In vitro evaluation of antimicrobial activity of chlorhexidine hexametaphosphate nanoparticle coatings on orthodontic elastomeric chains

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1. Introduction

Fixed orthodontic appliance is composed of orthodontic archwires secured on brackets and bands that are bonded onto tooth enamel with composite resin and banding adhesive material and are held in position by elastomeric ligature ties and elastomeric chains. Introducing a fixed orthodontic appliance into the oral cavity encourages colonization of microorganisms around the brackets, elastomeric chains and elastomeric ligature ties. Excess composite material at the bracket-enamel interface is a critical site for bacterial plaque accumulation [1]. Placing orthodontic bands can also cause increase in gingival pocket probing depth and increase in number of gram-negative Bacteroides intermedius (B. intermedius) and gram-positive Actinomyces odontolyticus (A. odontolyticus) species [2]. The most common microorganisms that can colonize and cause white spot lesions (WSL) and dental caries on enamel surface are gram-positive Streptococcus mutans (S. mutans) and gram-positive Lactobacilli [3]. When poor oral hygiene is maintained after fixed orthodontic appliance placement, this
can result in formation of WSLs and periodontitis. WSLs are defined as ‘subsurface enamel porosity from carious demineralization’ [4]. Development of WSLs is due to prolonged plaque accumulation, which causes the decalcification of enamel [4, 5]. WSLs, if left untreated can progress to dental caries [6]. An earlier study showed that teeth ligated with orthodontic elastomeric rings exhibited a slightly greater number of microorganisms (S. mutans and Lactobacilli) than teeth ligated with stainless steel ligature wires [7]. It has been reported that the incidence of new carious lesions formed during orthodontic treatment in patients is 45.8% and the prevalence of carious lesions in patients undergoing orthodontic treatment is 68.4% [4]. The number of WSLs increased the most during the first 6 months of treatment and continued to rise at a slower rate to 12 months [5].

Current practices in WSL management by dental professionals were investigated in earlier studies [8, 9]. The most commonly recommended method by orthodontists to prevent WSL formation was the use of a fluoride mouth rinse after brushing [8]. Patients were encouraged to use a fluoride mouth rinse by 85% of orthodontists and 69% of general dentists and 76% of orthodontists recommended in-office fluoride treatment for patients with severe WSLs immediately after orthodontic treatment [9]. This treatment may cause additional problems since use of fluoride treatment after the formation of WSL can result in the formation of fluorapatite crystals which prevents the remineralization of WSLs. As an alternative, MI pasteTM has been suggested for treatment of WSL. RecaldentTM, the active ingredient of MI PasteTM, is a complex of casein phosphopeptides and amorphous calcium phosphate (CPP-ACP) that increases the level of calcium phosphate in dental plaque to promote remineralization of enamel [10].

Prevention of microbial buildup is a preferred alternative to treatment. Chlorhexidine (CHX) is an antimicrobial agent which belongs to the biguanide class of drugs that is efficacious against gram-negative, gram-positive bacteria and yeasts [11]. The mechanism of action of CHX is attributed to generalized membrane damage involving phospholipid bilayers at low concentration and CHX also causes congealing of cytoplasm at high concentrations [11, 12]. As a broad-spectrum antimicrobial and antifungal agent, CHX does not promote the development of bacterial resistance and it is widely used in medicine and in dentistry, as a mouthrinse in the form of CHX digluconate [11, 12]. The chemical structure of CHX digluconate is shown in figure 1 [13].

