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RESEARCH PAPER

The Development of Drug-Free Therapy for Prevention of Dental Caries

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ABSTRACT

Purpose The purpose of this study was to develop a novel, drug-free therapy that can reduce the over-accumulation of cariogenic bacteria on dental surfaces.

Methods We designed and synthesized a polyethylene glycol (PEG)-based hydrophilic copolymer functionalized with a pyrophosphate (PPi) tooth-binding anchor using "click" chemistry. The polymer was then evaluated for hydroxyapatite (HA) binding kinetics and capability of reducing bacteria adhesion to artificial tooth surface.

Results The PPi-PEG copolymer can effectively inhibit salivary protein adsorption after rapid binding to an artificial tooth surface. As a result, the *in vitro S. mutans* adhesion study showed that the PPi-PEG copolymer can inhibit saliva protein-promoted *S. mutans* adhesion through the creation of a neutral, hydrophilic layer on the artificial tooth surface.

Conclusions The results suggested the potential application of a PPi-PEG copolymer as a drug-free alternative to current antimicrobial therapy for caries prevention.

KEY WORDS biofilm · caries · click chemistry · hydroxyapatite · tooth-binding

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ABBREVIATION

CFU	Colony forming units
Click-PEG	Multifunctional polyethylene glycol copolymer synthesized using click chemistry
DCC	N,N'-dicyclohexylcarbodiimide
DCM	Dichloromethane
DMAP	4-dimethylaminopyridine
DMF	Dimethylformamide
EDTA	Ethylenediaminetetraacetic acid
GRAS	Generally regarded as safe
HA	Hydroxyapatite
HMHP	PPi-PEG copolymer with high MW and high pyrophosphate content
HMLP	PPi-PEG copolymer with high MW and low pyrophosphate content
IRB	Institutional review board
LMHP	PPi-PEG copolymer with low MW and high pyrophosphate content
LMLP	PPi-PEG copolymer with low MW and low pyrophosphate content
MW	Molecular weight
PDI	Polydispersity index
PEG	Polyethylene glycol
PPi	Pyrophosphate
PPi-PEG	Pyrophosphate modified click-PEG
S. mutans	*Streptococcus mutans*
TBAP	Tris(tetra-n-butylammonium) hydrogen pyrophosphate
THYE	Todd Hewitt-yeast extract

INTRODUCTION

The microflora of the oral cavity creates a diverse biome, with more than 700 bacterial species being identified (1,2). These

bacteria contribute to the health of the host by preventing exogenous, and potentially pathogenic micro-organisms from becoming established in the mouth (colonization resistance), and by regulating the host's inflammatory response to oral commensal bacteria (3). Alterations to the healthy flora of the oral cavity, in terms of bacteria species and population distribution, can lead to the onset of dental diseases such as dental caries, gingivitis, and periodontitis (4).

Löe and coworkers (5) established the unequivocal role of dental plaque as the etiological agents of gingivitis since the 1960s. A previously published meta-analysis of antiplaque agents provided strong evidence in favor of using antimicrobial agents, such as triclosan and chlorhexidine, as adjuncts to mechanical plaque control (6). However, there are major concerns regarding the use of these antimicrobials, which include: a. Greatly reduced efficacy of antimicrobials immediately after application, due to their lack of retention in the oral cavity (7–9); b. The indiscriminative reduction of all plaque microflora, due to lack of selectivity (10,11); c. The development of resistant microorganisms, due to repeated exposure to antimicrobials (12). Consequently, technology that can reduce dental plaque accumulation, while also having a minimal impact on the healthy/beneficial oral microflora, is highly desirable.

Dental plaque accumulation starts with the initial attachment of early colonizers, such as *Streptococcus mutans* (*S. mutans*), to the saliva-derived enamel pellicle (13). The acquired enamel pellicle is formed through adsorption of salivary proteins onto the dental surface, which promotes the adhesion of *S. mutans* by specific (antigen I/II) and nonspecific mechanisms (14). Therefore, if the adsorption of salivary proteins and acquired enamel pellicle formation can be reduced and interrupted, the diseases caused by over-accumulation of dental biofilm may be better controlled.

