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Chlorhexidine hexametaphosphate as a coating for elastomeric ligatures with sustained antimicrobial properties: laboratory study

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Keywords: chlorhexidine, chlorhexidine hexametaphosphate, fixed appliances, elastomeric ligatures, orthodontic ligatures

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Declarations of interests:

Michele Barbour is named inventor on patents describing chlorhexidine polyphosphate technologies and is founder and director of Pertinax Pharma Ltd., a University spin-out company formed to commercialise these materials. The other authors have no competing interests to declare.

Keywords: chlorhexidine, chlorhexidine hexametaphosphate, fixed appliances, elastomeric ligatures, orthodontic ligatures

Highlights:

- Elastomeric ligatures were coated with chlorhexidine hexametaphosphate
- CHX-HMP-coated ligatures provide sustained release of ionic chlorhexidine
- CHX-HMP uptake is enhanced by ethanol but not acetone conditioning
- Ligatures release CHX continually over a period of 8 weeks
- Coating does not affect ligature extension, force delivery or dimensions

Abstract

Introduction: White spot lesions are a common side effect of orthodontic treatment. The aim of this laboratory study was to explore the suitability of chlorhexidine hexametaphosphate (CHX-HMP) as a coating for orthodontic elastomeric ligatures to provide sustained chlorhexidine release.

Materials and methods: dissolution kinetics of CHX-HMP were **firstly** explored using spectroscopy and a colorimetric phosphate assay. Elastomeric ligatures were divided into three groups: acetone conditioned, ethanol conditioned or as received, and were then immersed in 5 mM CHX-HMP suspension, or 5 mM chlorhexidine digluconate (CHXdg) solution, then rinsed. Chlorhexidine (CHX) release was measured over 8 weeks, and the effects of conditioning and immersion on elastomeric force and extension at rupture and **surface topography were investigated.**

Results: **CHX-HMP exhibited a gradual equilibration that had not reached equilibrium within 8 weeks, releasing soluble CHX and a mixture of polyphosphate and orthophosphate.** CHXdg treated ligatures showed no CHX release, whereas CHX-HMP treated ligatures showed varying degrees of release. As received, CHX-HMP treated ligatures showed a modest release of CHX up to 7 days. Acetone conditioning did not enhance CHX-HMP uptake or subsequent CHX release and caused a deterioration in mechanical properties. Ethanol conditioning enhanced CHX-HMP uptake (x6) and led to a sustained CHX release over 8 weeks without affecting mechanical properties.

Conclusion: Within the inherent limitations of this *in vitro* study, CHX-HMP leads to a sustained release of CHX from orthodontic elastomeric ligatures following ethanol conditioning. Conditioned and coated elastomeric ligatures may ultimately find application in the prevention of WSL in orthodontic patients.

Introduction

White spot lesions (WSL) are a common iatrogenic effect of orthodontic treatment, since orthodontic appliances compromise oral hygiene and promote plaque accumulation¹.

Demineralisation around orthodontic brackets and bands can be rapid, with WSLs developing after as little as 4 weeks of appliance placement². Although some remineralisation usually occurs after orthodontic treatment is concluded, baseline pre-treatment levels are never regained³. Furthermore, orthodontic appliances can increase the proportions of pathogenic microbes, increasing the likelihood of caries developing⁴. Figures quoted for WSL in orthodontic patients vary widely, with incidences ranging from 25% to 73%^{5,6,7,8} and these differences are most likely due to inconsistent definition and reporting of WSL rather than necessarily genuine differences in occurrence.

Oral hygiene practices are accepted as the single most important factor in determining whether WSL will develop⁹, but this preventative method relies heavily on patient compliance. It is known that oral health and hygiene often deteriorate rapidly after a fixed orthodontic appliance is fitted, which is usually explained by the patient experiencing pain and becoming accustomed to their new appliance¹⁰. For this reason, orthodontic biomaterials such as adhesives, bands and brackets, which are inherently antimicrobial or anticariogenic, have been the subject of attention in the research community. Orthodontic ligatures are a potentially useful vector for localised antimicrobial delivery to prevent WSLs in orthodontic patients. The ligatures are in close proximity to the enamel surface and are regularly replaced during a course of orthodontic treatment. Sustained, appropriately dosed antimicrobial delivery could therefore reduce dependence on patient compliance. This is not

a new concept. Elastomeric ligatures are easy to apply, cheap and relatively hygienic, making them the most common form of orthodontic ligation. The ligatures used in orthodontics are usually made from latex or polyurethane and are replaced at each orthodontic appointment, owing to their limited resistance to the oral environment.

