}C	degree(s) Celsius
$	dollar(s)
>	greater than
<	less than
$[NO]_m$	maximum NO concentration
%	percent
±	standard deviation of the mean
x *g*	times the force of gravity
ADP1	adepantin 1
AEAP3	*N*-(2-aminoethyl)-3-aminopropyltrimethoxysilane
Ag	silver
Ag^+	silver ion(s)
AHAP3	*N*-(6-aminohexyl)aminopropyltrimethoxysilane
AI-2	autoinducer 2
AMP	antimicrobial peptide
APO	adverse pregnancy outcome
Ar	argon gas
Au	gold
β	beta
BHI	brain heart infusion

BuOH	butanol
C	carbon
CFU	colony forming units
cGMP	cyclic guanosine monophosphate
CHX	chlorhexidine
CO_2	carbon dioxide
COPD	chronic obstructive pulmonary disease
CSP	competence-stimulating peptide
Cu_2O	cuprous oxide
CuO	copper oxide
d	days
DAF-2 DA	5,6-diaminofluorescein diacetate
DET3	(3-trimethoxysilyl)diethylenetriamine
DMEM	Dulbecco's modified Eagle's medium
DMF	*N*,*N*-dimethylformamide
DNA	deoxyribonucleic acid
e.g.	exempli gratia (for example)
EB	epoxy butane
ED	epoxy dodecane
EDRF	endothelium-derived relaxing factor
EH	epoxy hexane
eNOS	endothelial nitric oxide synthase
EO	epoxy octane

et al.	et alia (and others)
EtOH	ethanol
FAP	fluorapatite
FBS	fetal bovine serum
G1	generation 1
G2	generation 2
G3	generation 3
G5	generation 5
h	hour(s)
H	hydrogen
H_2	hydrogen gas
H_2O	water
H_2O_2	hydrogen peroxide
HA	hydroxyapatite
HCA	hydroxyl-carbonate apatite
HGF-1	human gingival fibroblasts
i.e.	id est (that is)
iNOS	inducible nitric oxide synthase
I_p	ion product
K_{sp}	solubility product constant
LPS	lipopolysaccharide
M	molar
MAP3	methylaminopropyltrimethoxysilane

MBC_{2h}	2 h minimum bactericidal concentration
MeOH	methanol
min	minute(s)
mg	milligram(s)
MOF	metal organic framework
mol	mole(s)
N	nitrogen
N_2	nitrogen gas
N_2O_3	dinitrogen trioxide
NH_4OH	ammonium hydroxide
nm	nanometer(s)
nM	nanomolar
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
O_2	oxygen
O_2^-	superoxide
PAMAM	poly(amidoamine)
PBS	phosphate buffered saline
PDI	polydispersity index
PELA	polyethylene oxide-co-lactic acid
pH	-log of proton concentration
pM	picomolar

PMS	phenazine methosulfate
PO	propylene oxide
PO_4^{3-}	phosphate ion
ppb	parts per billion
PPI	polypropylenimine
PROLI/NO	*N*-diazeniumdiolate-modified L-proline
PS	penicillin streptomycin
QA	quaternary ammonium
RITC	rhodamine b isothiocyanate
ROS	reactive oxygen species
RSNO	*S*-nitrosothiol
s	second(s)
SEM	scanning electron microscope
SI	selectivity index
SRP	scaling and root planing
STAMP	specifically targeted antimicrobial peptide
$t_{1/2}$	half-life
t[NO]	total NO release
$t[NO]_{2h}$	2 h total NO release
TEA	triethyl amine
TEOS	tetraethyl orthosilicate
THF	tetrahydrofuran
TiO_2	titanium dioxide

TMOS	tetramethyl orthosilicate
Tris	tris(hydroxymethyl)aminomethane
µL	microliter(s)
µM	micromolar
µmol	micromole(s)
UV	ultraviolet
v/v/v	volume to volume to volume ratio
w/w	weight to weight ratio
wt	weight
Zn^{+}	zinc ion(s)
ZnO	zinc oxide

Chapter 1: Treating Dental Plaque to Mitigate Oral Diseases

Since the days of Louis Pasteur, bacteria have been studied in single species, nutrient-rich batch cultures *in vitro* to generate data for *in vivo* applications.[1] However, infectious microbes do not typically exist in this pure, planktonic (i.e., free-floating) state in nature, but instead adhere to surfaces and ultimately organize into multispecies biofilms for survival in less than ideal (nutrient-deficient) conditions.[1-3] Although studying planktonic bacteria cultures according to classical germ theory has proved beneficial for identifying pathogens and developing antibiotics, the prevalence and virulence of infections caused by bacterial biofilms, particularly for oral diseases including dental caries, demands immediate attention.[1-6] In this chapter, dental plaque biofilms and associated oral diseases (i.e., dental caries and periodontitis) will be discussed, followed by existing treatment schemes, the current research into eradicating dental biofilms, and finally the design of nitric oxide (NO)-releasing therapeutics for the management of oral health.

1.1 Dental Plaque Biofilms

The human oral cavity is inhabited by a diverse collection of >700 microorganisms, with an estimated increase into the thousands coinciding with advances in mass sequencing and identification techniques.[7-9] Oral bacteria colonize a variety of surfaces in the mouth, including teeth, mucosal surfaces, and artificial structures (i.e., implants) as dental plaque. Dental plaque is a complex microbial community comprised of numerous bacteria embedded within an extracellular matrix of polymers characterized by high structural heterogeneity, complex

interspecies interactions, protection from host defenses, and enhanced resistance to antimicrobials.[10] Although dental research spans a period of >100 years, investigating plaque as a microbial community functioning within a biofilm rather than planktonic cultures is a fairly new practice.[9] Dental plaque biofilms are dynamic, multifarious, and variable from person to person dependent on both external factors (i.e., pH, oxygen, bacterial co-adhesion and nutrients) and host genetics rendering them even more difficult to treat. While certain commensal bacteria may support host defenses against infection, the accumulation of dental pathogens leads to illnesses such as dental caries and periodontal disease. The ailments caused by plaque biofilms are a major component of the $104.8 billion spent on dental care in 2010, representing a dramatic increase over the past 10 years, with an expected 5% increase through 2021.[11]

1.1.1 Dental plaque biofilm formation

Despite plaque exhibiting a high degree of variation from person to person, the general process by which these oral biofilms form is universal, consisting of a highly ordered sequence of events in which bacteria adhere to a surface with ensuing co-aggregation of microbes and biofilm proliferation.[8] These key steps can be grouped loosely into attachment, colonization, and biofilm development.[8]

As shown in Figure 1.1,[8] the formation of oral biofilms is initiated by bacterial attachment to a surface. Moments after a thorough cleaning, a proteinaceous pellicle comprised of salivary proline-rich proteins, amylase, peroxidase, and mucin forms on surfaces.[12] This film interacts with bacteria via weak, long-range physiochemical interactions causing a net attraction to the surface that results in reversible attachment to the pellicle. However, a subset of bacteria will adhere to the pellicle via stronger, close-range irreversible interactions as they bind to complementary host

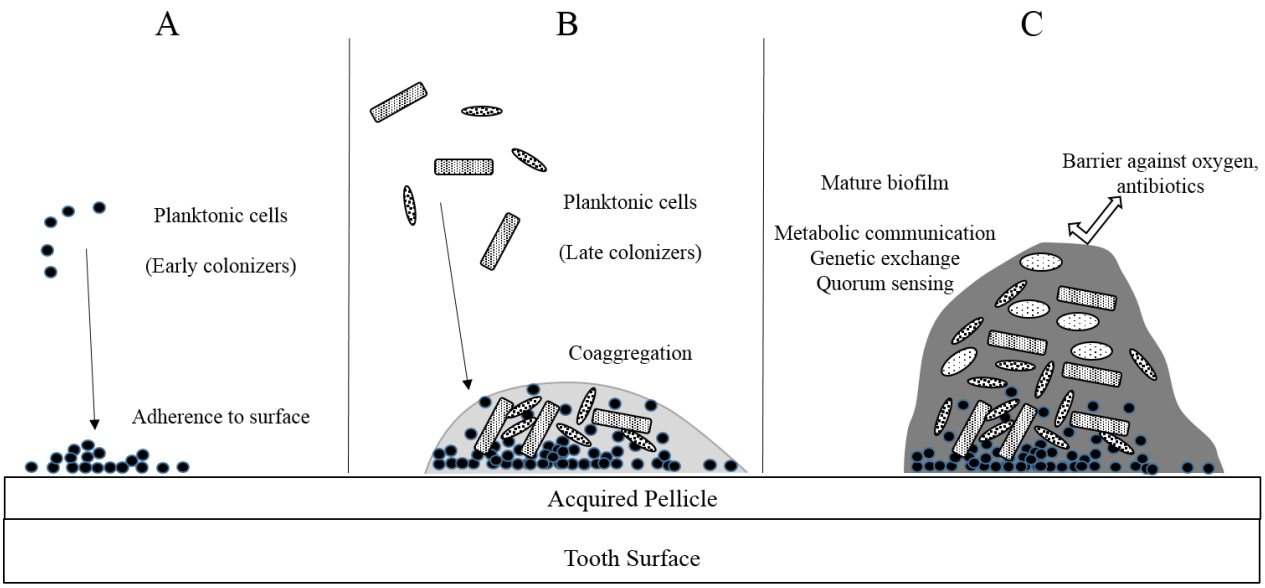

Figure 1.1 Key stages implicated in the formation of mature dental plaque biofilms. Processes are referred to as: A) attachment B) colonization and C) biofilm development. Figure adapted from Hojo et al.[8]

molecules present in the pellicle (e.g., bacterial glucotransferases binding with amylase).[12] Differences in pellicle composition govern the subsequent microbial interactions with this surface and thus dictate the bacterial composition of this layer of dental plaque. In general, oral streptococci species (e.g., *Streptococcus gordonii* and *Streptococcus sanguinis*) are considered initial colonizers as they can strongly bind with proline-rich proteins and glycoproteins that are abundantly present in the protein pellicle.[8]

Following initial attachment, bacterial co-aggregation and colonization occurs and plays a pivotal role in the development, structure, diversity, and functional organization of the biofilm. Planktonic cells unable to colonize the tooth pellicle directly can bind to bacteria already present on the film through various, yet specific, cell-to-cell interactions.[8] Co-aggregation of microbes to both surface streptococci and non-native cells permits late colonizers (particularly Gram-negative anaerobes) to be included within the developing biofilm.[4] For example, *Fusobacterium nucleatum* can interact with both streptococci species as well as Gram-negative anaerobes, serving as a link between the early and late colonizers of dental plaque.[8,13] Sequential bacterial binding of this nature promotes the formation of diverse, multispecies biofilms with unique morphologies that incorporate otherwise non-adherent cells into the biofilm matrix. Once integrated, the close proximity to oxygen-consuming aerobic cells allows for the survival of obligate anaerobes. Once a multispecies consortium of bacteria have anchored themselves to the tooth surface, active metabolism and cell division contribute to the formation of a mature biofilm.

1.1.2 Protective mechanisms of plaque biofilms

The persistent growth of numerous bacteria species, along with continued polymer production, creates a robust and thriving biofilm community embedded within a fortified exopolymer matrix. The benefits to constituent bacteria within the mature biofilm extend beyond physical protection from external factors such as oxygen (for obligate anaerobes), the host immune response, and antibiotics. The intimate interaction between cells allows for more efficient cellular metabolism, genetic exchange, and cellular signaling to ensure bacterial survival and success of the biofilm.[8] For example, oral bacteria can obtain nutrients for survival from endogenous substrates in saliva and gingival crevicular fluid (GCF) or exogenous sources such as sugars and food debris. However, their metabolism involves the concerted action of many bacterial species working together in metabolic communication. This process can involve bacteria breaking down larger compounds into biologically active substrates for other cells or metabolites from one bacteria functioning as nutrients for another.[8,13] This symbiotic metabolism has been observed for *Streptococcus* and *Veillonella* species, where lactates secreted from Gram-positive streptococci serve as energy sources for veillonellae and thus, these species are commonly detected together in the oral cavity.[14,15] In fact, veillonellae are unable to colonize tooth surfaces without streptococci present.[14]

Another example of bacterial symbiosis in dental plaque includes the production of growth-stimulating quinones for bacterial survival. Quinones are utilized as electron acceptors in bacterial metabolism, thus promoting bacterial growth, but are not produced endogenously.[8] Bacteria in the oral cavity can capture quinones produced by neighboring bacteria in order to survive in the mouth without an endogenous source, allowing for colonization of the oral cavity by species that would

typically be excluded. For example, veillonellae produce menaquinone (vitamin K_2) which facilitates the growth of adjacent bacterial species such as *Porphyromonas*.[8]

Besides benefiting metabolically from close spatial proximity to one another, oral bacteria communicate with each other via cell signaling known as quorum sensing. Quorum sensing regulates gene expression for survival under stress conditions and is often observed in cases of high cell density, such as in dental plaque biofilms.[8] These chemical signaling pathways are vital to the survival of the biofilm and regulate many cellular functions including acid tolerance, virulence, and biofilm formation. Two primary signaling molecules involved in quorum sensing are autoinducer-2 (AI-2) and competence-stimulating peptide (CSP).[8] The synthesis of AI-2 is directly linked to the preservation of oral bacteria (e.g., *Porphyromonas gingivalis*) as it protects against environmental stresses such as temperature, hydrogen peroxide, and pH.[8,16] Indeed, streptococci AI-2 (*luxS*) knockouts are characterized by an impaired ability to form biofilms with other species (e.g., *P. gingivalis*) once adhered to the tooth supporting the role of communication via AI-2 in successful dental plaque formation.[17] Similarly, CSP is produced by many oral streptococci species. It has been proposed that *S. mutans* produces CSP in acidic environments to warn other bacteria about the potential lethal threat of low pH and thus serves to protect other non-acidophilic species within the biofilm.[8,10] Collectively, communication through quorum sensing allows bacteria to coordinate their behaviors with other species to ensure microbial survival and preservation of the biofilm.

In addition to symbiosis and the protective effects of quorum sensing, bacteria residing within dental plaque biofilms possess enhanced resistance to antibiotics.[3] This phenomenon is due in part to the exopolymer matrix encompassing the bacteria, which acts as a physical barrier and limits drug diffusion into the biofilm. However, this mechanism alone does not account for the

drastic increase in drug concentration needed to eradicate plaque biofilms versus planktonic cultures. Plaque can function as a "genotypic reservoir," facilitating the sharing of genes between bacteria to further enhance antimicrobial resistance across species in the biofilm. Indeed, CSP-facilitated gene transfer for antibiotic resistant genes can play a significant role in propagating resistance among biofilm bacteria species.[8,10] On a physical level, the phenotypic differences and heterogeneity of environment cause a decrease in efficacy of classic antimicrobials against dental biofilms.[3] When bacteria are attached to a surface, the drug target may be modified or even nonexistent, rendering the drug less effective. Furthermore, bacteria in nutrient-depleted environments (e.g., biofilms) can exhibit reduced or altered metabolism, which can greatly reduce drug efficacy.[3] In addition, the local environmental conditions (e.g., decreased oxygen and lower pH) within the biofilm may be unfavorable for the optimal action of a drug. Finally, biofilm retention of drug-degrading enzymes can protect resident flora. For example, β-lactamase produced by oral bacteria and present in gingival crevicular fluid can degrade β-lactam drugs like penicillin.[18] Additionally, the penicillin-sensitive *Streptococcus pneumoniae* can be protected from antibiotics by β-lactamase producing *Moraxella catarrhalis* and thus may still cause infection despite no inherent antibiotic resistance.[19]

1.2 Plaque Biofilm-Related Diseases

Once established, mature dental plaque biofilms are relatively stable over time in terms of their microbial composition and beneficial function within the mouth.[20] In fact, healthy dental plaque, which consists primarily of Gram-positive cocci species and few Gram-negative bacteria, is important to the development and wellbeing of the host.[4] The presence of dental biofilms consisting of "protective" bacteria is important to maintain health through "colonization

resistance," where resident microbes outcompete foreign pathogens for nutrients and attachment sites.[4,21,22] Furthermore, native bacteria can produce inhibitory metabolites or create unfavorable environments for colonization by exogenous pathogenic bacteria.[4,23] While the importance of the resident microflora has been studied primarily in the gut, where germ-free animals are significantly more susceptible to disease than their healthy microflora-inhabited counterparts, this phenomena is important in the oral microbiome as well.[20]

As with environmental conditions (e.g., temperature, oxygen, pH and nutrients) for bacterial growth, the microbial composition and critical point at which these biofilms become virulent are host dependent.[9] Changes in environmental parameters are typically the driving force for oral disease, as they force the microbiome out of homeostasis with the host. Other parameters, such as smoking, disease, and compromised immune systems, can also promote disease states within the oral cavity.[4] Regardless of the contributing factors, the shift from health to disease is characterized by either the overpopulation of previously minor components of the biofilm or colonization by exogenous pathogens. The progression from a health-associated oral microbiome to a disease state is common and costly. Indeed, oral diseases in America have been coined a "silent epidemic" where 7.4% of total healthcare spending in 2006 was devoted to dental care, mainly due to dental caries and periodontal disease.[11]

1.2.1 Dental caries

Also known as tooth decay, dental caries is one of the most costly and prevalent infectious diseases worldwide. With 94% of the adult population (18 years or older) experiencing treated or untreated carious lesions,[24] tooth decay is considered ubiquitous amongst civilized populations.[25] Dental caries is defined as the localized destruction of supragingival tooth enamel caused by acid

produced via bacterial fermentation of dietary sugars.[25] Both the process leading to destruction as well as the lesions and cavities formed by the deterioration of enamel can be classified as caries.[26] Although the oral microbiome is diverse in microbial identity, a select few bacteria are overwhelmingly acidogenic and strongly correlated to the etiology of dental caries. These cariogenic bacteria tend to be Gram-positive and belong to the lactobacilli or streptococci genus. A number of these bacteria have been studied as cariogenic bacteria linked to dental caries (Table 1.1),[27] but *Streptococcus mutans* is the main focus of research and considered the key etiological agent of dental caries.

Acidogenic microorganisms within the dental plaque biofilm (i.e., *S mutans*) metabolize dietary carbohydrates (e.g., glucose, fructose, sucrose) to produce lactic acid, which causes a local drop in pH.[12] With the continuous production of acid, the local environment shifts to favor acidophilic bacteria and limits the survival of other microbes that cannot endure the harsh conditions. This, in turn, promotes the colonization of the biofilm by greater numbers of acidogenic bacteria, thus fostering the cariogenic process leading to tooth decay. Dental caries is a delicate balance between the constant demineralization and remineralization of the tooth surface with several factors, besides the presence of *S. mutans*, playing a role in demineralization of the enamel.

Although elevated concentrations of *S. mutans* is a risk factor for dental caries, its presence alone does not necessitate the formation of cavities. Instead, the process is extremely complex, occurs over a broad time frame, and is dependent on several host factors that are both innate and behavioral, complicating the prevention and treatment of the disease. Biological risk factors include salivary flow, salivary composition, high numbers of cariogenic bacteria, and genetic

Table 1.1 Bacteria studied for their implication in dental caries.[a] Table adapted from Silva et al.[27]

Cariogenic Bacteria[b]
Streptococcus mutans
Streptococcus sanguinis
Streptococcus gordonii
Streptococcus sobrinus
Streptococcus mitis
Lactobacillus casei
Lactobacillus acidophilus
Lactobacillus fermenti

[a]As found in a review of literature pertaining to antimicrobials against dental pathogens.
[b]Listed in decreasing order of appearances in articles reviewed. *Streptococcus mutans* was most prevalently studied bacteria amongst dental caries relevant pathogens.

factors.[26] The behavioral aspects of the host contribute to risk as poor oral hygiene, diet (i.e., consuming large amounts of carbohydrates), and smoking can promote the formation of dental caries.[26,28] In terms of diet, the frequency of sugar consumption more so than the total amount of sugar consumed has been shown to dictate the localized drop in pH leading to cavities.[12]

Also related to dietary behaviors, is salivary ion content which dictates the "critical pH" of the host. The critical pH refers to the pH where a solution is saturated with respect to a particular mineral.[29] Although acid produced by cariogenic bacteria (e.g., *S. mutans*) causes cavities, the dissolution of enamel only occurs if the local pH is below the critical point required for demineralization. When the pH is above the critical point, minerals will precipitate out of solution. Alternatively, when the pH is below the critical point, minerals will dissolve into solution.[29] Tooth enamel is comprised primarily of hydroxyapatite (HA; $Ca_{10}(PO_4)_6(OH)_2$) with an infinitesimally small solubility product constant (K_{sp} ~10^{-117}). As such, the dissolution process is dictated by the ion product (I_p) or the concentration of calcium and phosphate in saliva.[29] When $I_p < K_{sp}$, the solution is unsaturated and dissolution will occur. Conversely, when $I_p > K_{sp}$, the equilibrium shifts to favor precipitation out of solution. Calcium concentration is unaffected by pH but the concentration of phosphate (PO_4^{3-}) is drastically reduced at lower pH. This decrease in pH results in a decrease in I_p and dissolution of the tooth unless salivary concentrations of PO_4^{3-} can be maintained. Although experts cite the critical decalcification pH as 5.5, the actual pH varies not just from person to person but even temporally within the same individual.[29] The critical pH required for dissolution could be as high as 6.5 for individuals with low salivary phosphate levels and as low as 5.1 for those with much greater ion levels.[29] Due to both genetics and diet, the ion content of salivary fluid is constantly fluctuating and highly host-specific. These factors highlight

the variable susceptibility between people to dental caries, even in the presence of cariogenic bacteria such as *S. mutans*.

1.2.2 Periodontal disease

In general, periodontal disease refers to inflammation of the periodontium and tooth supporting structures caused by dental plaque biofilms. It encompasses the non-destructive, reversible inflammation of the gums known as gingivitis as well as periodontitis, the irreversible destruction of tooth-supporting tissue and bone.[30] Periodontitis can be further classified as chronic, typified by the slow and gradual destruction of bone, or aggressive periodontitis, which results in rapid, fast-acting detachment of tooth-supporting structures. Gingivitis is a fairly common ailment, affecting up to 90% of the world's population, and always precedes periodontitis.[30] However, gingivitis does not always lead to periodontitis, a much more severe condition, affecting roughly 30% of the US adult population.[30,31] Left untreated, periodontitis can result in tooth loss as well as stroke, coronary heart disease, chronic obstructive pulmonary disease (COPD) and adverse pregnancy outcomes (APOs).[32-38]

Periodontal disease is caused by an excess of Gram-negative bacteria within subgingival plaque biofilms. Although a number of bacteria are implicated in periodontitis, two key pathogens involved in the initiation and progression of the disease are *Aggregatibacter actinomycetemcomitans* (aggressive periodontitis) and *Porphyromonas gingivalis* (chronic periodontitis).[39] As illustrated in Figure 1.2,[40] these bacteria elicit a strong inflammatory response from the host, which initiates a cascade of events responsible for the breakdown of soft tissue and bone. Briefly, these Gram-negative bacteria contain endotoxins in their membrane known as lipopolysaccharides (LPS) that initiate an inflammatory cascade. The LPS recruit

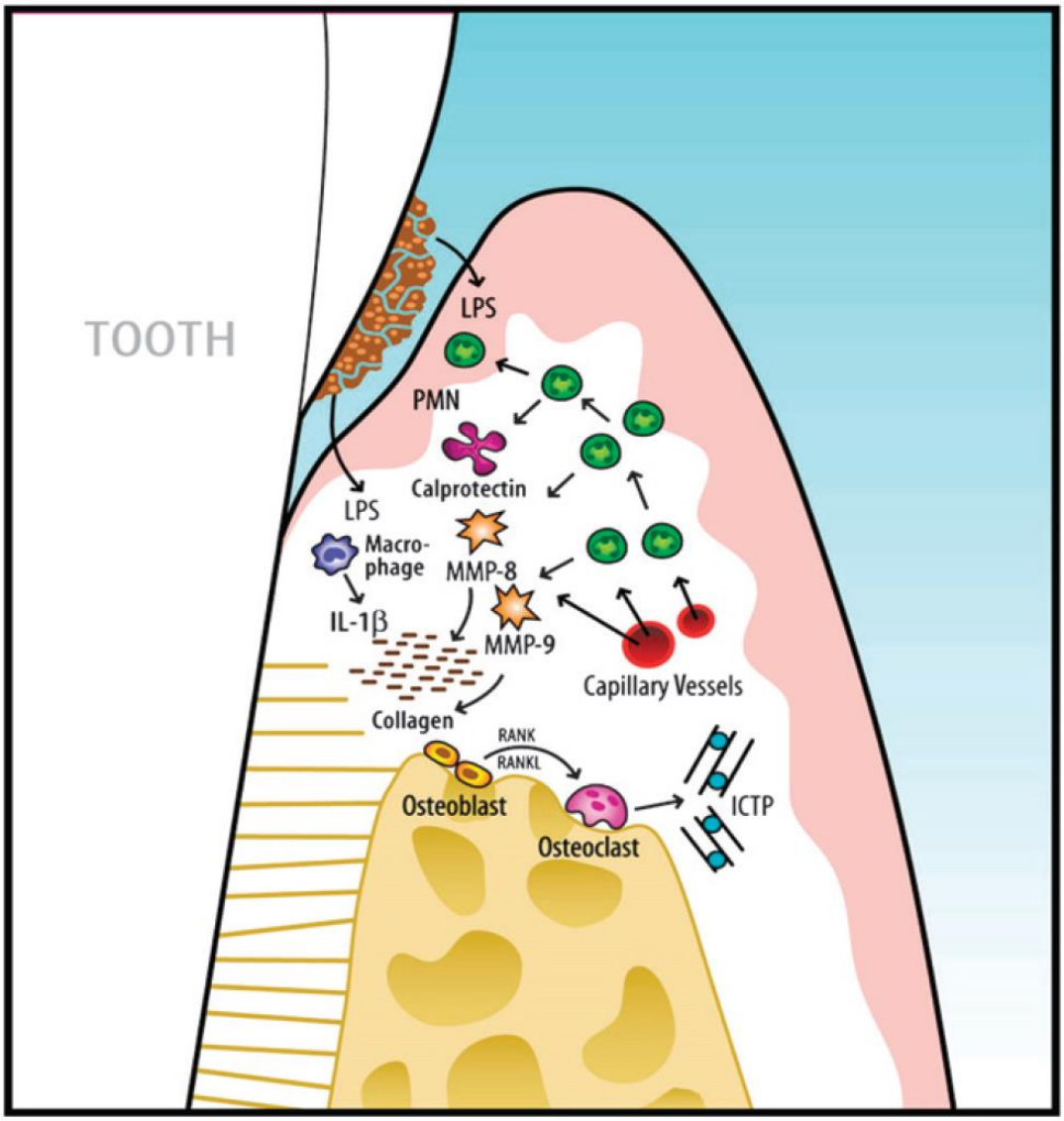

Figure 1.2 Schematic of host immune response cascade, initiated by Gram-negative lipopolysaccharide (LPS), responsible for the breakdown of bone and soft tissue associated with periodontal disease. Reprinted with permission from Giannobile et al.[40] as adapted from Kinney et al.[41] Copyright 2007, John Wiley and Sons.

polymorphonuclear leukocytes (PMNs), that subsequently release matrix metalloproteinases (MMPs) acting as powerful collagenases that destroy connective tissues.[40] Activated macrophages respond to LPS by releasing tumor necrosis factor (TNF)-alpha and interleukin (IL)-1β pro-inflammatory cytokines which mediate osteoclastogenesis and the breakdown of bone.[40] *P. gingivalis*-derived LPS also inhibits osteoblastic differentiation and thus retards periodontal tissue regeneration.[42] In this manner, the host response to the dental plaque biofilm is responsible for the destruction of the bone and tissue structures supporting the tooth. Persistent bacterial infection with concomitant host immune response fosters the progression of periodontal disease to destructive periodontitis, eventually resulting in tooth loss.

Apart from the physical and social ramifications of tooth loss, periodontitis can also affect an individual's health in a number of ways including an increased risk for respiratory disease, heart disease, stroke, and even adverse pregnancy outcomes (APOs).[32-38] Although confounded by smoking and weight, there is a relationship between periodontitis and respiratory issues. Oral bacteria can be aspirated into the lungs, leading to infection (e.g., pneumonia), reduced lung volumes, and limited airflow.[38,43] Periodontitis has also been implicated in the advancement of diseases such as chronic obstructive pulmonary disease (COPD). Indeed, evidence exists that periodontal therapy may be important in reducing COPD exacerbations supporting the link between these two diseases.[38] Periodontal bacteria can also enter the bloodstream and accumulate as arterial plaque, posing a risk for conditions such as coronary heart disease (CHD), a leading cause of mortality in the United States.[44,45] In fact, the periodontopathogens *A. actinomycetemcomitans* and *P. gingivalis* have been detected in arterial plaque within blood vessels.[46] Thus, the incidence of periodontitis increases the risk of CHD and, in one study, patients with chronic apical periodontitis were 2.79 times more likely to develop CHD.[35] Whereas the link

between cardiovascular disease and periodontal disease is well established, the connection between periodontal disease and stroke has been investigated more recently. It has been observed that periodontal disease is associated with recurrent vascular events in stroke patients, but it is unclear at this time whether periodontal treatment can reduce such risks.[32] Other major risks involving oral bacteria entering blood circulation are APOs, which are caused by periodontal pathogens or their byproducts eliciting an immune response upon reaching the placenta and amniotic fluid (Figure 1.3).[33] The production of inflammatory cytokines in response to virulence factors (e.g., LPS) can lead to premature birth or miscarriage. Additionally, infection and inflammation can cause impaired nutrient transport via pre-eclampsia, leading to low birth weight and possibly miscarriage.[33] This phenomena has been observed utilizing a subcutaneous chamber *P. gingivalis* infection model in both pregnant hamsters and mice in which both bacteria and inflammatory markers caused complications with pregnancy.[47,48] It was postulated that periodontal biofilms can serve as bacterial reservoirs that can translocate infectious properties systemically to negatively impact pregnancy outcomes.[47,48]

1.3 Current Oral Disease Treatments

The goals of oral therapies are to kill infection-causing bacteria, mitigate disease, and ideally prevent the need for invasive clinical procedures. Unfortunately, current treatments fall short of eliminating malignant conditions (i.e., dental caries and periodontal disease), causing persistence of the ailment and resulting in painful and costly dental procedures including surgery. This is particularly true for periodontal disease in which bacteria are organized in subgingival biofilms, making them especially difficult to access and treat. This section will discuss current

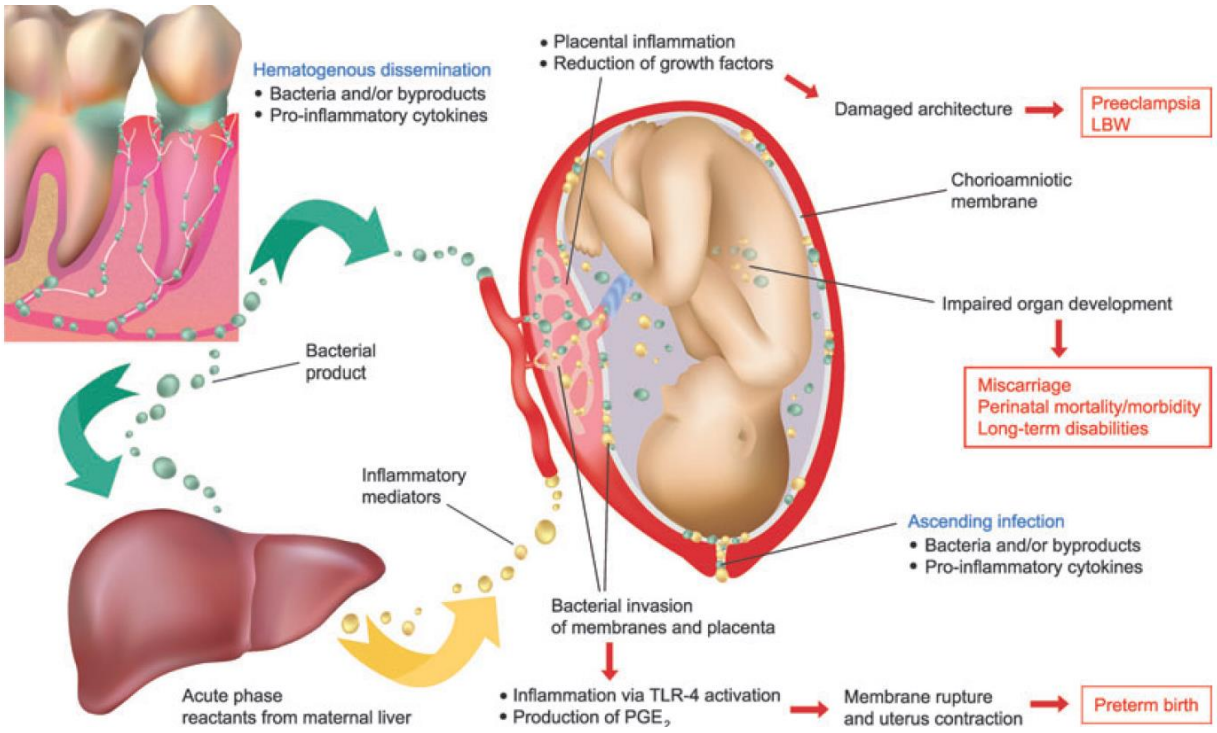

Figure 1.3 Possible mechanisms/pathways associating periodontal disease with adverse pregnancy outcomes. Reprinted with permission from Madianos et al.[33] Copyright 2013, European Federation of Periodontology and American Academy of Periodontology.

methods utilized to treat oral disorders, their shortcomings, and improvements that have resulted in marginal gains in terms of clinical outcomes.

