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RESEARCH PAPER

The Development of Dentotropic Micelles with Biodegradable Tooth-Binding Moieties

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ABSTRACT

Purpose Development of dentotropic (tooth-binding) micelle formulations to improved efficacy and safety of antimicrobial therapy for dental plaque prevention and treatment.

Methods Because of their excellent biocompatibility and biodegradability, diphosphoserine peptide and pyrophosphate were selected as the tooth-binding moieties to replace alendronate, which was used previously. Diphosphoserine peptide was conjugated to Pluronic P123 using "click" chemistry, whereas pyrophosphate was attached to P123 through an ester bond. The tooth-binding micelles (TBMs) were prepared by self-assembly of the modified P123 with the antimicrobial agent triclosan. The influence of human saliva and/or its components on TBMs' drug-releasing profile, tooth-binding potential and binding stability was evaluated *in vitro*. *S. mutans* UA159 biofilm formed on hydroxyapatite (HA) discs was used to evaluate the TBMs' therapeutic potential.

Results Saliva does not affect triclosan release from TBMs. More than 60% of TBMs' HA binding capacity was maintained in the presence of saliva. Less than 5% of TBMs bound to HA was released over 24 h in human saliva, protease or phosphatase, suggesting the retention properties of the TBMs will not be compromised due to the biodegradable nature of the binding moieties. In both *in vitro* biofilm prevention and treatment studies, the TBM treated group showed significantly lower CFU per HA disc compared to the controls (2-log reduction, $p < 0.05$).

Conclusion The data from these studies suggest that the novel dentotropic micelle formulations bearing biodegradable tooth-binding moieties can be used as an effective and safe delivery tool for antimicrobials to improve dental plaque prevention and treatment.

KEY WORDS antimicrobial · biofilm · dental plaque · dentotropic · Pluronic · micelle

INTRODUCTION

Mature dental plaque is a complex multispecies biofilm that grows on the tooth surface and is embedded in a protective matrix of host and bacterial polymers (1). It initiates dental caries (decay) and periodontal inflammation (gingivitis and periodontitis) by converting dietary carbohydrates into demineralizing acids (caries) or stimulating uncontrolled inflammation and bone resorption in surrounding tissues (periodontitis) (2,3). In addition, recent publications suggest that dental plaque bacteria and periodontal inflammation may contribute to the pathogenesis of cardiovascular disease (4–6), diabetes (7,8), rheumatoid arthritis (9) and respiratory diseases (10). It is clear that uncontrolled accumulation of dental plaque has profound public health implications.

Despite the effort that has been devoted to searching for novel anti-plaque methods such as vaccines and probiotics (11), antimicrobial therapy is still one of the most effective management strategies against dental plaque related diseases (12). One major concern with antimicrobial therapy is, however, the inability to maintain a minimum inhibitory concentration (MIC) of the drug in the dental microenvironment. Thus, there is an urgent need to improve antimicrobial delivery in the oral cavity, especially to the tooth surface (11). Recently, our group has successfully developed a mineral-binding micellar drug delivery system, which could

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quickly bind to the tooth surface and release encapsulated drug over a prolonged period of time. This was accomplished by covalently conjugating a tooth-binding moiety, alendronate, to the termini of Pluronic copolymer using "click" chemistry (13,14). This approach not only increased the water solubility of the non-ionic antimicrobial drug, triclosan, but also enhanced retention of the drug to the tooth surface.

The use of alendronate as a binding moiety, however, has raised concerns on the safety of these tooth-binding micelles (TBM). As a member of the bisphosphonate (BP) drug class, alendronate is clinically used as an inhibitor of bone resorption. Over the years, BPs have become the treatment of choice for a variety of bone metabolic diseases (e.g. Paget disease, cancer bone metastasis and osteoporosis, etc.) (15). Recently, a possible link between the use of BP (including alendronate) and a condition called osteonecrosis of the jaw (ONJ), characterized by necrotic bone exposure in the maxillofacial region, has been identified (16). Although it is unlikely that the daily use of a very small amount of alendronate-Pluronic conjugate as an excipient in oral hygiene products would lead to ONJ, it is prudent to replace alendronate in TBM with non-toxic, biodegradable dentotropic moieties to avoid any safety concerns and the potential hurdles in the translation of this technology into a clinical application.

To address this safety concern, we recently identified two biodegradable tooth binding moieties to replace alendronate. Statherin is a double phosphorylated human saliva protein, containing two phosphoserine residues that interact strongly with hydroxyapatite (HA). Sep3 chelates a Ca^{2+} ion in a fashion similar to that of phosphate groups in the bulk of the HA crystal, and Sep2 forms an intermolecular hydrogen bond where statherin is the proton acceptor and HA is the donor (17). Inspired by the phosphorylated structure of statherin and its high affinity for HA, we decided to replace alendronate with diphosphoserine (DPS) and conjugated it to the chain termini of Pluronic P123.

The other biodegradable tooth-binding moiety we considered is pyrophosphate (PPi). Pyrophosphate has been widely used in the food industry for decades. It has also been extensively used in oral care products for the purpose of abrasion (18), whitening (19), and anti-tartar activity (20) because of its strong affinity to enamel, dentin and tartar (21). In addition, pyrophosphate is hydrolysable *in vivo*. The combination of these properties makes it an excellent tooth-binding moiety for the TBM delivery system.

Using these two new binding moieties, we prepared triclosan-containing TBMs and characterized the HA binding kinetics and drug release profiles. Furthermore, the influence of human saliva on TBM binding capacity and stability was also investigated. Finally, the anti-plaque efficacy of TBMs was evaluated using an *in vitro* biofilm model.

