A novel, nitric oxide-releasing elastomeric chain for antimicrobial action: proof of concept

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Keywords: chain, elastomeric, nitric oxide, orthodontic, antibacterial, SNAP

Abstract

Introduction. Microbial colonization of orthodontic elastomeric chains is a two-fold problem: (1) plaque promotes carious lesions during orthodontic treatment and (2) bacterial by-products can degrade a chain’s mechanical properties. Efforts to combat this colonization have included the development of materials with antimicrobial activity. Recently, biomedical research has focused on the antibacterial properties of S-Nitroso-N-acetylpenicillamine (SNAP), a synthetic Nitric Oxide (NO) donor that exhibits extended NO release when incorporated into low water-uptake polymers. The objective of this study is to generate an antibacterial orthodontic elastomeric chain using this technology.

Methods. Elastomeric power chain is impregnated with the SNAP molecule and chains are evaluated for their Nitric Oxide release kinetics. The chains are then tested for their antibacterial efficacy against a common oral pathogen, Streptococcus mutans. Results. Existing elastomeric chains are successfully impregnated with SNAP and show NO-release over a three-day period. Experimental chains demonstrate good S. mutans inhibition on and around the chain surface over a 48 h period.

Conclusions. Nitric Oxide-Release technology can be applied to products in the dental field and may allow for biomimetic materials that would help to reduce bacteria-related pathologies such as white spot lesions and gingivitis.

1. Introduction

Since the advent of bonded fixed appliances for orthodontic treatment, poor oral hygiene, and the associated development of white spot lesions (WSLs) has been a major concern. If careful hygiene is not practiced, adolescents and adults alike can develop lesions around brackets which, at times, can warrant the removal of braces and interruption of treatment. Patients who develop one or more WSLs during fixed orthodontic treatment have been reported to be as high at 46% [1]. This incidence is concerning in a profession whose main goal is to improve the health of the dentition, not damage it. Though efforts have been made to reduce the risk for WSLs, no modality has been successful in completely eradicating this problem, and further research is needed to elucidate more effective preventative and treatment options.

Recent research into the prevention of WSLs has included the development and testing of materials with antimicrobial activity [2–4]. Efforts have focused on the fabrication of materials such as gels, varnishes, elastomeric ties, cements, etc. that incorporate molecules such as fluoride, chlorohexidine, nanoparticles of silver, and hydroxyapatite in hopes of creating an anti-cariogenic environment near the bracket interface [2...
Some studies incorporating nanotechnology have been particularly successful in demonstrating biofilm inhibition in vitro. However, many of these materials have not been translated into clinical practice due to constraints such as short duration of action (i.e. fluoride releasing cements can only act for the first few days after bonding), poor biocompatibility (i.e. chlorhexidine releasing cements have been shown to initiate inflammatory reactions), and lack of defense against specific oral pathogens such as Streptococcus mutans [8].

Recently, biomedical research has focused on a novel technology, nitric oxide-releasing polymers and nanoparticles. Nitric oxide (NO), a gaseous free radical, is innately present in the human body making it an optimal, biocompatible substance for use in biomaterials. NO is produced enzymatically by nitric oxide synthase (NOS) in the presence of oxygen from L-arginine [9]. There are three nitric oxide synthases produced by eukaryotic cells: endothelial NOS (eNOS), neuronal NOS (nNOS), and the inducible form, iNOS [10]. It is the iNOS form found in epithelial, endothelial, and inflammatory cells, whose expression is upregulated by cytokines, microbes, or bacterial products [10]. This gaseous free radical innately present in the human body displays a variety of physiological functions such as prevention of platelet activation and adhesion, signaling the immune system response, promoting angiogenesis in wound healing and inhibition of bacterial proliferation and biofilm formation [11–14]. NO exhibits antibacterial properties that help to break up and prevent surface biofilms. In the oral environment, Carossa et al. showed that NO levels increased during periods of plaque deposition, likely due to a host defense mechanism against bacterial proliferation [15]. NO exhibits nitrosative and oxidative actions [16]. Also, upon reaction with oxygen or reactive oxygen intermediates (i.e. superoxide and hydrogen peroxide) species including peroxynitrite, RSNOs, nitrogen dioxide, dinitrogen trioxide, and dinitrogen tetroxide can form [16]. These reactive species may disrupt cellular functions and structures by interacting with microbial proteins, DNA and metabolic enzymes [11]. Additionally, gaseous NO and nitrite (NO’s form in water or saliva) have also shown to effectively inhibit the growth of oral microbes such as Streptococcus mutans and Lactobacillus casei, particularly in an acidic environment [17]. As such, a material that might be able to mimic the body’s NO-release response could be of use for preventing oral bacteria-related pathologies.