1.3.1 Non-surgical treatments

The most common non-surgical treatments for oral ailments besides tooth brushing include disinfectant mouth rinses, fluoride treatments for dental caries, and scaling and root planing for periodontal disease.[49-51] Oral rinses consist of ethanol-based mouth washes (e.g., Listerine) and chemical antiseptics (e.g., chlorhexidine (CHX)). Chemical antiseptics are more potent than ethanol-based rinses and are therefore prescription based and not available over the counter. Both classes of mouth rinses are characterized by direct membrane disruption and bacterial killing via cellular leakage.[52] Additionally, Listerine's antibacterial efficacy is enhanced via enzyme inhibiton.[53] However, the limited broad-spectrum activity of each rinse combined with their inability to penetrate deep within plaque restricts killing to the outer layer of the biofilm, rendering them ineffective treatment options.[53,54] Chemical antiseptics in particular are associated with adverse side effects including staining of teeth, desquamation of mucosal surfaces, altered taste, and calcified deposits.[55,56] Furthermore, the long-term daily use of ethanol-based mouthwashes has been linked to oral cancers.

Topical fluoride therapy for the prevention of dental cavities through the remineralization of enamel via fluorapatite (FAP; $Ca_{10}(PO_4)_6F_2$) formation is well established.[24,57,58] Maintaining ample salivary fluoride concentration is essential for properly mitigating demineralization and is thus most effectively administered via toothpastes, gels, slow-release devices, and dietary supplements.[26,57,58] Cariostatic effects of fluoride treatment have also been attributed to a reduction in adhesion forces of oral cocci species to hydroxyapatite upon treatment with sodium

fluoride solution.[59] However, fluoride is not considered an antimicrobial agent for the prevention of caries due to the large concentrations necessary to produce such an effect. High concentrations of fluoride can have negative side effects such as gastrointestinal discomfort, dental fluorosis, and skeletal fluorosis when applied systemically.[60,61]

Scaling and root planing (SRP), the current "gold standard" non-surgical periodontal treatment, involves the physical removal of dental plaque above and below the gum line.[30,51] Scaling refers to tartar removal whereas planing involves smoothing the tooth via scraping to reduce bacterial adhesion. However, even successful treatment is often accompanied by a high probability of reinfection, leading to periodontal surgery. The use of mouth rinses such as CHX in conjunction with SRP has shown no significant differences in microbial composition over time, making it a poor candidate as an adjunct to SRP therapy.[62] While localized delivery of antibiotics (e.g., minocycline) have somewhat improved clinical outcomes and represent possible adjuncts to SRP,[63] their systemic use has undesirable side effects (e.g., pseudomembranous colitis) and may also foster the development of antibiotic-resistant bacteria.[64,65]

1.3.2 Surgical therapies

When non-invasive techniques fail, oral surgery is typically required to remove infection and eliminate disease. Untreated dental caries can cause decay down to the tooth root, requiring a root canal to prevent the spread of infection. During a root canal, the tooth is opened to remove decay, sometimes removing the associated nerve. The canals are then filled with gutta-percha and sealed with a crown. Despite the high success rate of root canals, a failed procedure may necessitate root canal re-treatment or extraction of the tooth.

Since the areas below the gum line are affected, periodontal diseases require significantly more invasive procedures. The most common procedure for persistent periodontal disease in which inflammation and deep periodontal pockets remain after SRP is flap surgery. This procedure involves physically lifting back the gum to remove deep periodontal tartar deposits and suturing the gums back into place when completed. This rids the area of infectious microbes and allows the gums to fit more snugly around the tooth, limiting bacterial infiltration to the periodontium, ideally preventing reinfection. In addition removing tartar, the periodontist may place a bone or soft tissue graft under the flap to promote bone and tissue regeneration, potentially restoring the destroyed periodontal structures.

The surgical processes listed above are costly and invasive, but necessary when non-surgical treatments fail. Therefore, the goal of current oral disease therapeutic research is focused on circumventing surgical procedures. This objective can be accomplished by improving the efficacy of non-surgical therapies in removing infection, reducing inflammation, and eliminating oral diseases before more extensive surgical intervention is necessary.

1.4 Current Research into Non-Surgical Oral Disease Therapies

As discussed in Section 1.3, the goal of current research into oral health therapeutics is to eradicate bacteria, reduce inflammation, and eliminate disease without the need for clinical intervention and surgery. In this section, the ongoing research into developing effective oral disease therapies will be discussed. Considerable research in this area has focused on using metal nanoparticles as antimicrobial dental plaque therapies, with antimicrobial peptides and silica materials receiving more attention recently.

1.4.1 Metal nanoparticles

With the increasing prevalence of antibiotic-resistant bacteria, metals that have been used as bactericidal agents for centuries such as gold, copper, zinc, and silver are being reevaluated for their utility as oral therapeutics.[66] As the activity of these metals is highly dependent on surface contact, the advent of nanoparticles with increased surface area has greatly improved their antimicrobial efficacy over bulk metals. Although gold (Au) and zinc oxide (ZnO) exhibit effective killing of *S. mutans*, silver (Ag) nanoparticles display the greatest efficacy.[66] Similarly, Ag is more bactericidal than cuprous oxide (Cu_2O), copper oxide (CuO), ZnO, and titanium dioxide (TiO_2) against the periodontal pathogens *P. gingivalis* and *A. actinomycetemcomitans*.[67] Due to the reliance on UV irradiation for the antimicrobial action of TiO_2 and the weak broad-spectrum activity of Au, neither of these materials have been heavily researched as oral antimicrobials.[66,68] Copper (CuO), although a more effective antibacterial than ZnO, is known for its severe toxicity to eukaryotic cells.[69] As such, the majority of dental research has focused on evaluating ZnO and Ag nanoparticles as potential oral antimicrobials.

As zinc (Zn^{2+} and ZnO) has previously been incorporated into toothpastes to control dental plaque, translation into more sophisticated dental therapeutics is relatively simple.[70,71] Zinc oxide (ZnO) nanoparticles have been used in dental composites for contact based killing with biocidal activity against *S. sobrinus* in both planktonic cultures and biofilms.[72] Zinc ions (Zn^{2+}) have been shown to limit glucosyltransferase activity and acid production by oral streptococci, thus limiting decalcification and impeding dental caries.[72] The generation of antibacterial hydrogen peroxide (H_2O_2) from the surface of ZnO particles has been repeatedly demonstrated with UV light initiation and regarded as the primary mode of biocidal action.[73-75] Although killing observed in the absence of UV light supports Zn^{2+} ion generation as an alternative biocidal mechanism,[68,72,76] the increase

in antibacterial efficacy with increased surface area (decreased particle size) and reduced bactericidal activity without UV light indicate the production of reactive oxygen species (ROS) (e.g., H_2O_2) as the principal bactericidal mechanism.[73-75] Most likely, it is the combination of ROS and Zn^{2+} interacting with bacteria contributing to bactericidal efficacy. However, Zn^{2+} is also essential in the formation of certain bacterial biofilms (e.g., *Staphylococcus aureus*).[72,77] Zinc's unclear antimicrobial action combined with its potential to promote biofilm formation warrants further examination against oral bacteria strains and has limited its utility as a potential oral therapeutic thus far.

Due to highly efficacious, broad-spectrum activity combined with a history of regulatory approval for biological applications,[78] silver has been the main research focus of metal nanoparticles as dental therapeutics. The multiple modes of action for silver killing are attributable to silver ions (Ag^+) and include both reaction with thiol groups to deactivate proteins and directly interfering with DNA replication.[68] Antimicrobial silver has been studied in many forms such as metallic silver, silver nitrate, silver sulfadiazine, and silver zeolites.[79] For example, when bacteria come in contact with silver zeolite, cellular uptake of silver ions exuded from the zeolite via ion exchange hinders cell function, damages the cell, and ultimately causes death.[79] Indeed, silver zeolites have proven effective against several dental bacteria including *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *S. mutans*, and *S. sanguinis*.[80] In general, the Gram-negative pathogens tested were more susceptible to Ag^+ treatment.[80] As the cell membranes of Gram-positive pathogens incorporate a greater amount of negatively charged peptidoglycan, Ag^+ binding to the membrane possibly reduces the amount of silver reaching the plasma membrane, suggesting a possible reason for the observed difference in susceptibility.[80]

Although most reports claim silver to be non-toxic, there is controversy surrounding its prolonged use. Silver exposure has been shown to result in argyria, or the blue-grey tint of the skin, particularly in large doses or after long-term accumulation. Furthermore, silver has been observed to hinder development in fish embryos and plants in a dose-dependent manner.[81] To reduce toxicity, silver has been impregnated in a number of materials to limit eukaryotic exposure. For example, dental resins loaded with silver ions have shown time-dependent efficacy against oral streptococci species *in vitro*.[82] Another study investigated silver-doped elastomers on oral streptococci species *in vivo* for long-term (12 week) caries prevention. Of note, no difference in bacterial composition between control elastomers and those loaded with silver was observed.[83] The lack of long-term clinical success, combined with potential *in vivo* and environmental toxicity over time, has limited the success of silver as an antimicrobial oral therapeutic.

1.4.2 Antimicrobial peptides

Antimicrobial peptides (AMPs) are a class of peptides ubiquitous in nature as defenses against pathogens in both plants and animals.[84,85] They are an essential component of the innate immune response, exhibiting broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, and viruses.[85] The multi-microorganism efficacy of AMPs is primarily attributed to direct membrane disruption, though they have been shown to inhibit DNA and protein synthesis, protein folding, enzyme function, cell wall synthesis, and metabolic turnover.[85] Typically, AMPs are cationic and attracted to negatively charged bacteria membranes. Once attached, they insert into the membrane creating transmembrane pores that allow for intracellular leakage leading to death.[84] The ability of AMPs to reduce infection via multiple pathways provides for a potent, naturally occurring antimicrobial with a limited probability of

bacteria developing resistance.[85] These findings, in concert with advancements in obtaining and purifying theses peptides, have propelled investigations into their use as dental antimicrobials to mitigate oral disease. For example, AMPs have been successfully applied as novel bactericidal agents against numerous dental pathogens *in vitro* as both stand-alone therapies and dental implant coatings. Peptides such as Chrysophsin-1 have been cited for their ability to eradicate *S. mutans* biofilms,[86] while others, like GL13K, are more effective against periodontopathogens such as *P. gingivalis*.[87,88] Certain peptides (GL13NH2 and GL13K) were found to exhibit anti-lipopolysaccharide (LPS) activity, demonstrating the potential to greatly reduce inflammation *in vivo* and, in turn, possibly mitigate periodontal disease.[87] However, each protein sequence is unique in form and function with slight derivations in structure resulting in dramatically different effects both *in vitro* and *in vivo*. For example, GL13NH2 and GL13K, despite having mutual anti-LPS activities and similar amino acid sequences (Figure 1.4),[89] exhibit dissimilar antimicrobial activity.

Limiting the clinical utility of AMPs are their observed hemolytic activity towards mammalian host cells and non-selective eradication of both pathogenic and commensal bacteria species. The latter can intensify the malignant condition or promote reinfection by non-native pathogens, potentially sustaining disease. As such, attempts to functionalize AMPs in order to target and selectively kill pathogenic over commensal microflora has been a focus of research. To this end, specifically targeted antimicrobial peptides (STAMPS), gram-selective AMPs, and environment-sensing AMPs are being investigated.[85] The STAMP technology involves attaching a pathogen-specific ligand, like competence stimulating peptide (CSP) for *S. mutans*, as a targeting domain. This strategy was shown to be effective at eradicating *S. mutans* while having no effect on the other streptococci tested.[90]

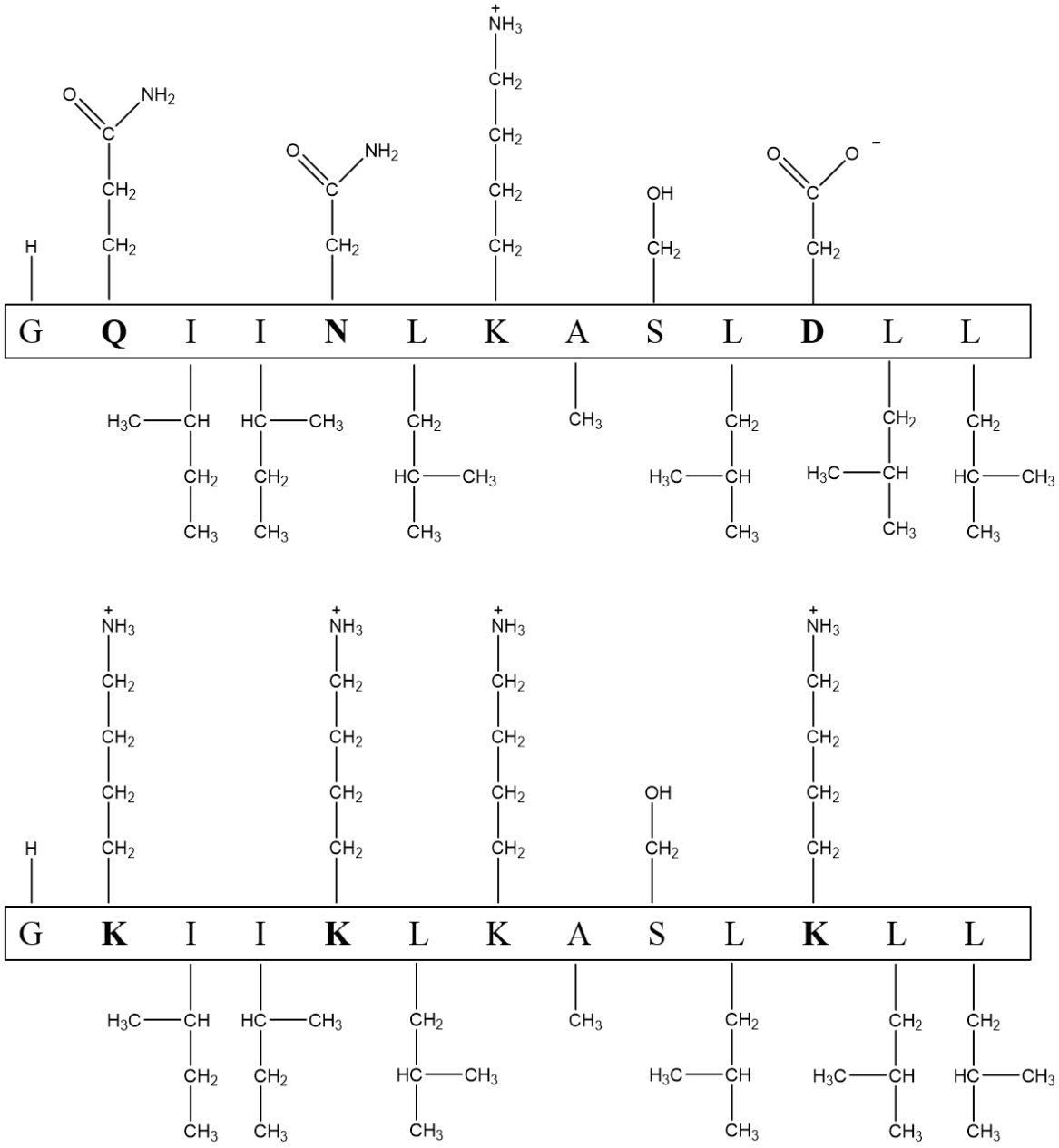

Figure 1.4 Structures of two antimicrobial peptides (AMPs). Bold letters designate altered amino acids. GL13-K is bactericidal while GL13NH2 is not. Both peptides shown exhibit anti-LPS activity. This highlights the small size of AMPs as well as the minute structural differences that greatly impact *in vivo* activity. Figure adapted from Gorr et al.[89]

Environment-sensing AMPs that exert antimicrobial activity under certain environmental stimuli such as acidic pH, were beneficial in selectively killing the acidogenic *S. mutans*.[91] For example, acid-activated peptide 2 (AAP2) demonstrated maximum antimicrobial activity at pH 5.5 but none at pH 7.5 highlighting its potential to selectively kill cariogenic *S. mutans*.[91] Lastly, Gram-selective AMPs have utility in selecting periodontal pathogens, which are primarily Gram-negative, over Gram-positive species using LPS-binding proteins (LBP), with >200-fold selectivity observed.[92] High LPS-inactivation was also noted, which can reduce inflammation, limit systemic endotoxin exposure, and potentially mitigate periodontal disease.[92]

The lack of AMP selectivity for bacteria over mammalian cells remains a major concern. Currently, cationic AMPs rely on electrostatic interactions with negatively charged bacteria for preferential association.[85,93] Mammalian cell membranes are also cholesterol-stabilized, providing rigidity and requiring greater concentrations for membrane disruption-based killing.[85] These factors contribute to the preferential, but not selective, killing of bacteria versus mammalian cells. Much attention has thus focused on bacteria-selective AMPs which attempt to limit hemolytic activity by reducing hydrophobicity and amphipathicity (hydrophobic moment).[94] One example of such a peptide is the glycine-rich adepantin 1 (ADP1), which has a significantly greater bacteria-human selectivity index (SI) than previously studied AMPs.[94] By modeling peptides to predict their hydrophobic moments, designer peptides can be synthesized with purpose as opposed to shotgun screening techniques to expedite their discovery.

The non-selective eradication of commensal bacteria along with pathogens, combined with their hemolytic activity towards mammalian cells, have limited the clinical utility of AMPs. Furthermore, AMP screening is arduous as innumerable peptide variations exist in nature, each with its own unique bioactivity. Indeed, novel AMPs are being isolated from the hydrolysis of

bovine milk proteins even after 30 years of investigation.[84] Designer AMPs have yet to be clinically utilized as their behavior *in vivo* is unpredictable. Much work is still necessary in the antimicrobial screening, design, and *in vivo* evaluation of designer AMPs prior to further pharmaceutical development.

1.4.3 Silica

Due to its biocompatibility and low cost,[95] silica has been used in toothpastes as an abrasive to remove dental plaque and polish teeth. Indeed, nearly all toothpaste formulations available include micron-sized silica in the form of hydrated silica or silicon dioxide. Recent research has focused on utilizing silica nanoparticles over microparticles for polishing teeth as finer particulate polishes result in smoother surfaces.[96] One study in particular compared nanoparticles (60 ± 4 nm) to both NuPro (1 – 180 μm) polishing paste and Crest toothpaste as polishing agents in terms of tooth surface roughness and strength of subsequent *S. mutans* adhesion.[96] The nanoparticle slurry resulted in single-nanometer surface roughness, an order of magnitude better than the professional polishing formula NuPro.[96] The smoothness obtained via polishing with silica nanoparticles led to reduced bacterial adhesion strength and easier removal of *S. mutans* from the tooth surface.[96] Thus, polishing with silica nanoparticles versus micron-sized particles, although not antimicrobial themselves, could potentially limit caries-induced tooth damage by reducing the numbers of cariogenic bacteria on teeth through routine personal oral hygiene (i.e., brushing).

Silica-based bioactive glass has been investigated for the restoration of eroded dental enamel caused by dental caries. Enamel reconstruction is challenging due to its acellular nature and 95 wt% mineral composition which prevents self-remodeling. Bioactive glass represents a possible *in vivo* dental implant material capable of depositing minerals on the tooth surface, thus

repairing carious lesions. Bioglass 45S5 is a bioactive implant that undergoes a series of reactions resulting in the formation of hydroxyapatite, stimulating dental bone repair.[97] This material was capable of forming a calcium-phosphate layer that resisted abrasion on enamel surfaces within 24 h.[97] At 14 d, this layer had transformed into hydroxyapatite crystals, highlighting its potential use for the treatment of incipient carious lesions formed by acidogenic bacteria like *S. mutans*.[97]

Antimicrobial activity can also be imparted on bioactive glass by incorporating biocides into the glass, allowing for the potential to kill cariogenic bacteria and simultaneously remineralize enamel. In one study by Palza et al., the bioactive glass formulation was shown to undergo chemical transformations resulting in the growth of crystalline hydroxy-carbonate apatite (HCA) that could potentially repair early carious lesions via remineralization.[98] Biocidal metal ions (i.e., silver and copper) were incorporated into the glass and demonstrated antibacterial activity against cariogenic *S. mutans*.[98] The bactericidal efficacy of such hybrid materials demonstrates the possibility of antimicrobial-doped bioactive glass to simultaneously kill cavity causing bacteria and repair minor enamel erosion. Bioactive glass represents a possible antibacterial/reconstructive therapeutic but is still limited by the use of antimicrobial metals known for their toxicity and adverse side effects.

1.5 Nitric Oxide

Nitric oxide is an endogenously produced gaseous, free radical species central to many physiological processes including neurotransmission, angiogenesis, vasodilation, wound healing, cancer biology and the innate immune response to invading pathogens.[99-104] Although NO's role in the immune response for eliminating bacteria will be the most pertinent in regards to this

dissertation, a thorough knowledge of NO's function in the body is essential to focusing its utility as a potential antimicrobial therapy.

1.5.1 Physiological roles of nitric oxide

Endogenously, NO is synthesized from L-arginine via NO synthase (NOS), which exists as three isoforms and can be classified into two broad categories. The constitutive class (cNOS) is comprised of the endothelium (eNOS) and neuronal (nNOS) isoforms that produce pM–nM levels of NO for signaling purposes, and the inducible (iNOS) isoform, which produces greater levels of NO (μM) for antimicrobial purposes.[104] The cNOS system functions continuously to regulate endogenous processes including angiogenesis, neurogenesis, and vasodilation, whereas the iNOS system is activated in neutrophils and macrophages by bacterial endotoxins during the immune response. This section will focus on the physiological roles of cNOS, while the antimicrobial effects of NO derived from iNOS will be discussed in the next section.

Calcium binding to the messenger protein calmodulin triggers cNOS to produce low levels of NO, which readily diffuses across cell membranes to react with molecular targets that ultimately dictate NO's function. Within the vascular endothelium, NO reacts with guanylyl cyclase, subsequently catalyzing the production of cyclic guanosine monophosphate (cGMP), which leads to smooth muscle relaxation and vasodilation similar to that of endothelium-derived relaxing factor (EDRF).[104] Further cardiovascular utilities of NO include regulation of vascular tone, myocardial contractility, endothelial-leukocyte interactions, endothelial integrity, vascular cell proliferation, and antithrombotic effects.[104] Deficiencies in NO production are directly linked to cardiovascular diseases such as hypertension, reperfusion injury, atherosclerosis, and stroke, further highlighting NO's role in the normal function and maintenance of mammalian vasculature.[99,105]

Apart from vascular effects, NO plays a pivotal role in the central nervous system of animals.[100] The firing of neurons and release of specific neurotransmitters such as acetylcholine and serotonin is modulated by NO. Other neuronal functions like long-term potentiation, long-term depression, and memory formation are also coordinated by NO. Reports suggest that a majority of NO effects on neurons are modulated via cGMP and glutamate. Evidence exists that NO can either excite or inhibit neuronal activity within major sections of the brain such as the hippocampus and hypothalamus.[100] Due to the opposing nature of NO-mediated effects on neuronal activity, its true role within all neuronal activity is still unclear. However, it is apparent that NO plays a significant role in neuronal function.

Additionally, NO plays a pivotal role in the progression of wound healing, as it is involved in modulating inflammation, angiogenesis, cell proliferation, matrix deposition, and tissue remodeling.[99,101,102,106,107] Although this process is complex and the exact role of NO in each phase is unclear with regards to its concentration and action, the importance of NO in wound healing is apparent. This can be observed for diabetic patients whose healing is impaired due to NO deficiencies at the wound site. Such effects have been verified by the partial reversal of impaired wound healing upon supplementation with exogenous NO or arginine.[106,107] Nitric oxide is paramount to successful wound healing and thus plays a crucial, irreplaceable role in this process.

1.5.2 Antimicrobial properties of nitric oxide

Nitric oxide is produced by macrophages and neutrophils in response to invading pathogens as part of the innate immune response. In this instance, larger concentrations of NO (μM) are produced by iNOS to provide an antimicrobial effect versus cell signaling and angiogenesis.[99] Nitric oxide produced in this manner exerts antimicrobial activity primarily

through nitrosative and oxidative stress, as depicted in Figure 1.5.[108] Outside of the cell, NO exerts nitrosative stress by reacting with oxygen to form dinitrogen trioxide (N_2O_3), which nitrosates amines on cell membrane and transmembrane proteins, thereby altering protein function.[99,108] Lipophilic in nature, NO and oxygen can both accumulate within the cell membrane, accelerating the formation of N_2O_3 and fostering nitrosative stress within the cell membrane. As a diatomic gas, NO can also rapidly traverse cell membranes and react with the bacteria respiration product superoxide (O_2^-) to produce the oxidative species peroxynitrite ($OONO^-$).[99,108] This species can cause damage to proteins and DNA, or react with nitrogen dioxide radicals in the form of peroxynitrous acid to cause lipid peroxidation within the cell membrane. Membrane destruction via $OONO^-$ lipid peroxidation is considered to be the primary antibacterial mechanism of NO.[108]

Along with its potent antimicrobial efficacy against Gram-positive, Gram-negative, and antibiotic-resistant pathogens, NO's selective biocidal activity against bacteria over mammalian cells and multiple killing pathways make it an attractive bactericidal agent. The selective action towards bacteria is due to the endogenous production of NO by mammals, rendering it a normal metabolite. As such, mammals deal with intracellular NO accumulation using superoxide dismutase, an enzyme that catalyzes the decomposition of superoxide into oxygen and hydrogen peroxide. This limits local peroxynitrite generation and minimizes NO cytotoxicity. Nitric oxide's multiple killing mechanisms contribute to its biocidal potency and diminish the chances of bacteria developing resistance to NO as an antimicrobial therapy. Privett et al. studied Gram-positive, Gram-negative, and antibiotic resistant bacteria that survived killing assays after several passages (20 d) and observed minimal increases in the NO concentrations required for growth inhibition.[109] The antimicrobial potency and lack of observed bacterial resistance highlight the therapeutic potential NO.

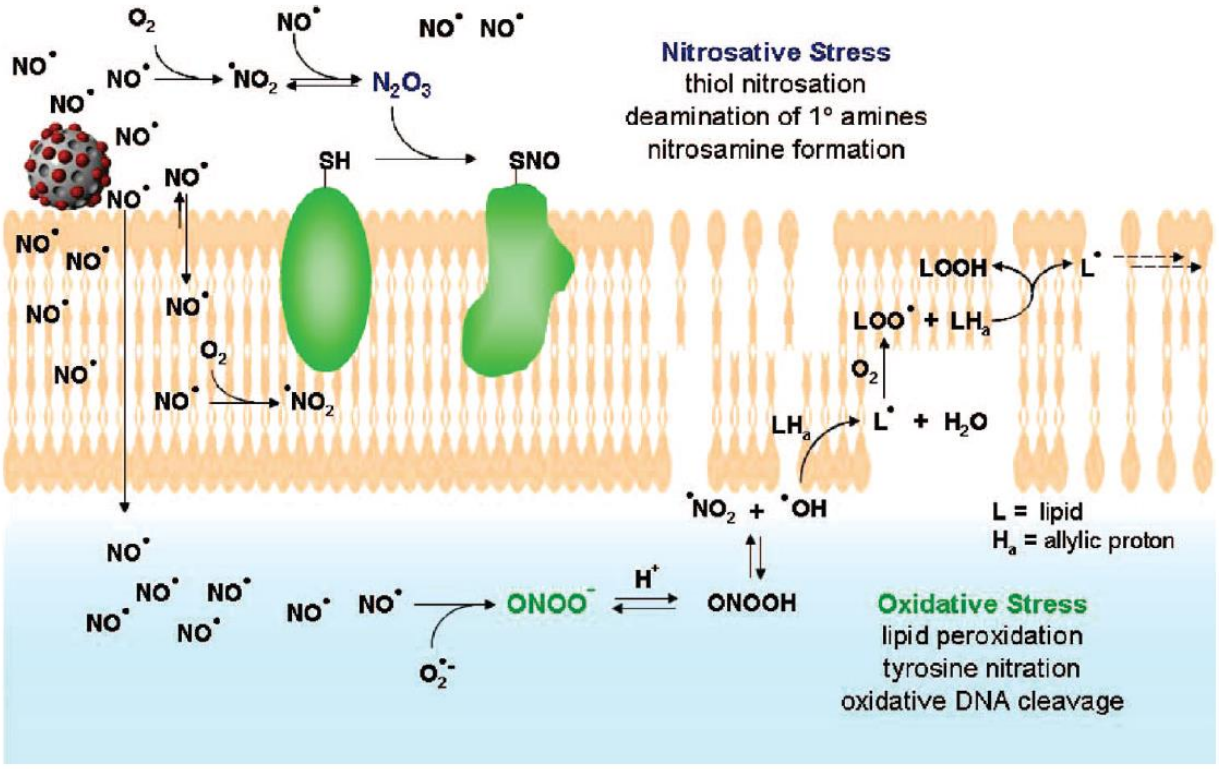

Figure 1.5 The multiple antimicrobial mechanisms of NO and its byproducts through both nitrosative and oxidative stress. Reprinted with permission from Hetrick et al.[108] Copyright 2008, American Chemical Society.

1.5.3 Nitric oxide as an antimicrobial against oral diseases

Endogenous NO production to fight infectious pathogens combined with its production by mucosal epithelial cells in response to pro-inflammatory stimuli initiated upon the deposition of plaque make it an ideal candidate for antimicrobial dental applications.[110] Indeed, studies have shown a correlation between high salivary nitrite and low incidence of caries, most likely due to the production of bactericidal NO from acidified nitrite.[111,112] Despite these findings, few studies have examined the antibacterial efficacy of exogenous NO against dental pathogens. Reports of NO as a dental antimicrobial have been limited to verifying the role of salivary nitrite in controlling oral bacteria levels, but have not investigated the delivery of exogenous NO as a biocidal therapeutic.

Of note, studies have shown that NO derived from acidified nitrite acts as an antimicrobial towards both cariogenic and periodontopathogenic bacteria.[113,114] Low concentrations of nitrite were added to bacterial suspensions at various pH levels to assess the acid-mediated production of NO and subsequent killing. Allaker et al. reported efficacy against periodontopathogenic *F. nucleatum* and *P. gingivalis*, while others reported antibacterial effects towards *S. mutans*.[113,114] These investigations were designed to examine the role of salivary levels of nitrite on bactericidal and bacteriostatic effects and were not intended to examine NO directly as a deliverable antimicrobial. However, this work supports the administration of exogenous NO to the oral cavity as a potent antibacterial agent for the treatment of oral diseases, namely caries and periodontitis.

1.6 Nitric Oxide-Releasing Materials

Nitric oxide has great potential as an antimicrobial therapeutic for oral applications. However, its gaseous and highly reactive nature make stable storage and localized delivery

challenging. This section will introduce NO-releasing scaffolds utilizing *N*-diazeniumdiolate NO donors for the chemical storage and delivery of NO. It should be noted several other NO donors exist, including metal nitrosyls, *S*-nitrosothiols (RSNOs), and organic nitrates/nitrites,[115] but this section will focus primarily on *N*-diazeniumdiolate NO donor storage and delivery chemistries. *N*-Diazeniumdiolates can be formed on amine groups through a base catalyzed reaction with gaseous NO at elevated pressure. As shown in Figure 1.6,[116] the proton-initiated decomposition of *N*-diazeniumdiolates yields two molecules of NO and the unmodified precursor scaffold. Stabilization of the *N*-diazeniumdiolate moiety by neighboring amines on the delivery scaffold allows for extended and controlled NO release. The potential for greater storage in conjunction with spontaneous, yet controllable, generation of NO under physiological conditions (e.g., the oral mucosa) makes this NO donor particularly amenable to application as an oral antimicrobial therapeutic.

1.6.1 Low molecular weight nitric oxide donors

Several low molecular weight small molecules have been modified with *N*-diazeniumdiolate moieties to serve as NO donors. The amino acid proline is well suited for NO donor functionalization. Indeed, "PROLI/NO" is capable of storing large quantities of NO (7.24 μmol/mg)[117] due to easily accessible secondary amines for *N*-diazeniumdiolate modification. Furthermore, the precursor scaffold (regenerated upon liberation of NO) has inherently low cytotoxicity. While PROLI/NO has been demonstrated to reduce arterial pressure for the treatment of pulmonary hypertension,[118] its antimicrobial therapeutic potential is limited due to minimal *N*-diazeniumdiolate stability, resulting in rapid NO release with a short half-life (1.8 s)[119] in physiological conditions. As such, a majority of the NO is released from the scaffold before

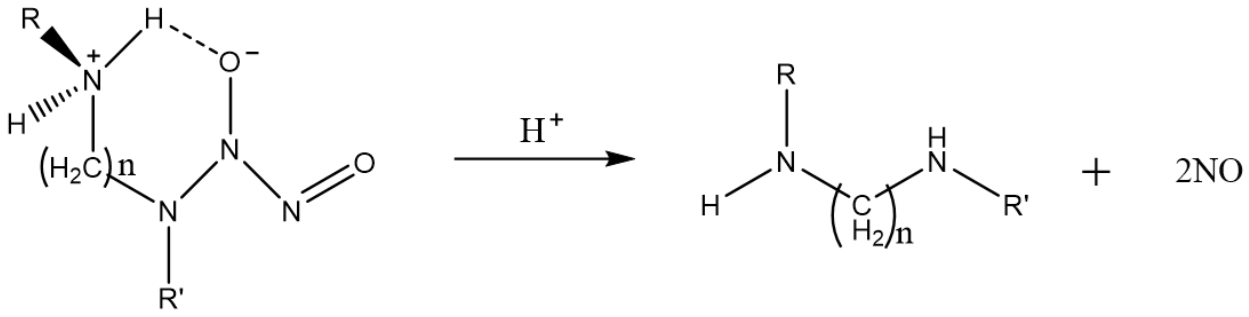

Figure 1.6 The spontaneous proton-initiated decomposition of an amine-stabilized *N*-diazeniumdiolate to yield two molecules of NO and the parent polyamine precursor. Adapted from Hetrick et al.[116]

reaching the target site in therapeutic applications, thus limiting both the effective NO dose and biological action. Despite high NO storage and direct application to the site of action, large concentrations of PROLI/NO are required to induce vasodilation, with even greater NO levels required to exert an antimicrobial effect. Furthermore, the potential cytotoxicity of NO at these concentrations hinders the utility of PROLI/NO and similar small molecule NO donors as potential antimicrobial therapeutics.