Studies were conducted by Wood et al [14, 15] and Barbour et al [16] to develop CHX releasing materials utilizing hexametaphosphate (HMP) nanoparticles. Sodium HMP is a cyclic inorganic phosphate widely used in the food industry and dental field due to its ability to inhibit the formation of dental calculus and prevent the formation of extrinsic stains [17, 18]. The chemical structure of Sodium HMP is shown in figure 2 [19]. CHX-HMP nanoparticles were prepared by adding aqueous sodium HMP to aqueous CHX digluconate under constant stirring and ambient conditions [14–16]. Specimens of glass [16], titanium [16], elastomeric wound dressing [16] and ethylene vinyl acetate polymer were successfully coated with CHX-HMP nanoparticle that provided continuous release of soluble CHX over a period of 50 days without reaching a plateau [15]. The antimicrobial activity of the released soluble CHX was shown by growth inhibition of methicillin-resistant Staphylococcus aureus (MRSA), Pseudomonas aeruginosa and Streptococcus gordonii [14–16]. These studies have shown that CHX-HMP nanoparticle can be affixed to materials with release of antimicrobially active CHX. Metal oxide nanoparticles like zinc oxide, copper oxide and iron oxide nanoparticles exhibit varying levels of
antibacterial activity against Gram-negative (Escherichia coli and Pseudomonas aeruginosa) and Gram-positive (Staphylococcus aureus and Bacillus subtilis) bacteria \[20\]. As CHX and Sodium HMP has been used widely in dentistry as antibacterial mouthwash and anticalculus agent and has been shown to be effective against oral microbes causing WSLs, coating of orthodontic materials such as orthodontic elastomeric chains (OEC) with antimicrobial CHX-HMP nanoparticles could provide the means to reduce WSLs by inhibiting the microbes causing the formation of the WSL. The antimicrobial effect of CHX-HMP nanoparticle on commercially available OEC has not been evaluated and the effect of such coatings on the force decay also needs to be evaluated.

The purpose of this in vitro study is to evaluate the (1) feasibility of coating of CHX-HMP nanoparticle on OECs, (2) CHX release from CHX-HMP nanoparticle coated OECs over the period of 28 days, (3) antimicrobial activity of the CHX released from CHX-HMP nanoparticle coated OECs and (4) the effect of coatings on force decay of OECs.

2. Materials and methods

2.1. Preparation of nanoparticle-coated orthodontic elastomeric chains

Solutions of 1 mM CHX (CHX-1), 5 mM CHX (CHX-5) were prepared from CHX digluconate salt (Sigma-Aldrich, St. Louis, MO, US). CHX-HMP nanoparticle colloidal solutions [1 mM HMP (CHX-HMP-1) and 5 mM HMP (CHX-HMP-5)] were prepared by adding aqueous sodium HMP (Sigma-Aldrich, St. Louis, MO, US) to aqueous CHX digluconate under constant stirring and ambient conditions. OECs of 6 rings in length (Energy Chain™ Elastics clear closed, J00120, Rocky Mountain Orthodontics, Denver, CO, US) were divided into groups as described in table 1. OECs were cleaned by ultrasonication for 10 min and then air-dried. Cleaned OECs were immersed in the above solutions and rapidly stirred for 30 s and then rinsed by deionized water for 10 s and air-dried.

2.2. Chlorohexidine release study

The amount of released CHX from CHX-HMP-nanoparticle coated OECs was studied for a period of 28 days. Each test group of 5 OECs were coated with solutions as indicated in table 1. The control group did not have any coating. A single OEC was placed in an individually labeled cuvette and 2.5 ml of deionized water was added to submerge the OEC. Cuvettes were kept sealed at ambient room temperature (24 °C) and medium was collected for evaluation of CHX release on day 1, 2, 3, 5, 7, 14, 21, and 28. The entire volume was collected on each time point and then the cuvette was refilled with 2.5 ml deionized water. Collected media were kept in sealed cuvettes and stored in the freezer at 0 °F until sample collection was completed. 200 μl of collected samples were placed into 96-well microplates with flat-bottom wells (36985, QIAGEN, Germantown, MD, US) and the absorption at 260 nm was measured by spectrophotometry (DTX 880, Beckman Coulter, Inc., Brea, CA, US) to determine the amount of released CHX. Standard solutions of 0–50 μM CHX were prepared as a reference and to calibrate the CHX concentrations.

2.3. Antimicrobial evaluation

Antimicrobial properties of coated OECs were tested for antimicrobial activity against Streptococcus mutans (KWIK-STIK S. mutans; ATCC® 25175™, American Type Culture Collection, Manassas, VA, US) and Lactobacillus rhamnosus (KWIK-STIK L. rhamnosus; ATCC® 7469™, American Type Culture Collection, Manassas, VA, US). S. mutans was cultured on Mitis Salivarius (M259, HiMedia Laboratories, Mumbai, India) agar plates and L. rhamnosus was cultured on MRS (de Man, Rogosa and Sharpe; M641, HiMedia Laboratories, Manassas, VA, US) agar plates.