Recent polymer research has aimed at controlling the release of NO from materials through methods such as photoinitiation [18], temperature regulation [19], and external molecule interactions [20]. These approaches have extended the duration of release. Polymers used for generating biomedical devices have proven capable of inhibiting biofilm formation for up to 4 weeks [21]. A particularly promising NO-donor molecule, S-nitroso-acetylpenicillamine (SNAP), has recently been incorporated into rubber-like materials including silicones and polyurethanes [21–23]. S-Nitrosothiols (RSNO) represent an endogenous class of NO donors and natural NO transporters within blood and tissues [24]. Belonging to this family, SNAP is reportedly one of the most stable NO donors available, due to its intermolecular hydrogen bonding and its promising display of fabrication of long-term NO releasing polymeric materials [25]. SNAP is retained in the biomedical polymers until it degrades into its component molecules, NO and NAP, both naturally occurring substances in the body. In fact, NAP has already been used to treat cystinuria at dosages of 2–4 g d −1 [21, 26]. Incorporated into devices such as urinary and arterial catheters, SNAP has shown good NO-release kinetics and positive antimicrobial action both in vivo and in vitro [27, 28], but this technology has not yet been translated into dental materials. Herein, we incorporate SNAP into an existing orthodontic elastomeric chain ligature, characterize the kinetics of NO flux from the chains, and test this new material for antimicrobial defense against S. mutans.

2. Material & methods

2.1. SNAP impregnation

SNAP (figure 1) was incorporated into pristine elastomeric chain (6 loops, 17 mm long) (Rocky Mountain Orthodontics, Denver, CO) using a swell-encapsulate-shrink method (figure 2). SNAP was dissolved in 5 ml glass vials in 4 ml THF to develop three solutions of concentrations 75, 125, and 200 mg mL −1, respectively. Controls vials contained 4 ml THF without SNAP. Chain ligatures (experimental and control) were suspended in the vials for 1 h, then removed from solution, washed with PBS buffer, and allowed to dry in the dark for 48 h in a fume hood to remove all excess solvent. Successful impregnation was verified visually by the characteristic green color of SNAP (figure 3). Elastomeric chains were stored in dry, amber glass vials in the dark.

2.2. NO release testing

Nitric Oxide flux from a single chain (from the 75 mg mL −1 concentration, surface area = 0.773 cm²) was measured daily for 4 d using a Sievers Chemiluminescence Nitric Oxide Analyzer (figure 4) to determine duration and magnitude of NO release. Chains were placed in a glass vessel (placed in a water bath at 37 °C) containing 4 ml of PBS (figure 3). Nitric oxide released from the chain was purged from the buffer and swept from the headspace using an N² sweep gas and bubbler into the detection chamber. Readings were continued
Figure 1. SNAP structural formula.

Figure 2. Schematic of swell-encapsulation-shrink method for fabricating chains.

Figure 3. Control chain and chains swelled in concentrations of 200, 125, and 75 mg ml$^{-1}$ SNAP in THF (top to bottom), surface area $= 0.776$ cm$^2$. 
until a steady state flux was achieved. When not being tested, chains were stored in amber glass vials in 1 ml PBS at 37 °C.