Polyethylene glycol (PEG) is a widely used biocompatible polymer, known to reduce protein and bacterial adhesion when chemically grafted to the surface of many materials, including tooth enamel (15–17). Chemical grafting, however, is inconvenient and impractical for daily oral hygiene procedures at home. Therefore, we designed and synthesized a dentotropic PEG-based hydrophilic copolymer as a novel oral hygiene product excipient. During a routine oral hygienic procedure, we envisioned the copolymer would quickly anchor itself to the enamel surface upon exposure and create a PEG "coating", which would reduce salivary protein adsorption, subsequent *S. mutans* colonization, and dental biofilm accumulation. Based on this approach, we developed a novel bioadhesive polymer derived from PEG, which can bind to the dental surface and reduce bacterial attachment.

METHODS

Synthesis of Bromoacetic Acid 2,2-Bis(azidomethyl) propane-1,3-diol Ester (Compound 1)

Briefly, 2,2-bis-(bromomethyl)propane-1,3-diol and NaN_3 were dissolved in DMF. This mixture was stirred at 120°C overnight and filtered. After the removal of DMF, residue was subjected to a standard diethyl ether/aqueous NaCl extraction. The crude product was further purified by silica gel column chromatography (chloroform/methanol = 20:1). Then the purified product and bromoacetic acid (1:1.3, mol/mol) were added to anhydrous DCM. The solution was cooled to 0°C and N,N'-dicyclohexylcarbodiimide (DCC, 1.1 eq.) and 4-dimethylaminopyridine (DMAP, 0.1 eq.) were added slowly to the reaction solution. The reaction was stirred for 4 h, filtered and evaporated to dryness under reduced pressure. The crude product was purified by silica gel column chromatography (hexane/ethyl acetate = 3:1). Yield: 75%. ^{1}H NMR (500 MHz, $CDCl_3$) δ=4.16 (s, 2H), 3.87 (s, 2H), 3.56 (s, 2H), 3.444 (s, 2H), 3.440 (s, 2H), 2.95 (br, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ=167.13, 64.39, 61.19, 50.87, 44.32, 25.24.

Synthesis of Pyrophosphate Monomer for "Click" Polymerization (PPi-Azide, Compound 2)

Tris(tetra-n-butylammonium) hydrogen pyrophosphate was dissolved in anhydrous acetonitrile, and bromoacetic acid 2,2-bis(azidomethyl)propane-1,3-diol ester (0.5 eq.) was slowly added to the solution. The reaction solution was stirred for 2 h to completion. The solvent was then removed by rotary evaporation, and the resulting residue was dissolved in 25 mM sodium chloride water solution (ion-exchange buffer). The solution was then slowly passed through a column containing 30 equiv. of Amberlite® IR120 Na form ion-exchange resin (Acros, Morris Plains, NJ) that had been equilibrated with ion-exchange buffer, at a flow rate of one column volume/15 min. The eluent was rotary evaporated to dryness at room temperature. Completeness of the ion-exchange was confirmed by the disappearance of tetra-n-butylammonium peaks from the ^{1}H NMR spectrum. The product was then further purified to remove any excess pyrophosphate, using cellulose flash chromatography (4.5:2:3 (v/v/v), isopropyl alcohol/acetonitrile/water). Fractions were combined and rotary evaporated to remove solvents at room temperature. The purified product was then stored at −20°C. Yield: 50%. ^{1}H NMR (500 MHz, D_2O) δ=4.59 (d, $\mathcal{J}$=8.8 Hz, 2H), 4.14 (s, 2H), 3.53 (s, 2H), 3.45 (s, 4H), 2.95 (br, 1H); ^{13}C NMR (125 MHz, D_2O) δ=167, 64.6, 63.1, 61.5, 51.4, 44.3; ^{31}P NMR (202 MHz, D_2O) δ=−10.55 (d, $\mathcal{J}$=20.2 Hz, 1P), −11.45 (d, $\mathcal{J}$=20.2Hz, 1P).

Synthesis of Acetylene-Terminated PEG (Acetylene PEG, Compound 3)

Briefly, PEG diol 2000 or PEG diol 600 was dissolved in dry toluene, refluxed, and dried in a vacuum to remove water. Phosgene solution (20% in toluene) was then added to the dried PEG while stirring. The reaction was allowed to continue overnight in a fume hood; subsequently, the excess phosgene was removed through a vacuum. Anhydrous DCM was used to dissolve the viscous residue. Propargyl amine was then added to the solution and the reaction was allowed to proceed for 7–8 h at room temperature. The product was precipitated into diethyl ether three times and purified by a LH-20 column and dialyzed against water. Yield: 81% (PEG 2000), 69% (PEG 600).