1.6.2 Macromolecular nitric oxide-releasing scaffolds

To circumvent the non-specific and premature NO-release characteristics of small molecule donors, macromolecular NO-release scaffolds capable of stabilizing the *N*-diazeniumdiolate NO donor moiety have been developed by our lab and others.[115,120-127] Liposomes have been investigated for the encapsulation of both gaseous NO and small molecule NO donors to prevent *N*-diazeniumdiolate degradation and sustain NO release.[115,128,129] These NO-release vehicles have the potential to produce a bolus of NO when liposome stability is compromised via stimuli.[115,129] Encapsulation of PROLI/NO within polyethylene oxide-co-lactic acid (PELA) microspheres has been achieved, but with limited success in terms of controlling the amount of NO donor encapsulated and NO-release kinetics.[115] Significant work still remains for the encapsulation of NO donors within both liposomes and microspheres to enhance total NO storage and control NO-release profiles.

Metal nanoparticles have also been investigated as NO-release scaffolds. Rothrock et al. reported on amine-functionalized gold nanoparticles as the first metal nanoparticle NO-release system with tunable storage and NO-release kinetics.[125] Despite their small size (~2 nm) and high surface area to volume ratio, these particles stored <0.04 μmol/mg due to poor *N*-diazeniumdiolate

conversion efficiency (<1%).[125] These factors, combined with toxicity concerns, have limited the utility of *N*-diazeniumdiolated metal nanoparticles as antimicrobial agents.

Zeolites and metal organic framework (MOF) NO donors exhibit improved NO storage (~1.2–1.3 μmol/mg for Zeolite-A) over metal nanoparticles, but are plagued by excessively rapid NO-release kinetics (half-life of ~340 s) due to chemisorbed NO storage.[115,130] Metal organic frameworks have achieved chemisorbed NO loading as high as 9 μmol/mg but have inaccessible NO stored within their structures, thus limiting the usable NO payload (~1 nmol/mg MOF).[115,131] *N*-diazeniumdiolates formed on primary amines of organic linkers between metal sites in MOFs improved NO-release totals (~0.51 μmol/mg), but were characterized by fast NO-release kinetics (total release <5 min).[123] The lack of stable secondary amine *N*-diazeniumdiolates, combined with the inherent toxicity of both zeolites and MOFs, have limited the potential of these scaffolds as antimicrobial therapeutics.

1.6.3 Nitric oxide-releasing silica nanoparticles

Silica has attracted attention as a drug delivery vehicle due to inherently low cytotoxicity[95] and facile synthesis, which provides for control over physical (size and shape) and chemical (composition) characteristics. Silica's use in dental applications such as toothpastes makes it easily translatable into existing dental therapies. Zhang et al. first reported on NO-releasing silica using aminosilane-grafted fused silica particles and subsequent *N*-diazeniumdiolate modification.[124] Subsequently, Shin et al. incorporated aminosilanes throughout the silica by synthesizing hybrid alkoxysilane/aminosilane NO-releasing nanoparticles, in which nanoparticle size, amine identity, and amine content were easily tunable,[126] thus greatly increasing the utility of NO-releasing silica particles as antimicrobial therapeutics.

Hetrick et al. compared the antimicrobial activity of NO-releasing *N*-(6-aminohexyl)aminopropyltrimethoxysilane (AHAP3) particles and the small molecule donor PROLI/NO against planktonic cultures of *Pseudomonas aeruginosa*.[108] The particles proved bactericidal at lower material concentrations and NO doses compared to PROLI/NO, highlighting the advantage of controlled NO storage and release from macromolecular scaffolds.[108] A subsequent study evaluated the efficacy of *N*-diazeniumdiolate-modified *N*-methylaminopropyltrimethoxysilane (MAP3) and AHAP3 silica particles against biofilms of Gram-positive and Gram-negative bacteria, as well as fungus, demonstrating the broad-spectrum anti-biofilm capabilities of these NO-release vehicles.[132] The particle concentrations tested were characterized by reduced toxicity to mouse fibroblasts (L929) compared to clinical concentrations of povidone iodine.[132] The two NO-releasing particle systems studied exhibited differences in bactericidal efficacy, signifying the importance of particle composition and *N*-diazeniumdiolate chemistry on the antibacterial activity of NO-releasing silica nanoparticles. Additionally, the role of physical and chemical properties of NO-release scaffolds on bactericidal efficacy including size, shape, and NO-release kinetics remained unclear. Nevertheless, the strong antimicrobial effect of NO and therapeutic potential of NO-releasing silica was validated.

Physical properties of NO-releasing silica nanoparticles, such as size and shape, are easily modified and may affect both particle-bacteria interactions and subsequent killing efficiency. Carpenter et al. investigated the role of NO-releasing silica particle size on bactericidal efficacy using AHAP-modified tetraethoxysilane (TEOS) silica particles with diameters of 50, 100, and 150 nm and similar NO totals.[133] Bactericidal activity was greatest for the smaller particles (50 nm), presumably due to faster particle-bacteria association attributable to enhanced diffusion. Visualization of particle association with the bacterial membrane via confocal microscopy

confirmed enhanced diffusion to the bacteria membrane for the smaller diameter particles.[133] The effects of shape on the efficacy of silica nanoparticles was also investigated. Lu et al. synthesized surfactant-templated silica nanorods of varying aspect ratios while maintaining constant particle volume and NO-release totals.[134] This study demonstrated enhanced NO delivery to bacteria for elongated, high aspect ratio particles over spherical particles as a result of the greater degree of particle-bacteria contact, requiring lower NO concentrations necessary for planktonic bacteria (*P. aeruginosa* and *S. aureus*) eradication.[134]

Chemical properties of silica particle NO donors have also been investigated as parameters for enhancing the antimicrobial efficiency of NO-releasing silica particles. For example, Lu et al. investigated the role of NO flux on the antibacterial efficacy of NO-releasing silica nanoparticles.[134] Utilizing the same silica nanorods described above, the maximum NO flux released from the particles was varied while holding the total NO storage constant.[134] As expected, greater maximum NO-release fluxes resulted in increased bactericidal efficacy against both planktonic *P. aeruginosa* and *S. aureus* due to greater instantaneous intracellular NO concentrations.[134] In addition to the apparent dependence of biocidal activity on NO-release kinetics, the incorporation of additional biocides into the macromolecular scaffold has been investigated as a means to enhance antimicrobial efficacy. Carpenter et al. reported on NO-releasing silica particles functionalized with quaternary ammonium (QA)-modified alkyl chains to impart an additional mechanism of killing.[135] Combining NO release with QA alkyl chains resulted in enhanced antibacterial activity and decreased the minimum bactericidal concentration of NO-releasing silica necessary to eradicate bacteria.[135]

Collectively, these previous studies have verified the potency of NO-releasing silica particles as antimicrobial agents, with particular emphasis on how their physical (size and shape)

and chemical (NO-release characteristic and additional biocides) properties affect bactericidal activity. At this stage, it is still unclear how NO-release kinetics (e.g., half-life) impact the antimicrobial efficacy of NO-releasing silica particles independent of particle size and NO totals. Furthermore, NO-releasing silica particles have only been tested against a limited scope of bacteria.

1.6.4 Nitric oxide-releasing dendrimers

Dendrimers are hyper-branched macromolecular scaffolds whose synthesis provides precise control over their size and exterior functionality. With each added generation, or layer of polymer growth, the number of functional end groups increases exponentially creating numerous sites for chemical modification while maintaining minimal molecular weight. As reported by Stasko et al., generation 3 and 5 (G3–G5) polypropylenimine (PPI) dendrimers were functionalized with *N*-diazeniumdiolate moieties at primary amine sites to impart NO-release capabilities.[127] As primary amine *N*-diazeniumdiolates are relatively unstable, dendrimers can easily be functionalized to include secondary amines and amides prior to *N*-diazeniumdiolate formation.[127] In this manner, the controllable storage and delivery of large NO payloads from dendrimers is greatly improved, which may enhance their therapeutic utility.

Due to increased NO storage, prolonged release kinetics, and exquisite synthetic control over resulting chemical properties, secondary amine-functionalized dendrimers have been researched as NO-releasing materials for therapeutic applications. Using a one-step ring-opening or conjugation reaction, Lu et al. converted primary amines on the periphery of PPI dendrimers to secondary amines imparting new surface chemistries and tunable NO-release properties, thus unlocking a series of novel NO-releasing materials.[136] This study resulted in dendrimer scaffolds

of varied generation (G2–G5) dendrimers with aromatic, hydrophobic, and hydrophilic exterior modifications that greatly affected NO storage (0.9–3.8 μmol NO/mg dendrimer) and release kinetics (0.3–4.9 h half-lives), highlighting the effect of both molecular weight and exterior functionality on proton-initiated NO-release characteristics.[136] Furthermore, the dendrimers proved capable of eradicating Gram-positive, Gram-negative, and antibiotic-resistant (i.e., methicillin-resistant *Staphylococcus aureus*) pathogens, with antibacterial efficacy dependent on generation (molecular weight) and exterior functionality.[137] Although bactericidal efficacy was observed for all modified dendrimers, greater efficacy was observed for higher generation and more hydrophobic functionalities, likely due to enhanced bacterial membrane damage.[137] In general, the addition of NO provided improved antimicrobial efficacy and lower cytotoxicity versus controls (i.e., modified dendrimer precursor scaffolds).[137]

Subsequently, Lu et al. reported on the bactericidal efficacy of amphiphilic NO-releasing poly(amidoamine) (PAMAM) dendrimers, characterized by simpler synthesis over previous PPI-based materials.[138] A ring opening reaction was used to modify the exterior primary amines of the dendrimers with varying ratios of hydrophilic to hydrophobic functionalities.[138] By maintaining constant NO-release totals across the dendrimer systems, this study examined the extent of exterior hydrophobicity on both bacteria killing and cytotoxicity.[138] Through tuning the exterior hydrophilic/hydrophobic ratio, a balance in the degree of hydrophobicity was discovered to maximize bactericidal efficacy while maintaining suitable biocompatibility with L929 mouse fibroblast cells.[138] Nitric oxide-releasing PAMAM dendrimers have also been functionalized with QA-modified alkyl chains in an attempt to increase the hydrophobic nature of the exterior.[139] The antimicrobial activity of QA-modified NO-releasing dendrimers increased considerably with increasing length of the alkyl chain. As observed previously, a balance in alkyl chain length (i.e.,

hydrophobic character) was required to maximize bactericidal efficacy while demonstrating minimal cytotoxicity.[139] The benefits of combining hydrophobic functionalities with NO release to improve the bactericidal efficacy of NO-releasing dendrimers is apparent. Future work must focus on exploiting alkyl chain-modified dendrimer antibacterial activity while mitigating cytotoxicity. Additionally, it is pertinent to evaluate NO-releasing alkyl-modified PAMAM dendrimers against different strains of bacteria and alternate cell lines for other biomedical applications including oral care.

1.7 Summary of Dissertation Research

The goal of my dissertation research was to design and develop NO-release vehicles for the treatment of oral diseases (i.e., dental caries and periodontal disease). In this capacity, I also sought to investigate the role of NO-release kinetics and exterior dendrimer modification on bactericidal action to enhance antibacterial efficacy against certain oral pathogens and minimize cytotoxicity. Specifically, my research aimed to:

1. Synthesize monodisperse silica particles with varying amine compositions and different NO-release properties (totals and kinetics) for subsequent bactericidal assays;
2. Evaluate the bactericidal efficacy of multiple NO-release vehicles against common strains of oral bacteria to assess the therapeutic utility of NO release;
3. Examine the role of silica-based NO-release kinetics on bactericidal efficacy against oral bacteria for the future development of NO-based dental therapeutics; and,
4. Utilize alkyl chain modified NO-releasing dendrimers as oral anti-biofilm agents.

In this introductory chapter, I sought to explain oral health diseases in terms of their detrimental impact on society and treatment challenges due to the complex nature of dental plaque biofilms. Inadequate current treatments and lack of satisfactory ongoing research in the area of dental plaque therapeutics has motivated the development and further study of NO-releasing materials, which have the potential to serve as future oral health pharmaceuticals. In Chapter 2, the Stöber method of silica particle synthesis is described for creating monodisperse similarly sized, spherical particles with varying amine content and identity. These NO-delivery vehicles allowed for microbiological studies in subsequent chapters. In Chapter 3, three distinct NO-release vehicles were evaluated against bacteria associated with dental caries (*Streptococcus mutans* and *Streptococcus sanguinis*) and periodontal disease (*Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*). In Chapter 4, silica particles with discrete compositions and unique NO-release kinetics were evaluated against oral pathogens to elucidate the role of NO-release kinetics on bactericidal efficacy. Chapter 5 describes the evaluation of NO-releasing dendrimers against both planktonic and biofilm cultures of *Streptococcus mutans* as a function of alkyl chain length and pH for use as dental caries therapeutics. Lastly, Chapter 6 provides a summary of my work and suggests future steps for the design and implementation of NO-release vehicles to mitigate oral diseases.

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Chapter 2: Stöber Synthesis of *N*-Diazeniumdiolate-Modified Nitric Oxide-Releasing Silica Nanoparticles

2.1 Introduction

Nitric oxide (NO) is an endogenously produced free radical central to many physiological processes.[1-6] At low concentrations (pM–nM), NO acts as a cell signaling molecule involved in angiogenesis, neurogenesis, vasodilation, and wound healing.[5-8] Greater NO concentrations (μM) produced by neutrophils and macrophages exhibit a potent antimicrobial effect, central to the innate immune response against infectious pathogens (e.g., bacteria).[9,10] Upon dental plaque deposition, pro-inflammatory endotoxins stimulate mucosal epithelial cells to release NO as an early antimicrobial against oral bacteria.[11] Several research groups have demonstrated that acidified nitrite is bactericidal against both cariogenic and periodontopathogenic bacteria.[12-14] Despite NO's promising antibacterial action,[15-17] the use of exogenous NO-release systems against oral pathogens has yet to be studied. As the administration of pure NO gas to the oral cavity is both impractical and inefficient, requiring a high pressure NO gas tank and lacking specific pathogenic targeting, methods for storing and releasing exogenous NO must be developed for clinical utility.[2]

Several NO donors are capable of stable NO storage and controllable, stimuli-induced release, including organic nitrates, metal nitrosyls, *S*-nitrosothiols, and *N*-diazeniumdiolates.[18] *N*-diazeniumdiolate NO donors undergo proton-initiated decomposition in physiological solutions (pH 7.4; 37 ^{o}C),[19] making them well-suited for *in vivo* applications. Two moles of NO are

produced per mole of *N*-diazeniumdiolate,[19] facilitating the storage and release of large NO payloads. The low molecular weight NO donor proline (PROLI/NO) is capable of high *N*-diazeniumdiolate conversion efficiency and large NO storage (7.24 μmol/mg).[20] However, PROLI/NO is plagued by incredibly rapid NO donor decomposition (PROLI/NO half-life = 1.8 s),[21] releasing a significant amount of NO indiscriminately upon contact with aqueous solutions. The inability of PROLI/NO, and other small molecular weight donors, to target NO to a specific site of action results in low effective NO doses and raises toxicity concerns, which hinders their clinical development.[18]

Macromolecular NO storage vehicles benefit from superior spatiotemporal control over exogenous NO-release payloads and release kinetics.[2,18,22-29] Silica nanoparticles are one such vehicle that exhibits great promise as a drug delivery system due to tolerance by mammalian cells and superior surface modification capabilites.[30] When considering drug delivery systems for oral therapeutics, silica (e.g., hydrated silica and silicon dioxide) is already present in toothpastes as an abrasive to remove dental plaque and polish teeth. Recent research has highlighted the advantage of using silica nanoparticles versus micron-sized silica as a polishing agent to reduce *S. mutans* adhesion strength to teeth, rendering bacteria easier to remove.[31] Utilizing silica nanoparticles as NO storage platforms could prove advantageous for delivering exogenous NO as an antibacterial therapeutic with the potential for simple translation into existing oral healthcare modalities.

Zhang et al. first reported on silica-based *N*-diazeniumdiolate NO storage and delivery using amine-grafted fumed silica.[28] Although NO storage was limited by the parent amine coupling efficiency to the silica surface (≤70%), the apparent half-life of the NO-releasing silica (43 min) was substantially lengthened compared to the solution phase *N*-diazeniumdiolate analogues, highlighting the benefit of silica-based NO storage and delivery vehicles.[28] To

overcome surface-grafting limitations, Shin et al. used the Stöber synthesis[32] to incorporate amines throughout silica particles via a high throughput, one-pot reaction.[29] Compared to previous silica-based NO-release scaffolds, greater NO storage totals and superior control over particle size, composition, and NO-release kinetics were achieved, greatly increasing the utility of NO-releasing silica nanoparticles as potential antibacterial agents.[29]

Our laboratory evaluated the use of NO-releasing silica nanoparticles against both planktonic and biofilm cultures of Gram-positive, Gram-negative, and fungal pathogens. Improved efficacy for these scaffolds was observed compared to the low molecular weight NO donor PROLI/NO.[33,34] Subsequent research focused on tuning the physical (e.g., size and shape) and chemical (e.g., NO-releasing kinetics) properties of the silica nanoparticles to further enhance bactericidal efficacy. For example, Carpenter et al. reported on a reverse microemulsion technique to synthesize aminosilane-functionalized silica of different sizes (50, 100, 200 nm). Greater bactericidal efficacy was observed for the smaller (50 nm) NO-releasing silica particles due to their more rapid diffusion, allowing for faster particle-bacteria association and more efficient intracellular NO delivery.[35] Lu et al. prepared high aspect ratio NO-releasing silica nanorods via surfactant-templated silane growth and subsequent surface grafting with aminosilanes. The improvement in antibacterial activity for the higher aspect ratio NO-releasing silica nanorods compared to spherical particles was attributed to the greater degree of particle-bacteria surface contact and intracellular NO delivery.[36] Additionally, Lu et al. observed that greater initial NO fluxes, independent of the total NO delivered, could reduce the concentration of NO-releasing particles necessary to kill bacteria.[36]

At this stage, physicochemical investigations into the resulting bactericidal efficacy of NO-releasing silica nanoparticles have involved complex and time-consuming synthetic methods for

modifying silica with aminosilanes for subsequent *N*-diazeniumdiolate modification. It is also unclear as to what effect NO-release kinetics (i.e., half-life) have on the bactericidal efficacy of NO-releasing silica nanoparticles. Furthermore, the antibacterial activity of NO-releasing silica in terms of these physicochemical properties have been investigated against a narrow scope of nosocomial pathogens (i.e., *Pseudomonas aeruginosa*). To this end, Chapter 2 describes the straightforward, one-step Stöber synthesis of NO-releasing silica particles for subsequent bactericidal evaluation against dental-relevant pathogens and cytotoxicity testing against human gingival cells. Both nanoparticle size and the NO-release properties (i.e., NO-release totals and kinetics) were varied systematically to isolate the role of physicochemical properties on the bactericidal efficacy of NO release.

2.2 Materials and Methods

Tetramethyl orthosilicate (TMOS) was purchased from Sigma-Aldrich (St. Louis, MO). 3-methylaminopropyltrimethoxysilane (MAP3), *N*-(6-aminohexyl)aminopropyltrimethoxysilane (AHAP3), *N*-(2-aminoethyl)-3-aminopropyltrimethoxysilane (AEAP3), and (3-trimethoxysilyl)diethylenetriamine (DET3) were purchased from Gelest (Morrisville, PA). Ethanol (EtOH), 1-butanol (BuOH), and ammonium hydroxide (NH_4OH; 30 wt%) were purchased from Fisher Scientific (Fair Lawn, NJ). Nitrogen (N_2), argon (Ar), and nitric oxide (NO) calibration (26.81 ppm, balance N_2) gases were purchased from National Welders (Raleigh, NC). Anhydrous methanol (MeOH), *N*,*N*-dimethlyformamide (DMF), and sodium methoxide (NaOMe; 5.4 M in MeOH) were purchased from Acros Organics (Geel, Belgium). Pure NO gas was purchased from Praxair (Sanford, NC). Distilled water (H_2O) was purified with a Millipore Milli-

Q Gradient A-10 water purification system (Bedford, MA) to obtain H_2O with ≤6 ppb organic content and a final resistivity of 18.2 mΩ·cm.

2.2.1 Synthesis of 150 nm silica particles with varying amine content

Hybrid alkoxysilane/aminosilane silica nanoparticles were produced using a modified Stöber method.[29] Briefly, particles were synthesized by combining TMOS with an aminosilane (MAP3, AHAP3, AEAP3, or DET3) (Figure 2.1) and bolus injection into a flask containing H_2O (27.84 mL), NH_4OH (9.8 mL), and EtOH as a co-solvent (balanced to make a final volume of 100 mL). Of note, DET3 required less H_2O (10.80 mL) and was still balanced with EtOH to a total volume of 100 mL. As shown in Table 2.1, the aminosilane precursor amounts were varied to synthesize different mol% (35, 50, 60, 70 mol% MAP3; 50, 60, 70 mol% AHAP3; 50, 60, 70, 80 mol% AEAP3; and 70 mol% DET3) nanoparticles (balanced TMOS). After 2 h of stirring at room temperature, the nanoparticles were collected via centrifugation (2907 x *g*; 4 ^oC; 10 min) and decanting of the supernatant. The pellet was resuspended in EtOH, centrifuged and decanted twice more to remove unreacted silanes and residual solvent. The resulting amine-functionalized silica particles were dried under vacuum overnight to remove residual EtOH.

2.2.2 Synthesis of 400 and 800 nm 70 mol% MAP3 silica nanoparticles

The size of the 70 mol% MAP3 particles was tuned by modifying the above procedure. Briefly, the total silane concentration was increased from 0.12 to 0.24 M and butanol used in place of ethanol as the co-solvent. A solution of TMOS (1.076 µL) was combined with MAP3 (3.360 µL) and injected as a bolus into a flask containing H_2O (27.84 mL), NH_4OH (9.8 mL), and BuOH (57.92 mL) at room temperature (~23 ^oC) to synthesize the ~800 nm 70% MAP3 silica.

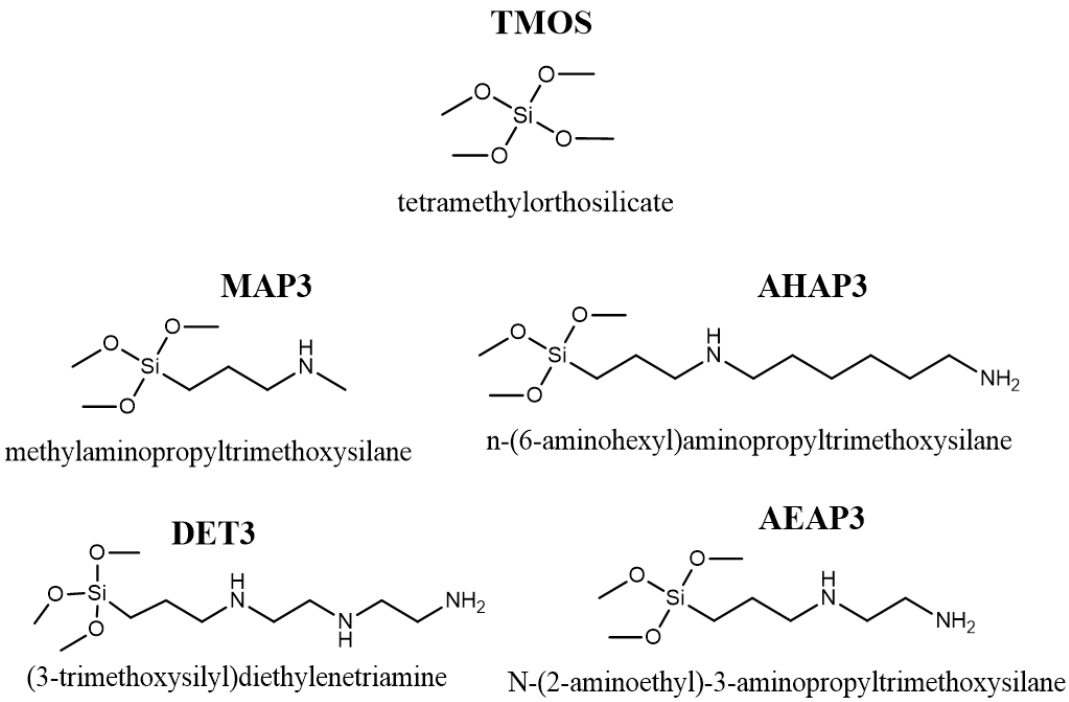

Figure 2.1 Structures of aminosilanes and backbone alkoxysilane (TMOS) used in the Stöber synthesis of NO-releasing silica nanoparticles.

Table 2.1 Concentrations and volumes of silanes used to synthesize 150 nm silica nanoparticles with varying amine content.

Aminosilane	Total Silane Concentration (M)	Mol% Aminosilane	TMOS (µL)	Aminosilane (µL)
MAP3	0.12	35	1.166	0.841
		50	0.897	1.201
		60	0.717	1.441
		70	0.538	1.680
		80	0.359	1.921
AHAP3	0.12	50	0.896	1.524
		60	0.717	1.829
		70	0.538	2.134
AEAP3	0.16	50	1.181	1.777
		60	0.945	2.133
		70	0.708	2.488
		80	0.472	2.844
DET3	0.20	70	0.886	3.610

The ~400 nm 70 mol% MAP3 silica nanoparticles were synthesized using the same synthetic mixture, but at increased reaction temperature (50 ^{o}C).

2.2.3 Silica nanoparticle characterization

The silica nanoparticles were characterized prior to *N*-diazeniumdiolate NO donor modification. Nanoparticle size and surface morphology were evaluated using a Hitachi S-4700 Scanning Electron Microscope (SEM; Chapel Hill Analytical and Nanofabrication Laboratory) and ImageJ software. Nanoparticles were suspended at 0.5 mg/mL in EtOH, cast on glass slides, and coated with 2 nm gold/palladium prior to imaging. Aminosilane content was confirmed and quantified as percent nitrogen per mg of nanoparticle using a Perkin Elmer 2400 Series II CHNS/O Elemental Analyzer operated in CHN mode (Waltham, MA). The monodispersity and hydrated diameter of the silica nanoparticles was measured in Milli-Q water via dynamic light scattering (DLS) using a Malvern Instruments Zetasizer Nano (Worcestershire, UK).

2.2.4 N-Diazeniumdiolate-modified nitric oxide-releasing silica nanoparticles

Amine-functionalized silica nanoparticles (30 mg) were suspended in 6 mL 9:1 DMF:MeOH with 25 µL of NaOMe (50 µL for 70 mol% MAP3, AHAP3, DET3). The resulting solution was sealed in a Parr reaction bottle, flushed with Ar thrice, followed by longer purges with Ar (3 x 10 min) to remove oxygen, before being pressurized with NO (10 bar). After 3 d of constant stirring, the Ar purges were again repeated to remove unreacted NO. Particles were collected via centrifugation (2907 x *g*; 4 ^{o}C; 10 min) and decanting of the supernatant. The resulting pellet was resuspended in EtOH before being centrifuged and decanted twice more to

remove residual solvent. The *N*-diazeniumdiolate NO donor-modified particles were dried under vacuum for 2 h and stored at -20 ^{o}C in a vacuum-sealed freezer bag until further use.

2.2.5 Characterization of nitric oxide-releasing silica nanoparticles

A Sievers 280i Chemiluminescence analyzer (Boulder, CO) was used to measure NO release in real-time to ascertain the most important NO-release parameters including total NO release (t[NO]), total NO release at 2 h ($t[NO]_{2h}$), maximum NO flux ($[NO]_m$), and NO-release half-life ($t_{1/2}$).[37] Briefly, ~1 mg NO-releasing silica nanoparticles was added to 30 mL of deoxygenated phosphate buffered saline (PBS; pH 7.4; 37 ^{o}C). The PBS solution was continuously bubbled with N_2 at 80 mL/min to carry liberated NO to the analyzer. The NO analysis was continued until NO levels fell below 10 ppb/mg. The *N*-diazeniumdiolate-modified silica nanoparticles were also visualized by SEM to ensure no changes in particle morphology or the formation of unwanted byproducts.

2.3 Results and Discussion

Previous reports have used core-shell or surface grafting techniques to study the connection between the physicochemical properties (i.e., size, shape, and maximum NO flux) and the resulting bactericidal efficacy of NO-releasing silica nanoparticles against *Pseudomonas aeruginosa*.[35,36] The effect of particle size, NO-release kinetics, and NO-release totals against other biomedically relevant pathogens remains avoid. Additionally, the previous synthetic burdens (e.g., multi-step and low yield) for tuning NO-releasing silica nanoparticle properties have limited both the breadth of physicochemical properties and bacteria strains investigated. As such, we sought to utilize a one-step, high throughput Stöber synthesis to create hybrid amino/alkoxysilane NO-releasing

nanoparticles for the investigation of physicochemical effects on dental bacteria killing and oral eukaryotic cell tolerance. Specifically, we synthesized similarly sized NO-releasing nanoparticles with different aminosilane identities and compositions, altering both NO-release totals and kinetics. We also attempted to tune particle size while maintaining amine identity and composition, thus holding both NO-release total and kinetics constant.

The formation of monodisperse, spherical silica particles synthesized via a one-step Stöber method is known.[32,38-40] Indeed, the ability to manipulate particle size based on feed rate of reactants, reaction temperature, co-solvent chain length, and molar ratios of silanes, water, and ammonia catalyst has been reported.[41-43] Previous studies have utilized tetraethyl orthosilicate (TEOS) or TMOS as single silane systems and did not attempt to create hybrid organic/inorganic silica particles. Building upon Shin et al.[29] and Riccio et al.,[44] dual silane hybrid particles were synthesized via the Stöber method. The optimized synthetic parameters and characterization of NO-releasing silica nanoparticles are detailed below. The successful synthesis of monodisperse, spherical NO-releasing silica nanoparticles in this chapter allowed for subsequent microbiological testing described in later chapters.

2.3.1 Varying aminosilane content of 150 nm silica nanoparticles

Bolus injection of silane mixtures (TMOS and aminosilane) into a mixture of water, ammonia, and ethanol allowed for the base-catalyzed formation of silica nanoparticles. To produce spherical, monodisperse particles with smooth surface morphologies, several reagent concentrations were varied, including total silane content, water, and ammonia in a total volume of 50 mL prior to being scaled up to 100 mL reactions. The total silane concentration used to synthesize MAP3 and AHAP3 nanoparticles was 0.12 M, whereas 0.16 and 0.20 M silane was

necessary for AEAP3 and DET3, respectively. The amount of water required for successful DET3 particle synthesis was 9.4 M. All other systems (i.e., MAP3, AHAP3, AEAP3) required 18.9 M. An ammonia concentration of 1.5 M proved useful for all particle systems. The initial goal was to synthesize 70 mol% aminosilane particles. As shown in Figure 2.2, SEM imaging revealed high sphericity, superior monodispersity, and smooth surface morphology, demonstrating the utility of a one-step Stöber synthesis for the co-condensation of TMOS with aminosilanes to create hybrid organic/inorganic silica nanoparticles.

The geometric diameters of the resulting nanoparticles were estimated using ImageJ software for at least n=3 syntheses with repeated measurements per synthesis ($n \geq 100$) to obtain a representation of absolute particle size and distribution. Nanoparticle hydrodynamic diameter and polydispersity index (PDI), a measure of particle size uniformity, were measured using DLS. The nanoparticle diameters measured in water by DLS were comparable, but slightly greater than the geometric diameters obtained via SEM. This phenomenon was expected, as the DLS measures hydrated (hydrodynamic) diameter, which includes both the particle size and the effective electrical double layer size based on mobility interactions with solvent. Table 2.2 displays both the geometric (SEM) and hydrodynamic (DLS) diameters, with coinciding PDI values, measured in H_2O for the 70 mol% silica nanoparticles, highlighting similar sizes (~150 nm) and small size distributions.

