Acta Biomaterialia xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Anti-biofilm action of nitric oxide-releasing alkyl-modified poly(amidoamine) dendrimers against *Streptococcus mutans*

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ARTICLE INFO

Article history:
Received 10 July 2015
Received in revised form 3 September 2015
Accepted 14 October 2015
Available online xxxx

Keywords:
Streptococcus mutans
Nitric oxide
Dendrimers
Dental caries

ABSTRACT

The effect of nitric oxide (NO)-releasing dendrimer hydrophobicity on *Streptococcus mutans* killing and biofilm disruption was examined at pH 7.4 and 6.4, the latter relevant to dental caries. Generation 1 (G1) poly(amidoamine) (PAMAM) dendrimers were modified with alkyl epoxides to generate propyl-, butyl-, hexyl-, octyl-, and dodecyl-functionalized dendrimers. The resulting secondary amines were reacted with NO to form *N*-diazeniumdiolate NO donor-modified dendrimer scaffolds (total NO ~1 μmol/mg). The bactericidal action of the NO-releasing dendrimers against both planktonic and biofilm-based *S. mutans* proved greatest with increasing alkyl chain length and at lower pH. Improved bactericidal efficacy at pH 6.4 was attributed to increased scaffold surface charge that enhanced dendrimer–bacteria association and ensuing membrane damage. For shorter alkyl chain (i.e., propyl and butyl) dendrimer modifications, increased antibacterial action at pH 6.4 was due to faster NO-release kinetics from proton-labile *N*-diazeniumdiolate NO donors. Octyl- and dodecyl-modified PAMAM dendrimers proved most effective for eradicating *S. mutans* biofilms with NO release mitigating dendrimer scaffold cytotoxicity.

Statement of significance

We report the antibacterial and anti-biofilm efficacy of dual-action nitric oxide (NO)-releasing dendrimers against *S. mutans*, an etiological agent in dental caries. This work was undertaken to enhance the anti-biofilm action of these scaffolds by employing various alkyl chain modifications. Furthermore, we evaluated the ability of NO to eradicate cariogenic biofilms. We found that at the lower pH associated with dental caries (pH ~6.4), NO has a more pronounced antibacterial effect for alkyl modifications less capable of biofilm penetration and membrane disruption. Of greatest significance, we introduce dendrimers as a new macromolecular antibacterial agent against the cariogenic bacteria *S. mutans*.

1. Introduction

Dental caries is one of the most costly and prevalent diseases worldwide, with 94% of the population experiencing cavities [1,2]. 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]. Although the oral microbiome is complex and exhibits extreme microbial diversity, a select few bacteria are overwhelmingly acidogenic and strongly associated with the etiology of dental caries. These cariogenic bacteria tend to be Gram-positive, often belonging to the lactobacilli or streptococci genus [2,9,10]. While multiple bacteria are cariogenic and linked to dental caries [9], *S. mutans* is considered the main etiological agent of dental caries. Current treatments are thus focused on eliminating acidogenic *S. mutans* biofilms.

Dental plaque biofilms are more difficult to treat than planktonic bacteria for a number of reasons [4,8,11]. Secreted exopolymers create a physical boundary that limits drug (e.g., antibiotics) penetration and prevents biofilm eradication [11]. Phenotypic differences in surface-attached bacteria alter potential antibiotic targets, ultimately mitigating drug-bacteria interactions [11]. The slower metabolic activity of biofilm bacteria also decreases antibiotic efficacy [8,11]. Lastly, antibiotic action is often inhibited by either the acidic environment or gingival fluid-derived

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http://dx.doi.org/10.1016/j.actbio.2015.10.021
1742-7061/

β-lactamases within oral biofilms [8,11–13]. 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) [14].

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 [15,16]. Antiseptic mouthwash rinses including chlorhexidine (CHX; 0.20% w/w) have been used to combat oral infections [17], but with modest success. Vitkov et al. reported only minor structural alterations to the exterior of mature biofilms upon CHX exposure [18]. Moreover, CHX may cause changes in taste, mouth discoloration, mucosal irritation, and desquamation of the gums [19,20]. Due to the undesirable side effects and insufficient biofilm suppression associated with CHX, the search for new anti-plaque therapeutics remains a continuing research focus.

Nitric oxide (NO) is an endogenous, diatomic radical that plays a pivotal role in wound healing, neurotransmission, and the immune response to pathogens [21,22]. 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 [23]. The multiple bactericidal pathways of NO [23,24] make it a potent broad-spectrum antimicrobial agent with low risk for promoting bacterial resistance [25,26]. 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 [27,28].