Synthesis of Dentotropic Click-PEG (PPi-PEG, Fig. 1)

Acetylene-terminated PEG 2000 or 600 (50 μmol), PPi-Azide or 2,2-bis(azidomethyl)propane-1,3-diol (50 μmol), and $CuSO_4 \cdot 5H_2O$ (5 μmol) were dissolved in H_2O with stirring; sodium ascorbic acid (10 μm dissolved in H_2O) was then added drop-wise to this solution. The reaction mixture was allowed to stir at room temperature for 4 h under an argon atmosphere. The reaction mixture was then rotary evaporated to remove the majority of water and then EDTA was added to remove Cu^{2+}. Yield before fractionation: 80%. ^{1}H NMR (500 MHz, D_2O) $\delta = 7.98$ (s, 2H), 4.56 (s, 2H), 4. 54 (d, 8.8, 2H), 4.46 (s, 2H), 4.37 (s, 4H), 4.19 (s, 4H), 3.71 (br, 4H), 3.66 (br, 180H); ^{31}P NMR (202 MHz, D_2O) $\delta = -10.82$ (d, $J = 20.7$ Hz, 1P), −11.45 (d, $J = 20.7$ Hz, 1P).

The product was then fractionated using the ÄKTA FPLC system (GE HealthCare) equipped with a Superose 12 preparative column. Fractions were collected with a 3 min interval and the weight average molecular weights (M_w) of the fractions were determined using a Superdex 200 analytical column with PEG calibration standards. Selected fractions were collected and lyophilized to obtain the final products.

Determination of Phosphate Content in PPi-PEG

Pyrophosphate (PPi) content in the copolymer was determined using a previously published method (18). Briefly, PPi-PEG was hydrolyzed in 1 M HCl for 1 h at 100°C to release phosphate. PEG backbone was then removed from the samples through ultrafiltration. To each sample, an equal volume of solution containing 0.5% (w/v) ammonium molybdate, 2% (w/v) ascorbic acid and 1 M HCl was added. The samples were then incubated at 37°C for 2 h and their absorbance at 820 nm was measured using a UV spectrophotometer and the resulting phosphate content was used to calculate the PPi content in PPi-PEG.

In Vitro Binding Kinetics of PPi-PEG to Hydroxyapatite (HA)

PPi-PEG (1 mg) was dissolved in PBS (1 mL) and mixed with HA particles (50 mg) in Eppendorf centrifuge tubes. The tubes were placed on a Labquake® rotator to allow binding at room temperature. At each predetermined time point, tubes ($n = 3$) were removed, centrifuged (12,000 rpm, 0.5 min), and UV absorption of the supernatant was measured at 218 nm and compared with that of the initial PPi-PEG solution. Click-PEG prepared using 2,2-bis(azidomethyl)propane-1,3-diol (which does not contain PPi) instead of PPi-Azide served as a non-binding polymer control.

Collection of Stimulated Human Parotid Salivary Secretion

Human saliva samples were obtained from five healthy volunteers and pooled together. The protocol was approved by the Institutional Review Board (IRB) for the Protection of Human Subjects at the University of Nebraska Medical Center. 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 filters (Whatman™, GE Healthcare). The salivary samples were used immediately for saliva and bacterial adhesion studies.

In Vitro Inhibition of Salivary Protein Adhesion to an Artificial Tooth Surface

Hydroxyapatite (HA) discs (model tooth surface) were first treated with the PPi-PEG solution (10 mg/mL) or control (click-PEG without PPi and non-treated) for 1 h, then washed with saline before being incubated with human saliva for 1 h. After the incubation, the discs were washed with saline and incubated in PBS (1 M) for 2 h to recover the surface bound protein. The PBS solution was then extracted with chloroform to remove detached PPi-PEG or click-PEG (without PPi) and subsequently analyzed by a micro BCA assay to determine saliva protein concentration ($n = 3$). HA discs incubated with saliva alone were used as untreated controls.