Once the synthetic parameters were optimized for 70 mol% silica nanoparticles, we sought to systematically vary the mol% aminosilane content of the particles. The percent ratio of each silane was varied without altering other synthetic parameters. In doing so, particle composition and potentially NO-release totals and kinetics could be tuned while maintaining spherical nanoparticles with smooth surface morphologies (Figure 2.3) and similar sizes across all particle

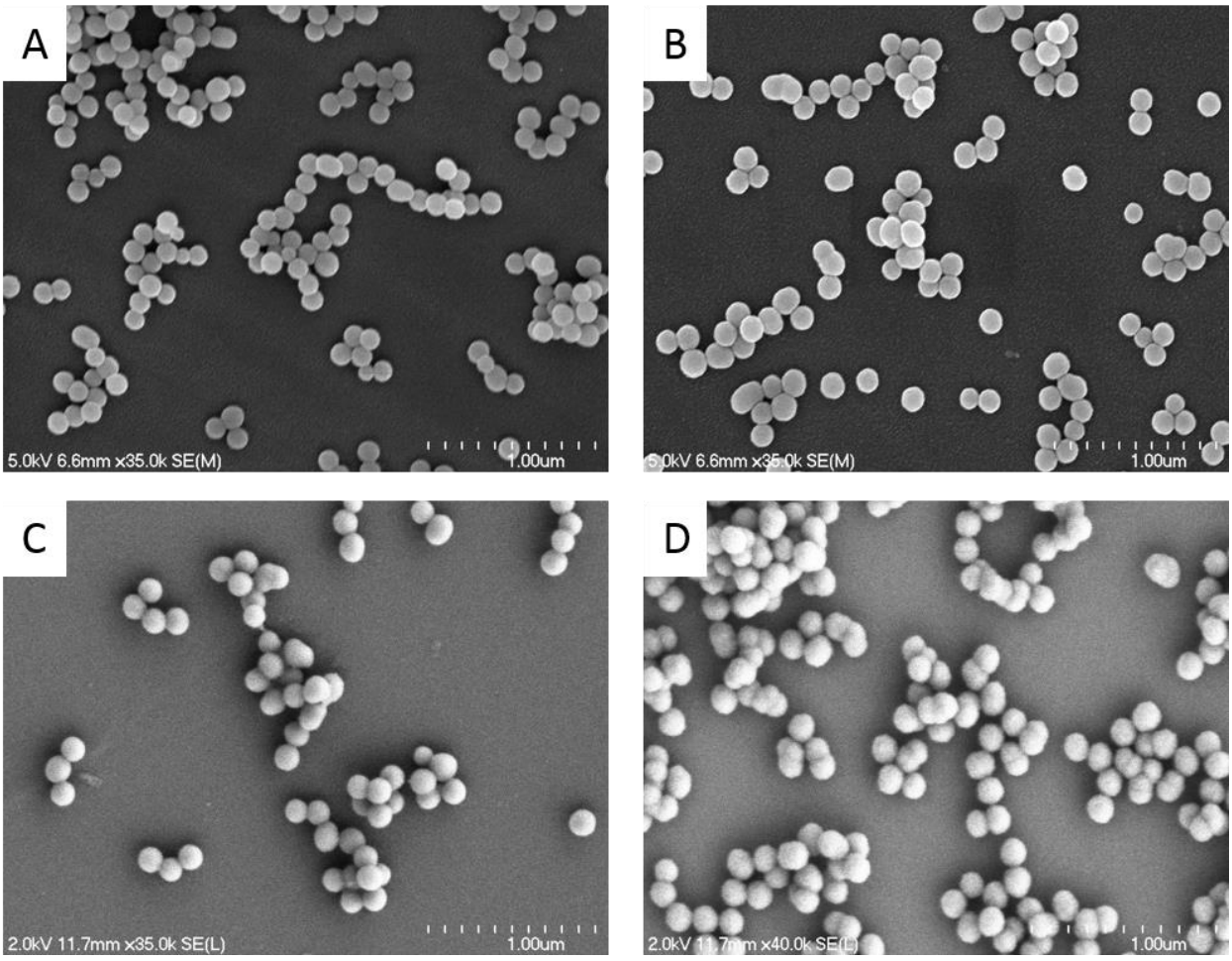

Figure 2.2 Scanning electron micrographs of 70 mol% silica nanoparticles consisting of (A) MAP3, (B) AEAP3, (C) AHAP3, and (D) DET3 (balance TMOS).

Table 2.2 Sizes for 70 mol% (balance TMOS) silica nanoparticles with different aminosilane compositions.

Aminosilane	Particle Diameter[b] (nm)	Particle Diameter[c] (nm)	Particle PDI[c]
MAP3	145 ± 15	195 ± 6	0.06 ± 0.02
AHAP3	161 ± 11	204 ± 25	0.05 ± 0.02
AEAP3	149 ± 14	169 ± 15	0.08 ± 0.06
DET3	157 ± 14	205 ± 5	0.03 ± 0.02

[a]Values shown as mean ± standard deviations for n ≥3 syntheses. [b]Geometric particle sizes measured using SEM. [c]Nanoparticle hydrodynamic diameter and PDI measured in water using DLS.

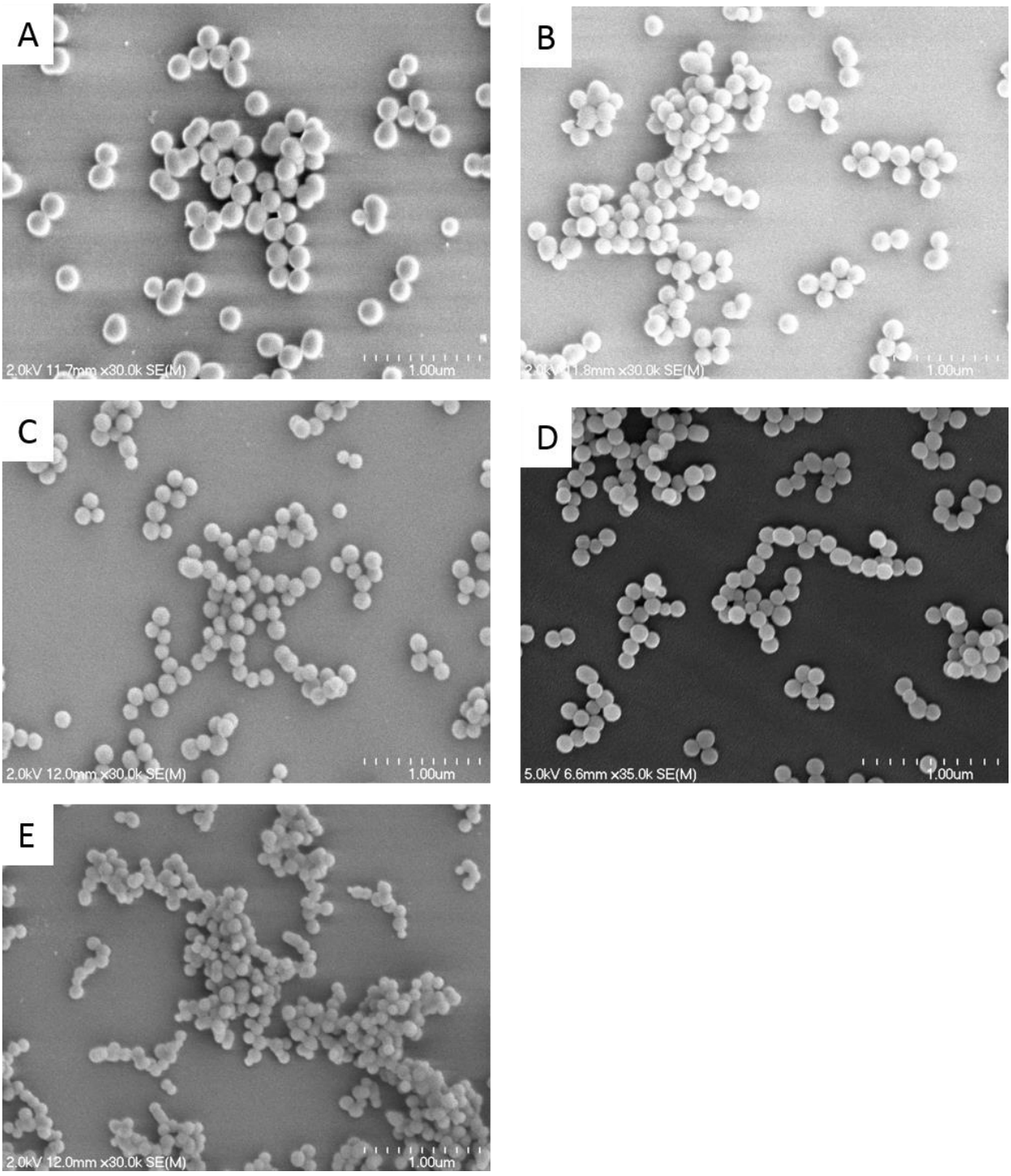

Figure 2.3 Scanning electron micrographs of (A) 35, (B) 50, (C) 60, (D) 70, and (E) 80 mol% MAP3 (balance TMOS) silica nanoparticles.

systems. The aminosilane content of the 70 mol% DET3 system was not varied systematically as control non-NO-releasing particles were bactericidal themselves (unpublished observations), rendering the evaluation of their NO-release kinetic-dependent bacterial killing ineffective. The diameter of nanoparticles (~150 nm) was again confirmed using SEM and DLS. Elemental analysis confirmed aminosilane incorporation into the nanoparticles by quantifying the percent nitrogen relative to the total nanoparticle mass. The size and percent amine content for all silica nanoparticle systems synthesized is provided in Table 2.3. Unexpectedly, the precursor aminosilane amounts do not directly relate to the degree of aminosilane incorporation within the particles between different silane systems. The aminosilane incorporation efficiency is inconsistent between silica systems, affecting the final aminosilane content of each particle type. Rather, CHN analysis confirmed the presence of amine functionality within the nanoparticles, with an observed increase in the nitrogen wt% for nanoparticles of increasing mol% aminosilane compositions for individual silica particle systems. The degree of amine functionalization could thus be tuned within a given aminosilane particle system, which could afford different NO-release properties.

2.3.2 Increasing 70 mol% MAP3 silica nanoparticle size

After synthesizing silica nanoparticles of different aminosilane compositions and similar size, attempts were made to increase particle diameter for the 70 mol% MAP3 system. Several strategies have been reported for increasing the size of single silane (TMOS or TEOS) silica particles, including altering the ammonia, water, or silane concentrations, and changing the chain length of the solvent or the reaction temperature.[41-43,45] These parameters were systematically varied for the hybrid silane systems and the resulting particles analyzed via SEM. Specifically,

Table 2.3 Diameters, PDIs, and nitrogen wt% for silica nanoparticles synthesized as a function of aminosilane type and concentration (mol%).[a]

Aminosilane	Mol% Aminosilane*	Particle Diameter[b] (nm)	Particle Diameter[c] (nm)	Particle PDI[c]	Nitrogen wt%[d]
MAP3	**35**	164 ± 21	208 ± 18	0.05 ± 0.03	2.49 ± 0.08
	50	160 ± 16	197 ± 7	0.04 ± 0.02	3.42 ± 0.17
	60	152 ± 15	210 ± 29	0.09 ± 0.04	3.84 ± 0.06
	70	145 ± 15	195 ± 6	0.06 ± 0.02	4.27 ± 0.13
AHAP3	**50**	176 ± 16	224 ± 18	0.03 ± 0.02	3.48 ± 0.28
	60	174 ± 15	239 ± 14	0.05 ± 0.02	4.28 ± 0.07
	70	161 ± 11	204 ± 25	0.05 ± 0.02	4.80 ± 0.03
AEAP3	**50**	170 ± 21	224 ± 7	0.07 ± 0.04	3.37 ± 0.32
	60	155 ± 16	209 ± 8	0.08 ± 0.04	3.87 ± 0.20
	70	149 ± 14	169 ± 15	0.08 ± 0.06	4.71 ± 0.27
	80	125 ± 13	143 ± 9	0.05 ± 0.02	5.83 ± 0.17
DET3	**70**	157 ± 14	205 ± 5	0.03 ± 0.02	4.95 ± 0.03

[a]Values shown as means ± standard deviations for n ≥3 syntheses. [b]Geometric diameters were evaluated using SEM. [c]Hydrodynamic diameter and PDIs were measured using DLS. [d]Nitrogen wt% was determined using CHNS/O elemental analysis. *Balance TMOS.

the silane concentration was increased from 0.12 to 0.16 M, the ammonia concentration was varied from 0.5 to 2.0 M, and the water concentration was decreased to 3.5 M (from the original 18.9 M). In each case, the silica nanoparticles were relatively unchanged in appearance as visualized using SEM (Figure 2.4). Nanoparticle sizes were maintained as the mean diameter ranged from 99–173 compared to the ~150 nm diameter of the original 70 mol% MAP3 silica nanoparticles.

Unlike other parameters, the co-solvent alkyl chain length had a profound effect on the resulting nanoparticle size. Initially, the alcohol co-solvent was changed to methanol, propanol, isopropanol, and mixtures of these alcohols with no success. Upon changing the co-solvent to butanol, which is immiscible with water, different sized particles were obtained as shown in Figure 2.5. The particles were much larger, but lacked the sphericity and smooth surface morphology of the original 70 mol% silica nanoparticles. Although some of the nanoparticles appeared spherical, many were variable in both size and shape. The total silane concentration was also increased to 0.24 and 0.36 M in an attempt to improve both the monodisperisty and surface morphology of the silica nanoparticles.[42] As shown in Figure 2.6, the increase in silane concentration improved the particle size distribution and surface characteristics at 0.24 M but negatively impacted the resulting particles at 0.36 M. The total concentration of silane impacts the amount of material deposited on the particle nuclei during the growth phase.[42] With too high of a silica concentration, the particles could suffer from an excess of material coating the particle nuclei. This could result in fused particles and particles with poor surface morphologies as observed with 0.36 M total silane concentration.

The 70 mol% MAP3 silica nanoparticles synthesized using a butanol co-solvent and 0.24 M silanes were further characterized based on these observations. The geometric size obtained via

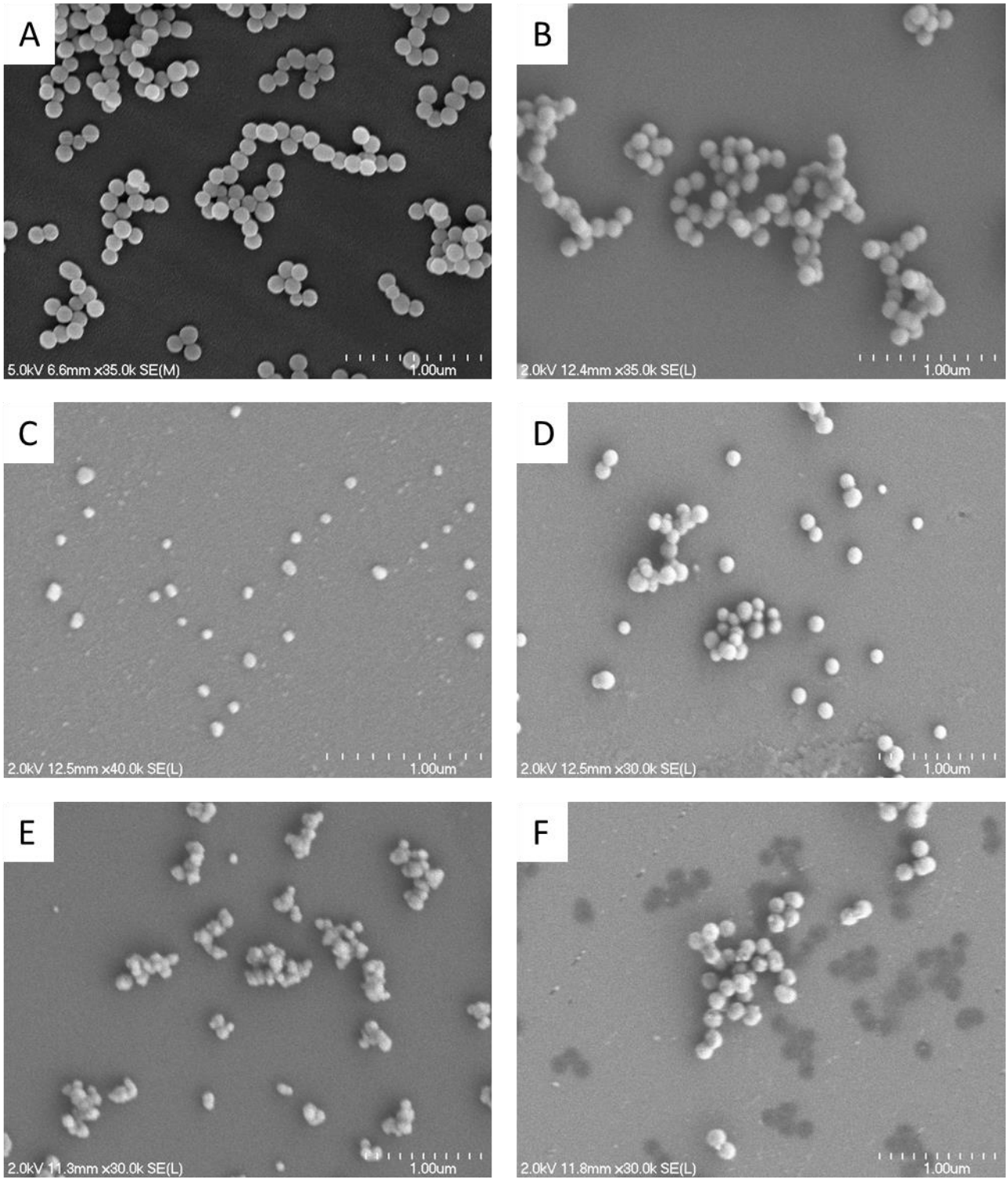

Figure 2.4 Scanning electron micrographs of 70 mol% MAP3 (balance TMOS) silica nanoparticles with (A) the original synthesis with 0.12 M silane, 1.5 M ammonia, and 18.9 M water, (B) 0.16 M silane, (C) 0.5 M ammonia, (D) 2.0 M ammonia, (E) 3.5 M water, and (F) 10.4 M water.

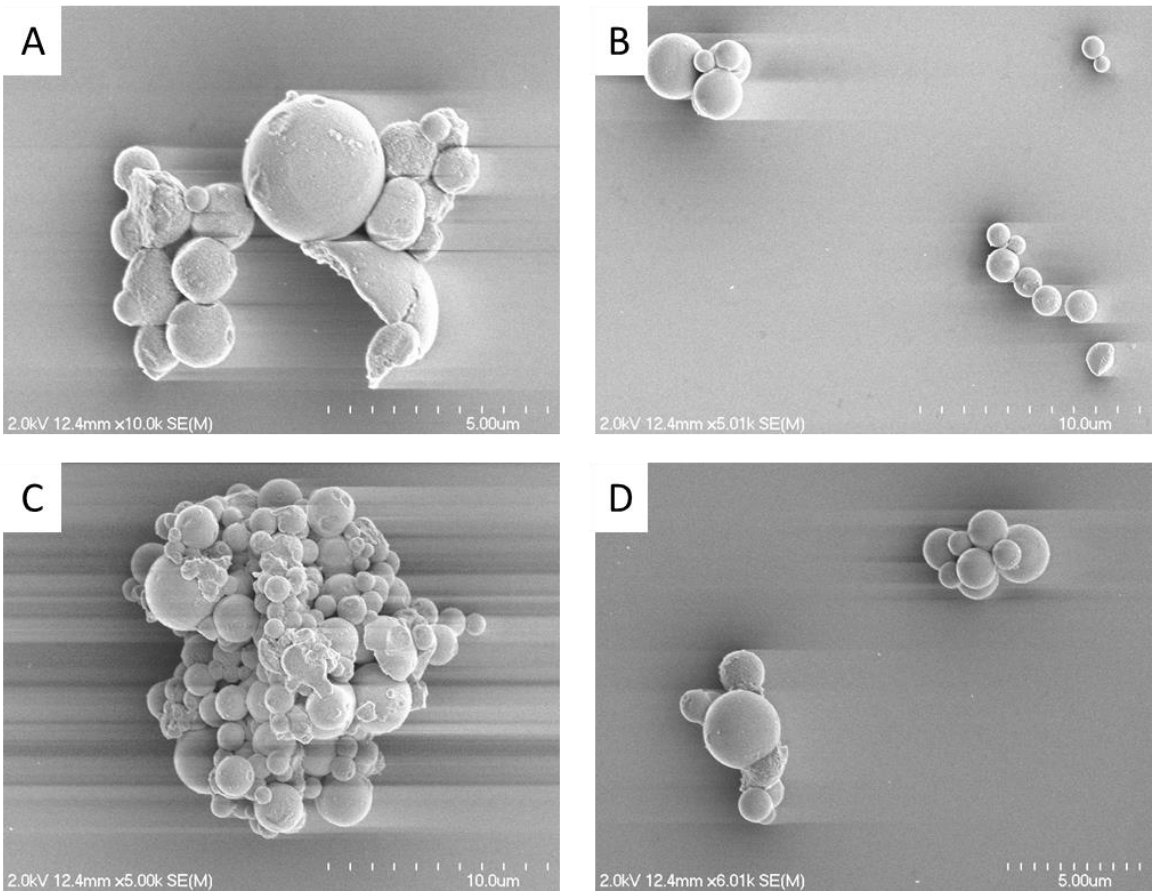

Figure 2.5 (A-D) Scanning electron micrographs of 70 mol% MAP3 (balance TMOS) silica nanoparticles synthesized in butanol instead of ethanol. Total silane concentration was 0.12 M.

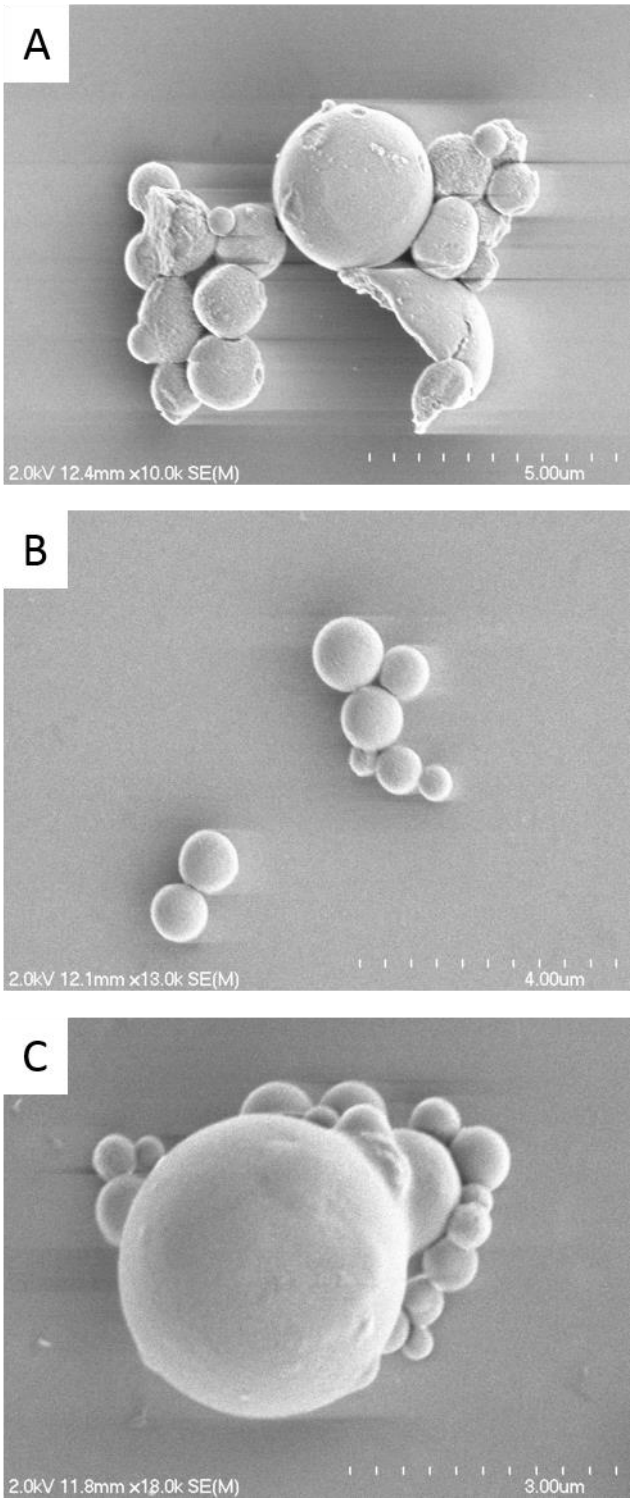

Figure 2.6 Scanning electron micrograph of 70 mol% MAP3 (balance TMOS) silica nanoparticles synthesized in butanol instead of ethanol with a total silane concentration of (A) 0.12 M, (B) 0.24 M, and (C) 0.36 M.

SEM and ImageJ was 855 ± 267 nm, which was the target size for this nanoparticle synthesis. However, the size distribution was quite large ranging from 500–1600 nm in diameter, possibly due to the biphasic nature of the reaction mixture created between immiscible butanol and water. Indeed, DLS measurements confirmed the highly polydisperse nature of particle size with excessively large PDI values (generally >0.50). Quality control parameters provided by the DLS also indicated that the large particle size distribution hindered accurate hydrodynamic size measurement. Thus, these ~800 nm silica nanoparticles were deemed unfit for a size-dependent bactericidal study.

Following the synthesis of larger hybrid silica nanoparticles with a non-uniform size distribution, improving the monodispersity was paramount for ultimately evaluating the size-dependent bactericidal efficacy of these silica scaffolds. Using a modified LaMer model for particle synthesis, Huang et al. proposed that size distribution is governed by the duration of the nucleation phase in which new particle nuclei are generated.[42] When the nucleation phase is long, particle nuclei appear at different times in solution and thus experience altered growth phase times, resulting in variable final particle sizes. At short nucleation windows (i.e., burst nucleation), the particles are initially formed at similar times and experience similar growth phases resulting in similarly sized particles. Huang et al. further demonstrated that an increase in temperature could be useful in speeding up the reaction kinetics to shorten the duration of the nucleation phase.[42] Upon increasing the reaction temperature from ambient (~23 ^{o}C) to 50 ^{o}C, the particle size distribution (monodispersity) was drastically improved (Figure 2.7). To corroborate the SEM images, DLS was then conducted in water to obtain a true sampling of both particle size and monodispersity. Unlike the silica synthesized using a butanol co-solvent at ~23 ^{o}C, the particles synthesized at 50 ^{o}C were monodisperse enough for accurate size assessment, with PDIs less than

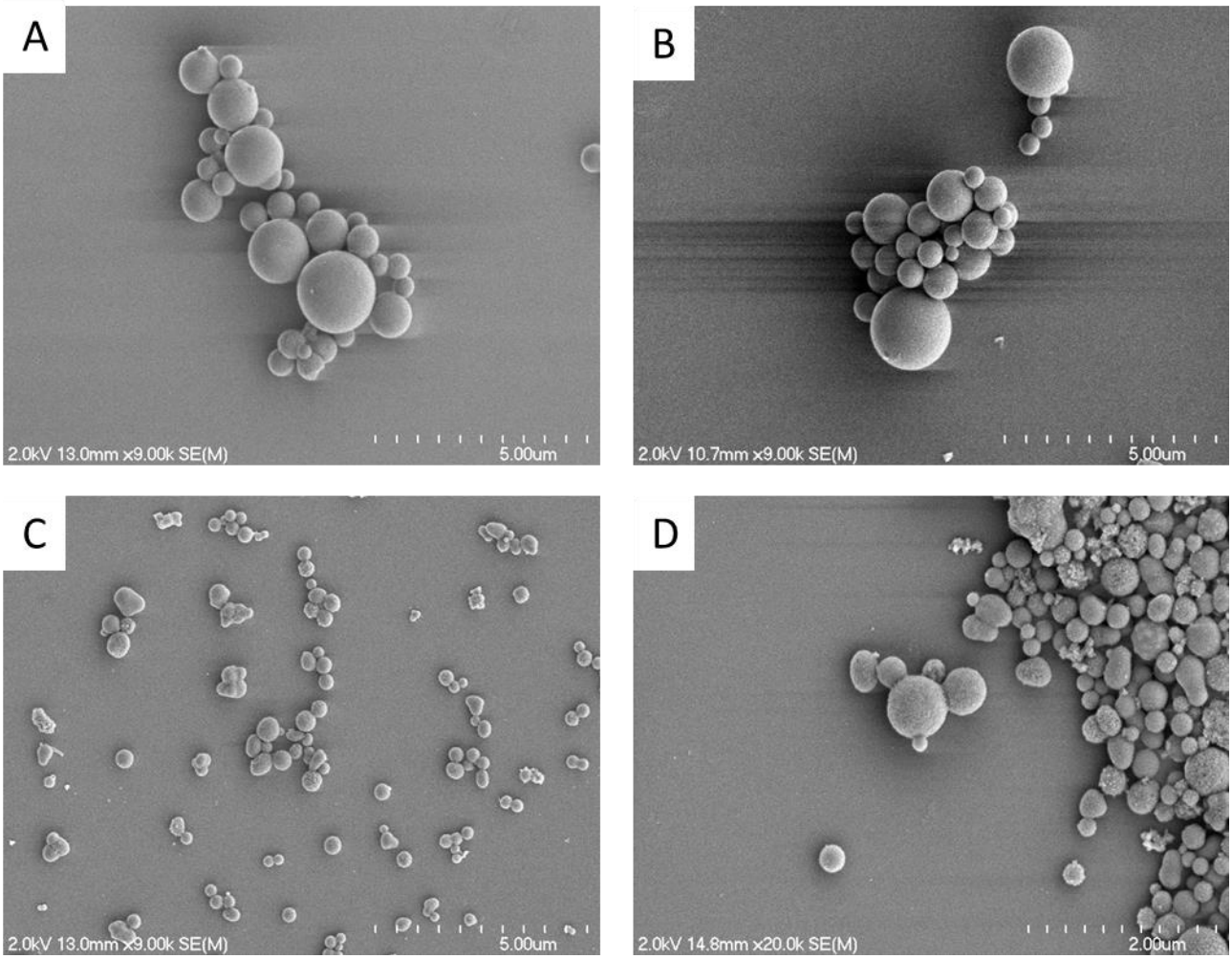

Figure 2.7 Scanning electron micrographs of 70 mol% MAP3 (balance TMOS) silica nanoparticles synthesized at (A,B) ambient temperature (~23 ºC) and (C,D) 50 ºC.

0.10. The size summary and elemental analysis for 70 mol% MAP3 silica nanoparticles synthesized with butanol at 50 ^{o}C is provided in Table 2.4.

2.3.3 N-Diazeniumdiolate NO donor-modified silica nanoparticles

Under basic conditions and high pressures of NO gas, *N*-diazeniumdiolate NO donors form at secondary amine sites.[19] In this work, sodium methoxide was used as a base to facilitate NO donor formation. Initially, an excess of sodium methoxide (NaOMe) was used to ensure maximum conversion efficiency. Unfortunately, *N*-diazeniumdiolate functionalization resulted in impurities observed in SEM images that were not present prior to modification (Figure 2.8). These byproducts were observed as crystal formations on the surface of the particles or in solution, and differed in both size and shape between particle systems, and even within repeat modifications using the same particle system.

Despite >100 years of contrary findings, DeRosa et al. reported on NO reacting with methoxide to ultimately produce sodium formate as an observable end product.[46] The generation of sodium formate may compete with the formation of desired products (i.e., *N*-diazeniumdiolates) and impair NO release from these materials. The presence of an unknown byproduct after washing with ethanol corroborated the formation of a salt, possibly sodium formate. The NO-releasing particles were washed with basic water to successfully remove the salt byproduct (Figure 2.9). Upon removal of the salt, the NO release from the particles was substantially lower, indicating that the salt itself was storing and releasing NO (Table 2.5). This was unexpected as we anticipated that the sodium formate could be removed without impacting the NO-release properties of the silica particles. This result indicates that carbon-bound bis or trisdiazeniumdiolates were potentially formed on the formate byproduct.[47] In contrast to other *C*-diazeniumdiolates,

Table 2.4 Comparison of material characteristics for 70 mol% MAP3 (balance TMOS) silica nanoparticles synthesized at ambient temperature (~23 ºC) in ethanol and at 50 ºC in butanol.[a]

Particle Diameter[b] (nm)	**Particle Diameter[c] (nm)**	**Particle PDI[c]**	**Nitrogen wt%[d]**
145 ± 15	195 ± 6	0.06 ± 0.02	4.27 ± 0.13
404 ± 184	444 ± 35	0.09 ± 0.06	4.68 ± 0.28

[a]Values shown as mean ± standard deviations for n ≥3 syntheses. [b]Silica nanoparticle geometric sizes were evaluated using SEM. [c]Nanoparticle hydrodynamic diameter and PDI measured using DLS. [d]Nitrogen wt% was determined using CHN elemental analysis.