Figure 2. Chemical structure of Sodium hexametaphosphate [19].

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| Group      | Coating solution                                      | Number of prepared specimens for CHX release study | Number of prepared specimens for microbiology evaluation | Number of prepared specimens for force decay evaluation |
|------------|-------------------------------------------------------|---------------------------------------------------|----------------------------------------------------------|-------------------------------------------------------|
| Control group | No coating                                            | 5                                                 | 3                                                        | 20                                                    |
| CHX-1      | Chlorhexidine digluconate 1 mM                       | 5                                                 | 3                                                        | 20                                                    |
| CHX-5      | Chlorhexidine digluconate 5 mM                       | 5                                                 | 3                                                        | 20                                                    |
| CHX-HMP-1  | Chlorhexidine hexametaphosphate 1 mM                 | 5                                                 | 3                                                        | 20                                                    |
| CHX-HMP-5  | Chlorhexidine hexametaphosphate 5 mM                 | 5                                                 | 3                                                        | 20                                                    |
Mumbai, India) agar plates. Antimicrobial evaluation was performed in triplicate per group for both *S. mutans* and *L. rhamnosus*. All cultures were incubated at 37 °C under aerobic conditions. 200 μl of eluate from day 1 of each of the test groups were placed at the center of agar plates, and then incubated at 37 °C for 4 days. After 4 days, the growth inhibition zones were measured by ImageJ Software (National Institute of Health, Bethesda, MD, US).

2.4. Microscopic analysis
Scanning electron microscopy (SEM; JSM-6700F, JEOL Ltd, Tokyo, Japan) was used to observe the surface of nanoparticle coated OECs. All 4 test groups and control group OECs were observed under SEM. After the CHX release study, test group OECs were observed under SEM to evaluate surface changes in the coatings.

2.5. Force decay measurement
The OECs were sectioned into six loop link lengths with the four middle loops actively utilized in experimentation to mitigate possible damage to the actively tested chain loops. The coated specimens were held on acrylic block jig with stainless steel pins set 25 mm apart (figure 3). The experimental distance simulated a first premolar extraction space closure acting as the distance between a first molar hook and the distal wings of a canine bracket. Over the course of the experimental protocol, the acrylic block jig was held in deionized water at ambient temperature. The force decay was measured in grams by an electronic force gauge (Lutron FG-5005; Taipei, Taiwan) at the following six-time points: Initial (immediately after coating; 0 h); 1 day; 7 days; 14 days; 21 days and 28 days.

In order to maintain a constant length during force measurement, the electronic force gauge was held at a constant vertical and horizontal relationship by use of acrylic transfer block and stainless-steel transfer jig.

Cumulative percentage of force decay was calculated in the following manner:
\[
\text{%FD} = \left( \frac{\text{IF} - \text{TF}}{\text{IF}} \right) \times 100
\]
where %FD: Percentage Force Decay, IF: Initial Force (grams) and TF: Force at a specific time interval (grams). Mean force measurements were calculated, and the standard deviation was determined for each of the control and experimental groups at all six-time points.

2.6. Statistical analysis
Release of chlorhexidine from four experimental categories was compared with one-way ANOVA, for each of the time periods under study. Multiple comparisons between the categories was performed by Tukey’s post-hoc test. One-way ANOVA along with Tukey’s post-hoc test was also used for comparing extent of zone of inhibition and force decay. The analysis was carried out by statistical package SPSS version 25.
3. Results