Chlorhexidine (CHX) is not commonly used as part of orthodontic treatment, although it is used widely in general dentistry. CHX is a cationic bisbiguanide with broad-spectrum antimicrobial activity, which is effective against a wide range of bacteria and yeasts. Being cationic, CHX is attracted to the negatively charged bacterial cell wall and binds to the inner membrane. This increases cell wall permeability, leading to loss of cell components, precipitation of the bacterial cytoplasm and cell death. CHX is most commonly used as the digluconate salt (CHXdg), which is readily soluble in water and therefore convenient to formulate into mouthrinses and other aqueous topical agents.

A novel salt of CHX, CHX-hexametaphosphate (CHX-HMP), has been reported as a material that provides sustained release of the constituent CHX when exposed to an aqueous environment¹¹. Owing to the physical and chemical properties of this salt, it can be used as a component of composite materials. Provided the composite has a degree of water permeability, it can provide sustained release of chlorhexidine under aqueous conditions. The dose and duration of release are influenced by a number of factors such as doping, local physicochemical conditions (flow, temperature, ionic strength, other ions) and host substrate. For instance, CHX release from glass ionomers doped with CHX-HMP can be

sustained for over 2 years¹², which is substantially longer than in other studies of CHX modified GICs and related materials^{13,14,15}.

The aim of the study described here was to explore whether CHX-HMP might be a useful component of elastomeric ligatures that could provide sustained release of CHX over the usual 4-8 week period between orthodontic appointments, and without adversely affecting pertinent mechanical and physical properties of the ligature.

Materials and methods

Analysis of CHX-HMP dissolution kinetics

Pure CHX-HMP powder for characterisation was supplied by Pertinax Pharma (Bristol, UK).

Saturated CHX-HMP suspensions were prepared using 10 g.L⁻¹ CHX-HMP in deionised water (DIW). The suspensions were stored in 250 mL lidded glass Schott bottles (n = 3 for each of CHX and phosphate analysis) and incubated at 37°C with constant stirring for the duration of the experiment. The pH of the suspensions were measured at regular intervals. Following centrifugation (21000 g, 15 minutes), the resulting supernatants were isolated, diluted as required to cause the expected CHX or phosphate concentration to fall within the range of the calibration standards, and transferred to sealed containers for further use.

For CHX analysis, 1 mL of the supernatant was placed in lidded, semi-micro polystyrene cuvettes, which were transparent in the UV for spectrophotometry at 255 nm. The reference were the calibration standards of aqueous CHX digluconate solutions (5-65 µM CHX), and with the deionised water background signal subtracted.

For phosphate analysis, 10 mL supernatant was combined with a commercial phosphate analysis reagent mixture (ammonium molybdate, potassium antimonyl tartrate, potassium disulfate and ascorbic acid; Hanna Instruments, Leighton Buzzard, UK). Measurements were also conducted using stock solutions of 25 μ M sodium dihydrogen orthophosphate (NaOP; Sigma Aldrich, Gillingham, UK) and 40 μ M sodium hexametaphosphate (NaHMP; Prayon, Lyon, France), where the concentration of NaHMP was calculated using the average phosphate chain length given by the manufacturer (16.5). After mixing by agitation for approximately 5 seconds, the mixture was left for 2 minutes before transferring a 1.5 mL aliquot into a lidded, semi-micro polystyrene cuvette. The reaction was monitored using absorbance values at 880 nm (λ_{max} of the blue phosphomolybdate complex) with the deionised water background signal subtracted at regular intervals over the course of 60 minutes.

Preparation of CHX-HMP and functionalised ligatures

For the purpose of functionalising ligatures, an aqueous suspension of CHX-HMP was prepared using the method described by Barbour *et al.*, (2013) ¹¹, whereby 10 mM solutions of CHXdg and NaHMP (nominal HMP chain length 17; both Sigma Aldrich, Gillingham, UK) were mixed by rapid stirring under atmospheric pressure at room temperature to yield a resultant equivalent CHX concentration, in CHX-HMP solid, of 5mM. For comparison a 5mM aqueous solution of CHXdg was also prepared.