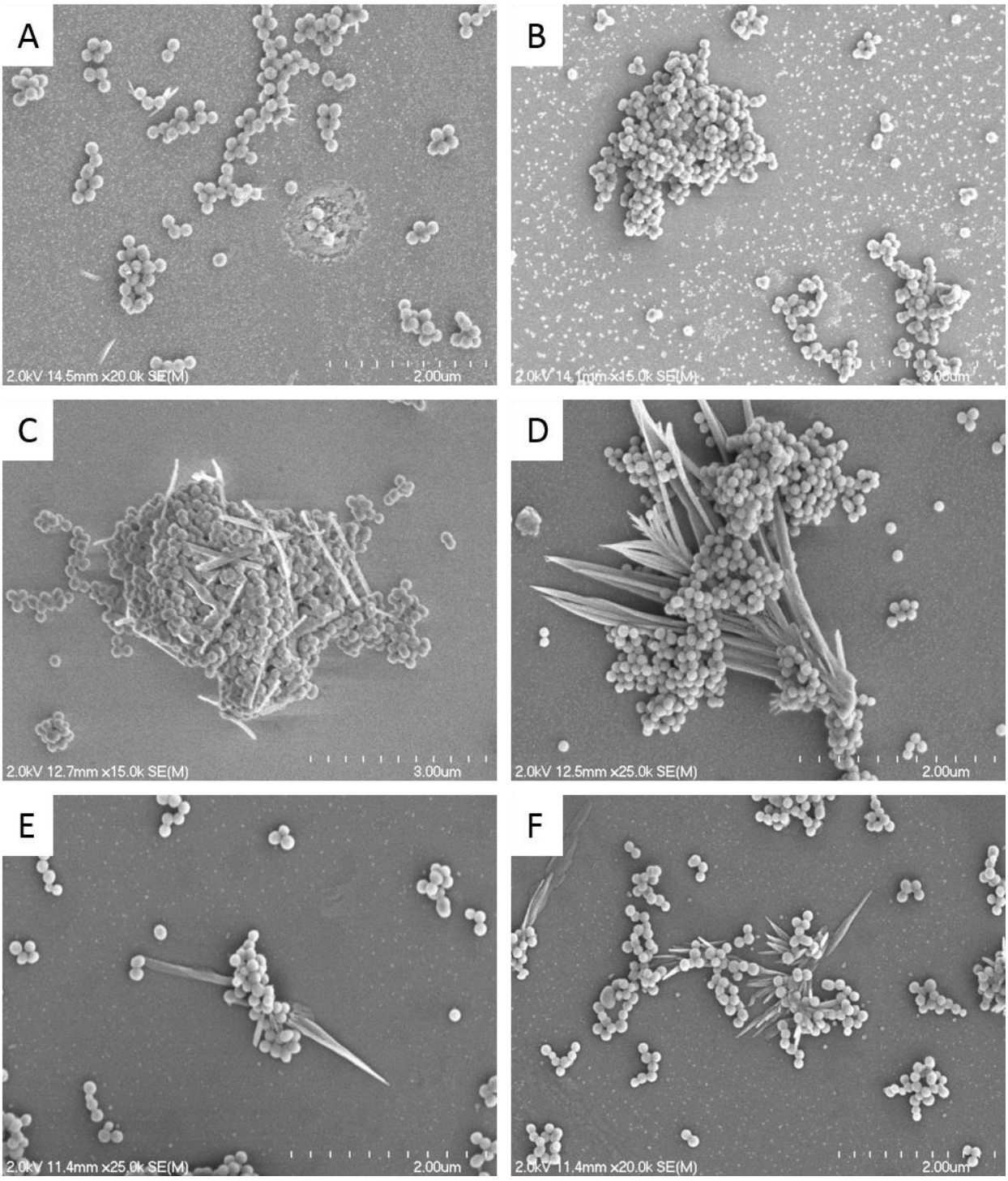

Figure 2.8 Scanning electron micrographs of (A) 70 mol% AEAP3 with 150 µL NaOMe (B) 70 mol% DET3 with 200 µL NaOMe (C) 50 mol% AHAP3 with 200 µL NaOMe (D) 50 mol% AHAP3 with 50 µL NaOMe (E) 70 mol% MAP3 with 100 µL NaOMe (F) 70 mol% MAP3 100 µL. (A-F) balance TMOS. In addition to the silica nanoparticles, the NO-releasing salt is also observed.

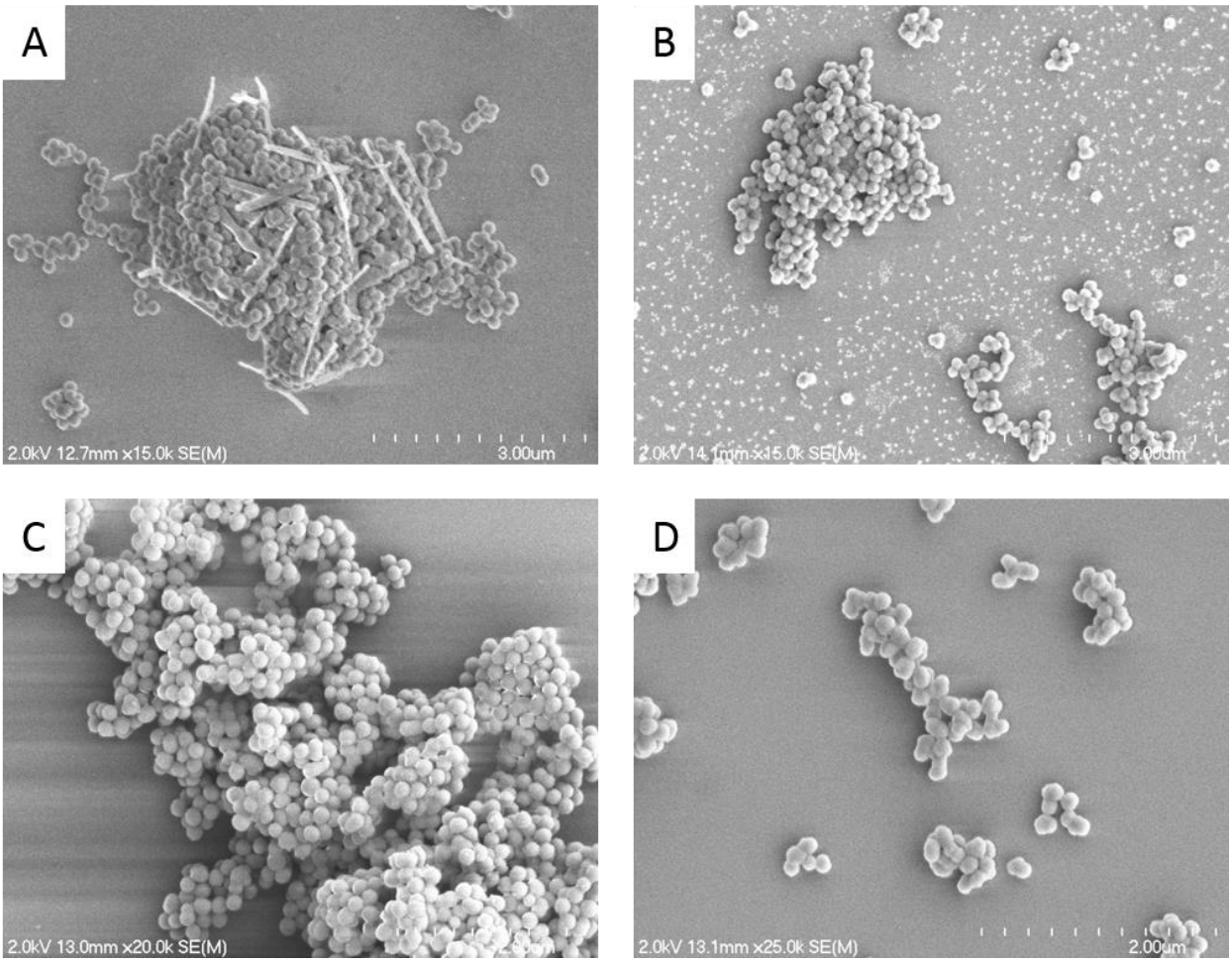

Figure 2.9 Scanning electron micrographs of (A) *N*-diazeniumdiolated 50 mol% AHAP3 (B) *N*-diazeniumdiolated 70 mol% DET3 (C) *N*-diazeniumdiolated 50 mol% AHAP3 washed with basic water to remove salt, and (D) *N*-diazeniumdiolated 70 mol% DET3 washed with basic water to remove salt. (A-D) balance TMOS.

Table 2.5 Silica nanoparticle nitric oxide release properties before and after washing with basic water.[a]

NO Release Scaffold*	t[NO][b] (μmol/mg)	$[NO]_m$[c] (ppb/mg)	$t_{½}$[d] (min)	$t[NO]_{2h}$[e] (μmol/mg)
50 mol% AHAP3 pre wash	0.31	5288	27.4	0.26
50 mol% AHAP3 post wash	0.14	585	36.7	0.12
70 mol% DET3 pre wash	0.23	2000	47.1	0.16
70 mol% DET3 post wash	0.06	141	59.0	0.05

[a]Results shown are from one trial to evaluate NO-releasing byproduct removal. [b]Total amount of NO released. [c]Maximum NO flux achieved. [d]Time to release half of total NO payload. [e]Total amount of NO released after 2 h. *Balance TMOS.

trisdiazeniumdiolates undergo rapid decomposition[47,48] and the NO release would thus be observable in our NO-release analysis.

Regardless of the identity and nature of the salt byproduct, the storage and release of NO from undesirable salt byproducts clearly hinders the study and application of NO-releasing silica nanoparticles as antibacterial therapeutics. As it is important to keep the sodium methoxide concentration large to maximize NO loading (and release), the amount of sodium methoxide used was systematically decreased until the formation of the salt byproduct was eliminated (as noted by SEM). At a sodium methoxide concentration of 74 mM, no salt was observed for the 50, 60, and 80 mol% silica nanoparticles. The 70 mol% formulations did not form salt up to 148 mM sodium methoxide. Once the maximum concentration of sodium methoxide was determined, the NO-release properties for the silica nanoparticles were characterized (Table 2.6). Of note, the total NO release and half-lives varied as a function of the aminosilane system, demonstrating the potential to alter NO release while maintaining constant particle size (~150 nm). Additionally, the NO-release totals and half-lives were similar for the 70 mol% MAP3 particles, demonstrating the ability to alter size while maintaining constant NO release. As described in subsequent chapters, the NO-releasing silica systems developed herein proved useful for microbiological testing.

2.4 Conclusions

The Stöber co-condensation of aminosilanes and TMOS in ethanol proved useful for synthesizing hybrid silica nanoparticles of spherical nature and high monodispersity. Amine incorporation into the silica particles was tunable by altering the aminosilane precursor mol%. Particle size could be altered by increasing the reaction temperature (23–50 ^{o}C) and replacing ethanol with butanol as the co-solvent for particle synthesis. The conditions necessary to maximize

Table 2.6 Summary of nitric oxide release properties for hybrid silica nanoparticles synthesized via the Stöber method.[a]

Aminosilane Scaffold	Mol% Aminosilane*	t[NO][b] (μmol/mg)	$[NO]_m$[c] (ppb/mg)	$t_{1/2}$[d] (min)	Conversion Efficiency[e] (%)
MAP3	**50**	0.19 ± 0.02	1490 ± 907	20.6 ± 5.5	3.9
	70	1.03 ± 0.13	24678 ± 12079	11.9 ± 4.3	16.9
400 nm	**70**	1.02 ± 0.19	20573 ± 6301	18.9 ± 4.1	15.3
AHAP3	**50**	0.17 ± 0.01	614 ± 160	42.9 ± 6.4	6.8
	60	0.25 ± 0.05	1721 ± 956	33.8 ± 5.5	8.2
	70	0.38 ± 0.01	4689 ± 758	29.5 ± 3.6	11.1
DET3	**70**	0.22 ± 0.04	793 ± 307	61.9 ± 6.2	9.3
AEAP3	**80**	0.33 ± 0.06	718 ± 496	122.8 ± 9.6	7.9

[a]Results shown as mean ± standard deviation for n ≥3 separate syntheses. [b]Total amount of NO released. [c]Maximum NO flux achieved. [d]Time to release half of total NO payload. [e]Conversion efficiency calculated from nitrogen wt% as determined via CHN elemental analysis and the total NO release from each particle system. *Balance TMOS.

NO storage and release while eliminating the formation of unwanted salt byproducts were determined to be a function of sodium methoxide concentration. The synthesis of silica scaffolds with distinct sizes and NO-release properties facilitated the bactericidal and cytotoxicity testing in subsequent chapters.

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Chapter 3: Antibacterial Efficacy of Exogenous Nitric Oxide on Periodontal Pathogens

3.1 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.[1] 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.[2] Left unmanaged, periodontitis can result in tooth loss and has been associated with increased risk for systemic conditions such as cardiovascular disease and stroke.[3,4]

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.[3] Other non-surgical treatments for periodontal disease, such as the use of chlorhexidine (CHX), have undesirable side-effects, including altered taste, discoloration of the mouth, and mucosal irritation.[5,6] In combination, CHX (0.2% w/w) with SRP does not significantly affect the microbial composition compared with SRP alone. Quirynen et al. suggested that CHX treatment should be re-evaluated as an adjunct to periodontal therapy.[7] While the use of antibiotics (e.g., minocycline and tetracycline) represents a possible complement to SRP, and local drug delivery systems can improve clinical and microbial outcomes,[8] their systemic use has undesirable side-effects (e.g., antibiotic resistance, pseudomembranous colitis).[9,10] 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.[11] For example, oral mucosal epithelial cells generate NO in response to bacteria and pro-inflammatory stimuli initiated upon the deposition of dental plaque.[12] While exogenous NO has been utilized as an antimicrobial agent with no previously observed resistance,[13,14] 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.[15,16] Previously, we have synthesized *N*-diazeniumdiolate-modified silica and dendrimer scaffolds capable of spontaneous NO release under biological conditions as potential *in vivo* antimicrobial agents.[17,18] The antimicrobial activity of such scaffolds was demonstrated against several Gram-positive, Gram-negative, and antibiotic-resistant bacteria[18,19] as well as biofilms.[20,21] 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.

3.2 Materials and Methods

3.2.1 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.[22] The bottle was purged with argon (Ar) and subsequently pressurized with NO gas (10 bar). After 3 days, 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 ^{o}C.

Hybrid tetramethyl orthosilicate (TMOS) (Sigma; St. Louis, MO, USA) MAP3 (Gelest; Morrisville, PA, USA) particles were synthesized via 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 aqueous ammonium hydroxide (30 wt%).[17] This solution was stirred for 2 h under ambient conditions, and then centrifuged at 2907 x *g* at 4 ^{o}C for 10 min. After decanting the supernatant, the pellet was resuspended 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) suspended in 6 mL 9:1 *N*,*N*-dimethylformamide (DMF):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 with NO. After 3 d, the bottle was flushed with Ar again to remove unreacted NO. The particles were collected by centrifugation and decanting of the supernatant. The pellet was resuspended in ethanol, centrifuged and decanted twice more to remove residual solvent and sodium methoxide. Particles were dried under vacuum to yield *N*-diazeniumdiolate NO donor-modified MAP3 silica and stored in a vacuum-sealed bag at -20 ^{o}C until use.

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).[21] 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 reaction bottle, sealed, flushed with Ar, and filled with NO (10 bar). After 3 d, the bottle was purged again with Ar to remove unreacted NO. The NO-releasing G1-PAMAM-PO dendrimers were stored at -20 ^{o}C.

3.2.2 Characterization of nitric oxide release

A Sievers 280i Chemiluminescence Nitric Oxide Analyzer (Boulder, CO, USA) was used to quantify NO release at 37 ^{o}C.[23] The NO-releasing material (~1 mg) was added to a flask containing 30 mL of deoxygenated phosphate-buffered saline (PBS; pH = 7.4). Nitrogen was used to purge this solution continuously and thus carry liberated NO to the analyzer (80 mL/min flow rate). The NO analysis was conducted in real-time and continued until the NO levels fell below 10 ppb.

3.2.3 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 A7463 was provided by the UNC School of Dentistry (Chapel Hill, NC, USA). Stock cultures, initially stored in 15% (v/v) glycerol in PBS at -80 ^{o}C, were grown overnight in brain heart infusion (BHI)

broth or Difco anaerobic broth for *P. gingivalis*. A 500 µL aliquot of this solution was reinoculated 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 h. Bacteria (10^8 CFU/mL) were resuspended 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 2h, 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 h. *A. actinomycetemcomitans* plates were incubated under microaerophilic conditions for 48 h. *P. gingivalis* plates were incubated for 96 h under anaerobic conditions.

3.2.4 Cytotoxicity evaluation

Human gingival fibroblasts (HGF-1) (ATCC #CRL-2014) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% v/v fetal bovine serum and 1 wt% penicillin/streptomycin. The cells were incubated in 5% v/v 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 h at 37 °C, the

supernatant was aspirated, and a mixture of DMEM, phenazine methosulfate (PMS), and [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) (100/20/1 v/v/v) was added to the wells. After incubation for an additional 2 h at 37 ºC, 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.

3.3 Results and 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,[18,19] its efficacy against dental microorganisms 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,[24,25] this work is the first to evaluate the effects of exogenous NO delivered via macromolecular scaffolds against periodontopathogens.

Nitric oxide-release half-life ($t_{1/2}$), maximum NO flux ($[NO]_m$), and total NO released over 2 h ($t[NO]_{2h}$) were determined for the three NO donor systems (Table 3.1). Of note, $t[NO]_{2h}$ represents the NO dose delivered during the MBC_{2h} bacterial killing assay. Of the three NO donors, *N*-diazeniumdiolate-modified proline (PROLI/NO) had the largest NO payload (6.29 ± 0.24 μmol/mg) and fastest NO release ($t_{1/2}$ = 2.3 ± 0.1 min). The $t[NO]_{2h}$ and the 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

Table 3.1 Characterization of nitric oxide-releasing materials.[a]

NO-Release Scaffold	t[NO][b] (μmol/mg)	$[NO]_m$[c] (ppb/mg)	$t_{½}$[d] (min)	$t[NO]_{2h}$[e] (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

[a]Results shown for particles (70 mol% MAP3), dendrimers (G1-PAMAM-PO), and the small molecule NO donor NO-releasing proline (PROLI/NO). [a]Values presented as means ± standard deviations for n = 3 or more pooled experiments. [b]Total amount of NO released. [c]Maximum NO flux achieved. [d]Time to release half of the total NO payload. [e]Total NO released after 2 h.

slowly ($t_{1/2}$ = 19.8 ± 3.3 min). Despite releasing an equivalent amount of NO ($t[NO]_{2h}$ = 0.77 ± 0.11 μmol/mg) to 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 NO-releasing silica or PROLI/NO (Figure 3.1). These scaffold concentrations correspond to NO doses of 3.2 and 25.2 μmol/mL, respectively. 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 (Figure 3.1). 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 Figure 3.2, 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* require 32 and 48 mg/mL PROLI/NO and NO-releasing dendrimers, respectively (NO doses = 201.3 and 37.0 μmol/mL).

Similar to an earlier study showing enhanced action against Gram-negative species,[19] 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 versus 3.2 μmol/mL for dendrimer and silica, respectively) and *P. gingivalis* (0.8 and 1.6 μmol/mL for dendrimers and silica, respectively) due to enhanced bacterial association of the dendritic scaffold.[18] Similar concentrations of PROLI/NO were required to kill periodontopathogens, albeit at larger NO levels (3.1 and 25.2 μmol/mL for *P. gingivalis* and

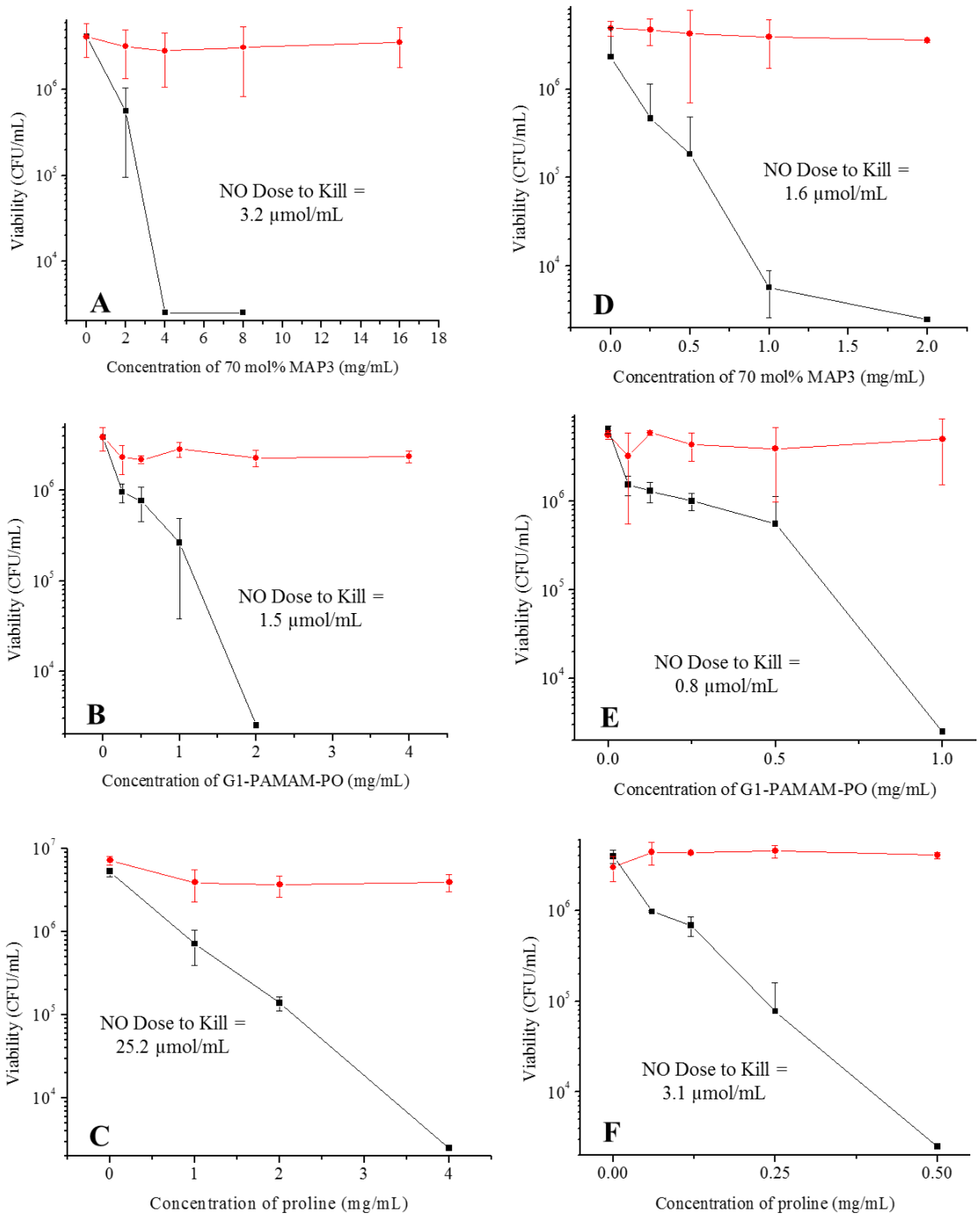

Figure 3.1 Bactericidal efficacy of (A) 70 mol% MAP3 particles, (B) G1-PAMAM-PO dendrimers, and (C) proline against *A. actinomycetemcomitans* in Tris-PBS after 2 h. Bactericidal efficacy of (D) 70 mol% MAP3 particles, (E) G1-PAMAM-PO dendrimers, and (F) proline against *P. gingivalis* in Tris-PBS after 2 h. 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.

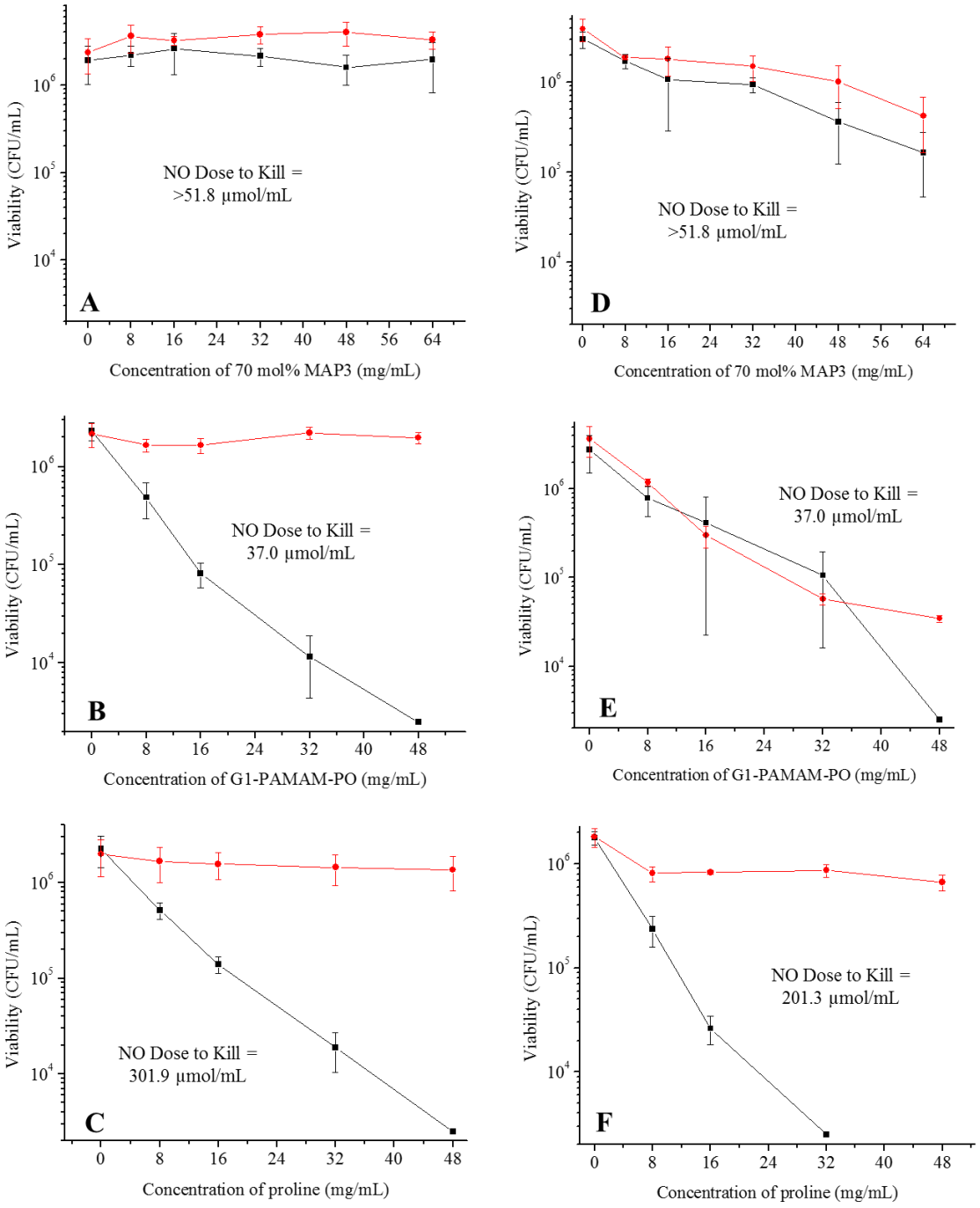

Figure 3.2 Bactericidal efficacy of (A) 70 mol% MAP3 particles, (B) G1-PAMAM-PO dendrimers, and (C) proline against *S. mutans* in Tris-PBS after 2 h. Bactericidal efficacy of (D) 70 mol% MAP3 particles, (E) G1-PAMAM-PO dendrimers, and (F) proline against *S. sanguinis* in Tris-PBS after 2 h. NO-releasing 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.

A. actinomycetemcomitans, respectively), despite storing and releasing more NO. This result would be expected, since the PROLI/NO produces NO without bacterial association – and thus indiscriminately – whereas macromolecular scaffolds associate with bacteria and localize (i.e., target) the NO.[19] 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 the Gram-negative 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 its survival in nitrite-rich, acidic environments.[26] Gusarov and Nudler reported that NO facilitates bacterial protection against oxidative stress ("NO-mediated cytoprotection") in *Bacillus subtilis*,[27] and this may be observed for other Gram-positive species. In this respect, *S. mutans* and *S. sanguinis* may tolerate NO due to its role as a metabolite rather than a biocidal agent.

Both NO-releasing dendrimers and silica showed reductions in human gingival fibroblast (HGF-1) viabilities (75 ± 3 and 61 ± 12% compared with untreated cells) at concentrations required to kill periodontopathogens (2 and 4 mg/mL, respectively), but only minimally when compared to clinical concentrations of chlorhexidine (0.12 and 0.20% (w/w)) (Figure 3.3). In contrast, PROLI/NO was more toxic to HGF-1 at the maximum concentration required to kill periodontopathogens (18 ± 2% compared with untreated cells at 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

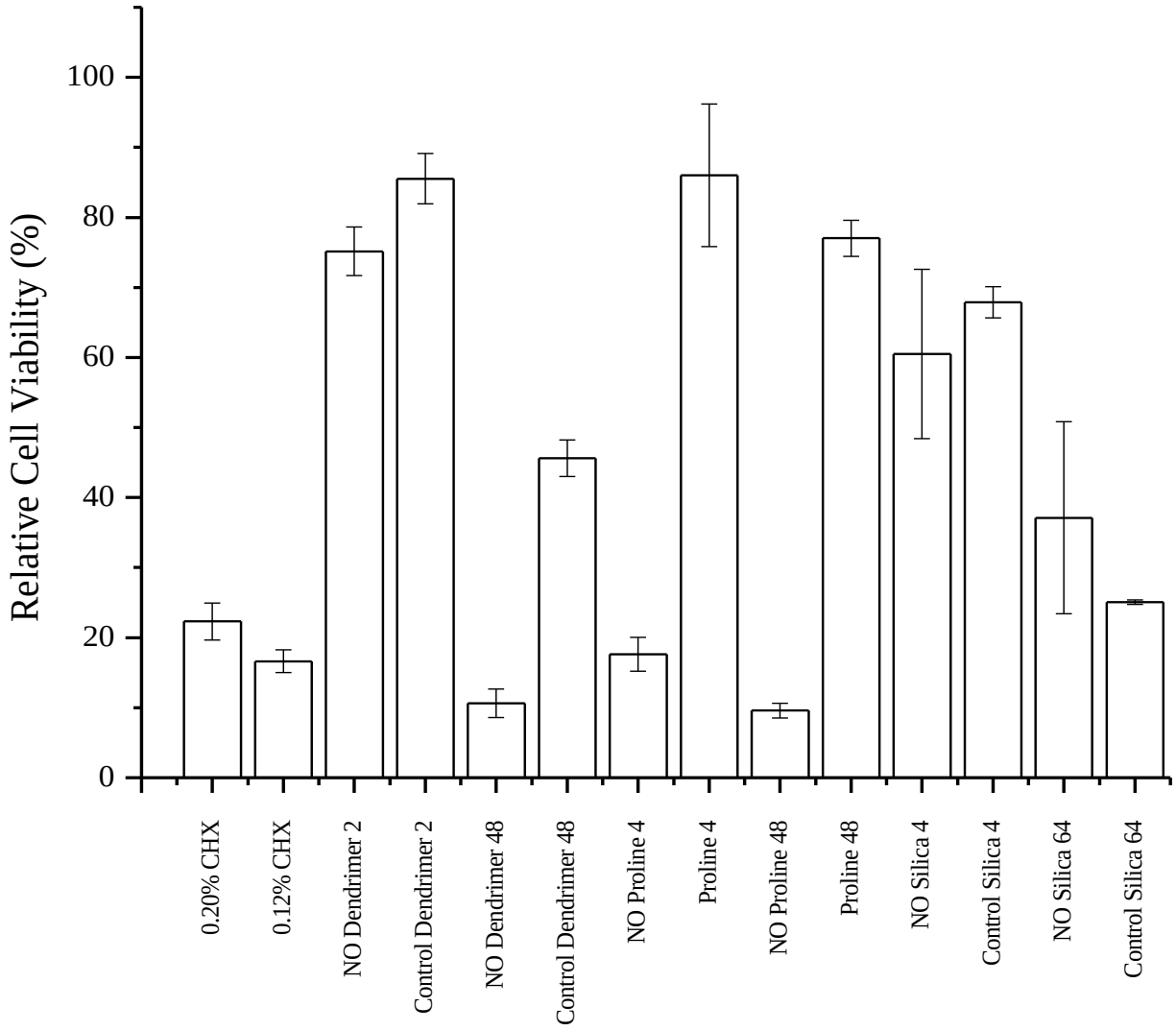

Figure 3.3 Cytotoxicity of NO-releasing materials and non-NO-releasing controls at MBC_{2h} concentrations (or maximum concentration tested) against HGF-1 cells. Viability measured as metabolically active fibroblasts using MTS and presented as normalized percent relative to untreated cells. Chlorhexidine 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.

molecule NO donor. Hetrick et al. noted similar toxicity to L929 murine fibroblasts for PROLI/NO concentrations ≥5 mg/mL.[19] 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). As expected, the relative cytotoxicity for all NO donor systems was much greater at the concentrations required for complete eradication of the cariogenic bacteria (*S. mutans* and *S. sanguinis*) due to exceedingly large material and NO concentrations.

3.4 Conclusions

The sharp disparity in NO susceptibility between the two classes of oral microbes investigated 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.[28] In this manner, NO-releasing macromolecular scaffolds may prove useful in killing periodontopathogenic Gram-negative bacteria over Gram-positive cariogenic microorganisms. 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 remains 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 susceptibility of periodontopathogens over cariogenic 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 exists as a complex biofilm. Last, *in vivo* models

of periodontal disease must be used to elucidate the full potential of NO release as an effective adjuvant therapy.

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Chapter 4: Kinetic-Dependent Bactericidal Efficacy of Nitric Oxide-Releasing Silica Nanoparticles against Oral Pathogens

4.1 Introduction

Due to stability and tolerability by mammalian cells,[1-3] silica particles are employed as abrasive scouring agents in toothpastes to facilitate the removal of bacteria. Recent evidence suggests that polishing with nanoparticles versus micron-sized silica helps decrease the surface roughness of teeth and further lessens the strength of bacterial adhesion, making *Streptococcus mutans* removal easier.[4] Although effective at reducing overall bacterial counts, silica nanoparticles are not inherently antimicrobial and thus dental plaque biofilms still form. Other materials including fibers, gels, polymers, and microspheres modified to release antibiotics (e.g., minocycline, doxycycline, and tetracycline) have demonstrated promise for the treatment of oral diseases.[5,6] Unfortunately, these scaffolds are less suitable for the physical removal of plaque-forming bacteria. While local drug delivery systems may limit the risk of systemic side effects (e.g., pseudomembranous colitis),[6,7] the overuse of antibiotics fosters antibiotic-resistant pathogens.[8] Therefore, the combination of silica (as a polishing agent) with new antibacterial agents that do not foster bacterial resistance could potentially lead to improved therapeutics for treating periodontal disease and dental caries that are easily implemented into existing dental hygiene practices (i.e., teeth brushing).