3.1. Microscopic analysis
SEM images were obtained at various levels of magnification and revealed coating of CHX-HMP nanoparticles on OEC surface (figures 4–7). SEM images at 2,000 × magnification showed smooth surfaces for the OECs of the control (figure 4(a)) and OECs coated with CHX-1 (figure 4(b)) and CHX-5 (figure 4(c)). No clear evidence of coated particles were found on the CHX-1 and CHX-5 surfaces. Large aggregates of nanoparticles were observed on OECs coated with CHX-HMP-1 and CHX-HMP-5 (figures 5(a)–(d)) at 2,000 × and 50,000 × magnification with the size of nanoparticles ranging from 37–71 nm measured at 170,000 × magnification (figure 5(e)). The size distribution histogram of the nanoparticles is shown in figure 5(f). Figure 6 shows SEM image of CHX-HMP-1 (a) immediately after coating and (b) after 28 days of CHX release study at 7,500 × magnification. Lesser nanoparticle aggregates were observed at day 28 when compared to the time point immediately after coating. Figure 7 shows SEM image of CHX-HMP-5 (a) immediately after coating where a denser coating can be observed and (b) after 28 days of CHX release study where the coating looks more porous at 7,500 × magnification.

3.2. Chlorohexidine release study
OECs coated with CHX-HMP-5 and CHX-HMP-1 released CHX over the 28 day period (figure 8), and ANOVA showing statistically significant differences for all testing period (p < 0.001). The OECs coated with CHX digluconate released smaller amounts of CHX over a shorter period of time. The CHX-HMP-5 group released the largest amount of CHX throughout the experimental period (figure 9). The amounts of CHX release determined spectrophotometrically on day 1 for the CHX-1, CHX-5, CHX-HMP-1 and CHX-HMP-5 coated OECs were 29.74 μmole, 62.36 μmole, 57.68 μmole and 98.82 μmole, respectively with all categories being statistically significantly different (p < 0.001) except CHX-5 and CHX-HMP-1. From Day 2 onwards (until the end of the study), the order of CHX level in categories from low to high was, CHX-1, CHX-5, CHX-HMP-1 and CHX-HMP-5, and the statistical significance was retained (P < 0.001) between all categories except CHX-5 and CHX-HMP-1. From Day 3 onwards, the difference of release level of CHX-5 was also statistically significantly

![SEM images](image-url)
lower than CHX-HMP-1 ($p = 0.001$), whereas all other categories retained the $p$ value of $<0.001$. Release of chlorhexidine ceased in CHX-1 category from day 5 onwards, and order of chlorhexidine release among other categories continued to be similar to as observed on day 2 with all categories staying statistically significantly different ($p < 0.001$). On day 28, CHX-HMP-5 group released 29.88 $\mu$ mole of CHX and CHX-HMP-1 group released 18.40 $\mu$ mole of CHX. No release of CHX was noted for CHX-1 and CHX-5 group on day 28. The cumulative amounts of CHX release for 28 days for the CHX-1, CHX-5, CHX-HMP-1 and CHX-HMP-5 coated OECs were 56.82 $\mu$ mole, 190.96 $\mu$ mole, 271.24 $\mu$ mole and 431.88 $\mu$ mole, respectively (figure 9).

### 3.3. Microbiological evaluation
After addition of 200 $\mu$l of eluate onto agar plates, a clear round zone of inhibition for *S. mutans* (MS agar plate, blue) and *L. rhamnosus* (MRS agar plate, yellow) was observed after day 4 (figure 10). The areas of the zones of inhibition (ZOI) for *S. mutans* and *L. rhamnosus* were largest in CHX-HMP-5 as shown in table 2 and figures 10 and 11. The zone of inhibition was larger for CHX-HMP-5 and CHX-5 than other two groups (figures 10 and

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**Figure 5.** Top row: SEM image of (a) CHX-HMP-1 and (b) CHX-HMP-5 at 2,000 $\times$ magnification immediately after coating. Aggregates of nanoparticles are observed in both groups with higher surface concentration of nanoparticles in CHX-HMP-5 group than the CHX-HMP-1 group. Middle row: (c) CHX-HMP-1 and (d) CHX-HMP-5 at 50,000 $\times$ magnification immediately after coating. Bottom row: (e) CHX-HMP-1 at 170,000 $\times$ magnification. Aggregates of nanoparticles varied from 37–71 nm in both CHX-HMP-1 and CHX-HMP-5 groups, (f) Size distribution histogram of the nanoparticles.
Figure 6. SEM image of CHX-HMP-1 (a) immediately after coating and (b) after 28 days of CHX release study at 7,500× magnification. Lesser nanoparticle aggregates are observed at day 28 when compared to the timepoint immediately after coating.