A total of 465 silver-coloured polyurethane elastomeric ligatures with occlusal guards (AlastiK™ 3M Unitek, UK) were rinsed in deionised water and allowed to air dry for 1 hour

prior to use. To test the effect of solvent conditioning on CHX-HMP release, the ligatures were either used in this as received but washed condition (stored in a dry environment), or were immersed in either ethanol or acetone for 60 min under agitation. Immediately after conditioning, the ligatures were then immersed in one of three solutions, namely: deionised water, 5mM CHXdg or 5mM CHX-HMP for 10 min under agitation. This was followed by a final immersion in DIW for 10 s in order to remove any unbound material and air drying for at least 1 hour prior to further use.

In this way, a total of 9 experimental groups of ligatures (n=180) were prepared and were referred to as N, E or A as prefix (none, ethanol or acetone as the solvent conditioning) and -DIW, -CHXdg or -CHX-HMP (deionised water, CHX digluconate or CHX-HMP as the coating treatment) as the suffix. Therefore, as an example N-DIW refers to no solvent conditioning and only DIW treatment, whereas E-CHX-HMP refers to ethanol conditioning and CHX-HMP treatment.

Chemical analysis of ligatures

Following functionalisation, the ligatures (n=20) were placed into individual UV-transparent cuvettes containing 2 mL DIW and sealed. The release of CHX as a function of time was measured using UV spectrophotometry as described above. Cumulative CHX release at the conclusion of the 8 week period was analysed using a two-way analysis of variance (ANOVA) with factors of pre-treatment and coating using a significance level of 0.05 and a Tukey HSD post-hoc test.

Physical and mechanical analysis of ligatures

Two samples from each group were examined using scanning electron microscopy (SEM) (Phenom, Eindhoven, Netherlands). The effect of just the solvents (water, ethanol and acetone) was also determined after drying for more than 24 hours, to avoid transient effects (n=5). Ligature width and lumen size were measured using light microscopy (4x magnification) and Cellsens computer software (Olympus, KeyMed (Medical & Industrial Equipment) Ltd, Southend-on-Sea, UK). The data were analysed using one-way ANOVA with a Tukey HSD post-hoc test.

To explore the effects of the treatments on the mechanical properties of the ligatures in the 9 groups, 30 specimens of each were tested using a universal testing machine (Zwick/Roell, Herefordshire, UK) with a 500 N load cell and a test rate of 100 mm per minute. Each ligature was mounted using a customised jig comprising two 0.6 mm stainless steel loops fastened to the clamps of the testing machine. Due to the asymmetric nature of the elastomeric ligatures under test, they were mounted with the occlusal guard facing in the same orientation. The data were analysed using a two-way ANOVA with a Tukey HSD post-hoc test.

Results

Analysis of CHX-HMP dissolution kinetics

The pH of the CHX-HMP suspensions varied between 5.7 and 6.0 over the period of measurement. There was no trend over time.

The aqueous CHX concentration in the CHX-HMP suspensions equilibrated in DIW as a function of incubation time as shown in **Figure 1**. There was an initial period (<1 day) during which CHX was released rapidly, reaching a pseudo-equilibrium concentration of 200 μM , followed by a period during which [CHX] the concentration changed little. After a lag of approximately 30 days the CHX concentration increased steadily at approximately 7 $\mu\text{M}/\text{day}$ for the remainder of the experiment.

