Tooth-binding Micelles for Dental Caries Prevention

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ABSTRACT

Maintenance of the effective local concentration of antimicrobials on the tooth surface is critical for management of cariogenic bacteria in the oral cavity. In this study, we report the design of a simple tooth-binding micellar drug delivery platform that would effectively bind on tooth surfaces. To achieve tooth-binding ability, the chain termini of biocompatible Pluronic® copolymers were modified with a biomineral-binding moiety (i.e. alendronate). Micelles formulated with this polymer were shown to be able to swiftly (< 1 min) bind to hydroxyapatite (HA, model tooth surface) and gradually release the encapsulated model antimicrobial (farnesol). These tooth-binding micelles were negatively charged and had an average effective hydrodynamic diameter ($D_{\text{eff}}$) of less than 100 nm. In vitro biofilm inhibition studies demonstrated that the farnesol-containing tooth-binding micelles were able to provide significantly stronger inhibition of *Streptococcus mutans* UA159 biofilm formation on HA discs compared to the untreated blank control ($p < 0.0001$). Upon further optimization, this delivery platform could provide an effective tool for caries prevention and treatment.
Dental caries is defined as the localized destruction of susceptible dental hard tissues by acidic by-products from bacterial fermentation of dietary carbohydrates (25). Overpopulation of the oral cavity by acid-producing bacteria is one of the three main pathological factors highlighted in the cariogenic process (6). Therefore, an important strategy to control or even eradicate dental caries is to target the bacterial aspect of the disease (5). Successful antimicrobial therapy against cariogenic bacteria largely depends on two major factors at the hard-tissue level: the specificity of the chemotherapeutic agent and the maintenance of its effective local concentration. Given that most of the available antimicrobial compounds do not have hard tissue specificity (19), it is critical to develop mechanisms for retaining these antimicrobials at the tooth surface, thereby maintaining their effective local concentration and improving their antimicrobial efficacy (4, 16).

Various delivery systems have been developed to maintain drug concentration in the oral cavity. These include bioadhesive tablets (1, 7, 18), bioadhesive patches/films (22, 26), and bioadhesive gels and semisolids (10, 24, 29). However, these formulations provide the highest drug concentration at mucosal epithelia instead of the tooth surface. Furthermore, local irritation at the site of adhesion and the uncomfortable sensation of a foreign object often lead to poor patient compliance (20, 27). To bring direct and long-lasting interaction of
antimicrobials with the tooth surface, varnish formulations have also been
developed (17, 28) which are generally applied by dental health practitioners
during routine office visits. However, the long-term benefit of these periodic
treatments is limited due to the episodic nature of dental caries.

We report here the design of a novel tooth-binding micellar delivery platform that
would effectively maintain drug concentration on the tooth surface. By covalently
attaching a prototype biomineral-binding moiety (alendronate) to the chain termini
of biocompatible Pluronic® copolymers (11), these micelles are predicted to bind
to hydroxyapatite (the model tooth surface used in these studies) immediately
upon exposure. The immobilized micelles should be able to act as a drug
reservoir and gradually release the encapsulated antimicrobial. Farnesol (3,7,11-
trimethyl-2,6,10-dodecatrien-1-ol), a recently identified anti-caries natural product
found in propolis with a MIC of 125 µM (28 µg/mL) for *S. mutans* UA159 (12-14),
was chosen as the model drug for micelle encapsulation. In the study presented
here, it was found that the micellar-farnesol formulation is capable of providing
near-complete inhibition of biofilm formation by the cariogenic bacterium *S.
mutans*. It is anticipated that the tooth-binding micelles have the potential to be
formulated into mouth rinses that may have the merits of simple application,
cultural acceptance and improved patient compliance.

MATERIALS & METHODS
Chemicals

Pentynoic acid 2,5-dioxo-pyrrolidin-1-yl ester (compound 1) and 1-hydroxy-4-pent-4-ynamidobutane-1,1-diylphosphonic acid (compound 2) were synthesized as previously described (15). Alendronate (ALN) was purchased from Ultratech India Ltd. (New Mumbai, India). Farnesol was obtained from TCI America (Portland, OR). Hydroxyapatite particles (HA, DNA grade Bio-Gel HTP gel) were purchased from Bio-Rad (Hercules, CA). Hydroxyapatite discs (0.5’ diameter × 0.04-0.06’ thick) were purchased from Clarkson Chromatography Products, Inc. (South Williamsport, PA). LH-20 resin was purchased from GE Healthcare (Piscataway, NJ). Pluronic® copolymers (P85 and P123) were generously provided by BASF Corporation (Florham Park, NJ). All other reagents and solvents if not specified were purchased from either Fisher Scientific (Pittsburgh, PA) or Acros Organics (Morris Plains, NJ).