Nitric oxide (NO) is a gaseous, free radical produced endogenously during the immune response to invading organisms.[9] In oral biology, both bacteria and pro-inflammatory stimuli present in dental plaque biofilms elicit the generation of NO from oral mucosal epithelial cells.[10] The antibacterial activity of NO is attributed to reactive byproducts causing oxidative and nitrosative stress on bacteria. For example, NO reacts with superoxide to form peroxynitrite, which elicits significant membrane damage via lipid peroxidation, while reaction with oxygen produces dinitrogen trioxide, a nitrosative species that disables protein function.[11] With broad-spectrum activity and no observed bacterial resistance to date,[12,13] the delivery of exogenous NO is a promising antibacterial for oral care. Although NO produced via the acidification of nitrite has bactericidal properties against cariogenic[14] and periodontopathogenic bacteria,[15] precise control over NO generation is pivotal in designing effective antibacterial delivery systems.[1,16]

Shin et. al first reported the Stöber[17] synthesis of hybrid NO-releasing silica nanoparticles of varying size (20–500 nm).[18] The aminosilane composition (10–87 mol%) of the silica nanoparticles was tunable, which enabled a thorough evaluation of NO's utility against Gram-positive, Gram-negative, and fungal biofilms.[18,19] Subsequent research has focused on studying the physicochemical properties (e.g., size, shape, and release kinetics) of NO donor-modified silica nanoparticles to maximize bactericidal efficacy. Carpenter et al. observed that ~50 nm NO-releasing silica particles were more effective at killing bacteria than larger particles (e.g., 100 and 200 nm) due to enhanced diffusion and faster bacteria-particle association.[20] Lu et al. described the synthesis of NO-releasing silica rods, noting improved bactericidal activity for oblong over spherical particles due to increased bacteria/particle surface contact and intracellular NO delivery.[21] In the same study, a greater initial flux of NO showed improved killing, suggesting that faster NO-release kinetics may enhance NO's antibacterial activity against *Pseudomonas*

aeruginosa.[21] To date, the antibacterial activity of NO-releasing silica nanoparticles as a function of NO-release kinetics has not been systematically evaluated, particularly against oral pathogens.

Herein, we investigated the influence of NO-release kinetics on bactericidal efficacy against common periodontopathogens independent of particle size and NO-release totals. Comparatively, the kinetic-dependent killing of a cariogenic strain was also examined using a single NO-releasing silica particle system with greater NO-release totals.

4.2 Materials and Methods

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). 3-methylaminopropyltrimethoxysilane (MAP3), *N*-(6-aminohexyl)aminopropyltrimethoxysilane (AHAP3), and *N*-(2-aminoethyl)-3-aminopropyltrimethoxysilane (AEAP3) were obtained from Gelest (Morrisville, PA). 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). Pure nitric oxide (NO) gas (99.5%) was purchased from Praxair (Sanford, NC). 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). 4,5-Diaminofluorescein diacetate (DAF-2 DA) was purchased from Calbiochem (San Diego, CA). Brain heart infusion (BHI) broth, BHI agar,

Wilkins-Chalgren agar, and anaerobe broth were obtained from Becton, Dickinson, and Company (Sparks, MD). *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). *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). All other solvents and reagents were analytical grade and used as received.

4.2.1 Synthesis of nitric oxide-releasing silica particles

The Stöber co-condensation of aminosilanes with alkoxysilanes to create secondary amine-modified silica particles has been reported previously.[18] Briefly, hybrid particles were synthesized by combining TMOS with an aminosilane (MAP3, AHAP3, AEAP3) and bolus injection of the resulting silane mixture into a flask containing water (27.84 mL), ammonium hydroxide (9.8 mL), and EtOH as a co-solvent to a total volume of 100 mL. After stirring for 2 h under ambient conditions, the particles were collected via centrifugation (2907 x *g*; 4 ^{o}C; 10 min) and decanting the supernatant. The pellet was resuspended in EtOH, centrifuged, and decanted twice more to remove unreacted silanes and residual solvent. 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 of MAP3 and 0.90 mL of TMOS.

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 of

NaOMe. This solution was sealed in a Parr reaction bottle, flushed briefly with Ar three times, and followed by three 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 before opening the reaction vessel to ambient conditions. Particles were then collected via centrifugation (2907 x *g*; 4 ^{o}C; 10 min) and decanting of the supernatant. The pellet was resuspended in EtOH, centrifuged and decanted twice more to remove residual solvent and NaOMe. The resulting *N*-diazeniumdiolate NO donor-modified silica particles were dried under vacuum for 2 h prior to storage in a vacuum-sealed freezer bag at -20 ^{o}C until further use.

4.2.2 Hybrid particle characterization

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 Perkin Elmer 2400 Series II CHNS/O Elemental Analyzer operated in CHN mode (Waltham, MA).

4.2.3 Characterization of nitric oxide release

Real-time NO release was measured at 37 ^{o}C using a Sievers 280i Chemiluminescence Nitric Oxide Analyzer (NOA; Boulder, CO) to quantify NO-release kinetics (i.e., half-life).[22]

Briefly, the NO-releasing material (~1 mg) was added to a flask containing 30 mL of deoxygenated phosphate buffered saline (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.

4.2.4 Bactericidal efficacy of NO-releasing silica nanoparticles

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, and Company; Franklin Lakes, NJ). *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). Initially stored in 15% (v/v) glycerol in PBS at -80 ^oC, bacterial cultures were grown overnight in BHI broth at 37 ^oC or Difco anaerobe broth (*P. gingivalis*). A 500 µL aliquot of this solution was reinoculated into 50 mL of fresh broth, incubated at 37 ^oC, 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 then pelleted via centrifugation (2355 x *g*) for 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. Nitric oxide's antibacterial activity on *S. mutans* was also tested in PBS (pH = 6.4), with 5% BHI (v/v). Bacteria solutions were then added to vials containing either NO-releasing or control (non-NO-releasing) silica, sonicated to disperse particles, and incubated aerobically at 37 ^oC with moderate shaking under static growth conditions. After 2 h, these solutions were diluted and spiral-plated using an Eddy Jet spiral plater (IUL; Farmingdale, NY) on BHI agar or Wilkins-Chalgren agar (*P. gingivalis*) and incubated at 37 ^oC under the aforementioned environmental conditions. Viable

colonies were enumerated on agar plates using a Flash and Go colony counter (IUL; Farmingdale, NY). The concentration resulting in a 3-log reduction in bacterial viability to below 2.5 x 10^3 CFU/mL (the limit of detection for this plating method)[23] was determined to be the minimal bactericidal concentration (MBC_{2h}).

4.2.5 Confocal microscopy for intracellular nitric oxide 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) 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 505–530 nm band-pass filter was used to obtain DAF-2 (green) fluorescence images. Both bright field and fluorescence images were collected using a N.A. 1.2 C-Apochromat water immersion lens with a 40x objective. Solutions of 50 mol% MAP3, 60 mol% AHAP3, and 80 mol% AEAP3 NO-releasing particles (2.0 mg/mL) in 1.5 mL Tris-PBS (containing 10 μM DAF-2 DA) were added to 1.5 mL of the *A. actinomycetemcomitans* bacterial suspension in the glass confocal dish to achieve 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 (containing 10 μM DAF-2 DA) were added to 1.5 mL of the *S. mutans* bacterial suspension in the glass confocal dish to achieve a final concentration of 0.25 mg/mL. Images were collected every 5 min to observe the changes in intracellular NO concentration over time. Of note, the fluorescence signal was color inverted for clarity. Confocal micrographs of *S. mutans* were noise filtered with Matlab (Natick, MA) using a

background subtraction threshold value equal to the average background pixel intensity. The resulting total fluorescence signal density was then summed for the image.

4.2.6 In vitro cytotoxicity

Human gingival fibroblasts (HGF-1) were grown in DMEM supplemented with both 10% (v/v) FBS and 1 wt% PS, and incubated at 37 ºC under humidified conditions in 5% CO_2 (v/v). After reaching 80% confluency, cells were trypsinized, seeded onto a tissue culture-treated 96-well plate, and incubated again at 37 ºC. Solutions of NO-releasing and control silica particles in DMEM were added to the cells. The supernatant was aspirated to remove particles following incubation for 2 h at 37 ºC. A DMEM, PMS, and MTS solution (120 µL; 100/20/1 v/v/v) was then added to the wells. After incubation for an additional 2 h at 37 ºC, the solutions were transferred to a new microtiter plate without cells. A Thermoscientific Multiskan EX plate reader (Waltham, MA) was used to measure the absorbance at 490 nm. The absorbance measurements were adjusted by subtracting blanks (i.e., DMEM/PMS/MTS without cells). Cell viability was then quantified relative to untreated cells.

4.3 Results and Discussion

We have previously reported the synthesis of hybrid silica nanoparticles capable of NO release,[18] with proven antibacterial and anti-biofilm activity against Gram-positive and Gram-negative bacteria.[19,24] Subsequent work evaluated the effects of size and shape on the bactericidal efficacy of NO-releasing silica nanoparticles against *Pseudomonas aeruginosa*.[20,21] In the present study, we synthesized NO-releasing silica nanoparticles of varying aminosilane identity and composition (50 mol% MAP3, 60 mol% AHAP3, and 80 mol% AEAP3) to investigate the role of

NO-release half-life on the killing of periodontal pathogens. For clarity, the particle systems are hereafter referred to as MAP3, AHAP3, and AEAP3.

4.3.1 Nitric oxide-releasing silica nanoparticle characterization

Hybrid alkoxysilane/aminosilane silica nanoparticles were synthesized via the Stöber method.[17] Through careful variation of the synthetic reaction parameters, MAP3, AHAP3, and AEAP3 silica nanoparticles were produced to have similar geometric diameters (~150 nm) and a high degree of monodispersity, as indicated by the low polydispersity index (PDI) values (≤0.06; Table 4.1). Maintaining a constant size was crucial as this parameter has been shown to influence bactericidal efficacy.[20] Successful incorporation of the aminosilanes within the silica nanoparticles was confirmed via CHN elemental analysis (Table 4.1). As expected, the nitrogen wt% increased with increasing mol% aminosilane precursor used for the particle synthesis (3.42, 4.28, and 5.83 wt% for MAP3, AHAP3, and AEAP3, respectively). The positively charged (i.e., protonated) surface amines of the particles resulted in positive zeta potentials, dependent on aminosilane identity (Table 4.1). In Tris-PBS (pH = 7.4), MAP3 and AEAP3 particles were characterized as having similar zeta potentials (~20 mV), while AHAP3 particles exhibited a greater positive surface charge (31.4 mV). The ability to tune both the identity and degree of silica nanoparticle aminosilane functionalization while maintaining constant geometric diameters enabled the decoupling of particle size-mediated killing from NO release and scaffold surface charge.

The kinetics of proton-initiated *N*-diazeniumdiolate decomposition from aminosilane NO donors is dependent on structure.[18] As such, MAP3, AHAP3, and AEAP3 NO-release was characterized in PBS (pH = 7.4; 37 ^{o}C) using a chemiluminescent NO analyzer to obtain

Table 4.1 Silica nanoparticle characterization.[a]

Aminosilane	Diameter[b] (nm)	Diameter[c] (nm)	PDI[c]	Zeta Potential[d] (mV)	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

[a]Results presented as mean ± standard deviation for n = 3 or more pooled experiments. [b]Geometric diameter estimated using SEM and ImageJ software. [c]Hydrodynamic diameter and particle PDI measured in water using DLS. [d]Zeta potential measured using DLS in Tris-PBS (pH = 7.4). [e]Quantified using CHN elemental analysis.

NO-release totals, 2 h NO-release totals, and NO-release kinetics (i.e., half-lives).[22] As shown in Table 4.2, the total NO release for the MAP3, AHAP3, and AEAP3 particles was 0.22, 0.25, and 0.39 μmol/mg, respectively. Of importance, the 2 h NO-release totals were equivalent (~0.20 μmol/mg) across all three particle systems and representative of the NO concentration delivered during the 2 h bacteria killing assays. The NO-release half-lives were also similar for MAP3 and AHAP3 (~32 min), but significantly longer for AEAP3 (112.5 min) due to *N*-diazeniumdiolate stabilization via hydrogen bonding with neighboring cationic amines on the AEAP3 aminosilane scaffold.[25] The bactericidal efficacy of MAP3 and AEAP3 was thus compared as a function of NO-release kinetics, independent of particle size, scaffold surface charge, and 2 h NO-release totals. The influence of scaffold particle charge on the antibacterial action of NO-releasing silica nanoparticles was compared between MAP3 (20.9 mV) and AHAP3 (31.4 mV), independent of NO-release kinetics. Lastly, we also evaluated the importance of increased positive scaffold surface charge (AHAP3) versus extended NO-release kinetics (AEAP3) on periodontopathogen killing.

4.3.2 Kinetic-dependent killing of periodontopathogens with NO-releasing particles

To study the role of NO-release kinetics and particle surface charge on bactericidal efficacy, 2 h bacteria killing assays were conducted against periodontopathogens in Tris-PBS (pH = 7.4; 37 ^{o}C). As expected from our previous work,[26] both *A. actinomycetemcomitans* and *P. gingivalis* were readily susceptible to NO, with bactericidal NO doses of <10 μmol/mL (Figure 4.1). However, we also observed differences in the bacteria killing efficiency dependent on both the NO-release kinetics and surface charge of the particles. For example, decreased minimum

Table 4.2 Characterization of NO-releasing silica particles in PBS (pH = 7.4 at 37 ^{o}C) by means of a chemiluminescent nitric oxide analyzer.[a]

NO Release Scaffold	t[NO][b] (μmol/mg)	$[NO]_m$[c] (ppb/mg)	$t_{½}$[d] (min)	$t[NO]_{2h}$[e] (μmol/mg)
MAP3	0.22 ± 0.04	900 ± 300	31.6 ± 6.7	0.19 ± 0.04
AHAP3	0.25 ± 0.05	1700 ± 1000	33.8 ± 5.5	0.21 ± 0.04
AEAP3	0.39 ± 0.05	1400 ± 200	112.5 ± 18.0	0.20 ± 0.02

[a]Results shown for 50 mol% MAP3, 60 mol% AHAP3, and 80 mol% AEAP3 NO-releasing silica particles and presented as mean ± standard deviation for n = 3 or more pooled experiments. [b]Total amount of NO released. [c]Maximum NO flux achieved. [d]Time to release half of total NO payload. [e]Total amount of NO released after 2 h.

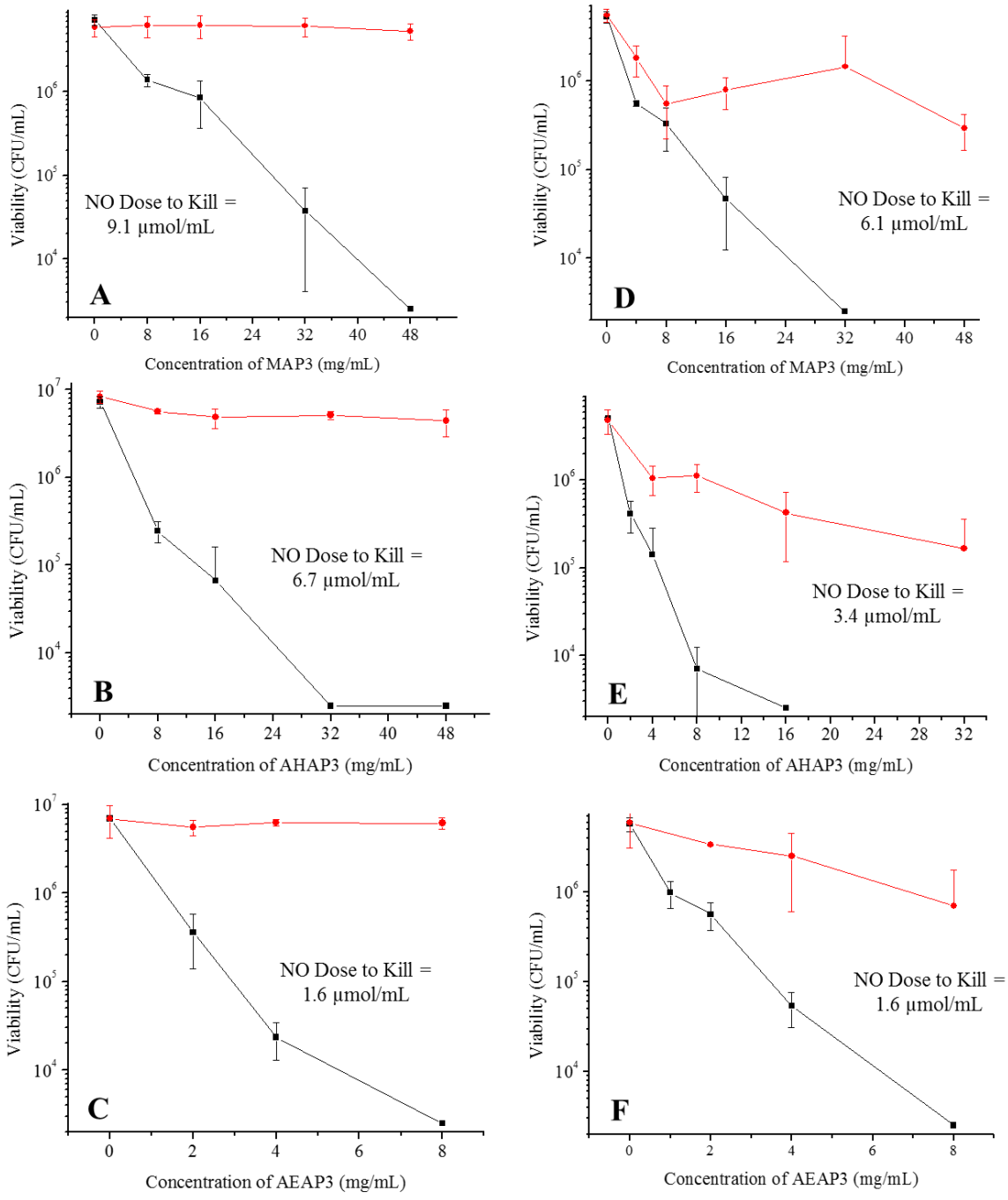

Figure 4.1 Bactericidal efficacy of (A) 50 mol% MAP3 particles, (B) 60 mol% AHAP3 particles, and (C) 80 mol% AEAP3 particles against *A. actinomycetemcomitans* in Tris-PBS (pH = 7.4) after 2 h. Bactericidal efficacy of (D) 50 mol% MAP3 particles, (E) 60 mol% AHAP3 particles, and (F) 80 mol% AEAP3 particles against *P. gingivalis* in Tris-PBS (pH = 7.4) after 2 h. 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.

bactericidal concentrations (MBC_{2h}) were observed for AHAP3 over MAP3 particles against both *A. actinomycetemcomitans* (32 versus 48 mg/mL, respectively) and *P. gingivalis* (16 versus 32 mg/mL, respectively). Due to the similar NO-release half-lives (~32 min) of these two systems, the improved bactericidal efficacy of AHAP3 was postulated to be a function of the increased particle surface charge (31.4 versus 20.9 mV for AHAP3 and MAP3 particles, respectively). Greater positive surface charge enhances the particle-bacteria association,[27] and most likely increases the buildup of intracellular NO and thus NO-mediated death. Confocal microscopy and the use of a fluorescent probe DAF-2 DA (to visualize intracellular NO levels) confirmed greater NO delivery to *A. actinomycetemcomitans* from AHAP3 versus MAP3 particles over a 30–120 min window (Figure 4.2). In contrast, intracellular NO from the MAP3 particle system was observed only after 120 min. The improved antibacterial activity of NO-releasing AHAP3 over MAP3 particles is thus attributed to the more efficient delivery of NO.

The most effective killing of periodontopathogens was observed for NO-releasing AEAP3 particles (Figure 1). Indeed, the minimum bactericidal concentration (MBC_{2h}) for NO-releasing AEAP3 was 8 mg/mL against both periodontopathogens, corresponding to a bactericidal NO dose of 1.6 μmol/mL. This NO dose is reduced from that of NO-releasing MAP3 (9.1 and 6.1 μmol/mL of NO to kill *A. actinomycetemcomitans* and *P. gingivalis*, respectively). We hypothesize that the extended NO-release kinetics (i.e., half-lives) characteristic of AEAP3 (112.5 min) compared to MAP3 (31.6 min) particles facilitates the enhanced killing. As shown in Figure 4.2, intracellular NO fluorescence was observed for the AEAP3 particle system after only 30 min of exposure to *A. actinomycetemcomitans* compared to MAP3, which required 120 min for any fluorescence to be seen. Furthermore, the intracellular NO from AEAP3 increased to a maximum at 60 min before decreasing at 120 min coinciding with outer membrane decomposition and bacterial death.[24,28]

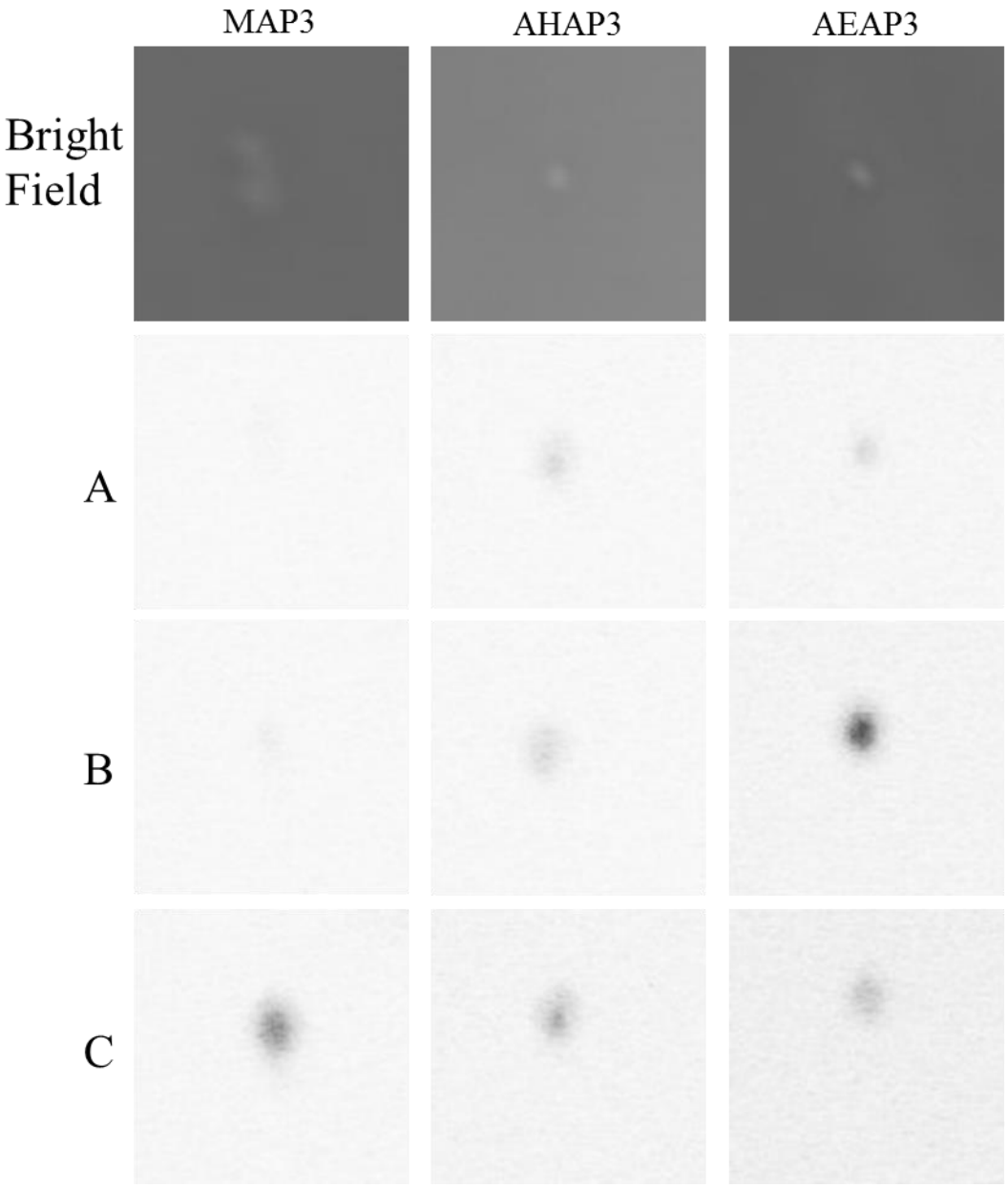

Figure 4.2 Confocal microscopy images of *A. actinomycetemcomitans* exposed to 1 mg/mL NO-releasing silica nanoparticles (50 mol% MAP3, 60 mol% AHAP3, and 80 mol% AEAP3) after (A) 30 min, (B) 60 min, and (C) 120 min of particle exposure. DAF-2 fluorescence is depicted as black in this image for clarity and represents intracellular NO concentrations.

The antibacterial activity of NO donor-modified AHAP3 and AEAP3 particles were compared to evaluate the relative importance of increased positive particle surface charge versus extended NO-release kinetics for enhancing the bactericidal efficacy of NO-releasing silica particles. Despite the decreased particle surface charge for AEAP3 (18.3 mV) versus AHAP3 (31.4 mV) particles, the antibacterial action of the NO-releasing AEAP3 (MBC_{2h} = 8) was enhanced over AHAP3 ($MBC_{2h} \geq 16$ mg/mL). The decreased minimum bactericidal concentration for NO-releasing AEAP3 suggests that extended NO-release kinetics may be more beneficial for killing periodontopathogens than a greater positive particle surface charge and enhanced particle-bacteria association. Confocal microscopy confirmed more efficient NO delivery to *A. actinomycetemcomitans* from NO-releasing AEAP3 versus AHAP3 particles (Figure 4.2). After only 30 min of exposure, intracellular NO was observable for both NO-releasing particle systems. Of note, the fluorescent signal intensity was greater for the NO-releasing AEAP3 particles at 60 min, indicating more accumulation of intracellular NO from AEAP versus AHAP3 particles. The fluorescence signal associated with the AHAP3 particles continued to rise up to 120 min, indicating the bacteria are able to handle this NO payload. In contrast, the NO-releasing AEAP3 particles deliver bactericidal levels of NO within 120 min resulting in cell death.[24,28] The visualization of intracellular NO concentrations confirms the benefit of slower NO-release kinetics over greater (positive) particle surface charge for enhancing the bactericidal action of macromolecular NO-release scaffolds against periodontopathogens. The combination of extended NO-release kinetics and greater positive surface charge might further optimize periodontopathogen killing.

The improvement in the antibacterial activity of NO-releasing silica against periodontopathogens with extended NO-release kinetics was somewhat surprising as it contradicts

previous reports. For example, Lu et al. observed that a greater initial NO-release flux and more rapid NO-release kinetics enhanced the bactericidal efficacy of silica-based NO release against *P. aeruginosa*.[21] Of importance, *P. aeruginosa* utilizes the enzyme nitric oxide reductase (NOR) as a defense against NO-based host immunity.[29] As NOR functions as an NO detoxification mechanism, it successfully mitigates low concentrations of intracellular NO. Under such conditions, larger instantaneous NO concentrations are required to overload protective processes and elicit NO-mediated killing. Conversely, *A. actinomycetemcomitans* and *P. gingivalis* do not possess these anti-oxidative pathways and are unable to effectively eliminate intracellular NO. Rather, NO accumulates within the bacteria over time, without the need for a substantial instantaneous NO concentration to induce NO-mediated cellular damage. Nitric oxide-releasing silica particles that can associate with bacteria prior to releasing a majority of their NO payload (slow NO-releasing AEAP3 particles) more effectively deliver bactericidal levels of NO than fast NO-releasing systems (MAP3 and AHAP3 particles) that release substantial amounts of NO prior to association with bacteria. The instantaneous NO concentration appears to be less critical for killing periodontal pathogens than total intracellular NO delivered over time. The benefit of extended NO-release kinetics for more effective periodontopathogen killing may influence the design of NO-releasing therapeutics for periodontal diseases treatments.

4.3.3 In vitro cytotoxicity

Human gingival fibroblasts (HGF-1) were used to evaluate the cytotoxicity of NO-releasing and control silica particles. As shown in Figure 4.3, the toxicity of control particles increased (i.e., decreased cell viability) as a function of the silica concentration. For the AHAP3 and MAP3 particle systems, NO release further decreased cell viability (31 and 21% relative to

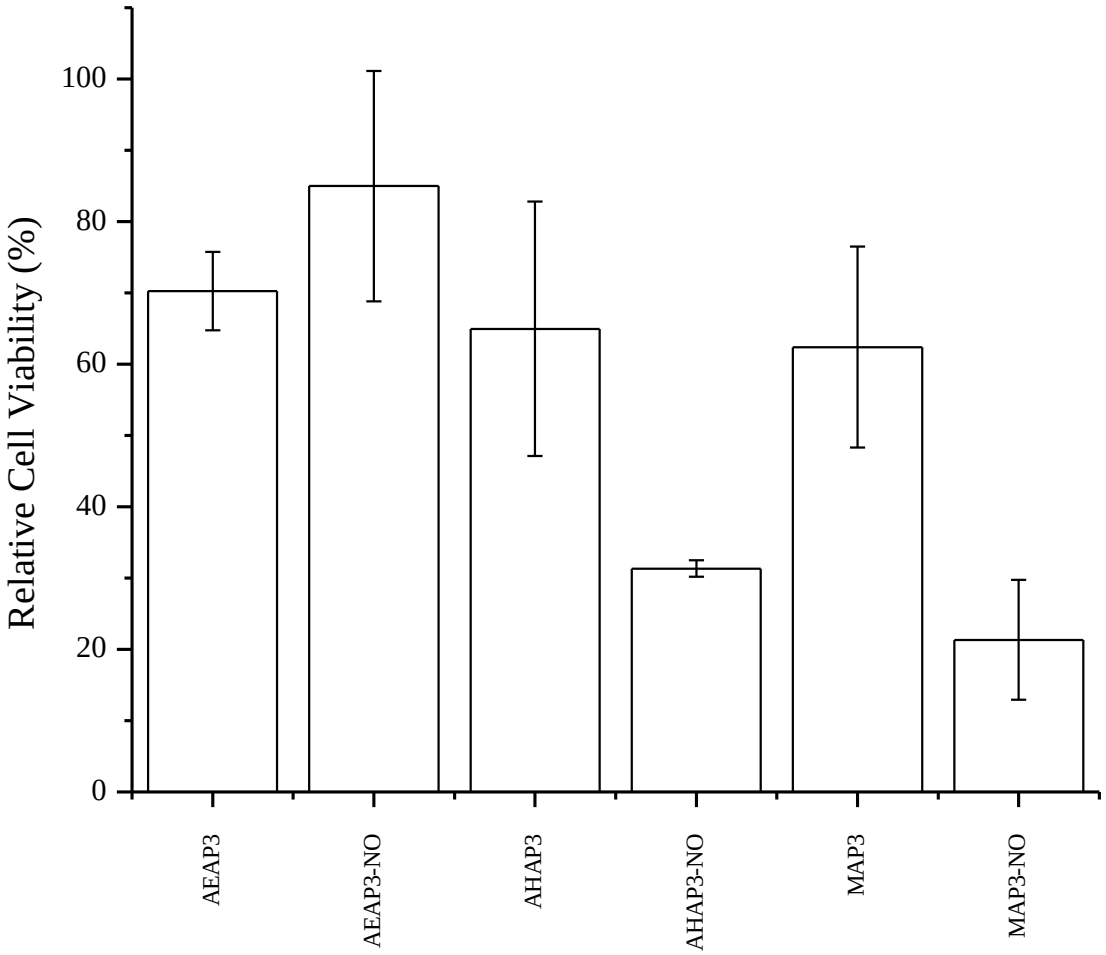

Figure 4.3 Cytotoxicity of NO-releasing and control silica particles against HGF-1 human gingival fibroblasts at the greatest MBC_{2h} values to kill periodontopathogens. The concentrations to kill *A. actinomycetemcomitans* were 8, 32, and 48 mg/mL for 80 mol% AEAP3, 60 mol% AHAP3, and 50 mol% MAP3, respectively. Viability measured as metabolic activity versus untreated cells. Error bars represent standard deviation of the mean. For all values, n = 4 replicate measurements.

untreated cells). Of note, NO release for the AEAP3 system improved HGF-1 viability. The ability of HGF-1 cells to tolerate low instantaneous levels of intracellular NO, such as those provided by AEAP3 particles, is attributed to the enzymatic breakdown of superoxide (O_2^-) by superoxide dismutase, an enzyme that limits the formation of reactive NO byproducts and concomitantly mitigates the nitrosative and oxidative stress associated with NO-mediated killing.[30] The increase in cell viability is likely the result of NO stimulating cell growth as reported previously.[31-33] The antibacterial characteristics and low cytotoxicity of the NO-releasing AEAP3 system warrants further investigation into slow NO-releasing silica-based materials as periodontal therapeutics. Increasing the total NO payload while maintaining slow release kinetics may prove optimal in the clinical implementation of NO-based oral therapeutics.

4.3.4 Kinetic-dependent killing of Streptococcus mutans with NO-releasing particles

We previously reported reduced sensitivity of *S. mutans* to NO treatment compared to periodontopathogens.[26] Even increasing the NO storage for the most rapid NO-releasing particle system (i.e., MAP3) to ~1 μmol/mg did not influence or enhance *S. mutans* killing (Figure 4.4). *S. mutans* is a unique pathogen that generates NO from nitrite via nitrite reductase to protect itself in nitrite-rich acidic environments such as the mouth.[26,34] Clearly *S. mutans* possesses a mechanism to mitigate intracellular NO, thus limiting the antibacterial activity of both NO and its reactive byproducts. We hypothesized that faster NO release might overload this protective mechanism and facilitate *S. mutans* killing. To accelerate the NO-release kinetics of MAP3, NO-release characterization and bacterial killing assays were conducted at lower pH (6.4).