Figure 2 shows an absorbance wavelength of 880 nm, the peak absorbance wavelength for the phosphomolybdate complex as a function of reaction time following mixing stock solutions of NaOP and NaHMP with the phosphate assay reagents. The data are expressed as percent of the maximum absorbance reached over the 60 minute period. When only orthophosphate was present there was no change in absorbance over the 60 minute period (**Figure 2a**). When only polyphosphate was present there was a material change of the absorbance at 880 nm by a factor of two (**Figure 2b**) and therefore in the concentration of the phosphomolybdate complex, over 60 minutes. This was attributed to hydrolysis of the polyphosphate to smaller phosphates, including orthophosphate, under the strongly acidic conditions of the phosphate assay. Therefore, when analysing unknown phosphate solutions, a stable reading over the 60 minute period following combining the reagents

indicates the presence of orthophosphate only, whereas an increasing reading is likely to indicate the presence of polyphosphate(s), possibly in combination with orthophosphate. Note, that not all of the polyphosphate was hydrolysed to orthophosphate over 60 minutes, and after longer periods the phosphomolybdate complex is unstable and breaks down. Therefore, this assay cannot under these conditions be used to ascertain concentration of polyphosphate in solution, only to infer its presence or absence.

Absorbance at 880 nm as a function of reaction time following mixing the supernatant from CHX-HMP suspensions with the phosphate assay reagents are shown in **Figure 3**. The data for the supernatant obtained after all CHX-HMP equilibration times (1 and 5h, 3, 7 and 24 days) show an increase in absorbance over the 60 minutes, starting at approximately 80% of maximum and reaching 100% within 15-20 minutes. This is intermediate between the two solutions illustrated in **Figure 2** and suggests the presence of both polyphosphate and orthophosphate in the solution.

Analysis of CHX-HMP functionalised orthodontic ligatures

The SEM images (Figure 4) reveal an inhomogeneous coating (of CHX-HMP) on the surfaces of the ligatures treated with CHX-HMP, whereas ligatures immersed in CHX-dg had no visible deposits and were physically indistinguishable from untreated ligatures (images not shown). There was no discernible difference between the surface CHX-HMP coating as a function of solvent conditioning. None of the conditioning or coating treatments resulted in a macroscopic change in the colour of the ligatures as judged by eye. Ethanol conditioning did

not affect ligature dimensions, but acetone conditioning resulted in a permanent swelling of the ligature, increasing the diameter and decreasing lumen size (Table 1).

CHX elution from the ligatures is shown in **Figure 5** and represents CHX release for a nominal “mouthful” of 20 ligatures. Uncoated ligatures (N-DIW, E-DIW and A-DIW) exhibited a small and short-lived release of a substance, absorbing radiation at 255 nm, which cannot be CHX since these ligatures are not exposed to CHX salts. Instead it was thought to be a by-product of the ligature manufacture. For this reason, **Figure 5** represents CHX release data that is normalised to controls by subtracting the signal at 255 nm from the DIW-treated ligatures. Ligatures “coated” with CHXdg were indistinguishable from uncoated ligatures, irrespective of solvent conditioning; that is, they exhibited no CHX release. Ligatures coated with CHX-HMP showed greater CHX release than DIW or CHXdg ligatures. Whereas CHX release was sustained for less than 10 days in the case of unconditioned and acetone-conditioned ligatures, for the ethanol-conditioned ligatures CHX release continued for the duration of the experiment i.e. 8 weeks.

The two-way ANOVA indicated that both the conditioning and the coating significantly affected CHX release. The Tukey HSD test indicated that the CHX-HMP coated ligatures resulted in greater CHX release than the CHXdg or DIW ligatures, and that the CHXdg ligatures could not be statistically distinguished from the DIW ligatures.

The values for force (N) and extension (mm) at rupture are shown in **Table 2**. Acetone had a statistically significant effect on both force and extension at rupture. Ethanol-conditioned

ligatures were indistinguishable from no conditioning. CHX-HMP had no effect on force at rupture ($p = 0.19$) or extension ($p = 0.69$).

Discussion

The widespread use of elastomeric ligatures and their close proximity to the enamel surface should make them the ideal and convenient vectors for the delivery of antimicrobials in orthodontics, with less reliance on patient compliance in the prevention of white spot lesions. This concept of localised delivery of an antimicrobial is not new, but the results of clinical and laboratory studies have both been somewhat equivocal. This might be down to the delivery system, or in some cases the experimental design. In one study fluoride-releasing ligatures were assessed in a randomised crossover clinical trial, but were found to have no statistically significant anticariogenic effect¹⁶, which was attributed to the short-lived nature of the fluoride release¹⁷. By contrast a separate case control study reported fluoride releasing elastomeric ligatures and chain to have a statistically significant effect in reducing the number of observed white spot lesions¹⁸. Silver nanoparticles have also been tested as an elastomeric ligature coating. In one *in vitro* study the silver nanoparticles provided local antimicrobial efficacy against microbes including *S. mutans* and *L. casei*, although the durability of the coating and the longevity of the efficacy were not explored¹⁹. In another study which assessed ligature colonisation by *S. mutans*, no significant inhibitory effect was observed²⁰.