Bacterial Culture Conditions

S. mutans UA159 (19) 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 of growth at 37°C and 5% CO_2, a single colony of bacteria was inoculated into THYE broth (20) and allowed to grow statically overnight in the presence of 5% CO_2 at 37°C. The next day, the overnight

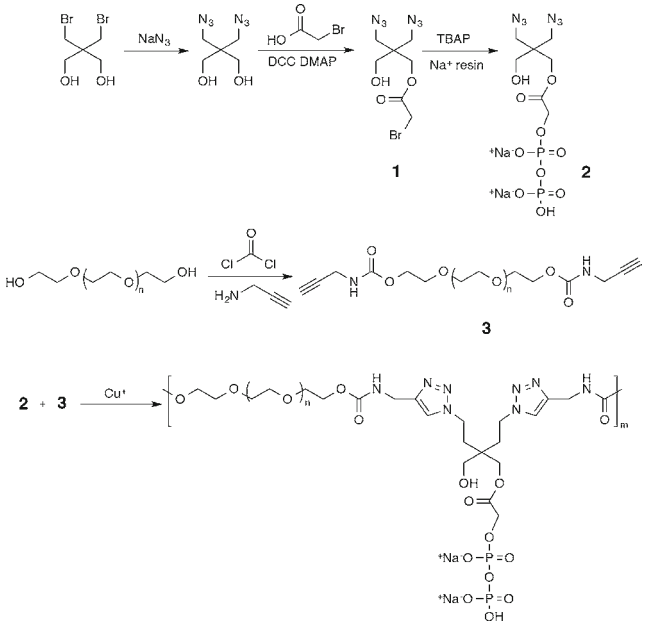

Fig. 1 Synthesis of dentotropic click-PEG (PPi-PEG).

culture was pelleted by centrifugation and resuspended to a density of approximately 1×10^9 CFU/mL (optical density 0.2 at 600 nm) in THYE culture media.

In Vitro Inhibition of *S. mutans* Adhesion on an Artificial Tooth Surface

Autoclaved HA discs (0.5 'diameter×0.04–0.06' thickness) were incubated with different PPi-PEG solutions or controls (3 discs per group) in a 24-well plate for 1 h and then washed with 40 mL of saline (with magnetic stirring at 200 rpm) for 5 s to remove unbound polymers. In two separate experiments, the HA discs were either treated with sterilized human saliva for 1 h or received no treatment before being transferred to wells containing 1 mL of *S. mutans* UA159 suspension (1×10^9 CFU/mL in THYE) and cultured statically for 1 h to allow bacterial attachment at 37°C and 5% CO_2, prior to quantification of bacterial growth. At the end of each experiment, the surface of each HA disc was gently scraped with a sterile spatula to harvest adherent cells. The removed cells were subjected to vortex mixing for 10 s and the number of viable cells in each sample was quantified using the track dilution method (21).

For statistical analysis, specific differences among experimental groups (for biofilm inhibition studies, log-CFU/biofilm of each experimental group were compared) were analyzed using the Student *t*-test. $P<0.05$ was considered as statistically significant.

RESULTS

Synthesis of PPi-PEG and Characterization

The synthetic strategy was designed as shown in Fig. 1. We anticipated that PPi content and molecular weight (MW) of the PEG copolymer would be the two main factors affecting PEG performance. Therefore, we used PEG building blocks with different MW (PEG 2000 or PEG 600) to obtain copolymers with different PPi contents. Column fractionation was used to achieve different MWs. A total of four PPi-PEG copolymers with different MW and PPi contents were obtained for the subsequent experiments and a click-PEG copolymer without PPi was used as the control. The characterizations of these copolymers are shown in Table I.

In Vitro Binding Kinetics of PPi-PEG to a Model Tooth Surface (HA)

The binding kinetic study presented in Fig. 2 was designed to demonstrate whether PPi-PEGs could efficiently bind to the model tooth surface (HA). Our results showed that PPi-PEG could effectively bind to HA within a two-min time period (Fig. 2), the maximum length of time that people brush their teeth or rinse with a mouthwash (22). The increase of both MW and PPi content had a significant positive impact on their binding capacity.

In Vitro Inhibition of Salivary Protein Adhesion to an Artificial Tooth Surface

As mentioned earlier, the salivary protein pellicle on the tooth surface plays an important role in dental biofilm initiation (13). To investigate whether PPi-PEG copolymers could effectively prevent salivary protein adsorption on the HA surface, an *in vitro* binding inhibition study was performed. The findings (Fig. 3) were consistent with the HA binding study (Fig. 2), whereby PPi-PEG with low MW and low PPi content (LMLP) which showed the lowest binding capacity, also demonstrated lowest inhibitory effect. The non-binding control copolymer (no PPi) showed a very weak reduction in protein binding when compared to the untreated group, likely due to the weak interaction between the hydroxyl groups or triazole rings (electron donor) and HA surface (electron acceptor) (23).