MATERIALS AND METHODS

Chemicals

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® P123 copolymer was generously provided by BASF corporation (Florham Park, NJ). Fmoc-Ser[PO(Obzl)OH]-OH and trityl chloride resin for standard solid phase peptide synthesis (SPPS) were obtained from Novabiochem® (Merck KGaA, Darmstadt, Germany). Tris(tetra-n-butyl-ammonium) hydrogen pyrophosphate was purchased from Sigma-Aldrich (St. Louis, MO). Triclosan was obtained from TCI America (Portland, OR). All other reagents and solvents if not specified were purchased from either Fisher Scientific (Pittsburgh, PA) or Acros Organics (Morris Plains, NJ).

Methods

^{1}H 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. Effective hydrodynamic diameters (D_{eff}) of the micelles were measured by photon correlation spectroscopy (DLS) using a "ZetaPlus" analyzer (Brookhaven Instrument Co.) 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 triclosan concentration analysis.

Synthesis of Dipeptide L-Phosphoserine—Pentynoic Acid Conjugate (DPS-PA)

A standard SPPS method was used to synthesize dipeptide L-phosphoserine—pentynoic acid conjugate (DPS-PA) (Fig. 1a). Fmoc-Ser[PO(Obzl)OH]-OH (100 mg, 0.201 mmol) was loaded to trityl chloride resin (500 mg, 0.5 mmol –Cl, 50% loading) for 3 h, washed with dimethylformamide (DMF)/methanol (MeOH)/diisopropylethylamine (DIPEA) (10:1:1) 4 times (5 min each), then DMF 4 times. For N-deprotection, 20% piperidine (5 mL) was added and allowed to react for 5 min at room temperature, followed by a second treatment for another 5 min. Acylation was then achieved by adding a solution containing Fmoc-Ser[PO(Obzl)OH]-OH (3 eq, 300 mg, 0.6 mmol) or 4-pentynoic acid (final step, 3 eq, 59 mg), HOBT (3 eq), PyBOP (3 eq) and DIPEA (6 eq, 0.21 mL) in DMF (5 mL) to the N-deprotected resin and agitated for 24 h. Dichloromethane (DCM) was used to wash the resin (5 × 2 min) prior to cleavage step. Resin was cleaved

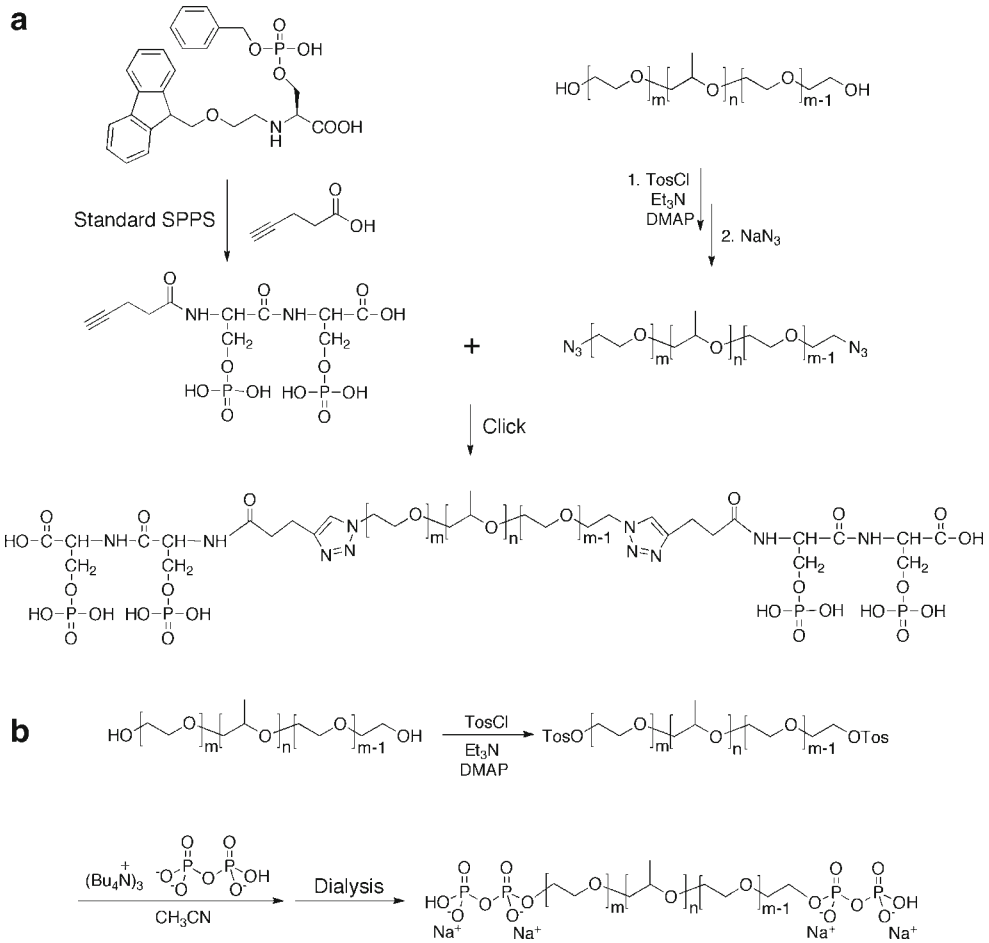

Fig. 1 Synthesis of (**a**) di-phosphoserine-Pluronic P123 conjugate (DPS-P123) and (**b**) pyrophosphate-Pluronic P123 conjugate (PPi-P123).