The aim of the present study was to determine whether it was possible to produce elastomeric ligatures capable of sustained chlorhexidine release. The utility of *topical* CHX products in reducing microbial colonisation and inhibiting the formation of WSL in

orthodontic patients is widely recognised^{21,22,23}. CHX digluconate and diacetate have both previously been incorporated into prototype ligatures comprised of three polyurethane layers. Although fabricating multilayer ligatures is complex, CHX release was sustained for up to 6 weeks. However, the CHX salts were incorporated at comparatively high loadings (10 and 20 %) and the effects on the mechanical and physical properties on the multi-layer structure of the ligatures were not assessed²⁴.

The ability of the novel salt, CHX-HMP, to release CHX over a period of several weeks or months under aqueous conditions has been reported elsewhere^{11,12,25}. However, the solubility properties of this material have not previously been reported, but are important to understand in order to inform the most appropriate deployment of this technology in anti-infective medical devices. The data presented here indicates that during a prolonged exposure to an aqueous environment, CHX-HMP continues to release CHX and does not reach equilibrium in a sealed system after 8 weeks. This was shown by the ongoing increase in CHX concentration at the conclusion of the experiments (**Figure 1**). An initial period of rapid CHX release was followed by a slower and much more gradual CHX release. The phosphate-based counter ions released appear to be a mixture of polyphosphate and orthophosphate, and there was no clear change in this as a function of equilibration time of the CHX-HMP (**Figure 2** and **Figure 3**). Orthophosphate is ubiquitous in the body and in consumer products. Polyphosphates, such as hexametaphosphates, are already in widespread use as emulsifiers in foods and in oral care products such as toothpastes, and so their potential release in small quantities from orthodontic ligatures is unlikely to present a health risk. **The conditions in this study were significantly simplified in comparison to the *in***

vivo situation and were not intended to replicate the complex and varying composition, temperature and pH of saliva and associated oral fluids. The CHX concentration that could result from the use of these materials *in vivo* would be less compared to what was reported in this study as the oral environment is an open system and hence some dilution of the CHX would occur during activities such as eating, swallowing and tooth brushing.

The microscopy images revealed that all ligatures incubated with CHX-HMP suspensions had an inhomogeneous coating of CHX-HMP (**Figure 4**), and this inhomogeneity is to some extent reflected in the CHX release data (**Figure 5**), particularly for the sparsely coated ligatures (no conditioning and acetone conditioning). For the ethanol conditioned CHX-HMP coated ligatures (E-CHX-HMP in **Figure 5**), the CHX release data suggest that the inhomogeneity was less pronounced. Nevertheless, the degree of control of the coating would need to be further improved for a product to be commercially realised.

Functionalisation of orthodontic ligatures with CHX-HMP was enhanced by solvent conditioning using ethanol. The use of organic solvents to impregnate biomedical polymers with antimicrobials has been reported previously, and in the case of silicone catheters it leads to sustained antimicrobial release of at least 30 days²⁶. Exposure of polyurethane orthodontic ligatures to ethanol for one hour has been shown to cause a degree of chemical degradation²⁷, although this was assessed not by chemical analysis but by the degree of discolouration. The actual nature of any degradation and its potential clinical significance were not determined. No such colour changes were observed in the current study.

Ethanol/water mixtures have been used for the explicit purpose of “artificial aging” in lieu of

in vivo use to explore how orthodontic ligatures respond to environmental factors²⁸.

Therefore, it is plausible that exposure to ethanol used in this study was sufficient to soften the surface layers of the ligature, without affecting the bulk, enhancing the uptake of CHX-HMP at the surface and in the near-sub-surface region. This might explain the greater CHX release from these ligatures without adverse effects on the mechanical properties. Other researchers have sought to confer lasting antimicrobial properties on orthodontic ligatures using CHX salts. A sandwich structure of polyurethane – CHX diacetate or CHX digluconate – polyurethane was constructed, and this yielded ligatures which resulted in release of CHX for the duration of the experiment (42 days), but which decayed over time, with 8-15 % of the total CHX released during the first day, and with only around 0.5 % of the CHX released per day at the end of the period of analysis (days 35-42)²⁴.