Figure 7. SEM image of CHX-HMP-5 (a) immediately after coating where a denser coating can be observed and (b) after 28 days of CHX release study where the coating looks more porous at 7,500× magnification.

Figure 8. CHX release evaluated spectrophotometrically on days 1, 2, 3, 5, 7, 14, 21 and 28 (n = 5/group). There was a gradual decrease of CHX release in all groups with continued release of CHX from CHX-HMP-5 throughout the 28 day period. CHX-1 ceased to release CHX after 5 days.
Post-hoc multiple comparison for *S. mutans*, showed the ZOI by CHX-HMP-5 to be statistically significant larger than all other categories (*p* = 0.002 versus CHX5 and < 0.001 for other two categories); although the ZOI by CHX5 was significantly larger than CHX1 (*p* = 0.038). Post-hoc Tukey’s test for *L. rhamnosus* showed CHX-HMP-5 ZOI to be statistically significantly larger than CHX-HMP-1 (*p* = 0.42), whereas the ZOI by CHX-5 was significantly larger than CHX-HMP-1 (*p* = 0.027).

### 3.4 Force decay measurement

For all groups, the largest mean loss of force occurred over the first 24 experimental hours in between time point 1 (initial) and time point 2 (24 h). The one-way ANOVA reported there was no significant difference in mean force at the following time points: 3 (7 days), 4 (14 days) and 6 (28 days) (figure 12). While at the following timepoints a significant difference (*P* < 0.05) was observed: 0 (Initial), 1 (1 day) and 5 (21 days). Average cumulative percentage of force decay was also reported revealing the largest percentage of force lost over the first
24 h followed by a relative plateau. All groups maintained >50% of the initial force over the experimental protocol as reported by the mean percentage decrease at the final time point (28 days) (figure 13).

4. Discussion

CHX and HMP combinations as test groups was chosen for coating OECs due to their reported capability of forming adherent nanoparticles, their aggregation on the specimen surface and release of CHX over long time periods [14–16]. We found that the CHX-HMP-nanoparticle coated OECs also showed similar results as previous studies.

SEM analysis showed that OECs were coated with CHX-HMP nanoparticles in aggregates. A greater surface area was covered by the CHX-HMP-5 than CHX-HMP-1. Importantly, aggregates were still present on day 28 of the release study for both CHX-HMP-1 and CHX-HMP-5 coated surfaces, suggesting that the CHX release from nanoparticle coated OECs could have continued even beyond Day 28. No coating was seen for the CHX-1 or CHX-5.

Deionized water was chosen for the release study even though it doesn’t mimic saliva. Artificial saliva solution (Biotene®, GlaxoSmithKline, London, England) was considered for the release study. Due to its chemical composition, it was not used in the study considering the potential interaction with the released CHX. Similar to earlier studies by Jeon et al [21] and Padois et al [22] the greatest amount of CHX release occurred on day 1 in all four test groups. CHX release on day 1 for the CHX-1, CHX-5, CHX-HMP-1 and CHX-HMP-5 coated OECs were 29.74 μmole, 62.36 μmole, 57.68 μmole and 98.82 μmole, respectively (p<0.001). The CHX release for CHX-HMP-5 and CHX-HMP-1 groups continued over the course of the experiment with approximately 3 times more CHX on day 1. On day 28, the CHX release for CHX-HMP-1 & CHX-HMP-5 were
about one-third of their release level on day 1. Although the CHX release on day 1 of CHX-5 OECs was marginally higher than CHX-HMP-1 OECs, the levels of release on day 2 to the end of the study diminished and CHX release was higher in the CHX-HMP-1 coated OECs than CHX-5 coated OECs (46.46 μmole versus 42.06 μmole on day 2). The difference between CHX released amount of CHX-1 and CHX-5 on day 1 was 2.09 times, whereas the difference between cumulative values at day 28 was 3.36 times. For CHX-HMP-1 and CHX-HMP-5, the difference between CHX released amounts on day 1 was 1.71 times, and that of day 28 was 1.59 times. In agreement with Wood et al. [15] the CHX-HMP-5 coated OECs showed the largest amount of CHX release over the 28 day period of the study (figure 8, p < 0.001) with potential for release beyond the period. As expected, there was less CHX release from the CHX-HMP-1 OECs than the CHX-HMP-5 OECs due to the observed lesser amount of nanoparticles on the surfaces of CHX-HMP-1 OECs.