In Vitro Inhibition of *S. mutans* Adhesion on Artificial Tooth Surface

To test the inhibitory effect of the PPi-PEG copolymers against *S. mutans* adhesion to HA, we first performed an inhibitory study on untreated clean HA discs. The results showed that PPi-PEG copolymers could effectively prevent *S. mutans* adhesion to a clean HA surface (Fig. 4a), with

Table I Characterization of PPi-PEG copolymers

Polymers[a]	MW (kDa)[b]	PDI[c]	Pyrophosphate content (mg/g)
HMHP	66	1.1	136
LMHP	6.0	1.2	141
HMLP	54	1.2	68
LMLP	7.4	1.2	61
Non-binding	Mixture	–	0

[a] HMLP, PPi-PEG copolymer with high MW and low pyrophosphate content; LMHP, PPi-PEG copolymer with low MW and high pyrophosphate content; HMHP, PPi-PEG copolymer with high MW and high pyrophosphate content; LMLP, PPi-PEG copolymer with low MW and low pyrophosphate content; Non-binding, control click-PEG copolymer which do not contain pyrophosphate

[b] Weight average molecular weight

[c] *PDI* polydispersity index

HMHP displaying the strongest effect (~90% reduction). Given that the tooth surface is covered by saliva pellicle most of the time, we also tested whether PPi-PEG could effectively inhibit *S. mutans* accumulation through reduced saliva protein adhesion. Thus, HA discs were first treated with PPi-PEG, further coated with human saliva and then tested for bacterial adherence. The results of this experiment showed that a saliva pellicle can indeed strongly promote *S. mutans* adhesion to an HA surface, as indicated by the significant increase in CFU/disc of the untreated control group (Fig. 4b). However, PPi-PEG copolymers successfully reduced *S. mutans* binding, whereas non-binding polymer controls showed no effect at all.

DISCUSSION

PPi-PEG copolymers were synthesized using adjustable PEG building blocks and functionalized linkers. The potential benefits of such design are in their predictable safety profile and flexibility of the polymer structure. Both PEG and PPi, the two major components of this copolymer and its major predictable metabolic products (i.e. phosphate and PEG), are listed as Generally Regarded As Safe (GRAS) by the FDA (24). Therefore, we anticipate that this novel polymer should have an excellent safety profile, which is important when considering the translational application of these polymers in oral hygienic products. Thorough toxicity study is yet to be done to validate this hypothesis. Additionally, the building block and multifunctional linker design provides great flexibility to the structure of the copolymer. The di-azide linker contains two hydroxyl groups that are very convenient for further modifications with different functionalities, such as binding anchors, drugs, diagnostic molecules, etc. This means, by introducing the linker into the product polymer back bone, multiple types of functional groups can be added into the polymer chain. The density of these functionalities can also be easily adjusted by using PEG building blocks with different MW.

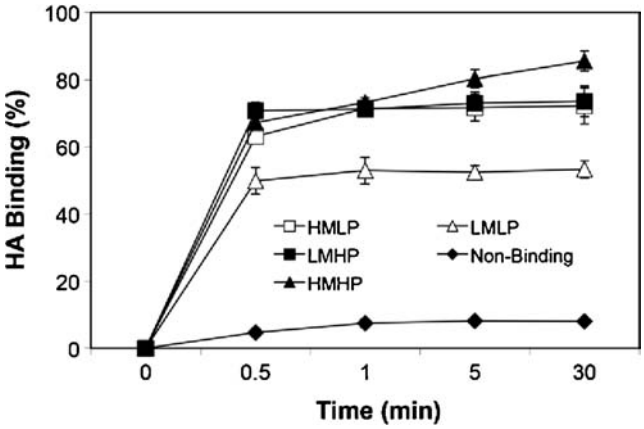

Fig. 2 The binding kinetics of PPi-PEG copolymer to HA surface. HMLP, PPi-PEG copolymer with high MW and low pyrophosphate content; LMHP, PPi-PEG copolymer with low MW and high pyrophosphate content; HMHP, PPi-PEG copolymer with high MW and high pyrophosphate content; LMLP, PPi-PEG copolymer with low MW and low pyrophosphate content; Non-binding, control click-PEG copolymer which do not contain pyrophosphate. *Data* are presented as the mean ± SD, $n = 3$. *Asterisks* indicate significant differences ($P < 0.05$).