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 [23,29–31]. More recently, we reported on the controlled delivery of exogenous NO to kill *S. mutans* [32,33]. In these studies, large instantaneous concentrations of NO were required to eradicate *S. mutans*. Such NO levels are generally toxic. To further enhance *S. mutans* killing, the use of non-depleting secondary biocides in combination with NO release was proposed to create dual-action NO-releasing antibacterial agents. Carpenter et al. previously functionalized NO donor-modified silica nanoparticles with long alkyl chain quaternary ammonium (QA) groups to improve bactericidal efficacy over the solely NO-releasing silica particles [34]. 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 (i.e., non-NO-releasing) QA-modified dendrimers and NO-releasing QA-modified silica nanoparticles [35]. Due to the reduced concentrations required to kill bacteria, NO-releasing QA-modified dendrimers proved less toxic to mouse fibroblasts than the dual-action silica particles [34,35]. The greater bactericidal action of the QA-modified NO-releasing dendrimers was attributed to both the increased cell damage via membrane intercalation of the hydrophobic alkyl chains and small size of the dendrimers allowing for greater interaction with bacteria [34–36]. Subsequently, we demonstrated that short (i.e., butyl and hexyl) alkyl chain-modified dendrimers were also effective at eradicating pathogenic *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilms, with the addition of NO release facilitating anti-biofilm action for the macromolecules not capable of biofilm penetration [37]. Due to their increased biocidal activity against nosocomial pathogens, we hypothesized that dual-action dendrimers would be more effective at eradicating the NO-resistant cariogenic pathogen *S. mutans* than NO-releasing agents alone. Herein, we report the bactericidal and anti-biofilm activity of NO-releasing alkyl-modified dendrimers against cariogenic *S. mutans* as a function of alkyl chain length, pH, and NO-release kinetics.

2. Materials and methods

2.1. Materials

S. mutans (ATCC #27517) and human gingival fibroblast cells (HGF-1; ATCC CRL-2014) were 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). Hydroxyapatite (HA) disks (5 × 2 mm) were purchased from Clarkson Chromatography Products, Inc. (South Williamsport, PA). Common laboratory salts and solvents, including Tris(hydroxymethyl)aminomethane (Tris) and Tris hydrochloride, were acquired from Fisher Scientific (Fair Lawn, NJ). Dulbecco's modified Eagle's medium (DMEM) and Dulbecco's phosphate buffered saline (PBS) were purchased from Lonza Group (Basel, Switzerland). Trypsin, penicillin streptomycin (PS), phenazine methosulfate (PMS), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS), 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). Sodium methoxide (NaOMe; 5.4 M solution in methanol) was bought 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.

2.2. Synthesis of alkyl chain-modified nitric oxide-releasing dendrimers

Generation 1 (G1) poly(amidoamine) (PAMAM) dendrimer core scaffolds were synthesized as described previously [38,39]. Primary amine-terminated 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 M ratio (TEA:alkyl epoxide: primary amines on the PAMAM dendrimer) to synthesize propyl-, butyl-, hexyl-, octyl-, and dodecyl-functionalized G1 PAMAM dendrimers, respectively [37]. After 3 d of constant stirring, the MeOH solvent and unreacted epoxides were removed under vacuum to purify the dendrimer product. Purification was confirmed via ^{1}H NMR as described previously [37].

Representative ^{1}H NMR data of alkyl chain-modified G1 PAMAM included the following peaks. G1 propyl: ^{1}H NMR (400 MHz, MeOD, δ) 2.28 (s, $NCH_2CH_2C(O)NH$), 1.08–1.03 (t, $NHCH_2CH(OH)CH_2CH_3$). G1 butyl: ^{1}H NMR (400 MHz, MeOD, δ) 2.28 (s, $NCH_2CH_2C(O)NH$), 1.41–1.35 (m, $NHCH_2CH(OH)CH_2CH_3$), 0.87–0.85 (t, $NHCH_2CH(OH)CH_2CH_3$). G1 hexyl: ^{1}H NMR (400 MHz, MeOD, δ) 2.28 (s, $NCH_2CH_2C(O)NH$), 1.34–1.20 (m, $NHCH_2CH(OH)C(H_2)_3CH_3$), 0.85–0.81 (t, $NHCH_2CH(OH)C(H_2)_3CH_3$). G1 octyl: ^{1}H NMR (400 MHz, MeOD, δ) 2.29 (s, $NCH_2CH_2C(O)NH$), 1.35–1.23 (m, $NHCH_2CH(OH)(CH_2)_5CH_3$), 0.83–0.80 (t, $NHCH_2CH(OH)C(H_2)_5CH_3$). G1 dodecyl: ^{1}H NMR (400 MHz, MeOD, δ) 2.30 (s, $NCH_2CH_2C(O)NH$), 1.40–1.20 (m, $NHCH_2CH(OH)(CH_2)_9CH_3$), 0.83–0.80 (t, $NHCH_2CH(OH)C(H_2)_5CH_3$).

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 M equivalent NaOMe (with respect to the primary amines on the

Please cite this article in press as: C.J. Backlund et al., Anti-biofilm action of nitric oxide-releasing alkyl-modified poly(amidoamine) dendrimers against *Streptococcus mutans*, Acta Biomater. (2015), http://dx.doi.org/10.1016/j.actbio.2015.10.021