with trifluoroacetic acid (TFA)/Water/triisopropylsilane (95:2.5:2.5) incubation for 3 h. The combined cleavage filtrate was evaporated under vacuum and precipitated into cold ether. The precipitate was then dissolved in water and precipitated in cold ethanol. Yield, 40%. The structure of the conjugate was confirmed with MALDI-TOF mass spectrometry and NMR. The m/z (negative ion) of the product is 430.92 (calculated 431.02). ^{1}H NMR (500 MHz, D_2O) δ (ppm)=4.54 (t, J=5.6Hz, 1H), 4.34 (t, J=6.1Hz, 1H), 4.03–4.10 (m, 4H), 2.55 (td, J=6.3Hz, 2.4Hz, 2H), 2.49 (td, J=6.8Hz, 2.0Hz, 2H), 2.37 (t, J=2.4Hz, 1H); ^{31}P NMR (202 MHz, D_2O) δ (ppm)=0.214 (s, 1P), 0.105 (s, 1P).

Synthesis of DPS-PA-Pluronic P123 Conjugate (DPS-P123)

Diphosphoserine-modified Pluronic P123 (DPS-P123) were synthesized using "click" chemistry as described previously (14) (Fig. 1a). Briefly, *p*-toluenesulfonyl terminated Pluronic P123 (Tos-P123) was synthesized by the reacting Pluronic P123 with *p*-toluene sulfonyl chloride using 4-dimethylaminopyridine (DMAP) as a catalyst in DCM at room temperature. Azide terminated P123 (Azido-P123) was synthesized by the reacting Tos-P123 with sodium azide at 110°C in DMF. DPS-P123 was then obtained by "click" reaction of DPS-PA with Azido-P123 using sodium ascorbate and copper (II) sulfate pentahydrate as a catalyst in EtOH/H_2O (1:1) at room temperature. The resulting Di-PS-P123 conjugate was purified by LH-20 column and then dialyzed against water. Yield, 85%. The serine content in the final lyophilized product was determined as 1.4 mol of diphosphoserine per mol of conjugate by amino acid analysis. The structure was also confirmed by ^{1}H NMR analysis. ^{1}H NMR (D_2O) δ (ppm)=7.81 (s), 3.04 (t, J=6.83 Hz), 4.55 (d), 3.80–3.39 (m), 2.70 (t), 1.12 (d, J=7.81 Hz).

Synthesis of Pyrophosphate-P123 (PPi-P123) Conjugate

As shown in Fig. 1b, Tos-P123 (725 mg, 0.125 mmol) was dissolved in anhydrous acetonitrile and tris(tetra-n-butyl-ammonium) hydrogen pyrophosphate (451 mg, 0.5 mmol) was slowly added to the solution. The reaction was stirred overnight at room temperature. The product was then purified by dialysis against NaCl and then against water. Yield,

90%. ^{1}H NMR (500 MHz, D_2O) δ (ppm) = 3.96 (m, 4H), 3.62–3.50 (br, 350H), 3.30–3.50 (br, 150H), 1.04 (s, 210H); ^{31}P NMR (202 MHz, D_2O) δ (ppm) = −10.56(d, J = 20.2Hz, 2P), −11.91 (d, J = 20.2Hz, 2P). Pyrophosphate content in the polymer was determined using a previously published method (23). Briefly, PPi-P123 was hydrolyzed in 1 M HCl for 1 h at 100°C to release phosphate. P123 was then removed by extraction with chloroform. An equal volume of a solution containing 0.5% (w/v) ammonium molybdate, 2% (w/v) ascorbic acid and 1 M HCl was then added to each sample. The samples were incubated at 37°C for 2 h and then their absorbance at 820 nm was measured using an UV/Vis spectrophotometer. The conjugate used in the following studies contained 1.9 mol of pyrophosphate per mol of conjugate. The result was confirmed using ^{31}P NMR analysis with Na_2HPO_4 as an internal standard.

Triclosan-Loaded TBM Formulation Using Novel Biodegradable Tooth-Binding Moieties

Direct dissolution method was used to form the micelle (13). DPS-P123 or PPi-P123 was dissolved in water (5 mL) and placed at 4°C overnight. The system was then equilibrated at 24°C for 24 h in order to allow micelle formation. An excess amount of triclosan (100 mg) was added into this solution and the dissolution of the drug was allowed under stirring for 24 h. The undissolved drug was removed by centrifugation at 12,000 rpm for 0.5 min, followed by filtration of the supernatant through a 0.2 μm filter. Non-binding control micelle solution (NBM) was prepared similarly according to the same procedure, where un-modified Pluronic P123 was used.

In Vitro Binding Kinetics of Triclosan-Loaded TBMs

Micelle formulation (200 μL each) was mixed with HA particles (Bio-Rad, NJ, US; 20 mg/tube) in Eppendorf centrifuge tubes. The tubes were placed on a Labquake® rotator to allow binding at room temperature. At each predetermined time point, 3 tubes were removed, centrifuged (12,000 rpm, 0.5 min), and 100 μL of the supernatant was collected. The concentration of triclosan in collected samples was then analyzed by HPLC with the conditions as described previously (13). The amount of triclosan bound to HA particles via the micellar formulation was calculated by subtracting the amount of triclosan left in the supernatant from the initial amount of triclosan added.

In Vitro Release of Triclosan from TBMs

Micelle formulations (2 mL) were each mixed with an equal volume of either PBS or human saliva and sealed into a dialysis bag (MWCO 12,000) and incubated in 16 mL of release medium (0.1 M PBS, pH = 7.4 containing 2% Pluronic P123) to release the encapsulated drug at 37°C with gentle shaking. At predetermined time intervals, samples (0.5 mL) were removed from the release medium and replaced with fresh medium. The collected samples were then mixed with an equal volume of acetonitrile, filtered (0.2 μm) and analyzed by HPLC with the conditions described previously (13).