Methods

1H NMR spectra were recorded on a Varian Inova Unity 500 NMR Spectrometer. UV-visible spectra were measured on a Shimadzu UV-1601PC UV-Visible Spectrophotometer. Electrophoretic mobility measurements were performed using a “ZetaPlus” analyzer (Brookhaven Instrument Co.). Zeta-potential (ζ) of the micelles was calculated from the electrophoretic mobility values using the Smoluchowski equation. Effective hydrodynamic diameters (D_{eff}) of the micelles were measured by photon correlation spectroscopy (DLS) using the same instrument equipped with a Multi Angle Sizing Option (BI-MAS). An Agilent 1100...
HPLC system with a quaternary pump (with degasser), an autosampler, a fluorescence detector and a diode-array based UV detector was used for drug release analysis.

**Synthesis of p-toluenesulfonyl terminated Pluronic® 123 (Tos-P123, 3).**

P123 (10.5 g, 2 mmol) was dried by azeotropic evaporation with toluene (3 × 50 mL) and dissolved under argon in anhydrous dichloromethane (DCM, 20 mL) together with 4-dimethylaminopyridine (DMAP, 0.122 g, 1 mmol) and triethylamine (TEA, 2.02 g, 20 mmol). The reaction mixture was cooled to 0°C and p-toluenesulfonyl chloride (3.81 g, 20 mmol) was added. After overnight reaction at room temperature, the mixture was washed with hydrochloric acid (0.1 M, 2 × 10 mL), water (2 × 10 mL), saturated NaCl solution (brine, 2 × 10 mL) and then dried over anhydrous magnesium sulfide. After removal of the solvent under reduced pressure, the crude product was further purified by LH-20 column. The yield of polymer was 60% (yield = weight of the purified product/theoretical weight of the product × 100%). The ratio of tosylation was determined as 100% by ¹H NMR. ¹H NMR (DMSO-d₆) δ (ppm) 7.79 (d, J = 8.29 Hz), 7.48 (d, J = 8.29 Hz), 4.11 (t, J = 4.88 Hz), 3.65-3.43 (m), 1.04 (d, J = 4.39 Hz).

**Synthesis of azide terminated Pluronic® 123 (Azido-P123, 4).** Tos-P123 (1.64 g, 0.27 mmol) was dissolved in dimethylformamide (DMF, 20 mL). Sodium azide (0.176g, 2.7mmol) was then added. The reaction proceeded with stirring at 100 °C for 1 day. After filtration and solvent removal, the crude product was
dissolved in DCM, washed with water (2 × 10 mL) and brine (2 × 10 mL) and then dried over anhydrous magnesium sulfide. After removal of the solvent under vacuum, the product was obtained. The yield of polymer was 96.2%. Complete azidation was supported by the disappearance of Tos signals in the $^1$H NMR spectrum. $^1$H NMR (DMSO-$d_6$) $\delta$ (ppm) 3.61 ($t$, $J = 4.88$ Hz), 3.56-3.43 ($m$), 1.04 ($d$, $J = 4.39$ Hz).

**Synthesis of Pluronic® 123-alendronate conjugate (ALN-P123, 5).** Azido-P123 (2.9 g, 0.5 mmol) and 1-hydroxy-4-pent-4-ynamidobutane-1,1-diylidiphosphonic acid (0.395 g, 1 mmol) were dissolved in EtOH/H$_2$O solution (1/1, 15 mL). Sodium ascorbate (0.198 g, 1 mmol) and copper sulfate pentahydrate (25 mg, 0.1 mmol) were then added separately under argon. The reaction mixture was allowed to stir for 3 days at room temperature. After removal of the solvent, the product was acidified and purified with a LH-20 column using methanol as the eluent. The yield of the polymeric product was 70%. The conversion ratio from azide to alendronate at P123 chain termini was estimated to be 90% by $^1$H NMR. See Figure 1 for the entire synthetic route. $^1$H NMR (D$_2$O) $\delta$ (ppm) 7.81 (s), 3.93 ($t$, $J = 4.90$ Hz), 3.80-3.39 ($m$), 3.14 ($t$, $J = 6.83$ Hz), 1.86 ($m$), 1.75 ($m$), 1.12 ($d$, $J = 7.81$ Hz).