2.3. Bacterial testing
Antibacterial action of the chains from the 75 mg SNAP 4 ml⁻¹ THF solution was tested against the common oral microbe, *Streptococcus mutans*, in three separate microbial studies to determine biofilm formation on surfaces surrounding the chain, planktonic growth surrounding the chain, and biofilm formation on the chain itself.

2.4. Biofilm on surrounding surfaces
In a 12-well plate, a standardized solution of *S. mutans* in PBS was added to a 1 ml solution of brain-heart infusion (BHI) supplemented with 2% sucrose. Each well contained either: (1) a single experimental chain (2) two experimental chains (3) a single control chain or (4) two control chains. Three of each combination were included for a total of 12-wells. All chains were suspended in the solution and incubated at 37 °C, anaerobically with shaking, for a period of 48 h. Chains were removed, wells were washed with 2 ml PBS, and 500 μl crystal violet stain was added to each well and incubated for 15 min. Wells were washed again with tap water and 2 ml acetic acid was added to each well. 0.2 ml of solution was removed and absorbance was measured at 570 nm (figure 5).

Additionally, the amount of Lactate Dehydrogenase (LDH), and enzyme present during cell lysis, was measured using a CytoTox 96® Non-Radioactive Cytotoxicity Assay. 1 ml of medium was removed from each of the wells and centrifuged. 100 μl of supernatant was added to 50 μl substrate and incubated at room temperature for 22 h. Absorbance was measured at 490 nm. All tests were performed in triplicate.

2.5. Planktonic growth in surrounding solution
A standardized solution of *S. mutans* in PBS (OD = 0.2) was added to a vial with 5 ml solution of BHI + 2% sucrose. Each vial contained either: (1) a single experimental chain (2) two experimental chains (3) a single control chain or (4) two control chains. Three of each combination was included for a total of 12 vials. Vials were incubated at 37 °C, aerobically, without shaking, in the dark. 1 ml of solution was removed at 2, 4, 6, 8, 10, 12, and 24 h, respectively and OD was measured and recorded.

2.6. Biofilm formation on chain surface
Experimental and control chains (3 of each) were place in chamber wells with BHI + 2% sucrose medium and a standardized solution of *S. mutans*. Chains were incubated at 37 °C for 48 h and cell viability was measured with
a Celltiter-Flo Luminescent Cell Viability Assay. Chains were removed, placed in 1.5 ml PBS and sonicated for 1 min with 30 s rest the another 1 min. Sonication was repeated in 1.5 ml fresh PBS and both solutions were centrifuged and cell pellets were removed and combined. Cell pellets were placed in a 96-well plate and 100 μl of CellTiter-Glo® Reagent was added to each well and incubated for 10 min. Luminescence was measured. Tests were performed in duplicate.

Confocal Laser Scanning Microscopy was completed with two experimental and two control chains.

3. Results

3.1. SNAP impregnation
SNAP was successfully incorporated into existing clear elastomeric chains at three concentrations. All experimental and control chains returned to the same dimensions after shrinking and experimental chains exhibited a light to dark gradient of green color from lowest to highest concentration, respectively (figure 3). Upon stretching the chains soaked in concentrations of 125 and 200 mg ml⁻¹ SNAP + THF, the material broke under tensile stress. Chains soaked in 75 mg ml⁻¹ solution as well as control chains maintained their elastic properties.

3.2. NO release testing
Experimental chain exhibited a large burst of NO release followed by a sharp decrease then steady-state NO flux at 2 h on each day testing with the NOA. On the first day of testing, the chain exhibited NO released above the reading capability of the machine, but returned to a steady state flux of 5.3 × 10⁻⁹ mol min⁻¹ cm⁻² (figure 6). This flux gradually decreased over the following three days. On the fourth day of testing, the EC showed no NO release over baseline (figure 6).
3.3. Bacterial testing

3.3.1. Biofilm on surrounding surfaces
Wells containing experimental chains had a statistically significant decrease in biofilm formation on the well surface ($p < 0.05$) compared to control chains (figure 7). LDH Assay demonstrated a significantly higher level of LDH activity ($p < 0.05$) in the medium with experimental chains compared to controls. (figure 8).