Collection of Stimulated Human Parotid Salivary Secretion

Human parotid saliva samples were obtained from 5 healthy volunteers and pooled together. The protocol was approved by the Institutional Review Board (IRB) for the Protection of Human Subjects of UNMC. Parotid salivary flow was stimulated by the application of lemon juice to the tongue. Samples were centrifuged at 12,000 rpm for 10 min to remove any insoluble material or cell debris. The supernatant was then filter sterilized using 0.45 μm filter. The salivary samples were used immediately for saliva and bacterial adhesion studies.

Influence of Human Saliva on TBM Binding to Artificial Tooth Surface

In this study, three different scenarios were investigated. 1) HA discs were treated with saliva (1 mL) first for 1 h, washed with saline and then incubated in TBM solution (1 mL) for 1 h. 2) HA discs were incubated with TBM solution for 1 h, washed with saline and then incubated with saliva for 1 h. 3) HA discs were incubated with the mixture of saliva and TBM solution (1:1, 1 mL) for 1 h. After the treatments, all discs were washed with saline. Triclosan remaining on HA discs was extracted with acetonitrile (1 mL), and its concentration was determined by HPLC. HA discs treated with empty micelle alone were used as control.

Influence of Human Saliva and Salivary Enzymes on the Stability of TBM Binding on HA Surface

TBMs prepared using novel biodegradable tooth-binding moieties were incubated with HA powder (20 mg) for 1 h. HA powder was then washed with saline to remove unbound micelle. Micelle-loaded HA powder was then suspended in media (0.5 mL) and incubated under 37°C with gentle shaking. For DPS-P123 micelle, PBS, PBS with MMP-3 (20 unit/mL), or human saliva was used as the incubation media. For PPi-P123 micelle, Tris-buffer, Tris-buffer with alkaline phosphatase (20 unit/ml), or human saliva was used as the incubation media. Samples were taken at predetermined time points. Micelle detachment from HA

surface was determined by measuring triclosan concentration in the supernatant using HPLC.

Bacterial Culture

S. mutans UA159 (22) 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 h growth at 37°C and 5% CO_2, a single colony of bacteria was inoculated into 3 mL of THYE broth (24) and allowed to grow statically overnight at 37°C and 5% CO_2. The next day, the overnight culture was diluted to a density of 2×10^4 CFU/mL in chemically-defined media containing 0.25% w/v glucose and 0.25% w/v sucrose (CDM), prepared as previously described (25). Human saliva used in bacterial culture studies was collected as stated above and sterilized by centrifugation following by filtration through a sterilized 0.45 μm filter.

In Vitro Prevention of *S. Mutans* Biofilm Formation on HA Discs

Autoclaved HA discs (0.5′ diameter × 0.04–0.06′ thick) were incubated with different micelle solutions or CDM in a 24-well plate for 1 h and then washed in 40 mL of saline (under magnetic stirring at 200 rpm) for 5 s to remove unbound micelles. In two separated experiments, these HA discs were either treated with sterilized human saliva for 1 h or with no treatment before they were transferred to wells containing 1 mL of *S. mutans* UA159 suspension (1×10^4 CFU/mL in CDM) and cultured statically for 48 h to allow biofilm growth at 37°C and 5% CO_2, prior to quantification of bacterial growth.

In Vitro Treatment of Preformed *S. Mutans* Biofilm Growth on HA Discs

Autoclaved HA discs were incubated in 1 mL of sterilized human saliva for 1 h before being transferred into 1 mL of *S. mutans* UA159 suspension (1×10^4 CFU/mL in CDM), and cultured statically for 48 h to allow biofilm growth at 37°C and 5% CO_2. These 48 h HA disc-associated biofilms were either treated with different micelle formulations for 5 min, or were left as untreated controls. After treatment, the HA disc biofilms were washed in 40 mL of saline (under magnetic stirring at 200 rpm) for 5 s to remove loosely attached micelles before each being transferred to wells of a 24-well plate containing sterile CDM media, and grown at 37°C, 5% CO_2. This same procedure was repeated at 72 and 96 h of growth, prior to quantification of biofilm growth.

Biofilm Analysis

At the end of each experiment, HA disc biofilms were individually transferred to a well containing 1 mL of THYE medium. The surface of each HA disc was gently scraped with a sterile spatula to harvest adherent cells. The removed biofilms were subjected to vortex mixing for 10 s and then serially diluted in THYE broth at a 1:10 ratio. The number of viable cells in each sample was quantified using the track dilution method (25). All plates were incubated for 48 h at 37°C with 5% CO_2 and then the CFUs recovered per biofilm were determined. Specific differences between the log-CFU/biofilm of each experimental group were analyzed using the Student *t*-test. A *p*-value of <0.05 was considered as statistically significant.

RESULTS

In Vitro Binding Kinetics of Triclosan-Loaded TBMs Prepared Using Biodegradable Tooth-Binding Moieties

The HA binding kinetics assay was carried out using a previously-described method (13,14). Encapsulation efficiency (triclosan dissolved/triclosan added) was found to be 73.5 ± 3.9% for both DPS-TBM and PPi-TBM. Results of this binding kinetic study (Fig. 2) showed that both DPS and PPi biodegradable tooth-binding moieties were able to give the TBMs very fast binding kinetics, where the majority (approximately 50% of the maximum binding) of HA-binding occurred within 1 min. TBMs prepared with PPi-P123 (PPi-TBM) showed a higher ($p<0.05$) binding capacity than TBMs prepared with DPS-P123 (DPS-TBM).