**Preparation and Characterization of Tooth-Binding Micelle**

Different amounts of farnesol were added to aqueous Pluronic solution. The mixture was subjected to vortex mixing for 30 seconds and placed at 37 °C.
overnight with gentle agitation to allow micelle formation. The resulting micelle solutions were filtered (0.45 µm filter) and then subjected to ζ-potential and $D_{eff}$ measurements using DLS. See Table 1 for details of all micelle formulations.

**Binding Kinetics of Tooth-Binding Micelle on HA Particles**

Micelle solution (preparation 2, 6, 7 and 8 in Table 1) were mixed with HA particles (20 mg/tube) in Eppendorf centrifuge tubes. The tubes were placed on a Labquake® rotator (Thermo Fisher Scientific Inc.) to allow binding at room temperature. At each predetermined time point, 3 tubes were taken out, centrifuged (12,000 rpm, 0.5 min), and 100 µL of the supernatant were collected. The collected samples were then diluted 100 times and analyzed by HPLC with the following conditions: Agilent C$_{18}$ reverse-phase column (4.6 × 250 mm, 5 µm) was used with mobile phase of acetonitrile/water (80:20, v/v) at a flow rate of 1 mL/min. The UV detection was set at 210 nm. The amount of farnesol bound to HA particles via the micellar formulation was calculated by subtracting the amount of farnesol left in the supernatant from the initial amount of farnesol added.

**In Vitro Release of Farnesol from Tooth-Binding Micelle Immobilized on HA Particles**

Micelle solutions (preparations 2, 3 and 4) were mixed with HA particles (100 mg) for 30 min to allow binding of the micelles to HA. The mixture was then centrifuged and HA particles were washed 3 times with water to remove unbound...
micelles. The total amount of farnesol loaded on HA particles was calculated by subtracting unbound farnesol from the amount of farnesol added. The micelle-loaded HA particles were then resuspended in 1 mL of releasing medium (0.1 M PBS, pH = 7.4) and placed on a Labquake® rotator to allow drug release at 37°C. At predetermined time intervals, samples were centrifuged and all the supernatant was removed and replaced with 1 mL of fresh medium, then resuspended. The collected supernatant (0.5 mL) was mixed with acetonitrile (0.5 mL), filtered (0.2 µm) and analyzed with HPLC.

In vitro Inhibition of *S. mutans* Biofilm Growth on HA discs

*S. mutans* UA159 (21) was used in this study. *S. mutans* frozen stock cultures were maintained in 25% (v/v) glycerol at -80°C. For each experiment, *S. mutans* was streaked from a frozen stock onto Todd Hewitt-Yeast Extract (THYE; Todd Hewitt broth containing 0.3% w/v yeast extract) agar (1.5% w/v). After 48 hours growth at 37°C and 5% CO₂, a single colony of bacteria was inoculated into 3 mL of THYE broth (2) and allowed to grow statically overnight at 37°C and 5% CO₂. The next day, the overnight culture was diluted to a density of 2×10⁴ CFU/mL in chemically-defined media containing 0.25% w/v of glucose and sucrose (CDM), prepared as previously described (2).

Autoclaved HA discs were incubated with different micelle solutions (preparations 2, 3, 4 and 5 in Table 1), farnesol solution (1 w/v % in ethanol) or CDM in a 24-well plate for 1 hr. The discs were then removed from the wells and dip-washed...
with CDM 3 times to remove unbound micelles. For the farnesol-ethanol solution
group, discs were dip-washed 3 times with ethanol and then followed by washing
with CDM. The HA discs were then transferred to wells containing 1 mL of the
diluted S. mutans, and cultured statically for 48 hr to allow biofilm growth at 37°C
with 5% CO₂. CDM was changed at 24 hr.

After 48 hours growth, the HA discs were dip-washed 3 times in THYE media to
remove loosely attached bacteria and then placed in 1 mL of THYE media. The
surfaces of HA discs were gently scraped with a sterile spatula to harvest
adherent cells, and the number of viable cells in each sample was quantified
using the track dilution method (9) to achieve countable colony-forming units
(CFUs). All plates were incubated for 48 hr at 37°C with 5% CO₂ prior to
counting colonies. These experiments were repeated three times.