The binding kinetics study demonstrated that both the increase of PPi content and polymer MW had a positive impact on binding capacity. Since the PPi molecule is known for its high affinity for HA crystals, it is not surprising that increased PPi density in the polymer chain appears to increase its HA binding capacity (Fig. 2). This effect was also demonstrated in our previous studies (23,25); however, it is not clear why the increase of MW also improve the binding capacity PPi-PEG. One explanation is that, since the increase in MW also increases the number of PPi molecules per polymer chain, a higher MW polymer would contain more PPi per chain to

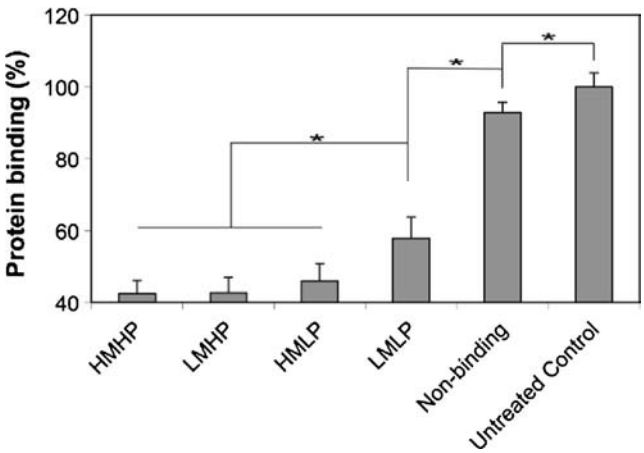

Fig. 3 *In vitro* inhibition of salivary protein adsorption on HA disc surface by PPi-PEG copolymers. HMLP, PPi-PEG copolymer with high MW and low pyrophosphate content; LMHP, PPi-PEG copolymer with low MW and high pyrophosphate content; HMHP, PPi-PEG copolymer with high MW and high pyrophosphate content; LMLP, PPi-PEG copolymer with low MW and low pyrophosphate content; Non-binding, control click-PEG copolymer which do not contain pyrophosphate. *Data* are presented as the mean ± SD, $n = 3$. *Asterisks* indicate significant differences ($P < 0.05$).