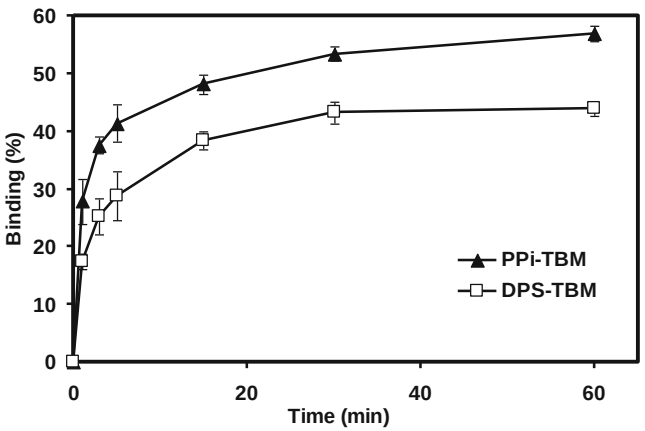

Fig. 2 TBM binding kinetics on HA powder. DPS-TBM, tooth-binding micelle prepared using DPS-P123; PPi-TBM, tooth-binding micelle prepared using PPi-P123. Data presented are mean ± SD, $n=3$.

In Vitro Release of Triclosan from TBMs

A release study was performed in either PBS or human saliva to investigate the influence of salivary proteins on triclosan release from the TBMs. The results of these experiments demonstrate that, for both PPi-P123 TBM and DPS-P123 TBM, triclosan was released in a similar manner throughout the entire 24 h duration of this experiment in PBS and saliva. Furthermore, the presence of salivary proteins did not appear to have any significant impact on triclosan release for both formulations (Fig. 3).

Influence of Human Saliva on TBM Binding to an Artificial Tooth Surface

TBMs and saliva were applied to HA disc surfaces in different orders to investigate the competition between TBMs and salivary proteins for binding sites on the HA discs surface. The results showed that no matter what the application order is, the TBMs competed effectively with the salivary proteins for binding to the HA discs. The binding capacity of TBMs remained above 60% of control (where HA discs were treated with TBMs alone). The highest binding capacity of TBM was achieved in the case where TBM was first loaded on HA surface and then exposed to human saliva (Fig. 4).

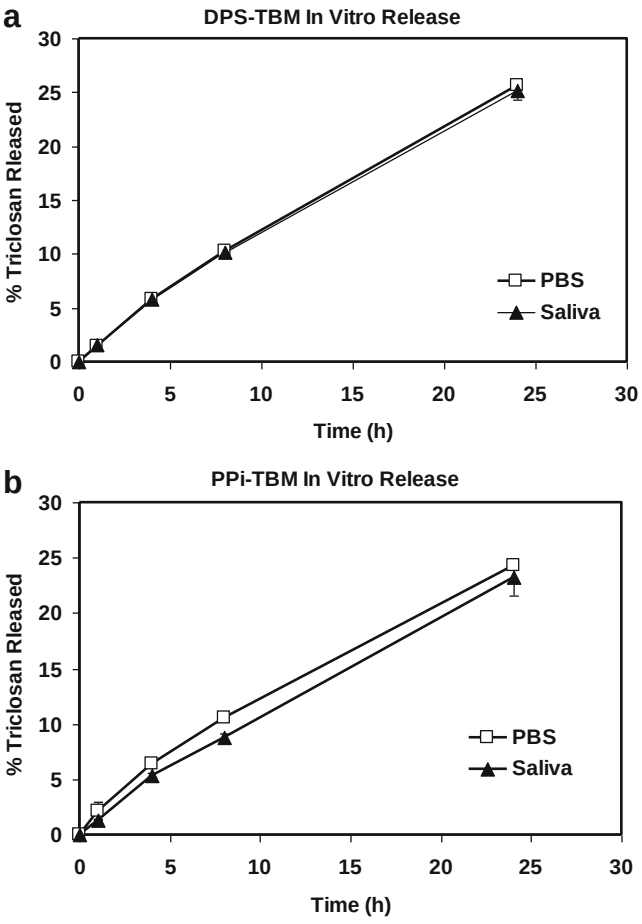

Fig. 3 Triclosan releasing profile from TBM in human saliva or PBS. (**a**) DPS-TBM, (**b**) PPi-TBM. DPS-TBM, tooth-binding micelle prepared using DPS-P123; PPi-TBM, tooth-binding micelle prepared using PPi-P123. Data presented are mean ± SD, $n = 3$.

Influence of Human Saliva and Salivary Enzymes on the Stability of TBMs Bound to HA Discs

To determine the influence of whole saliva as well as purified salivary enzymes on TBM stability on HA, triclosan release studies were performed in the absence of any solubilizing agent, whereby only a limited amount of triclosan can be