During clinical use elastomeric ligatures are subject to tensile forces, particularly during placement. However, it is important they maintain adequate tensile strength during treatment in order to sustain full engagement of archwires within the bracket slot and to elicit the desired tooth movement. Therefore, it is important that any alteration, such as conditioning and the addition of an antimicrobial, must not detrimentally affect the mechanical/physical properties of the elastomeric ligature. Conditioning in ethanol and subsequent coating with CHX-HMP in this experiment did not result in any statistically significant changes to the physical properties under investigation. Whereas conditioning using acetone caused changes in to both the dimensions and the elastic behaviour. For this reason, coupled with the observation that it did not enhance CHX-HMP uptake, acetone conditioning was not considered suitable.

1. Conclusions

The following conclusions can be drawn from this study investigating the potential use of CHX-HMP to functionalise orthodontic elastomeric ligatures:

- CHX-HMP incubated with water for 10 weeks continually released aqueous CHX and via a mixture of orthophosphate and polyphosphate, both of which are used widely in oral care and other consumer products.
- Elastomeric ligatures can be functionalised with CHX-HMP via immersion coating, creating a coating that subsequently releases soluble CHX.
- The uptake of CHX-HMP and subsequent CHX release was enhanced by conditioning with ethanol, but not acetone.
- Ethanol conditioning and CHX-HMP coating did not significantly affect the maximum extension, maximum force delivery or dimensions of the ligatures.
- Further work should focus on attaining a homogeneous CHX-HMP coating.

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Figures and Tables

Figure 1. CHX_{aq} concentration in a suspension of CHX-HMP in water. Note the (1) fast equilibration (<1 day) to ~200 μ M CHX, (2) the period during which the CHX concentration changes little to over the next 20-30 days and (3) the slow and sustained release of CHX following this period.

Figure 2. Absorbance at 880 nm, the peak absorbance wavelength for the phosphomolybdate complex, as a function of reaction time following mixing stock solutions of (a) sodium orthophosphate or (b) sodium hexametaphosphate with the phosphate assay reagents, expressed as % of the maximum absorbance during the 60 minute period.

Figure 3. Absorbance at 880 nm as a function of reaction time following mixing supernatants from CHX-HMP suspensions equilibrated for different times (see legend) with the phosphate assay reagents and diluting such that the concentration of phosphate fell within the linear range of the calibration standards, expressed as % of the maximum absorbance during the 60 minute period. For all equilibration times there is an initial increase in absorbance at 880 nm over 15-20 minutes. This is less than the proportional change expected for a pure polyphosphate solution (Figure 2b), and is followed by a period of little or no change, indicating the presence of both orthophosphate and polyphosphate in the solution.

Figure 4. Scanning electron micrographs of elastomeric ligature surfaces after no (N), ethanol (E) or acetone (A) conditioning then coating with CHXdg or CHX-HMP. Image width 200 μ m.

Figure 5. Cumulative CHX elution from ligatures as a function of conditioning method (none, ethanol or acetone), coating modality (DIW, CHXdg or CHX-HMP) and time.

Table 1. Mean lumen and ligature diameter (μm) as a function of conditioning treatment.

Standard deviations are given in parentheses. Superscript letters that are the same indicate no statistically significant difference between the groups. With acetone conditioning there was a statistically significant effect on both lumen size and diameter compared with the controls. No such effect was seen with ethanol conditioning.

Table 2. Force and extension at rupture for ligatures as a function of conditioning method (none, ethanol or acetone), and coating modality (DIW, CHX-HMP). Standard deviation shown in parentheses. Coating (DIW vs CHX-HMP) had no statistically significant effect on force or extension at rupture. Acetone significantly increased both force and extension at rupture whereas ethanol affected neither parameter. Ligatures treated with CHXdg were not investigated for mechanical properties as investigations reported elsewhere in the manuscript had already revealed that they did not exhibit significant CHX release.