Statistical Analysis
The Kruskal-Wallis test, a nonparametric alternative to one-way analysis of
variance that does not assume that data follow normal distribution, was used for
overall comparison of the mean ranks of the CFUs under different types of
treatments. To evaluate specific differences between experimental groups, the
Tukey’s nonparametric multiple comparison method was used; p < 0.05 was
considered as significant in both tests.
RESULTS

Preparation and Characterization of the Tooth-Binding Micelle

The multi-step synthesis of Pluronic® 123-alendronate conjugate (ALN-P123) is critical for the success of tooth-binding micelles. Each reaction step was accomplished with reasonable yield of polymer at least 60%. For the Pluronic® 123 chain termini modification, both tosylation and azidation steps were completed with 100%. For the last step of conjugation to alendronate with click chemistry, the conversion is 90% or an average of 1.8 alendronate per Pluronic® 123 chain (Figure 1). DLS analyses (Table 1) suggest that both empty micelles and farnesol loaded non-binding micelles had the biggest particle size (~100 nm). Farnesol loaded tooth-binding micelles had a relatively smaller size, which increased as the farnesol loading was raised. In the loading range tested, however, the $D_{eff}$ of farnesol loaded tooth-binding micelles is < 100 nm.

Binding Kinetics of Tooth-Binding Micelle to HA Particles

As shown in Figure 2, the amount of micelle bound to HA particles was increased by raising the ALN-P123 content. However, the HA binding kinetics did not change significantly after ALN-P123 was increased to above 0.1% w/v (preparation 7). Preparations 7, 8 and 2 (containing 0.1% w/v, 0.2% w/v or 0.4% w/v of ALN-P123 respectively) bound quickly (< 1 min) to the HA particles and reached a binding plateau within 5 min.
In Vitro Release of Tooth-Binding Micelles

The in vitro release profile of farnesol from tooth-binding micelles that bound to HA particles was evaluated over a 48 hr period. About 10-20% of farnesol was released into the release media by 48 hours. Sustained drug release profiles were observed (Figure 3) regardless of the farnesol loading amount in the micelle formulation.

Inhibition of S. mutans Biofilm Growth on HA Discs

As shown in Figure 4, all farnesol-containing tooth-binding micelle groups (TBM, containing low, medium and high dose of farnesol) showed significantly (p < 0.0002) higher levels of biofilm inhibition when compared to untreated blank control (UBC), empty tooth-binding micelle (EBM), farnesol-containing non-binding micelle (NBM, containing same amount of farnesol as high dose TBM) and ethanol solution of farnesol (ESF, containing same amount of farnesol as high dose TBM). No significant differences were observed between the three groups treated with farnesol-containing TBM (low, medium, and high dose), as each displayed an approximate 4-log decrease in CFU/biofilm recovered from the HA discs. A rather weak but significant (p < 0.006) biofilm inhibitory effect was also observed when HA discs were treated with non-binding farnesol-containing micelle (NBM), relative to the untreated blank control (UBC), empty tooth-binding micelle (EBM) and ethanol solution of farnesol (ESF) groups. This may have been due to non-specific interaction of the micelle with the HA disc and the subsequent release of residual farnesol.
DISCUSSION

The purpose of this study was to develop a simple yet efficient drug delivery system that would maintain a long-lasting effective concentration of drug on the tooth surface. Such an approach, in combination with potent antimicrobials, may effectively prevent biofilm formation on the tooth surface by cariogenic bacteria such as *S. mutans*, and subsequent development of dental caries. The important and novel element of this delivery system is the introduction of a tooth-binding moiety that would anchor the delivery system of choice onto the tooth surface.

Alendronate, a bisphosphonate, was chosen as a prototype binding moiety because of its high affinity for hydroxyapatite crystals (the main component of tooth enamel), and the fact that it has been used clinically for the treatment of osteoporosis for many years (23). However, a potential risk of alendronate usage in an oral rinse formulation would be the systemic ingestion of the drug. Concerns have been raised about the long-term impact of bisphosphonates on bone resorptive activity and its rare association with osteonecrosis of the jaw (3). These risks would likely be minimal with a mouth rinse formulation using the micellar delivery system described here, due to (1) the chemical conjugation of alendronate to the chain termini of Pluronic® and (2) the expectoration of the vast majority of the formulation, which would result in a very low systemic dose. Biodegradable biomineral-binding moieties (e.g. acidic peptides) have been used...
Farnesol is a hydrophobic compound with a water solubility of merely 1.2 mM (8). In previous investigations, organic solvents (ethanol and DMSO) were required to assist the dissolution of farnesol in water (12-14). Using the Pluronic® block copolymers, which can readily self-assemble into micelles in aqueous solutions with a core (hydrophobic)-shell (hydrophilic) structure, farnesol can be encapsulated into the hydrophobic core of Pluronic® micelle (PPO segment) which acts as the hosting reservoir that dissolves and readily disperses the hydrophobic farnesol in water. Therefore, the benefits of this formulation may also include the prevention of irritation caused by organic solvents, and potentially-improved patient compliance.