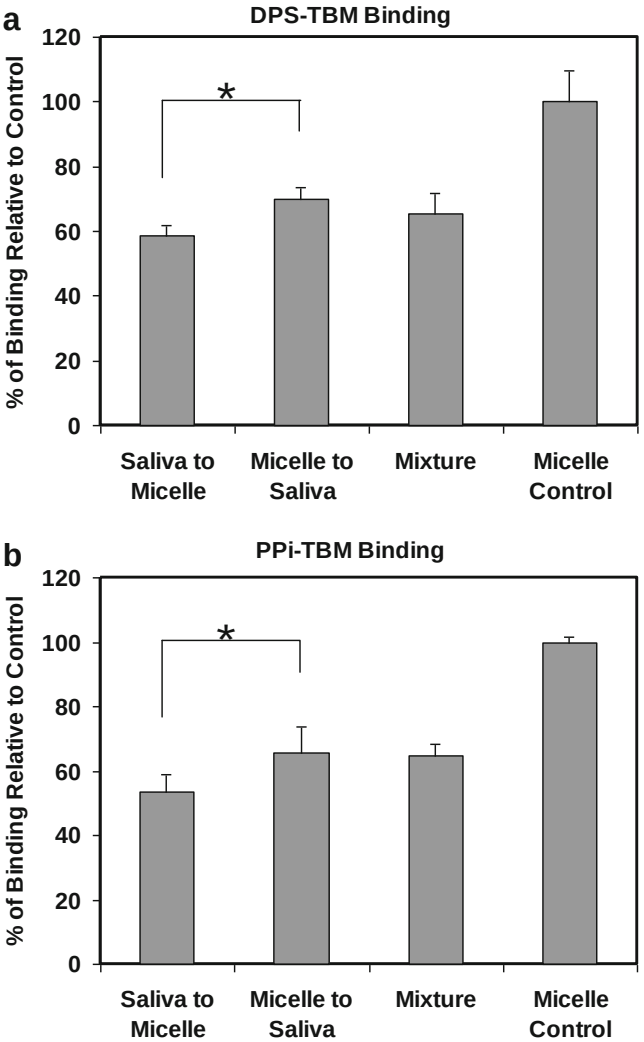

Fig. 4 Influence of salivary protein on TBMs binding capacity; (**a**) DPS-TBM, (**b**) PPi-TBM. DPS-TBM, tooth-binding micelle prepared using DPS-P123; PPi-TBM, tooth-binding micelle prepared using PPi-P123. Saliva to Micelle, HA discs were treated with saliva for 1 h first then micelle for 1 h before analysis; Micelle to Saliva, HA discs were treated with micelle for 1 h first then saliva for 1 h before analysis; Mixture, HA discs were treated with the mixture of micelle and saliva for 1 h before analysis. Data presented are mean ± SD, $n = 3$. *Asterisk* indicated significant difference ($p < 0.05$).

released into the release media due to its poor water solubility. Therefore under these experimental conditions, any presence of triclosan in the release media could only be attributed to the detachment of TBMs from the HA surface. By detecting triclosan concentration in the release media, the detachment of TBMs and stability of the binding can be estimated. As shown in Fig. 5, less than 5% of TBMs (both DPS-TBM and PPi-TBM) were released from HA surface in the presence of different salivary enzymes or human saliva after 24 h, suggesting that the TBMs displayed strong and stable binding on the HA surface during the tested period of time.

In Vitro Prevention of *S. Mutans* Biofilm Formation on HA Discs

To test the ability of the TBM formulations to prevent biofilm development, two separate experiments were performed. In the first experiment, HA discs were treated with the micelle formulations and then incubated directly with bacteria culture where the influence of saliva was not incorporated in the test. In line with previously published findings using ALN-P123 TBMs (13,14), the results of these experiments showed that both DPS and PPi TBMs strongly inhibited biofilm formation on HA surface when compared to the control. The non-binding micelle (NBM) treated group also showed a weak inhibitory effect. The effect, however, was significantly ($P<0.05$) less than the TBM treated group (Fig. 6a). In the second experiment, the influence of saliva competition and pellicle formation on the biofilm inhibitory efficacy of the micelle formulations was considered. HA discs were treated with micelle formulations, washed, and incubated in saliva for 1 h before incubated in bacteria culture. The results show that TBMs still demonstrated a significant ($P<0.05$) inhibitory effect on biofilm growth when compared to the control. In this setting, the NBM showed no effect against biofilm formation (Fig. 6b).

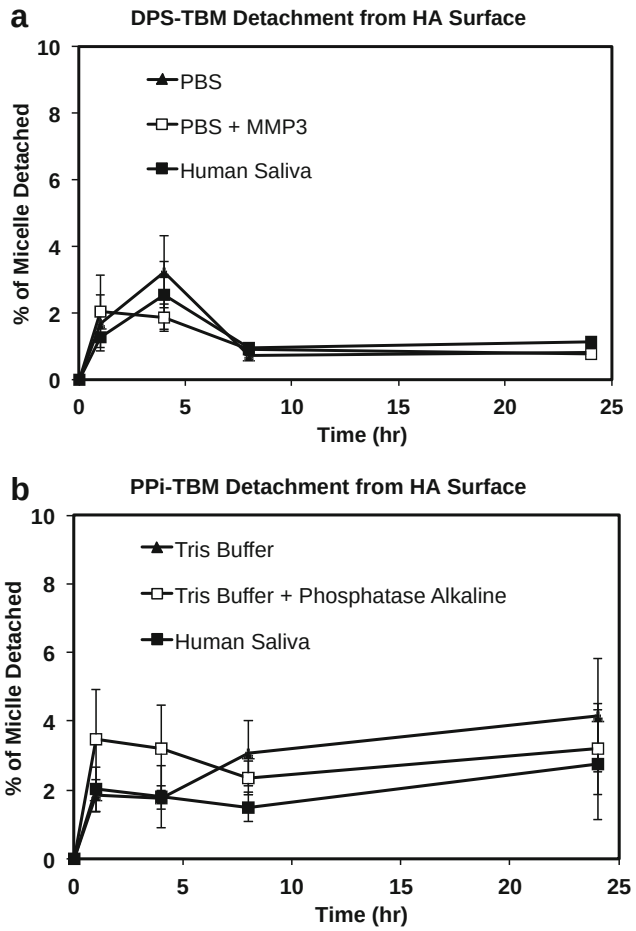

Fig. 5 Influence of saliva and salivary enzymes on TBM binding stability. (**a**) DPS-TBM, tooth-binding micelle prepared using DPS-P123; (**b**) PPi-TBM. Tooth-binding micelle prepared using PPi-P123; Data presented are mean ± SD, $n=3$. MMP3, matrix metalloproteinase-3.