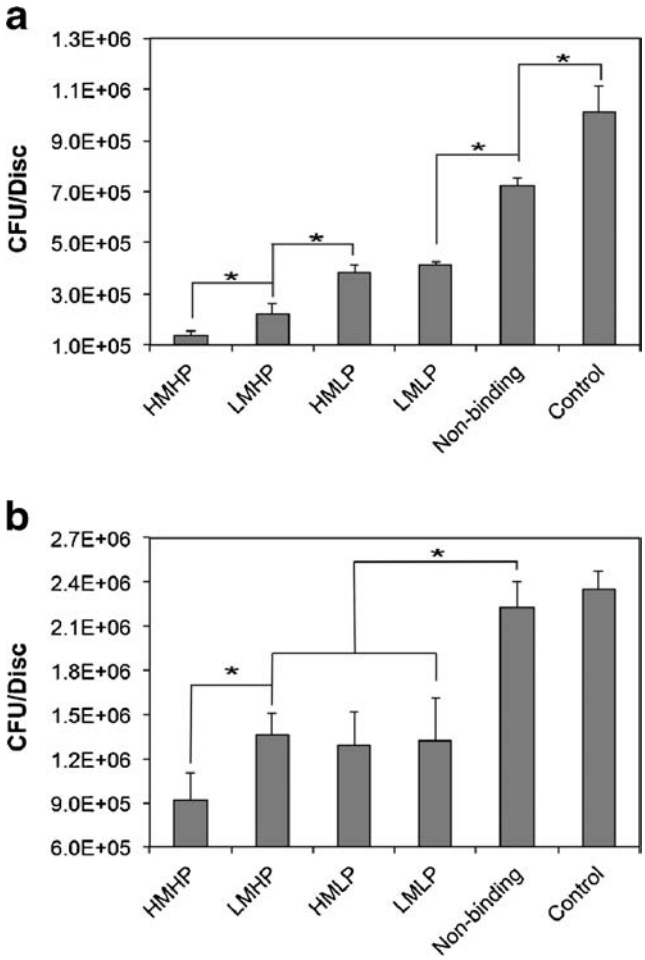

Fig. 4 *In vitro* inhibition of *S. mutans* adhesion on (**a**) clean HA disc surface; (**b**) saliva-coated HA disc surface. HMLP, PPi-PEG copolymer with high MW and low pyrophosphate content; LMHP, PPi-PEG copolymer with low MW and high pyrophosphate content; HMHP, PPi-PEG copolymer with high MW and high pyrophosphate content; LMLP, PPi-PEG copolymer with low MW and low pyrophosphate content; Non-binding, control click-PEG copolymer which do not contain pyrophosphate. *Data* are presented as the mean ± SD, $n = 3$. *Asterisks* indicate significant differences ($P < 0.05$).

provide stronger binding to HA than a lower MW polymer, due to the multivalent binding nature of these copolymers.

Further tests using HA discs suggested that PPi-PEG copolymer could effectively inhibit saliva protein adsorption to artificial enamel surface. The inhibitory effect of PPi-PEG copolymers involves two possible mechanisms. The first mechanism is the occupation of binding sites. Specifically, the HA surface possesses two different sites for protein binding, one of which is a positively charged calcium site (C site) and the other a phosphate site (P site), lacking calcium ions, which attaches to the basic groups of proteins (26). PPi-PEG can effectively occupy the C site through PPi groups, which provide a much stronger binding affinity than the carboxyl groups on the protein molecules (27). The second possible mechanism is the creation of a neutral hydrophilic layer on the HA surface. Due to the hydrophilic nature of the PEG polymer, the binding of PPi-PEG copolymers to HA could dramatically change the HA surface properties and create a neutral, hydrophilic layer, which in turn, could effectively reduce hydrophobic interactions of salivary protein and bacteria with the HA surface (28,29).

S. mutans is one of the early bacterial colonizers, that initiates the plaque forming process in the oral cavity (30), and also plays a major role in dental caries. Our PPi-PEG copolymer demonstrated strong inhibition of *S. mutans* adhesion on both clean HA surfaces, as well as that covered by a saliva pellicle. However, it should be noted that, in previous studies, phosphorylated polymers were unable to replace an existing saliva pellicle (14,31), suggesting that these polymers should be used during or immediately after dental hygiene procedures. The inhibitory mechanism of these polymers against *S. mutans* adhesion was likely through prevention of salivary protein adsorption as well as creation of a neutral, hydrophilic PEG layer on the HA surface. Non-binding polymer controls showed weak inhibition against *S. mutans* adhesion on a clean HA surface. However, this effect diminished immediately after incubation with human saliva, indicating that the presence of PPi is likely vital for the effectiveness of these polymers in the oral cavity. Interestingly, LMHP, which showed superior binding capacity as well as an inhibitory effect of protein adsorption, failed to demonstrate significantly better protection against *S. mutans* adhesion on saliva coated HA surface than HMLP and LMLP. However, HMHP, which performed the best in all evaluation studies, also showed the best efficacy in preventing *S. mutans* adhesion, suggesting that both high MW and PPi content are likely important for the efficacy of inhibiting *S. mutans* adhesion.

CONCLUSION

A linear PEG-based hydrophilic copolymer functionalized with a PPi tooth-binding anchor was successfully synthesized by "click" chemistry, using acetylene terminated PEG as building blocks and PPi-modified di-azide monomer as linkers. These PPi-PEG copolymers showed rapid binding kinetics and high binding capacity to the HA surface. They also effectively inhibited salivary protein adsorption by nearly 60%. *In vitro S. mutans* adhesion studies showed that the PPi-PEG copolymer could not only significantly prevent *S. mutans* adhesion to a clean HA surface, but also inhibited saliva protein-promoted *S. mutans* adhesion through reduced salivary protein adsorption and the creation of a neutral, hydrophilic layer on the HA surface. These results support the applications of PPi-PEG copolymer as a novel excipient in oral hygienic products. As an alternative or complimentary strategy to the existing antimicrobial therapies, it may help to further reduce the dental plaque accumulation and cariogenesis. This type of approach may also help to address

the safety issues associated with currently employed antimicrobial therapy and avoid potential detrimental impacts on beneficial oral microflora.

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