3.4. Planktonic growth in surrounding solution
Growth curves in solutions containing experimental chains versus control exhibited markedly lower growth by 6 h and inhibition was maintained for 24 h (figure 9). Statistically significant inhibition was present in the vials with two chains at 8 h and was maintained for 24 h ($p < 0.05$). Single experimental chains exhibited an inhibition of 28% at 24 h. Two chains exhibited an inhibition of 49% at 24 h. At each time point, inhibition exhibited by two chains was nearly twice that of a single chain. (figure 10).

3.5. Biofilm formation on chain surface
Cell viability assay demonstrated a nearly 3-log decrease in cell viability on the experimental chains compared to controls (figure 11). Scanning microscopy demonstrated lower and thinner biofilm formation on the experimental chains compared to controls (figure 12).
4. Discussion

An effective, long-acting antibacterial biomaterial would be of great benefit in the effort to combat bacteria-related pathologies such as white spot lesions and gingivitis in orthodontics. This study demonstrates the successful incorporation of a nitric oxide releasing molecule, SNAP, into the polymer matrix of an existing elastomeric chain. These chains’ antibacterial effectiveness will be largely dependent on three factors including (1) amount of SNAP that can be incorporated into the material (2) SNAP’s degradation rate and thus how much nitric oxide is released over time and (3) the susceptibility of the microbe to the nitric oxide molecule. We explored each of these factors in this study.

**SNAP Loading** In generating the chains, the two solutions with the highest SNAP concentrations generated chains that did not maintain their elastic properties, likely due to the amount of SNAP molecule loaded into the chains and its interactions with the polymeric network within the material. The chains soaked in solution of 75 mg ml\(^{-1}\) SNAP + THF, however, did maintain their ability to stretch. We thus hypothesize that the limit for SNAP loading is somewhere between 75 and 125 mg ml\(^{-1}\). Future testing will be needed to determine whether the SNAP molecule has any effect on the chain’s force degradation rate, as the chains will still need to be effective clinically.
NO-Release
The kinetics of NO-release was characterized by a large burst of NO on the first day at a flux of $5.3 \times 10^{-9}$ mol min$^{-1}$cm$^{-2}$ with a gradual decrease of flux over the following three days and no significant release by the fourth day of testing (figure 5). Physiological nitric oxide flux levels from human endothelial tissue has been reported to be in the range of $1-5 \times 10^{-10}$ mol cm$^{-2}$ min$^{-1}$ [29]. The flux generated by our chain over the first three days was consistently higher, by a factor of 10–50, than these physiologic flux levels. While this amount of nitric oxide is likely to be effective against microbes, it poses two problems in that (1) it depletes that SNAP stores quickly, so release is only sustained over the first three days and (2) the amount of NO released could become toxic to native tissues such as oral mucosa. Efforts in future research will focus on decreasing this flux by means of a secondary polymeric coating after SNAP loading or use of a different, potentially less degrading solvent to generate the chains. Ideally, flux would be tailored so that it is clinically effective but is also long acting and nontoxic.

Antimicrobial Defense
The experimental chains showed good effectiveness in defense against the oral pathogen, *Streptococcus mutans*. Previous reports have noted that cariogenic microbes such as, *S. mutans* and *S. sanguis*, are less susceptible to NO than periodontal pathogens such as *Porphyromonos gingivalis* [30]. This is likely due to the organism’s use of the enzyme, nitrite reductase, which converts NO to nitrite in NO-rich environments [31]. Regardless, the high levels of NO-release over a three-day period in our study appeared to

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Figure 11. ATP cell viability assay of bacterial cells attached to chain surface at 48 h.