In Vitro Treatment of Preformed *S. mutans* Biofilm Growth on HA Discs

The treatment effect of TBMs on preformed biofilm growth was tested. Biofilm was allowed to grow for 48 h on saliva pellicle coated HA discs before TBMs treatment. The results presented in Fig. 6c demonstrate that even a short exposure of TBM treatment (5 min per day) could greatly reduce the viability of the 48 h old biofilm formed on a saliva protein covered HA surface. The effect was significantly stronger ($P<0.05$) than that of NBM treatment, indicating both DPS and PPi tooth-binding moieties could effectively retain TBM on the disc surface, even when the surface is covered by saliva pellicle layer and biofilm (Fig. 6c).

DISCUSSION

The goal of this study was to address one of the most critical issues related to the translation of dentotropic micelle technology into a clinical application: the biocompatibility or safety of the dentotropic Pluronics. Due to the concern of the potential link between the clinical use of bisphosphonates and osteonecrosis of the jaw (ONJ), we have selected diphosphoserine peptide and pyrophosphate to replace alendronate for use in chain termini modification of Pluronic P123.

Solid phase peptide synthesis (SPPS) was used to synthesize di-phosphoserine peptide. Though the di-peptide is sufficient to serve as the HA-binding moiety for TBM, the synthesis of tetra-phosphoserine and hexa-phosphoserine were also explored to investigate whether the binding potential of the micelles could be further improved. Building phosphoserine peptide longer than 3 units, however, was found to be extremely challenging using the SPPS method (data not shown). Presumably, this difficulty may be explained by the strong steric hindrance from the Fmoc

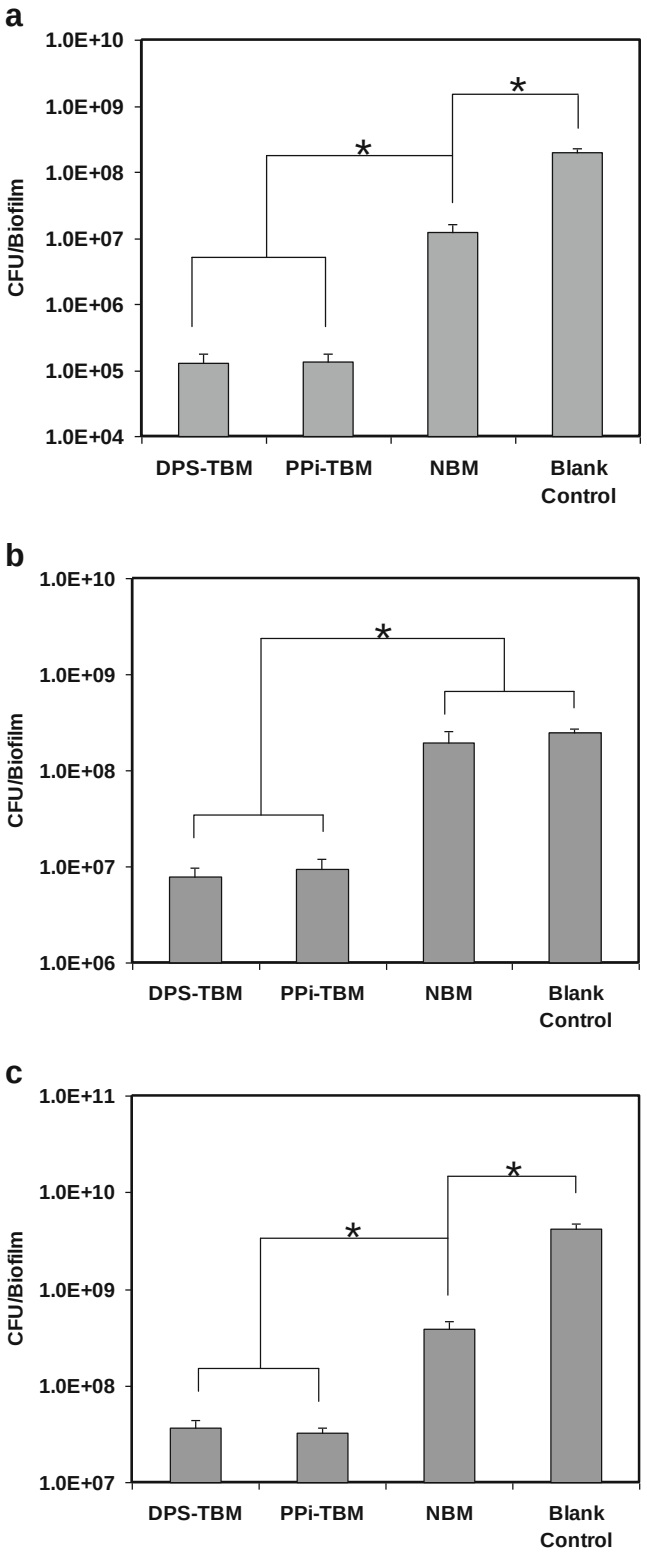

Fig. 6 *In vitro* biofilm inhibition study. (**a**) *In vitro* biofilm inhibition study without the presence of saliva; (**b**) *In vitro* biofilm inhibition study with the presence of saliva pellicle; (**c**) *In vitro* biofilm treatment study with the presence of saliva pellicle. DPS-TBM, tooth-binding micelle prepared using DPS-P123; PPi-TBM, tooth-binding micelle prepared using PPi-P123; NBM, non-binding micelle, micelle formulation prepared with Pluronic P123. Data presented are mean ± SD, $n = 3$. *Asterisk* indicated significant difference ($p < 0.05$).