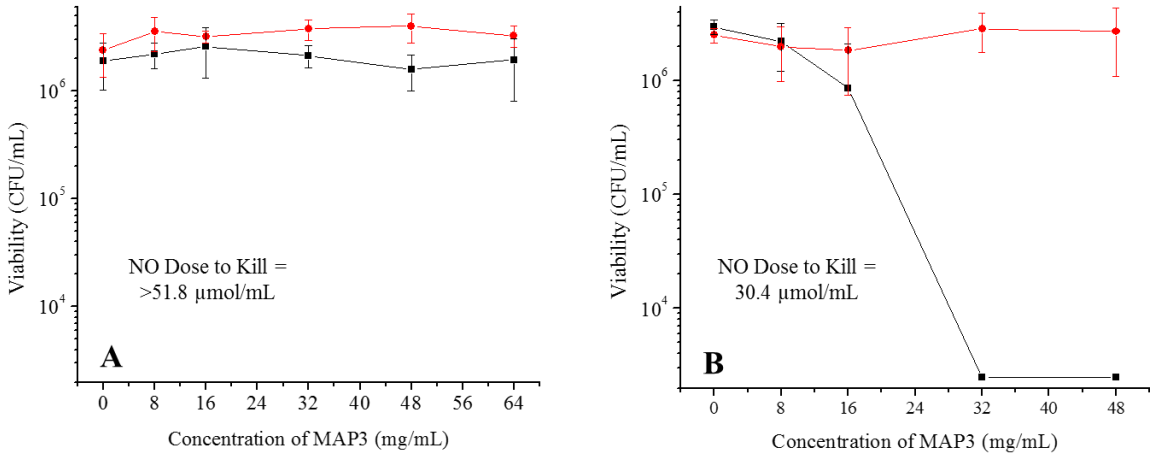

Figure 4.4 Bactericidal efficacy of 70 mol% MAP3 particles against *S. mutans* in (A) Tris-PBS (pH = 7.4) and (B) PBS (pH = 6.4) after 2 h exposure. NO-releasing material denoted by rectangles (■) and non-NO-releasing controls denoted by circles (●). Error bars signify standard deviation of the mean bacterial viability (CFU/mL). For all measurements n = 3 or more pooled experiments.

Table 4.3 Characterization of MAP3 NO-releasing silica particles in PBS (37 ºC) at different pH values (7.4 and 6.4) using a chemiluminescent nitric oxide analyzer.[a]

pH	t[NO][b] (μmol/mg)	$[NO]_m$[c] (ppb/mg)	$t_{½}$[d] (min)	$t[NO]_{2h}$[e] (μmol/mg)
7.4	0.84 ± 0.14	9200 ± 4300	19.8 ± 3.3	0.81 ± 0.15
6.4	0.95 ± 0.17	25800 ± 7500	4.5 ± 0.2	0.95 ± 0.17

[a]Results shown for 70 mol% MAP3 NO-releasing particles and presented as mean ± standard deviation for n = 3 or more pooled experiments. [b]Total amount of NO released. [c]Maximum NO flux achieved. [d]Time to release half of total NO payload. [e]Total amount of NO released after 2 h.

As expected, MAP3 NO-release kinetics were significantly enhanced at pH 6.4 because the NO donor system (i.e., *N*-diazeniumdiolate) decomposes more rapidly with increasing proton concentration (Table 4.3). Indeed, the NO-release properties included a greater initial NO flux (9200 to 25800 ppb/mg) and shorter half-life (19.8 to 4.5 min). The zeta potential for MAP3 particles at pH 6.4 (26.3 mV) also indicated a greater positive charge, which would be expected given that the NO donor precursors would be protonated to a larger extent at lower pHs. This combination (i.e., faster NO release and positive surface charge) resulted in *S. mutans* killing (3-log) at pH 6.4 with 32 mg/mL MAP3 particles. As shown in Figure 4.5, confocal microscopy confirmed more efficient NO delivery at this pH. For example, greater intracellular NO levels were observed at pH 6.4 compared to pH 7.4, as evidenced by a larger DAF-2 fluorescence intensity at 60 min.

4.4 Conclusions

Our study details the synthesis of ~150 nm NO-releasing silica nanoparticles with tunable NO-release kinetics and their antibacterial activity. Slower NO-release kinetics enhanced the bactericidal efficacy of NO-releasing silica particles against periodontopathogens while only minimally impacting human gingival fibroblast viability. The enhanced antibacterial action and reduced cytotoxicity for a macromolecular NO-release scaffold that slowly release NO warrants careful attention with respect to the development of NO-releasing therapeutics against periodontopathogens. A differential sensitivity to NO-release totals and kinetics was observed between periodontopathogens and cariogenic bacteria. The incorporation of additional biocides onto NO-releasing silica nanoparticles would be required to facilitate more complete killing of *S. mutans*.[32]

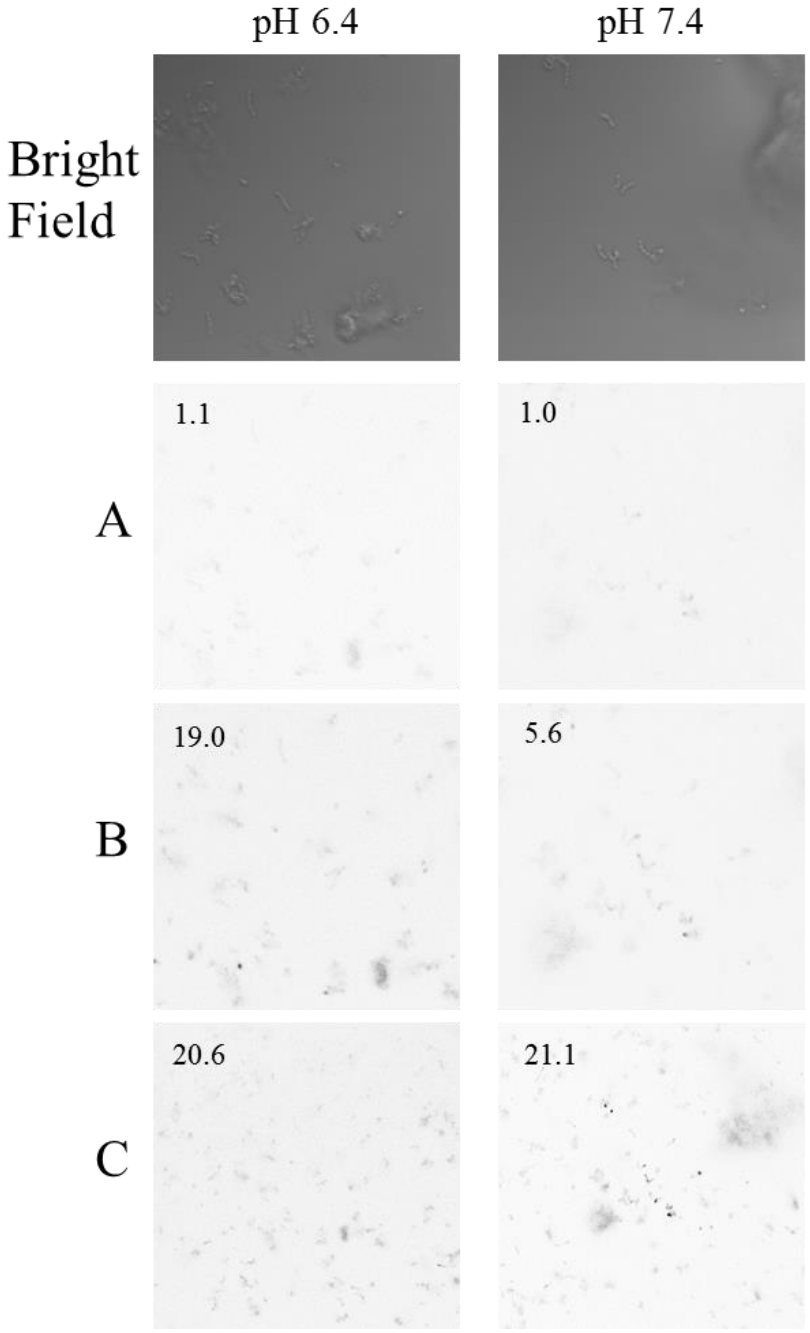

Figure 4.5 Confocal microscopy images of *S. mutans* exposed to 0.25 mg/mL 70 mol% MAP3 NO-releasing silica nanoparticles at (A) 40, (B) 60, and (C) 90 min post particle exposure. DAF-2 fluorescence is depicted as black in the images for clarity. Numbers in images represent the relative summed signal intensities normalized to the fluorescence intensity at 0 min and pH 7.4.

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Chapter 5: Anti-Biofilm Efficacy of Poly(amidoamine) Alkyl-Modified Nitric Oxide-Releasing Dendrimers against *Streptococcus mutans*

5.1 Introduction

Dental caries is one of the most costly and prevalent diseases worldwide, with 94% of the population experiencing cavities.[1,2] Oral infections are caused by dental plaque biofilms, and the presence of Gram-positive lactobacilli and streptococci acidogenic species is considered a risk factor for dental caries.[3-6] Cariogenic bacteria like *Streptococcus mutans* metabolize dietary sugars and produce lactic acid, which demineralizes tooth enamel.[6,7] The resulting acidic environment promotes preferential biofilm colonization by acidophilic species over native flora, furthering tooth decay.[8] Current treatments for dental caries are thus focused on eliminating acidogenic *S. mutans* biofilms.

Dental plaque biofilms are more difficult to treat than planktonic bacteria for numerous reasons.[4,8,9] Secreted exopolymers create a physical boundary that limits drug (e.g., antibiotics) penetration and prevents biofilm eradication.[9] Phenotypic differences in surface-attached bacteria alter potential antibiotic targets, ultimately mitigating drug/bacteria interactions.[9] The slower metabolic activity of biofilm bacteria also decreases antibiotic efficacy.[8,9] Lastly, antibiotic action is often inhibited by either the acidic environment or gingival fluid-derived β-lactamases within oral biofilms.[8-11] Collectively, these factors require greater drug concentrations to effectively eradicate dental biofilms at the expense of undesirable systemic side effects (e.g., pseudomembranous colitis and promoting antibiotic-resistant bacteria).[12]

Although the use of local antibiotic delivery systems has been shown to reduce systemic toxicity, continued emergence of antibiotic-resistant bacteria remains a concern, necessitating alternative treatments for dental caries.[13,14] Antiseptic mouthwash rinses including chlorhexidine (CHX; 0.20% w/w) have been used to combat oral infections,[15] but with modest success. Vitkov et al. reported only minor structural alterations to the exterior of mature biofilms upon CHX exposure.[16] Moreover, CHX may cause changes in taste, mouth discoloration, mucosal irritation, and desquamation of the gums.[17,18] Due to the undesirable side effects and insufficient biofilm suppression associated with CHX, the search for new anti-plaque therapeutics is a continuing effort.

Nitric oxide (NO) is an endogenous, diatomic radical that plays a pivotal role in wound healing, neurotransmission, and the immune response to pathogens.[19,20] Nitric oxide's antimicrobial activity results from its reaction with superoxide and oxygen to form peroxynitrite and dinitrogen trioxide, respectively. These species kill bacteria through lipid peroxidation, DNA cleavage, and protein dysfunction.[21] The multiple bactericidal pathways of NO make it a potent broad-spectrum antimicrobial agent with low risk for promoting bacterial resistance.[22,23] Due to the highly reactive nature of NO gas, the design of storage vehicles that controllably release biocidal levels of NO is crucial to its application as a dental therapeutic.[24,25]

Our laboratory has previously reported on the synthesis of silica and dendrimer-based macromolecular NO-release scaffolds capable of eradicating planktonic and biofilm cultures of Gram-positive, Gram-negative, and fungal pathogens.[21,26-28] More recently, we reported on the controlled delivery of exogenous NO to kill *S. mutans*.[29] To further enhance *S. mutans* killing, the use of non-depleting secondary biocides was proposed to create dual-action NO-releasing antibacterial agents. Carpenter et al. previously functionalized NO donor-modified silica with long

alkyl chain quaternary ammonium (QA) groups to improve bactericidal efficacy over the single action (i.e., solely NO-releasing) silica particles.[30] Likewise, Worley et al. described the modification of NO-releasing poly(amidoamine) (PAMAM) dendrimers with alkyl chain QA moieties, which exhibited improved antibacterial activity over both single action QA-modified dendrimers and NO-releasing QA-modified silica.[31] Due to the reduced concentrations required to kill bacteria, QA-modified NO-releasing dendrimers were less toxic to mouse fibroblasts than QA-modified silica.[30,31]

The superior bactericidal efficacy of the QA-modified NO-releasing dendrimers has been attributed to increased cell damage via membrane intercalation of the hydrophobic alkyl chains.[30,31] Due to the highly cationic nature inherent to PAMAM dendrimers, we hypothesized that alkyl chain functionalization without QA groups would reduce the synthetic burden to achieve dual-action killing while still enhancing the bactericidal efficacy over single-action NO-releasing dendrimers. Herein, we investigated the bactericidal and anti-biofilm activity of NO-releasing alkyl-modified dendrimers against cariogenic *S. mutans* as a function of alkyl chain modification, pH, and NO-release kinetics.

5.2 Materials and Methods

5.2.1 Materials

Streptococcus mutans (ATCC #27517) was purchased from the American Type Tissue Culture Collection (Manassas, VA). Brain heart infusion (BHI) broth and agar were purchased from Becton, Dickinson, and Company (Franklin Lakes, NJ). Murine fibroblasts (L929) were obtained from the UNC Tissue Culture Facility (Chapel Hill, NC). Hydroxyapatite (HA) disks (5 x 2 mm) were purchased from Clarkson Chromatography Products, Inc. (South Williamsport, PA).

Tris(hydroxymethyl)aminomethane (Tris), and Tris hydrochloride were acquired from Fisher Scientific (Fair Lawn, NJ). 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, fetal bovine serum (FBS), rhodamine B isothiocyanate (RITC), triethylamine (TEA), propylene oxide (PO), epoxybutane (EB), epoxyhexane (EH), epoxyoctane (EO), and epoxydodecane (ED) were purchased from Sigma-Aldrich (St. Louis, MO). Methanol (MeOH), sodium methoxide (NaOMe; 5.4 M in MeOH), and tetrahydrofuran (THF) were obtained from Acros Organics (Geel, Belgium). Argon (Ar), carbon dioxide (CO_2), nitrogen (N_2), and nitric oxide (NO) calibration (25.87 PPM, balance N_2) gases were acquired from National Welders (Raleigh, NC). Nitric oxide gas (99.5%) was purchased from Praxair (Sanford, NC). Distilled water was purified with a Millipore Milli-Q Gradient A-10 water purification system (Bedford, MA) to ≤6 ppb organic content and a final resistivity of 18.2 mΩ·cm. Other solvents and reagents were analytical grade and used as received.

5.2.2 Synthesis of alkyl chain-modified nitric oxide-releasing PAMAM dendrimers

Generation 1 (G1) poly(amidoamine) (PAMAM) dendrimer core scaffolds were synthesized as described previously.[32,33] Primary-amine functionalized G1 PAMAM dendrimers (200 mg) were then dissolved in MeOH (2 mL) with TEA and an alkyl epoxide (i.e., PO, EB, EH, EO, or ED) at a 1:1:1 molar ratio (TEA:alkyl epoxide:primary amines on the PAMAM dendrimer) to synthesize propyl-, butyl-, hexyl-, octyl-, and dodecyl-functionalized G1 PAMAM dendrimers, respectively. After 3 d of constant stirring, the MeOH solvent and unreacted epoxides were

removed under vacuum to purify the dendrimer product. Following purification, dendrimers were resuspended in MeOH and stored at -20 ^{o}C.

Secondary amines on the alkyl-modified dendrimers were converted to *N*-diazeniumdiolate NO donors by combining alkyl-modified G1 PAMAM dendrimers (30 mg) with 1 molar equivalent NaOMe (with respect to the primary amines on the unmodified G1 PAMAM scaffold) in 1mL MeOH/THF. The mixtures were placed in a stainless steel Parr reaction vessel, flushed with Ar three times to a pressure of 7 bar, and then filled with Ar three times for longer durations (10 min) to remove trace oxygen from the solutions. Following deoxygenation, the reaction chamber was filled with NO and held at 10 bar with constant stirring. After 3 d, the chamber was flushed again with Ar three times at short durations prior to extended Ar purges (3 x 10 min) to remove unreacted NO. The solutions were then collected and excess solvent was removed under vacuum. The NO-releasing dendrimers were resuspended in MeOH and stored at -20 ^{o}C until further use.

5.2.3 Characterization of nitric oxide release

Real-time NO release was measured using a Sievers 280i Chemiluminescence Nitric Oxide Analyzer (Boulder, CO) to quantify total NO release (t[NO]), total NO released after 2 h ($t[NO]_{2h}$), NO-release half-life ($t_{1/2}$), and maximum NO flux ($[NO]_{max}$).[34] The NO-releasing dendrimers were added to a flask containing 30 mL of deoxygenated phosphate-buffered saline (PBS; 37 ^{o}C) at either pH 7.4 or 6.4. Nitrogen was bubbled continuously through the solution to carry liberated NO to the analyzer at a flow rate of 80 mL/min. The NO analysis was terminated when NO levels fell to <10 ppb/mg dendrimer.

5.2.4 Planktonic bactericidal assays

To evaluate the bactericidal efficacy of the alkyl-modified dendrimers against planktonic *S. mutans*, the 2 h minimum bactericidal concentration (MBC_{2h}) (i.e., the minimum dendrimer concentration required to achieve a 3-log reduction in bacterial viability) was determined under static growth conditions. Briefly, bacteria was cultured overnight at 37 ^{o}C in ~3 mL BHI broth from a frozen stock (15% (v/v) glycerol in PBS at -80 ^{o}C). The next day, a 1 mL aliquot of the resulting bacterial suspension was inoculated into 50 mL fresh BHI broth and grown at 37 ^{o}C with moderate shaking to a concentration of 10^8 colony forming units per milliliter (CFU/mL) as confirmed by optical density (OD; 600 nm). The bacteria were then collected by centrifugation (2355 x *g*; 10 min), resuspended in distilled water, and diluted to 10^6 CFU/mL in either Tris-PBS (pH 7.4) with 1% (v/v) BHI broth or PBS (pH 6.4) with 5% (v/v) BHI broth. The bacterial solutions were then added to vials containing NO-releasing or non-NO-releasing dendrimers, mixed, and incubated at 37 ^{o}C with moderate shaking. After 2 h, the bacteria suspensions were diluted and plated on BHI agar using an IUL Eddy Jet spiral plater (Farmingdale, NY). Plates were incubated at 37 ^{o}C for 72 h before viable colonies were enumerated using a Flash & Go colony counter (IUL; Farmingdale, NY). The dendrimer concentration resulting in bacterial viability below 2.5 x 10^3 CFU/mL (the limit of detection for this plating method)[35] from the initial 10^6 CFU/mL culture was taken to be the MBC_{2h}.

5.2.5 Biofilm-killing assays

Suspensions of *S. mutans* (10^8 CFU/mL) were prepared as described above and collected by centrifugation (2355 x *g*; 10 min). Following centrifugation, bacteria were resuspended in distilled water and diluted to 10^6 CFU/mL in 40 mL of 50/50 BHI/distilled water with 1% w/v

glucose and sucrose. The bacterial suspension (3 mL) was added to a 12-well culture plate with each well containing one hydroxyapatite (HA) disk. Each disk was incubated with bacteria at 37 ^{o}C and gentle shaking. After 24 h, the disks were removed, rinsed in distilled water, and added to test suspensions of NO-releasing or non-NO-releasing dendrimers in Tris-PBS (pH 7.4) or PBS (pH 6.4). After 2 h of moderate shaking under static growth conditions, the disks were removed, rinsed in distilled water again, and sonicated in 3 mL of distilled water at low power for 10 min. The resulting bacterial solutions were then plated on BHI agar using the aforementioned spiral plating system, and incubated further at 37 ^{o}C. After 72 h, viable colonies were enumerated as described above. The dendrimer concentration resulting in a 3-log or greater reduction in viable bacteria to below 2.5 x 10^4 CFU/mL (the limit of detection for this plating method)[35] was determined to be the 2 h biofilm killing concentration $(BKC)_{2h}$.

5.2.6 Confocal microscopy to assess dendrimer-bacteria association

Fluorescently-labeled G1 PAMAM dendrimers were synthesized as described previously.[26,31,36] Briefly, 100 mg G1 PAMAM were added to a vial containing one molar equivalent of RITC (3.75 mg) per mole of dendrimer in methanol (2 mL). One mole equivalent of triethylamine (with respect to the number of dendrimer primary amines) was then added to the vial and the solution was stirred for 24 h in the dark. After removal of the solvent under vaccum, dendrimers were dissolved in water, purified via dialysis against water (1 d), and then lyophilized. The dendrimers were functionalized with alkyl chains per the above procedure in the dark to create fluorescently-labeled butyl-modified G1 PAMAM dendrimers.

S. mutans was cultured as described above and diluted to 10^6 CFU/mL in Tris-PBS (pH 7.4) or PBS (pH 6.4), each containing 3% (v/v) BHI broth. Aliquots of the bacteria solution were

incubated in a glass bottom confocal dish (MatTek Corporation; Ashland, MA) at 37 °C for 45 min. A Zeiss 510 Meta inverted laser scanning confocal microscope with a 543 nm HeNe excitation laser (1.0 mW, 25.0% intensity) and a 560–615 nm band-pass filter was used to obtain fluorescence images of the RITC-modified dendrimers. Both bright field and fluorescence images were collected using an N.A. 1.2 C-Apochromat water immersion lens with a 40x objective. Solutions of RITC-labeled butyl-modified dendrimers (200 μg/mL) in Tris-PBS or PBS (1.5 mL) were added to 1.5 mL of the respective bacteria solution in the glass confocal dish to achieve a final concentration of 100 μg/mL. Images were collected in 2 min intervals to temporally monitor dendrimer association with the bacteria. The image colors were modified and inverted for clarity using ImageJ software.

5.2.7 In vitro cytotoxicity

Murine fibroblasts (L929) were grown in DMEM supplemented with 10% (v/v) FBS and 1 wt% PS. The cells were incubated at 37 ^{o}C under humidified conditions in 5% CO_2 (v/v). After reaching confluency, cells were trypsinized and seeded onto a 96-well plate. The supernatant was then aspirated, and solutions of both NO-releasing and non-NO-releasing dendrimers in DMEM were added to the wells. Following incubation for 2 h at 37 ^{o}C, the supernatant was aspirated, and 120 μL of a DMEM, PMS, and MTS (100/20/1 v/v/v) mixture was added to the wells. After incubation for an additional 2 h at 37 ^{o}C, the supernatant was transferred to a new 96-well plate. Absorbance was measured at 490 nm using a Thermoscientific Multiskan EX plate reader (Waltham, MA) and subtraction of blanks (i.e., DMEM/PMS/MTS without cells). A DMEM solution containing 10% DMSO (v/v) served as a positive control. Cell viability was quantified with respect to untreated cells.

5.3 Results and Discussion

We have previously reported on the modification of PAMAM dendrimers to create secondary amine-functionalized scaffolds that store and release NO.[26] The chemistry of NO release was also combined with a non-depleting, membrane-disrupting QA biocide, which proved highly effective at killing nosocomial pathogens (i.e., *Pseudomonas aeruginosa* and *Staphylococcus aureus*).[31] In the present study, we investigated the effects of exterior dendrimer hydrophobicity, pH, and NO release on the antibacterial and anti-biofilm activity of NO-releasing propyl-, butyl-, hexyl-, octyl-, and dodecyl-modified G1 PAMAM dendrimers against *S. mutans*, an etiological agent of dental caries.

5.3.1 Characterization of nitric oxide-releasing dendrimers

N-Diazeniumdiolate NO donor-modified dendrimers were synthesized by 1) modifying PAMAM to contain secondary amines; and 2) reacting the dendrimer scaffold with NO gas at high pressure under basic conditions.[37] Nitric oxide release was measured in PBS at both pH 7.4 and 6.4 (Table 5.1). At pH 7.4, the total NO released (0.90–1.12 μmol/mg) and maximum instantaneous NO concentrations (3980–5700 ppb/mg) were similar for each dendrimer system, independent of alkyl chain modification. The NO-release half-life increased slightly (~21 to 37 min) with longer alkyl chains (i.e., increased hydrophobicity), which we attributed to impeded water diffusion to the proton-labile *N*-diazeniumdiolate NO donors. Of importance, the total NO released after 2 h was similar (~0.9 μmol/mg) regardless of alkyl chain length, allowing us to examine the effect of exterior hydrophobicity on bactericidal efficacy and anti-biofilm activity, independent of NO-release totals and kinetics.

Table 5.1 Characterization of NO-releasing alkyl-modified G1 PAMAM dendrimers in PBS (37 ^{o}C) at pH 7.4 and 6.4 by means of a chemiluminescent nitric oxide analyzer.[a]

pH	Dendrimer Modification	t[NO][b] (μmol/mg)	t[NO]$_{2h}$[c] (μmol/mg)	$t_{1/2}$[d] (min)	[NO]$_{max}$[e] (ppb/mg)
7.4	**Propyl**	0.90 ± 0.20	0.82 ± 0.10	21.8 ± 3.9	5700 ± 2293
	Butyl	1.06 ± 0.10	0.97 ± 0.10	21.7 ± 5.0	4933 ± 689
	Hexyl	0.98 ± 0.11	0.90 ± 0.14	23.5 ± 5.5	4344 ± 1069
	Octyl	1.07 ± 0.13	0.88 ± 0.08	29.8 ± 5.4	3980 ± 936
	Dodecyl	1.12 ± 0.10	0.90 ± 0.05	37.3 ± 6.3	4200 ± 952
6.4	**Propyl**	1.09 ± 0.19	1.09 ± 0.19	4.2 ± 0.8	25975 ± 3783
	Butyl	0.98 ± 0.22	0.98 ± 0.22	4.4 ± 0.5	21433 ± 6962
	Hexyl	1.04 ± 0.34	1.03 ± 0.34	4.3 ± 0.2	22600 ± 6222
	Octyl	1.06 ± 0.24	1.04 ± 0.24	5.0 ± 0.1	20467 ± 4839
	Dodecyl	1.10 ± 0.31	1.07 ± 0.29	8.9 ± 0.8	15067 ± 1848

[a]Results shown as mean ± standard deviation for n=3 or more pooled experiments. [b]Total amount of NO released. [c]Total amount of NO released after 2 h. [d]Time to release half of total NO payload. [e]Maximum NO flux achieved.

To more accurately represent the *in vivo* environment associated with dental caries, NO release from alkyl-modified dendrimers was also characterized at pH 6.4 (Table 5.1). As expected with proton-initiated decomposition of *N*-diazeniumdiolate NO donors, faster NO-release kinetics were observed under acidic conditions. The more rapid NO release at pH 6.4 is evident by the greater maximum NO release (15067–25975 ppb/mg) versus pH 7.4 (3980–5700 ppb/mg) and the shorter NO-release half-life (4–9 versus 21–37 min, respectively). Despite the faster NO-release kinetics, the 2 h total NO release was similar at pH 6.4 (~1.0 μmol/mg) compared to pH 7.4 (~0.9 μmol/mg).

5.3.2 Bactericidal efficacy of alkyl-modified dendrimers

The bactericidal efficacy of alkyl-modified G1 PAMAM dendrimers against planktonic cultures of *S. mutans* was evaluated at both pH 7.4 and 6.4 by determining the minimum bactericidal concentration (MBC_{2h}) required to elicit a 3-log reduction in bacterial viability relative to untreated cultures (Table 5.2). At pH 7.4, the non-NO-releasing short alkyl chain (i.e., propyl and butyl) dendrimers were ineffective at killing *S. mutans*, with no reduction in bacterial viability up to a concentration of 48 mg/mL. This result was somewhat expected as minimal membrane intercalation by less hydrophobic tails should limit the bacterial membrane damage caused by these scaffolds.[30,31] Compared to the shorter alkyl chains, the longer alkyl chain (i.e., hexyl, octyl, and dodecyl) dendrimers were considerably more bactericidal (MBC_{2h} values $\leq$2 mg/mL). Clearly, membrane intercalation causes significant membrane damage, as has been reported previously.[26,30,31] Under more acidic conditions (pH 6.4), little to no antibacterial action was again observed for the propyl-modified dendrimers (MBC_{2h} >48 mg/mL). The bactericidal efficacy of butyl-modified dendrimers was improved at pH 6.4, but still required large dendrimer

Table 5.2 Comparison of minimum bactericidal concentrations (MBC_{2h}) and bactericidal NO doses of alkyl-modified G1 PAMAM dendrimers against planktonic *S. mutans* at pH = 7.4 and pH = 6.4.[a]

	pH = 7.4		**pH = 6.4**	
	MBC_{2h} (mg/mL)	**NO Dose (μmol/mL)**	**MBC_{2h} (mg/mL)**	**NO Dose (μmol/mL)**
Propyl	>48		>48	
Propyl-NO	48	39.4	8	8.7
Butyl	>48		32	
Butyl-NO	48	46.6	8	7.8
Hexyl	2		1	
Hexyl-NO	2	1.8	1	1.0
Octyl	0.05		0.012	
Octyl-NO	0.1	0.1	0.025	<0.1
Dodecyl	0.05		0.025	
Dodecyl-NO	0.05	0.1	0.025	<0.1

[a]Results of n ≥3 pooled experiments.

concentrations (32 mg/mL) to elicit killing. The lack of exterior hydrophobic character associated with short alkyl chains seems to limit the biocidal activity of the dendrimer scaffolds, regardless of pH. In contrast, the bactericidal action of longer alkyl chain-modified dendrimers was enhanced at lower pH (6.4), as evidenced by the reduced MBC_{2h} values relative to those at pH 7.4 (Table 5.2). The increased bactericidal action at pH 6.4 is likely due to greater protonation of pendant amines (i.e., increased positive surface charge) on the dendrimer scaffold promoting more efficient dendrimer-bacteria association and improving membrane intercalation of the hydrophobic chains.

Dendrimer diffusion to bacteria in real-time was visualized with confocal microscopy to confirm enhanced dendrimer-bacteria association at lower pH (Figure 5.1). At pH 6.4, a substantial fluorescence signal from RITC-labeled butyl-modified dendrimers associating with *S. mutans* was observed after only 30 min of dendrimer exposure. This signal continued to increase in intensity up to 60 min. In contrast, the fluorescence signal intensity at pH 7.4 was negligible, and did not change through the same period of study. The greater fluorescence signal at pH 6.4 both initially and over time suggests more rapid and extensive dendrimer-bacteria association under acidic conditions. Enhanced dendrimer-bacteria association would translate to more extensive bacterial damage as alkyl chain-induced death is contact-based.[30,31] This hypothesis is consistent with the lower MBC_{2h} values observed for butyl-, hexyl-, octyl-, and dodecyl-modified dendrimers at pH 6.4 compared to pH 7.4.

The incorporation of NO release did not improve the biocidal action of the hexyl-, octyl-, and dodecyl-modified dendrimer scaffolds (Table 5.2). The hydrophobic alkyl chains of these PAMAM analogues likely compromise the bacterial membrane prior to significant accumulation of intracellular NO, thus limiting NO-induced killing.[31] Upon lowering the pH to 6.4, similar behavior was again observed, with NO-releasing and control dendrimers exhibiting no difference

in killing efficiency. The highly efficient membrane disruption of hydrophobic alkyl chains clearly precludes intracellular NO accumulation, alleviating NO-mediated cell death.[31]

The addition of NO release moderately improved the bactericidal activity for propyl- and butyl-modified dendrimers at pH 7.4, albeit requiring large doses of NO (39.4 and 46.6 μmol/mL, respectively) to elicit a 3-log reduction in bacterial viability (MBC_{2h} = 48 mg/mL). In contrast to the long alkyl chain-modified dendrimers, the antibacterial efficacy of NO-releasing propyl- and butyl-modified dendrimers was significantly enhanced at pH 6.4. Indeed, the bactericidal concentration was 6-fold lower at pH 6.4 (8 mg/mL) versus pH 7.4 (48 mg/mL) for both NO-releasing propyl- and butyl-modified dendrimers. The lower MBC_{2h} values translate to reduced bactericidal NO doses for propyl-modified (39.4 to 8.7 μmol/mL) and butyl-modified (46.6 to 7.8 μmol/mL) dendrimers. As shown in Figure 5.1, the bacterial association of butyl-modified dendrimers was enhanced at lower pH. However, the lack of antibacterial activity by the butyl-modified controls (MBC_{2h} = 32 mg/mL) at pH 6.4 suggests minimal membrane disruption via alkyl chain intercalation. Similarly, no antibacterial activity was observed up to 48 mg/mL for the propyl-modified controls at pH 6.4. The disparity in killing efficiency between the NO-releasing and control propyl- and butyl-modified dendrimers at pH 6.4 unequivocally demonstrates that rapid NO release in combination with enhanced dendrimer-bacteria association are responsible for the improved bactericidal efficacy. These findings suggest that large instantaneous NO concentrations are more effective for eradicating *S. mutans*, an important consideration when using single-action NO-releasing macromolecular scaffolds to kill *S. mutans* and possibly other cariogenic bacteria as well.