The binding kinetics of the tooth-binding micelles, as demonstrated in Figure 2, indicates that even at relatively low binding moiety content, these micelles exhibit swift binding kinetics to HA particles. Additional studies will be performed in the future to clarify the impact of other in vivo factors (e.g. salivary pellicle) on the binding kinetics of these micelles. The small size (~ 100 nm) of the micelles developed in this study may also facilitate their ability to access locations on and between teeth that normal tooth-brushing misses and are unlikely to cause uncomfortable foreign object sensations in patients. As shown in Figure 3, the
newly designed delivery system has the potential to provide a sustained drug releasing profile and maintain a long-lasting effective drug concentration on the tooth surface. It is important to keep in mind, however, that the release of drug may become faster in the oral cavity due to the presence of physiological factors such as saliva flow.

One of the biggest challenges in dental antimicrobial therapy lies in the fact that most antimicrobials have low, if any, retention in oral cavity (especially the tooth surface). As a consequence, the level of cariogenic bacteria can be readily reestablished between each antimicrobial exposure. As demonstrated in Figure 4, neither the ethanol solution of farnesol nor the farnesol loaded non-binding micelle could very effectively prevent S. mutans biofilm formation due to the lack of farnesol retention on the HA disc surface. On the other hand, because of their ability to bind to the HA surface, tooth-binding micelles at all dosing levels demonstrated near-complete biofilm inhibition, even after extensive washing. Although only one strain of S. mutans was tested in this proof-of-principle study, future testing of the inhibitory effect of these tooth-binding micelles will extend to clinical strains of S. mutans and other cariogenic bacteria.

In summary, a tooth-binding micelle delivery platform has been designed and prepared in this study to prevent/treat dental caries. It was found to bind to the HA surface swiftly and release the encapsulated antimicrobial (farnesol) in a sustained manner. HA discs treated with this formulation could effectively inhibit
in vitro biofilm formation by cariogenic *S. mutans*. Further optimization and exploration are needed to translate this novel delivery platform into clinical application in dental caries prevention and treatment.

ACKNOWLEDGMENT

This work was supported in part by NIH grants AR053325 (DW) and AI038901 (KWB). We also acknowledge Dr. James Booth for the helpful discussion at the beginning of the project.

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Table 1. Composition and characterization of different micelle preparations.

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<th>Preparations</th>
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<th>ALN-P123 (%)*</th>
<th>Farnesol (%)*</th>
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* Percentages are all presented in weight/volume.
FIGURE LEGENDS

**Figure 1.** The synthesis of Pluronic® 123-alendronate conjugate (ALN-P123).

**Figure 2.** In vitro binding kinetic study of the tooth-binding micelle formulations (as listed in Table 1) to hydroxyapatite (HA) surface. All data were the mean ±SD, n=3. Color insert: HA particles treated with non-binding micelles (left) and tooth-binding micelles (right). Both micelles were labeled with rhodamine B (a pink dye). All data are expressed as mean ± SD, n=3.

**Figure 3.** In vitro release of farnesol from tooth-binding micelle formulations (as listed in Table 1) bound to hydroxyapatite (HA) particles. All data are expressed as mean ± SD, n=3.

**Figure 4.** Average number of colony forming units (CFU) of *S. mutans* recovered per hydroxyapatite (HA) disc after 48 hr incubation. HA discs were pretreated with the following formulations. TBM: Tooth-binding micelle (2, 3 and 4 corresponding to low, medium and high dose of farnesol); EBM: Empty tooth-binding micelle; NBM: Non-binding micelle (containing same amount of farnesol as high dose TBM); ESF: Ethanol solution of farnesol (containing same amount of farnesol as high dose TBM); UBC: Un-treated blank control. The numbers in the figure refer to the preparation # as listed in Table 1. P < 0.0001 (Kruskal-Wallis test). All data are expressed as mean ± SD, n=3.