and benzyl protecting groups of the amino acid building block. For the synthesis of longer phosphoserine peptides, the NCA (N-carboxyanhydride) polymerization method may be considered (26). Comparing to the conjugation with diphosphoserine peptide, the method used to modify the chain termini of Pluronic with pyrophosphate is rather straightforward (27,28). The two-step reaction is very simple and effective with a high yield of the final product (almost 100% conversion to pyrophosphate ester). The tetra-n-butyl-ammonium salt of pyrophosphate was used to increase its solubility in organic solvents, and the product can be easily converted to the sodium salt form through ion exchange by dialysis against a NaCl solution where excessive NaCl can be removed through a second dialysis against water.

The TBMs were then formed using a previous method with a high loading capacity (13). The TBMs were then characterized for binding kinetics. As shown in Fig. 2, Both TBMs prepared using DPS-P123 and PPi-P123 as the binding moieties can swiftly bind to HA powders. The binding kinetics are similar to that of TBM prepared using ALN as the binding moiety (13), suggesting the high affinity of diphosphoserine and pyrophosphate for HA and their potential to replace alendronate. More interestingly, PPi-TBM showed a slightly higher binding capacity than DPS-TBM, which may be explained by the higher efficiency of PPi to occupy the HA surface binding site than PO_4^{3-} (29).

To assess the long-term effect of human saliva on triclosan release from the TBMs, we also did *in vitro* drug release assays in both PBS and human saliva. A sustained release profile was observed in both media, suggesting that saliva proteins have minimal influence on triclosan release from the TBMs and a prolonged therapeutic effect may be achieved using these formulations.

When used clinically, the influence of saliva on TBM binding to tooth surfaces is vital for the success of the treatment. Due to the fact that both diphosphoserine and pyrophosphate binding moieties need to compete with salivary proteins for binding sites (calcium ion) on the tooth surface, the influence of saliva on the TBM binding capacity cannot be ignored. Therefore, we further evaluated the influence of human saliva on TBM binding before, after and during TBM treatment of HA discs. The results showed that in all the simulated situations, the majority of the TBM binding capacity (~60%) was maintained, suggesting very strong binding affinities of these TBMs for the HA surface. Such strong binding strength may be explained by the multivalent binding of TBM where multiple diphosphoserine peptides or pyrophosphates bound to different binding sites on the HA surface, which can be very difficult to be replaced simultaneously by salivary proteins. When the TBMs were applied to HA discs after saliva pellicle formation, its binding capacity was found to be the lowest, which may be explained by the reduced accessibility of the TBMs to the HA surface. As

the binding capacities of the TBMs are the highest when they were applied before saliva treatment, the optimal application window for TBM containing oral hygiene products should be immediately after tooth brushing.

The most important feature of the two novel biodegradable tooth-binding moieties is their superior safety potential. Even if they are accidentally swallowed, TBMs will be degraded into harmless amino acids and phosphates, as well as Pluronics (which are classified as Generally Regarded As Safe or GRAS by the FDA) by gastric acidity and enzymes. However, enzymes found in human saliva such as proteinase or phosphatase might also degrade these binding moieties and cause unstable binding and premature detachment of the TBMs from the enamel surface. Therefore, we further tested the stability of the TBMs on an HA surface in the presence of human saliva or enzymes found in saliva. The results demonstrated that both TBMs exhibited stable binding within the time frame we tested, probably because it is difficult for the salivary enzymes to gain access to the degradable tooth-binding moieties of the TBMs after they are bound to HA surface.

As part of this functional study, the new triclosan-containing TBMs were evaluated in an *in vitro* biofilm model. In the first experiment, when HA discs were not pretreated with human saliva, the TBMs were found to be able to strongly prevent biofilm growth. To appreciate the influence of human saliva on this prevention effect, we then treated the HA discs with human saliva before TBM treatment to allow pellicle layer formation. The results showed that the TBMs were still able to significantly inhibit biofilm growth when compare to the non-binding micelle control. This finding confirmed the results of our binding and release studies demonstrating that the influence of saliva on TBM performance was limited. To mimic the situation where dental plaque cannot be completely removed by routine dental hygiene procedures, we tested whether TBMs could treat preformed biofilm on a saliva pellicle layer. The results showed a significant reduction in CFU number with only 5 min of TBM treatment per day, suggesting that the TBMs can be retained on the HA disc surface even when it was covered by saliva pellicle and biofilm. These findings are consistent with previous studies using alendronate as the TBM binding moiety (13).

CONCLUSION

In this study, dentotropic micelles using phosphoserine dipeptide and pyrophosphate as targeting moieties have been successfully developed. The new TBMs showed fast binding kinetics and high binding capacities. Human saliva had a limited influence on the TBMs binding capacity, *in vitro* release profile and binding stability on HA surface. The new TBMs were shown to be able to effectively prevent *S. mutans* biofilm formation and reduce the viability of preformed biofilm even in the presence of a saliva pellicle layer. These properties of the newly developed TBMs are very similar to our previous findings using alendronate-bearing TBMs. Due to the outstanding biocompatibility of the newly developed dentotropic Pluronics, the phosphoserine dipeptide or pyrophosphate-bearing TBMs are well positioned for a swift clinical translation to further improve the performance of the current oral hygiene products.

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