The importance of the nanoparticles for sustained release was demonstrated by the rapid initial release of CHX when OECs were coated with the CHX without nanoparticles and then no release after day 5 (figure 8). This is contrasted to the prolonged CHX release over the 28 days of the study with a gradual decrease for the CHX-HMP-1 and CHX-HMP-5 coated OECs (figure 8, p < 0.001). The cumulative amount of release of CHX over the 28 days by CHX-HMP-5 was 1.6 times and 2.3 times greater than that of CHX-HMP-1 and CHX-5 respectively with a total amount of CHX released by CHX-5 that was 3.4 times greater than CHX-1 (figure 9). This result indicates that more CHX was bound to the OECs using the HMP-nanoparticles and that the HMP-nanoparticles promoted a slow and steady release of CHX which extends the treatment period with the antimicrobial. This shows that HMP-nanoparticle is effective as a carrier for CHX coatings and for slow, long-term continual release. The microbiological evaluation also confirmed that active and sufficient CHX is released of CHX-HMP-nanoparticle coated OECs to be effective against S. mutans and L. rhamnosus. S. mutans and L. rhamnosus, both gram positive bacteria are the primary microbes that cause WSLs and dental decay. Hence both these bacterial strains were used in this study to evaluate the antibacterial activity of the released CHX. One of the limitations of this study is the use of zone of inhibition calculation for measurement of antibacterial activity. Future studies should be supplemented with one more type of antibacterial assay to evaluate the antibacterial activity of the released CHX.

In terms of force decay, the results showed that all OEC specimens regardless of treatment had the highest force loss over the first 24 h followed by a steady gradual loss and a relative plateau similar to previous studies [23–28]. Also, over the first 24 h, 18%–36% of the mean initial force was lost in all specimen groups. In all groups, 37%–49% force loss was observed at the termination of the experiment. Over the majority of the experiment, the control group had the smallest force loss and the 5 mM CHX-HMP nanoparticle group the largest. However, the 1 mM CHX-HMP nanoparticle group had the smallest initial 24-hour force loss and the largest change over the first week. The 5 mM CHX-HMP nanoparticle group did not have the largest force loss at a single time point, 24 h were the 5 mM CHX was the largest at 36%. The ranges of average cumulative force loss in this experiment are similar to those reported widely in the literature [23–25, 27, 28]. However, other studies have shown an increased range over the first 24 h and the 4-week experimental protocol [29, 30]. These differences are often attributed in the literature to experimental conditions [31].
The results of a one-way ANOVA comparing the OEC mean force values in grams showed that a significant difference at half of the time points (initial, 1 day and 21 days). Despite the statistically significant differences, the mean force of all samples remained above optimum force for orthodontic tooth movement as established by Prollit et al with the lowest significant different mean of 215.45 g of the 5 mM CHX group at the 21st day is greater than the 70–120 g of optimum translational force. In fact, all groups of OEC mean force (g) remained above established optimum forces for all types of orthodontic tooth movement over the entire experimental protocol. The finding indicates that all experimental coatings of OECs provided a clinically acceptable orthodontic force over the 28-day protocol.

5. Conclusions

In conclusion, both groups of CHX-HMP nanoparticle coated OECs releases CHX over a period of at least 28 days. The eluate from day 1 is capable of inhibiting the growth of S. mutans and L. rhamnosus in vitro. The coatings did not alter the force decay of the OECs. Use of such a coating on OECs can exhibit antibacterial effect and reduction of biofilm buildup and prevent white spot lesions and offers promising clinical applications, which needs to be explored further.

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Conflict of interest

None to declare.

Ethical statement

The research protocol was reviewed by the University’s Institutional Review Board (IRB) and was exempt as the study did not involve human or animal subjects.

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