Several factors may contribute to the increased bactericidal efficacy observed with faster NO-release kinetics. For example, *S. mutans* makes use of nitrite reductase, an enzyme capable

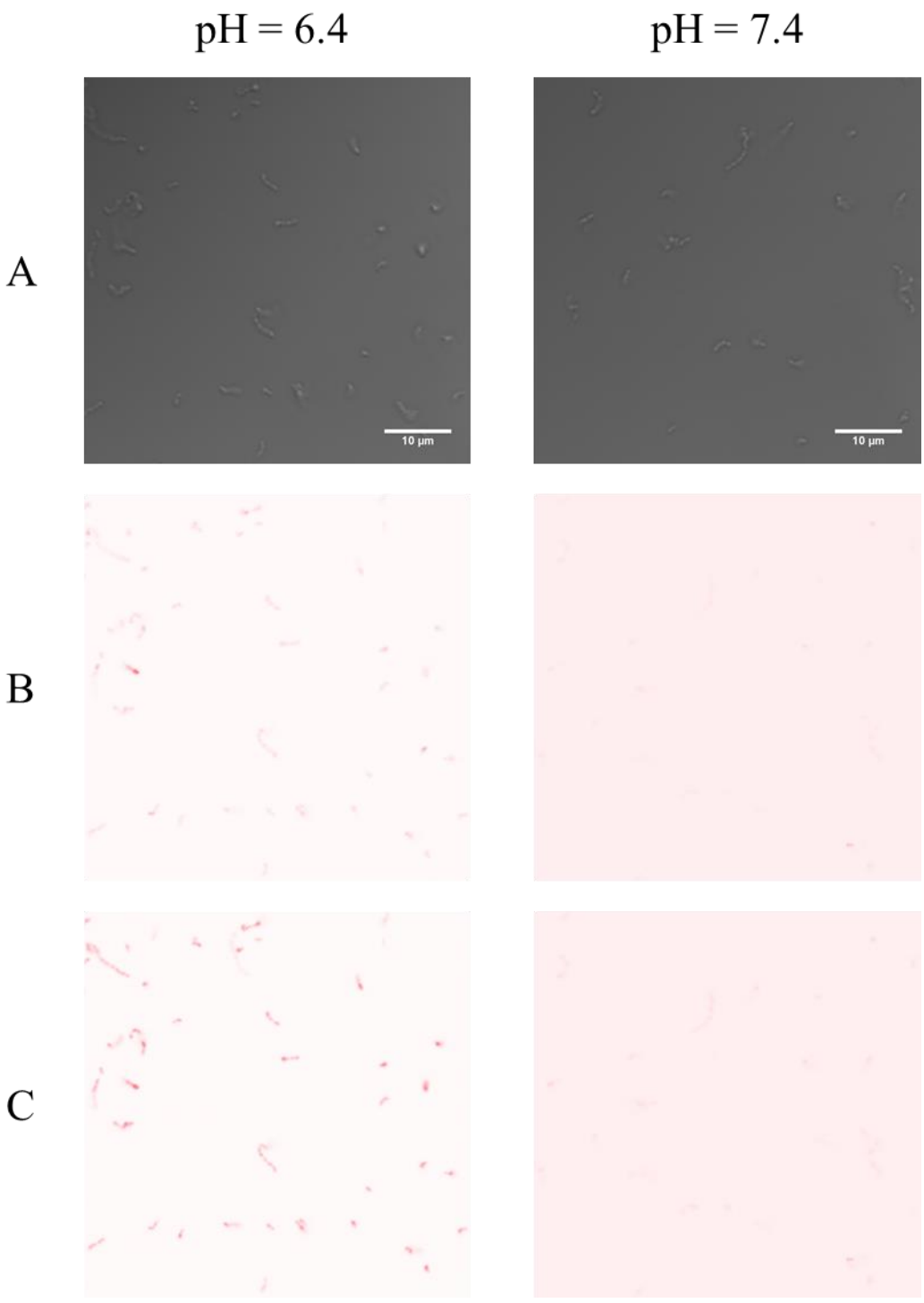

Figure 5.1 Confocal microscopy images of *S. mutans* exposed to 100 µg/mL RITC-labeled G1 butyl dendrimers at pH 6.4 and 7.4 imaged at (A) 0, (B) 30, and (C) 60 min. Image colors modified and inverted for clarity. Scale bar = 10 µm.

of generating NO from nitrite to promote survival in nitrite-rich acidic environments.[38] Gusarov and Nudler reported on the ability of *Bacillus subtillus* to utilize NO for protection against oxidative stress ("NO-mediated cytoprotection") and suggested other Gram-positive species may utilize this cellular detoxification mechanism as well.[39] In this regard, low instantaneous concentrations of NO may act as a metabolite rather than a biocidal agent for Gram-positive bacteria such as *S. mutans*, thus necessitating more rapid delivery of large NO payloads to induce NO-mediated killing.

5.3.3 Anti-biofilm activity of alkyl-modified dendrimers

Although studying microbes in planktonic, nutrient-rich batch cultures is useful for initial drug activity screening, *in vivo* infections are typically caused by bacterial biofilms.[5,40] Dental caries is associated with persistent biofilms composed of mainly streptococci and lactobacilli, with *S. mutans* considered a primary causative agent.[2,7,41-44] The eradication of cariogenic *S. mutans* biofilms is thus important for the treatment of dental caries. The concentration of NO-releasing and control alkyl-modified dendrimers required to elicit at least a 3-log reduction in bacterial viability for *S. mutans* biofilms (BKC_{2h}) was thus evaluated at pH 7.4 and 6.4 to more accurately represent the *in vivo* environment associated with dental caries.

As expected, the anti-biofilm activity for control (non-NO-releasing) propyl- and butyl-modified dendrimers was negligible at pH 7.4 (BKC_{2h} >64 mg/mL) due to insufficient membrane disruption by the shorter alkyl chains (Table 5.3).[30,31] Biofilm killing was substantially improved by increasing the alkyl chain length, with significantly reduced BKC_{2h} values for hexyl- (8 mg/mL) and octyl- and dodecyl-modified dendrimers (2 mg/mL). Similar to planktonic killing, this enhancement in anti-biofilm activity is attributed to increased membrane disruption by the long

Table 5.3 Comparison of 2 h biofilm killing concentrations (BKC_{2h}) and bactericidal NO doses of alkyl-modified G1 PAMAM dendrimers against *S. mutans* biofilms at pH = 7.4 and pH = 6.4.[a]

	pH = 7.4		**pH = 6.4**	
	BKC_{2h} (mg/mL)	**NO Dose (μmol/mL)**	**BKC_{2h} (mg/mL)**	**NO Dose (μmol/mL)**
Propyl	>64		>48	
Propyl-NO	64	52.5	8	8.7
Butyl	>64		48	
Butyl-NO	48	46.6	8	7.8
Hexyl	8		8	
Hexyl-NO	8	7.2	8	8.2
Octyl	2		2	
Octyl-NO	2	1.8	2	2.1
Dodecyl	2		1	
Dodecyl-NO	2	1.8	2	2.1

[a]Results of n ≥3 pooled experiments.

hydrophobic chains compared to minimal membrane damage caused by shorter alkyl chains.[30,31] Of note, Lu et al. previously reported decreased *P. aeruginosa* biofilm killing with the greatest hydrophobic dendrimer exterior tested due to less efficient penetration of the exopolysaccharide matrix.[26] Such a phenomenon was not observed with *S. mutans*, as biofilm eradication was continually improved with increasing exterior dendrimer hydrophobicity. The addition of NO improved the anti-biofilm activity of propyl- and butyl-modified dendrimers with BKC_{2h} values of 64 and 48 mg/mL, respectively. However, significant NO doses (52.5 and 46.6 μmol/mL, respectively) were required for biofilm eradication. Again, the combination of NO release and longer chain (i.e., hexyl, octyl, and dodecyl) dendrimer modifications did not improve biofilm killing over control dendrimers at pH 7.4, most likely due to efficient membrane disruption precluding significant intracellular accumulation of NO and mitigating NO-mediated killing.[31] Additionally, the efficient removal of intracellular NO by *S. mutans* may have further hindered bacterial killing via NO.[38,39]

At pH 6.4, the longer alkyl chain-modified control dendrimers were characterized as having similar BKC_{2h} values to those at pH 7.4. The highly efficient bacterial membrane disruption caused by longer alkyl chain-modified dendrimers at pH 7.4 likely prevents any further enhancement in killing with greater dendrimer-bacteria association at lower pH.[30] Similarly, the difference in biofilm killing for longer alkyl chain-modified NO-releasing dendrimers at pH 7.4 versus 6.4 was negligible, indicating that greater dendrimer-bacteria association in combination with faster NO release does not enhance biofilm killing.

Unlike the long alkyl chains, the addition of NO release to propyl- and butyl-modified dendrimers significantly enhanced biofilm killing at pH 6.4. While BKC_{2h} values for NO-releasing propyl and butyl dendrimers at pH 7.4 were 64 and 48 mg/mL, respectively, substantial killing

was observed at pH 6.4 (8 mg/mL). The corresponding NO doses required to eradicate the *S. mutans* biofilms were decreased from 52.5 to 8.7 μmol/mL for propyl- and 46.6 to 7.8 μmol/mL for butyl-modified dendrimers. The disparity in BKC_{2h} values cannot be attributed to increased membrane damage via enhanced dendrimer-bacteria association, as control propyl- and butyl-modified dendrimers displayed little to no antibacterial activity ($BKC_{2h} \geq 48$ mg/mL) at either pH. Rather, the enhanced anti-biofilm activity of propyl- and butyl-modified NO-releasing dendrimers at pH 6.4 must be the result of more efficient dendrimer-bacteria association (Figure 5.1) combined with faster NO-release kinetics (Table 5.1). This dramatically improved killing of *S. mutans* biofilms could have implications for the future design of NO-releasing materials to treat cariogenic dental plaque biofilms.

As biofilms often require greater concentrations for eradication compared to planktonic cultures,[4,8,9] it was not surprising that the BKC_{2h} values for longer alkyl chain (i.e., hexyl, octyl, and dodecyl) dendrimers were greater than the MBC_{2h} values (Tables 5.2 and 5.3). Unexpectedly, this trend was not apparent for the propyl- and butyl-modified NO-releasing dendrimers, as no difference between the planktonic killing and biofilm eradication concentrations was observed at pH 6.4. The amounts of NO from the propyl- and butyl-modified dendrimers for eradicating *S. mutans* biofilms and planktonic cultures were equivalent (8.7 and 7.8 μmol/mL, respectively) at lower pH (6.4). These two shorter chain alkyl modifications are unique in that their NO-mediated antibacterial activity against biofilms is not diminished relative to planktonic cultures as is observed for many antibacterial agents, including antibiotics and CHX.[4,8,9,16] This result could lead to the development of NO-releasing materials with no decrease in bactericidal efficacy against dental biofilms, an important consideration when designing antibacterial agents for treating oral infections.

5.3.4 In vitro cytotoxicity

To evaluate dendrimer cytotoxicity, murine fibroblasts (L929) were exposed to NO-releasing and control dendrimers for 2 h at the maximum concentrations required to kill *S. mutans* biofilms at both pH 7.4 and 6.4 (Figure 5.2). As postulated, short alkyl chain (i.e., propyl and butyl) dendrimer controls were non-toxic to L929 fibroblasts (>90% cell viability relative to untreated cells) at the concentrations required for anti-biofilm activity (8 and 64 mg/mL at pH 6.4 and 7.4, respectively). However, the addition of NO to these scaffolds proved lethal to mammalian cells (0% cell viability), even at the concentration necessary for biofilm eradication at pH 6.4 (8 mg/mL). The toxicity of NO-releasing short alkyl chain dendrimers is attributed to the large dose of NO required to eradicate *S. mutans* biofilms, as evidenced by the tolerance of mammalian cells to the control dendrimers at similar concentrations. Of note, microscopic examination of the tissue-culture plate revealed that the cells were actually removed from the surface when treated with 8 mg/mL of NO-releasing propyl- and butyl-modified dendrimers. As such, these materials may not be as toxic as indicated.

The cells also showed poor tolerance to hexyl-modified control dendrimers (3 ± 1% cell viability) at 8 mg/mL, likely the result of membrane disruption by the hydrophobic alkyl chains. Not surprisingly, hexyl-modified NO-releasing dendrimers also revealed significant toxicity (0% cell viability relative to untreated cells). The octyl- and dodecyl-modified control dendrimers proved less toxic, with cell viabilities of 18 ± 3 and 11 ± 3%, respectively. The lower material concentrations (2 mg/mL) necessary for anti-biofilm activity compared with hexyl-modified dendrimers (8 mg/mL) made this an expected result. Interestingly, the addition of NO to the octyl- and dodecyl-modified dendrimers further improved the tolerance of the cells to these dendrimers,

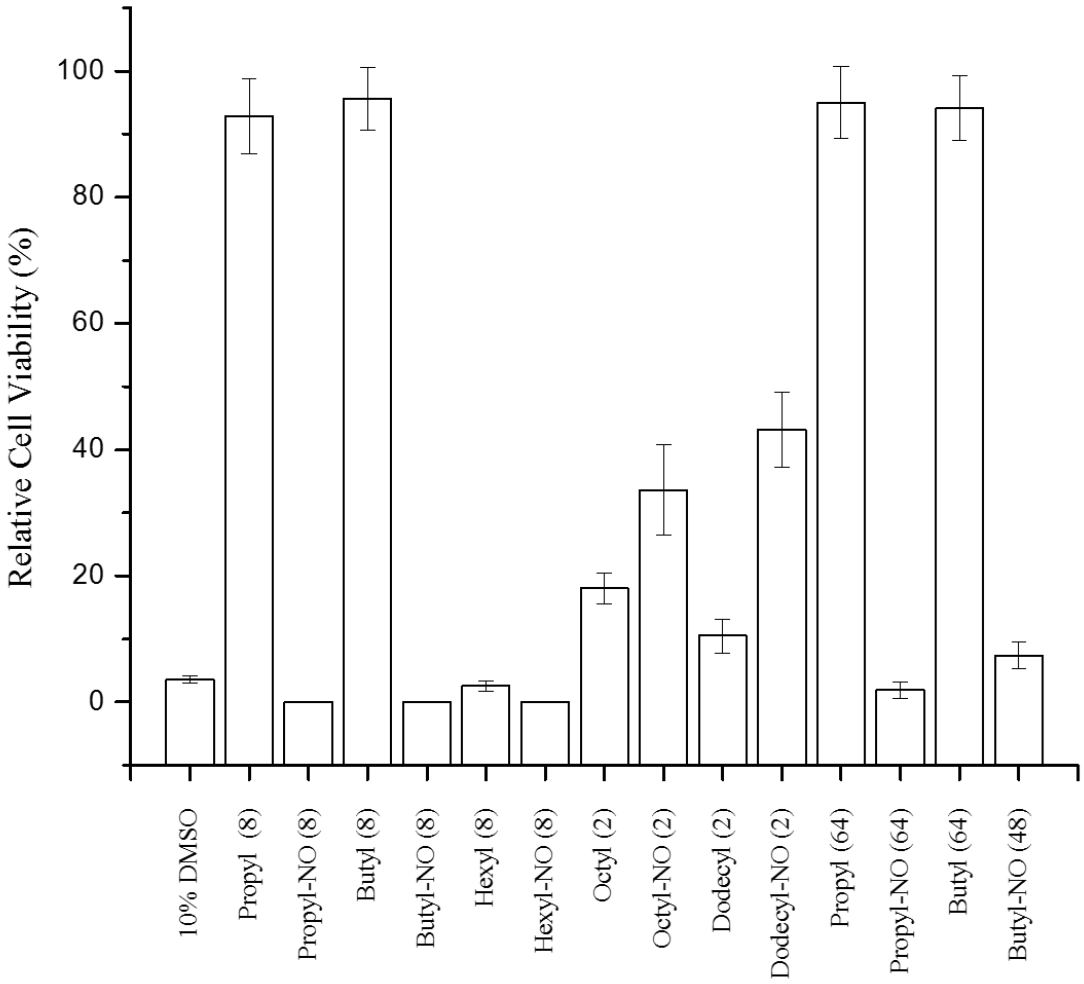

Figure 5.2 Cytotoxicity of NO-releasing and non-NO-releasing dendrimers against L929 mouse fibroblasts at the concentrations required for biofilm killing. 10% DMSO was used as a positive control. Numbers following each dendrimer modification (i.e., 2, 8, 48, and 64) represent the BKC_{2h} in mg/mL. Error bars represent the standard deviation of the mean. For all values n = 3 or more replicate measurements.

with cell viabilities of 34 ± 7 and 43 ± 6%. We have previously observed that low levels of NO are capable of improving cell viability for NO-release systems over controls.[30,31] The reduced toxicity observed for NO-releasing long alkyl chain dendrimers, while maintaining superior anti-biofilm activity, demonstrates their potential utility as dental caries therapeutics.

5.4 Conclusions

The bactericidal and anti-biofilm activity of NO-releasing PAMAM dendrimers was increased with greater exterior hydrophobicity at both pH 7.4 and 6.4. Improved bactericidal action was observed at lower pH (6.4) as a result of enhanced dendrimer-bacteria association and faster NO-release kinetics. Long alkyl chain (i.e., octyl and dodecyl) dendrimer modifications demonstrated superior anti-biofilm activity and NO release was found to partially mitigate the cytotoxicity of the dendrimer scaffold against mammalian cells. Collectively, these results demonstrate the potential utility of NO-releasing long alkyl chain-modified dendrimers as anti-biofilm agents for the treatment of dental caries. Studies involving complex, poly-microbial biofilms that more closely resemble dental plaque are necessary to better predict the clinical utility of alkyl chain-modified NO-releasing dendrimers.

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Chapter 6: Summary and Future Directions

6.1 Summary

The synthesis and subsequent microbiological testing of NO-releasing silica nanoparticles and dendrimers with different chemical and physical properties was described. Chapter 1 provided an overview of specific oral health ailments (i.e., dental caries and periodontal disease), current research into treating oral diseases, and the need for alternative therapies to effectively combat oral infections. The introduction established the potential advantages to using NO-release vehicles as dental therapeutics, with an emphasis on tuning their physical and chemical properties to maximize bactericidal efficacy while minimizing cytotoxicity. In Chapter 2, similarly sized (~150 nm), monodisperse NO-releasing silica particles with tunable NO storage and release kinetics were synthesized via the Stöber method. Optimization of *N*-diazeniumdiolate modification solution parameters (i.e., sodium methoxide concentration) was performed to maximize silica particle NO storage while limiting the formation of unwanted byproducts. Alteration of synthetic parameters provided 70 mol% MAP3 silica particles with increased size (~400 nm), but with similar NO-release totals (~1 μmol/mg) and release kinetics (half-life of ~15 min) to the 150 nm MAP3 particles. Additional *N*-diazeniumdiolate modification studies afforded ~150 nm NO-releasing silica particles with different NO-release half-lives allowing the effect of NO-release kinetics on bacterial killing, independent of both particle size and 2 h NO-release totals, to be studied. The bactericidal efficacy and cytotoxicity for a portion of the NO-releasing nanoparticles developed in Chapter 2 was described in subsequent chapters.

In Chapter 3, the antibacterial activity of NO-releasing silica nanoparticles, dendrimers, and proline (PROLI/NO) was evaluated against pathogenic oral bacteria. Despite storing less NO, the bactericidal efficacy of macromolecular NO-release vehicles (i.e., dendrimers and silica) was greater than that of the small molecule NO donor PROLI/NO. The stabilization of the *N*-diazeniumdiolate donor (i.e., longer half-lives) by macromolecular NO-release scaffolds provided more efficient biocidal NO delivery to bacteria. Cariogenic bacteria (i.e., *S. mutans* and *S. sanguinis*) were less susceptible to macromolecular-based NO release (≥37.0 μmol NO/mL required to kill) than periodontopathogens (i.e., *A. actinomycetemcomitans* and *P. gingivalis*), which required ≤3.2 μmol NO/mL for eradication. The greatest concentrations of NO-releasing dendrimers and silica required to kill periodontopathogens (2 and 4 mg/mL, respectively) proved relatively non-toxic to human gingival fibroblasts (HGF-1) compared to untreated cells (viabilities ≥60%) and especially when compared to chlorhexidine (~20% cell viability). These results suggested a differential sensitivity between cariogenic bacteria and periodontopathogens to NO treatment and supported further investigation into macromolecular NO-release vehicles as potential periodontal disease therapeutics.

Chapter 4 described the bactericidal efficacy of NO-releasing silica particles with varied NO-release kinetics (i.e. half-lives) against oral bacteria. Extended NO-release kinetics (half-lives of ~130 min versus ~32 min) proved to be more effective at killing periodontal pathogens (i.e., *A. actinomycetemcomitans* and *P. gingivalis*) and reduced the bactericidal NO dose by at least half. Confocal microscopy using the intracellular NO-sensitive dye DAF-2 DA confirmed more efficient NO delivery to *A. actinomycetemcomitans* from silica particles with extended NO-release. Furthermore, the slower NO-release kinetics combined with the lower silica concentrations necessary to kill bacteria reduced HGF-1 cytotoxicity, supporting the use of extended NO-release

vehicles to kill periodontopathogens. As observed previously, *S. mutans* was significantly less sensitive to NO treatment, with no observed reduction in bacterial viability up to 51.8 μmol NO/mL at pH 7.4. However, faster NO-release kinetics obtained under acidic conditions (pH 6.4) increased the bactericidal efficacy of NO-releasing silica particles and reduced the NO dose required to kill (30.4 μmol NO/mL). Confocal microscopy using DAF-2 DA confirmed more rapid intracellular NO accumulation and greater bacterial killing with faster NO-release kinetics at pH 6.4.

In Chapter 5, NO-releasing propyl-, butyl-, hexyl-, octyl-, and dodecyl-modified PAMAM dendrimers were synthesized to create dual-action antibacterial agents. The bactericidal efficacy and anti-biofilm activity of NO-releasing alkyl-modified dendrimers were evaluated against *S. mutans* with respect to pH, NO-release kinetics, and alkyl chain length. Greater bactericidal and anti-biofilm efficacy was observed for longer alkyl chain (i.e., hexyl, octyl, dodecyl) dendrimers versus shorter alkyl chain (i.e., propyl and butyl) modifications, attributed to more extensive bacterial damage from membrane intercalation of the longer hydrophobic chains. The addition of NO did not improve the bactericidal efficacy of longer alkyl chains, as the highly efficient membrane damage caused by the hydrophobic tails resulted in bacterial death prior to significant intracellular NO accumulation. At lower pH, confocal microscopy using RITC-modified butyl dendrimers confirmed greater dendrimer-bacteria association. The enhanced bactericidal efficacy of longer alkyl chain dendrimers at lower pH was thus due to increased pendant amine protonation, resulting in improved dendrimer-bacteria association and greater bacterial membrane damage. Since the shorter alkyl chain dendrimer scaffolds demonstrated minimal bactericidal efficacy at pH 6.4, the enhanced antibacterial and anti-biofilm activity at lower pH was attributed to faster NO-release kinetics. The greater instantaneous concentration of NO resulted in lower NO doses

required to kill *S. mutans*. Short alkyl chain dendrimers proved to be non-toxic against L929 mouse fibroblasts, but were extremely toxic (0% viability relative to untreated cells) with the addition of NO release due to the large NO dose required to kill bacteria. Although the octyl- and dodecyl-modified dendrimer scaffolds were toxic to murine fibroblasts, the addition of NO release increased cell viability, partially mitigating the cytotoxicity of the scaffolds. The superior bactericidal efficacy of octyl- and dodecyl-modified dendrimers, combined with the reduced cytotoxicity observed with integrated NO release, supports the development of NO-releasing long alkyl chain-modified dendrimers as anti-biofilm dental caries therapeutics.

6.2 Future Directions

Although macromolecular NO-release vehicles have been employed as bactericidal and anti-biofilm agents previously,[1-6] this is the first report investigating their use for the management of oral health diseases. Both the physical and chemical properties of NO-release vehicles were systematically altered to maximize bactericidal and anti-biofilm activity for the treatment of dental caries and periodontal disease. While the results described herein represent foundational research toward achieving successful oral disease therapeutics, much work is still required to utilize NO-release vehicles in a clinical setting. The concerted effort of NO-release chemists and oral health microbiologists may realize the full potential of NO-releasing materials for the treatment of oral health diseases.

6.2.1 Studies to increase the synthetic versatility of nitric oxide-releasing silica nanoparticles

In Chapter 2, silica particles were synthesized with various aminosilane precursors, different mol% aminosilane compositions, and two different sizes (i.e., 150 and 400 nm) to create

a toolbox of NO-releasing materials for subsequent microbiological testing. While the Stöber method[7] provides a one-step, high-throughput approach to achieve hybrid aminosilane/alkoxysilane NO-releasing nanoparticles,[8] the co-condensation of different silanes in a bolus fashion limits the physical and chemical flexibility of the resulting nanoparticles. After extensive systematic alteration of synthetic parameters, the particles created were still restricted to dual-silane systems (i.e., TMOS and one aminosilane) of only two sizes (i.e., 150 and 400 nm). Furthermore, the dense silica particle core limited *N*-diazeniumdiolate modification to the exterior, restricting the total NO storage for non-porous particles. To circumvent these issues, alternative synthetic procedures to create more versatile NO-releasing silica nanoparticles for biomedical applications are necessary.

Mesoporous silica nanoparticles are synthesized using a surfactant-templated method to create an alkoxysilane core with channels that traverse the inside of the particle and provide superior control over size, shape, and pore size/structure.[9-12] Surface-grafting techniques can incorporate aminosilanes into both the channels and the exterior of the particle, thereby increasing total NO storage compared to non-porous silica particles. Furthermore, surface-grafting of targeting ligands (e.g., sugars, imidazoles, positive charges) on the particle exterior prior to surfactant removal and aminosilane functionalization can potentially improve particle-bacteria association while maintaining NO-mediated antibacterial activity, thus improving the overall bactericidal efficacy of NO-releasing silica particles.[13-16] Similarly, selective aminosilane functionalization of the particle exterior and subsequent internal modification with a distinct aminosilane could create dual-aminosilane NO-releasing particles, with the advantage of more finely controlled NO-release kinetics (i.e., both burst and extended NO-release capabilities).

Indeed, the ability to tune both the chemical and physical properties of mesoporous NO-releasing nanoparticles is superior to nonporous silica-based systems.

6.2.2 Susceptibility of cariogenic and periodontopathogenic bacteria to nitric oxide

Chapter 3 highlighted the advantage of macromolecular NO-release vehicles versus small molecule NO donors (PROLI/NO) as bactericidal agents against oral pathogens. Furthermore, periodontopathogens (i.e., *A. actinomycetemcomitans* and *P. gingivalis*) were more susceptible to NO treatment than cariogenic bacteria (i.e., *S. mutans* and *S. sanguinis*). In Chapter 4, slower NO-release kinetics were shown to be superior for killing periodontopathogenic bacteria, whereas faster NO-release kinetics were beneficial for killing cariogenic bacteria. These findings should be expanded to include more bacteria from each disease class (i.e., cariogenic versus periodontopathogens) to validate these results. Relevant cariogenic bacteria are *Streptococcus gordonii*, *Streptococcus mitis*, *Streptococcus sobrinus*, *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Lactobacillus fermenti*.[17] Periodontopathogens of interest include *Fusobacterium nucleatum*, *Treponema denticola*, *Actinomyces viscosus*, *Tannerella forsythia*, and *Veillonella atypica*.[17] Furthermore, periodontopathogens are typically microaerophilic or anaerobic organisms, but bactericidal assays described herein were conducted under aerobic exposure conditions. As the reactive byproducts of NO are produced via reaction with oxygen or superoxide,[1] killing assays against periodontopathogens under strictly anaerobic environments to validate NO's efficacy against these bacteria in an environment more closely resembling the periodontal pocket would be of interest.

6.2.3 Alternate nitric oxide-releasing materials for periodontal disease treatment

In Chapter 4, extended NO-release kinetics were shown to improve the bactericidal efficacy of NO-releasing silica nanoparticles against periodontopathogens. However, silica nanoparticles may not be the ideal scaffold for achieving extended NO release to treat periodontal disease. Although our laboratory has reported on O^2-protected NONOate-based silica particles with sustained NO release ($t_{1/2}$ = 23 d),[18] the large size of these particles (>100 nm) limits penetration into subgingival periodontal biofilms and thus prevents NO delivery to the desired site of action. Due to their small size (<10 nm) and ability to penetrate tissue/biofilms, dendrimers may be a more viable option for the treatment of subgingival plaque biofilms associated with periodontal disease.[4] With large NO storage (>1 μmol/mg) and extended NO-release capabilities (>4.5 h),[19] NO-releasing dendrimers may serve as superior antibacterial agents against periodontal biofilms. Results to date suggest that dendrimers may prove cytotoxic against fibroblast cells, limiting their utility as periodontal disease therapeutics.[4] However, optimization of the chemical and physical properties of NO-releasing dendrimers could potentially create potent anti-biofilm agents with reduced gingival fibroblast cytotoxicity.

Although *N*-diazeniumdiolates store large payloads of NO, they undergo spontaneous decomposition in aqueous environments (i.e., the oral cavity). The rapid NO release from *N*-diazeniumdiolate NO donors upon initial application in the mouth may preclude effective NO delivery to the subgingival milieu where periodontal biofilms reside. Therefore, *S*-nitrosothiols may represent a more suitable NO donor, as they primarily undergo light-activated decomposition to release NO. These donors may reach subgingival plaque prior to the light-initiated release of their NO payload.[20,21] Targeting ligands for specific periodontopathogens may also be used to enhance scaffold-bacteria association prior to NO release. Once associated, *S*-nitrosothiol NO-

donor scaffolds can be irradiated with light to initiate NO release and disrupt biofilms. Suci et al. reported on a similar method in which *A. actinomycetemcomitans*-specific antibodies were used to target bacteria and accumulate a photosensitizer within the biofilm.[22] Light was then used to trigger the release of reactive species, resulting in significantly greater biofilm killing than without the targeting antibody. Similar methodologies using light-triggered *S*-nitrosothiols may prove beneficial for killing periodontal biofilms.

As discussed in Chapter 1, periodontal disease involves the host immune response breaking down periodontal tissue and bone, which can result in tooth loss. Therefore, the clearance of periodontopathogens is desirable to prevent further breakdown of periodontal structures, but does not reverse the damage caused by infection. The combination of extended NO-release kinetics with tissue regenerative strategies should be a primary focus for next generation NO-releasing periodontal disease therapeutics. Nitric oxide-releasing chitosan has been shown to have superior anti-biofilm activity against *P. aeruginosa* biofilms with no significant L929 mouse fibroblast toxicity *in vitro*.[23] Chitosan has also been shown to stimulate cell proliferation in gingival fibroblasts both independently and synergistically with specific growth factors (e.g., platelet-derived growth factor).[24] Additionally, chitosan is small and biodegradable, making it well-suited as a periodontal pocket insert for localized and extended NO delivery.[23,24] Chitosan can also be electrospun to form fibrous inserts with excellent osteoblast adherence properties that preserve cell phenotype and activity.[25] Therefore, electrospun chitosan nanofibers have great potential as drug release platforms for tissue engineering and remodeling applications, specifically as NO-releasing periodontal disease therapeutics.

6.2.4 Polymicrobial biofilms

As discussed in Chapter 1, dental plaque biofilms are complex, multi-organism communities comprised of both aerobic and anaerobic bacteria.[26-29] As such, a more accurate representation of *in vivo* biofilms would include polymicrobial biofilms versus the over-simplified monomicrobial *S. mutans* biofilms studied herein. Streptococci species (e.g., *S. mutans*) represent the initial colonizers,[27] but other bacteria can act as co-colonizers (i.e., *F. nucleatum*) that serve as a bridge between early and late colonizers (i.e., anaerobic bacteria).[27,30] Therefore, the layered, multi-step growth of complex polymicrobial biofilms is essential to more accurately study the utility of NO-releasing dental therapeutics. A possible challenge will be quantifying the individual bacteria species present in a polymicrobial biofilm both before and after treatment to accurately assess NO-releasing material performance on biofilm disruption and killing. Fluorescence in situ hybridization (FISH), in conjunction with confocal microscopy, can be used to visualize and quantify individual bacterial species within a complex, polymicrobial community. Bacteria specific probes are used in conjunction with FISH that can selectively target dental disease-relevant streptococci species, *A. actinomycetemcomitans*, *T. forsythensis*, *P. gingivalis*, and *T. denticola*.[31-33] Fluorescence measurements can then identify and quantify individual bacteria strains amongst a consortium of pathogens. Additionally, specific growth conditions (i.e., aerobic versus anaerobic) and selective plating methods could provide accurate bacteria colony counts of distinct species within the polymicrobial biofilm. For example, trypticase soy-serum-bacitractin-vacomycin (TSVB) and malachite green bacitracin (MGB) agar plates are selective for *A. actinomycetemcomitans*,[34,35] whereas *P. gingivalis* will form black colonies on Brucella agar plates supplemented with 5% defibrinated horse blood.[36] The polymerase chain reaction (PCR) with

strain-specific primers can also be used to isolate, amplify, and selectively detect pathogens within a mixed bacterial population (e.g., *A. actinomycetemcomitans* and *P. gingivalis*).[36,37]

6.3 Conclusions

The work described herein describes the potential utility of NO-release vehicles for the management of oral health diseases (i.e., dental caries and periodontal disease). Altering the physical and chemical properties of the NO-release scaffolds significantly affected the resulting bactericidal efficacy, anti-biofilm activity, and cytotoxicity. Macromolecular NO-release scaffolds were more efficient than small molecule NO donors at delivering lethal doses of NO to bacteria. Regardless of NO-release vehicle, Gram-positive cariogenic bacteria were less susceptible to NO treatment than Gram-negative periodontopathogens. Slower NO-release kinetics proved to be beneficial for killing periodontopathogens, whereas faster NO-release kinetics were beneficial in eradicating cariogenic bacteria. The low concentrations of slow NO-releasing silica particles proved relatively non-toxic to HGF-1 cells, supporting further investigation into extended NO-release for the treatment of periodontal disease. Alkyl-modified NO-releasing dendrimers were capable of eradicating *S. mutans* biofilms and bactericidal efficacy was increased with longer alkyl chain modifications and at lower pH (6.4). The NO-releasing long alkyl chain (i.e., octyl and dodecyl) dendrimers demonstrated superior anti-biofilm activity and reduced cytotoxicity versus non-NO-releasing long alkyl chain dendrimer scaffolds, supporting additional studies for their use as dental caries therapeutics. While this research represents preliminary work in the area, the results described herein have implications to guide the future development of NO-releasing materials for the treatment of oral infections.

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