Figure 12. Confocal scanning light microscopy of control chains (top) and SNAP-incorporated chains (bottom) demonstrating biofilm formation on the chain’s surface.
supersede the microbe’s ability to avoid NO’s bactericidal effects, as the chains were effective in decreasing bacterial growth both around and on the chains themselves. If this level of nitric oxide proves to be toxic to mucosal cells, however, chains with slower NO release will need to be fabricated, and it may be that the effectiveness against S. mutans decreases. If so, these chains may, instead, prove to be more effective against a more susceptible microbe such as P. gingivalis.

Biofilm formation on surfaces adjacent to the chain and planktonic growth in solution were both significantly decreased with experimental chains compared to controls and higher levels of cell lysis, as indicated by LDH levels, was seen in solutions with the experimental chains. This inhibition becomes important when considering the chains’ application for reducing plaque on tooth and gingival surfaces. Furthermore, when two chains (12 links total) were placed in solution, nearly twice the growth inhibition was achieved compared to with single chains (6 links total). This demonstrated the importance of total surface area for NO release and influences the application and adaptability of this technology to dental materials; for example, elastomeric chains with NO-release will likely be more effective than single elastomeric ties due to their increased surface area.

Biofilm formation on the chain itself was also reduced significantly for the experimental chains. Elastic chains have been shown to have higher rates of force degradation in the mouth due to environmental factors. It is hypothesized that a portion of this force degradation is due to the acidic bacterial bi-products that are produced by the surface biofilm [32–34]. The acidic molecules tend to degrade the polymer structure and thus decrease effectiveness over time. Our chain’s inhibition of biofilm formation on the chain’s surface, therefore, may be of use in reducing the rate of force degradation clinically.

Similar studies have been conducted using S-nitroso-acetylpenicillamine to demonstrate its anti-microbial action. Many showcased effective nitric oxide leaking and antagonistic properties to common microbes. In a study by Brisbois et al SNAP incorporated into Elasteon E2As polymer catheters using a dip coating method and the trilayer configuration exhibited nitric oxide release at physiological levels ($>0.5 \times 10^{-10}$ mol cm$^{-2}$ min$^{-1}$) for up to 20 d [22]. In 2013, Vogt et al, fabricated a nanofibrous gelatin matrix functionalized with SNAP [35]. Effective clearing of S. aureus was observed as the SNAP−gelatin successfully created a zone of inhibition when light activated the NO release [35].

Colleta et al also impregnated catheters with SNAP, characterized their NO flux and biofilm formation against Staphylococcus epidermidis and Proteus mirabilis [23]. The proposed catheters generated NO surface-fluxes $>0.7 \times 10^{-10}$ mol cm$^{-2}$ min$^{-1}$ for over one month. S. epidermidis adhered on the surface of SNAP catheter tubing after growing biofilms for 7 and 14 d was reduced by 2.5 and 3.5 log units, respectively, compared to controls. Cell counts for 7 and 14 d P. mirabilis biofilms grown on the surface of SNAP-impregnated catheter tubing were 5 and 6 log units lower than those for controls [23].

There have been reports of successful storage and sterilization of technologies incorporated with SNAP [21]. Goudie et al demonstrated retention of approximately 90% active SNAP content in SNAP + E2A polymers after ultraviolet radiation, ethylene oxide, and hydrogen peroxide vapor sterilization. The SNAP-incorporated E2As stored at room temperature for over 6 months retained 87% of its initial SNAP content, as well [21].

SNAP is reportedly one of the most stable NO donors available, due to its intermolecular hydrogen bonding and its promising display of fabrication of long-term NO releasing polymeric materials [25]. Although the chains in our study demonstrated NO flux consistently higher than physiologic flux levels for the first three days, future research focusing on extending the nitric oxide release may prove beneficial against bacteria-related oral pathologies.

5. Conclusions

The nitric-oxide releasing molecule, S-Nitroso-N-acetylpenicillamine, can be successfully incorporated into an existing Rocky Mountain orthodontic elastomeric chain. These chains achieve NO-release over a three-day period and inhibit Streptococcus mutans growth both on and around the chain’s surface for the first 24–48 h of use. Future research will focus on extending nitric oxide release and investigating the chains’ cytotoxic effects and mechanical properties.

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

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