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The anti-bacterial effect of zinc-doped phosphate-based glasses (Zn-PBG) and its role in the demineralisation and remineralisation process of bovine enamel

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1.0 Abstract

The anti-bacterial effect of zinc-doped phosphate-based glasses (Zn-PBG) and its role in the remineralisation and demineralisation process of bovine enamel by Sindhuja Rajadorai

Aims

This is a laboratory-based experimental study that aims to assess the anti-bacterial effect of zinc-doped phosphate based glasses on the growth of *Streptococcus mutans* and the role of zinc in the demineralisation and remineralisation process of bovine enamel. Enamel demineralisation is highly prevalent during orthodontic treatment. Newly developed zinc doped phosphate-based glasses (Zn-PBGs) are controlled delivery agents for zinc ions that may be effective in reducing the incidence of white spot lesions.

Materials and Methods

Zinc doped phosphate-based glasses (3mol% zinc and varying calcium concentrations, denoted as C11, C12 and C13) and control glasses which are zinc free phosphate-based glasses (C-PBG) rods (5 x 2mm) were produced using a conventional melt quenching method, at 1100°C for 1 hour. The calcium in the glass serve to control and alter the degradation rate of the glasses which contributes to a controlled manner manner of ion delivery. The glass characteristics were assessed through glass degradation studies (0-56h), which were carried out using a weight loss method in dH_2O at 37 ± 1^0C and a starting pH of 7 ± 0.1. A pH analysis was conducted and calcium, zinc, sodium and phosphate concentrations remaining in solution were measured by inductively- coupled optical-emission spectrometry (ICP-OES).

The anti-bacterial effects were assessed through disc diffusion assays and liquid broth analysis. Disc diffusion assays were conducted on isosensitest (IST) agar with standardised cultures of *S.mutans* NCTC10449. The plates were incubated for 24 hours aerobically at 37°C. The diameters of the zones of inhibition that developed around each of the glass samples were measured. A liquid broth assay was conducted in phosphate buffered saline (PBS) using *S.mutans* suspension to a standardised optical density (OD_{600}=0.03) exposed to C11, C12 and C13 and controls (a positive control of 0.2% chlorhexidine and a negative control of C-PBG). At 2, 4, 6 and 24 hours, samples were removed, diluted appropriately in PBS, spread on BHI agar to assess viable colony-forming units (CFU) present.

The effect of Zn-PBG on bovine enamel was investigated under pH cycling and utilising a constant depth film fermentor (CDFF) model. pH cycling of enamel blocks allocated to 1 of 5 conditions (Zn-PBG, C-PBG, $ZnSO_4$, NaF and H_2O) over 5 days of cyclical acidic challenge of immersion in demineralising solutions for 6h (2.0 mM $CaCl_2$, 2.0 mM KH_2PO_4, 0.04 ppm NaF and 0.075 M acetic acid adjusted to pH 4.7 with 1.0 M NaOH;) and remineralising solutions for 18h (1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4, 150 mM KCl, 0.05 ppm NaF and 20 mM HEPES adjusted to pH 7.0 with 1.0 M NaOH) followed by 2 days in remineralising solution. Data obtained from the pH cycled samples were analysed using non-contact surface profilometry, (NCSP) (Proscan 2000) to assess surface roughness (Iso Ra) of the bovine enamel samples. Biofilms were grown in the CDFF model on bovine enamel discs using artificial saliva. On the 5th day and 12th day, the pans were removed aseptically from the CDFF system and the discs containing biofilms were removed, subjected to 10 minutes exposure of Zn-PBG (C11) compared with 0.2% diluted chlorhexidine, 0.05% sodium fluoride or water. Transverse micro-radiography (TMR), to quantify and assess mineral loss (ΔZ) from bovine enamel, was used for bovine enamel samples exposed to dissolving acids in the CDFF model and the pH cycling experiment. All tests were conducted in triplicate.

Statistical Analysis

Statistical analyses were conducted using the Prism GraphPad software (San Diego, California, USA). Paired T-tests, a one-way analysis of variance (ANOVA), and Tukey Kramer multiple comparison tests were used to compare values and p values <0.05 were considered statistically significant.

Results

The average pH values over time were 6.95 ± 0.19 for C-PBG, 6.87 ± 0.17 for C11, 6.71 ±0.04 for C12 and 6.71 ± 0.06 for C13. pH analyses showed a statistically significant difference ($p <0.05$) for C12 and C13 when compared with C-PBG. The degradation rates of the Zn-PBGs were significantly ($p<0.05$) different (28.94µg mm^{-2} h^{-1} for C11, 25.56µg mm^{-2} h^{-1} for C12 and 19.72µg mm^{-2} h^{-1} for C13) compared with C-PBG (10.17µg mm^{-2} h^{-1}). Zinc ion release was decreased as calcium concentration increased in the glasses, which showed a strong correlation with the weight loss of the glasses.

Zones of inhibition from disc diffusion assays were found to decrease (from 18± 1.7, 16± 1.6 to 15.0 ± 1.1 mm) as the calcium concentration in the glasses increased from 11, 12

and 13 mol% of calcium. Zones of inhibition were significantly increased for C11, C12 and C13 ($p < 0.01$) when compared with C-PBG. Viable cells in the liquid broth assay were found to decrease as the calcium concentration in the glasses increased from 11, 12 and 13 mol% of calcium. Viable cell counts in the untreated group and C-PBG group were significantly increased ($p< 0.01$) when compared with groups C11, C12 and C13.

From the constant depth film fermentor (CDFF) model, there was statistically significant mineral loss ($p <0.05$) in the bovine enamel samples that were exposed to C-PBG, NaF, Chx and enamel discs (ED) at day 12 compared with day 5. There was no statistically significant difference in mineral loss ($p>0.05$) in the bovine enamel samples that were exposed to C11 between day 5 and day 12. NCSP analysis of the bovine enamel samples that were subjected to alternating remineralisation and demineralisation solutions through pH cycling was carried out. It was revealed that there were no significant differences in surface roughness (Iso Ra) of the bovine enamel samples between C11, C12, C13, C-PBG, $ZnS0_4$, NaF and H_20. Transverse microradiography (TMR) analyses showed significant ($p<0.05$) mineral loss in the pH-cycled H_20 group when compared with pH-cycled C11, C12, C13, C-PBG, $ZnS0_4$ and NaF.

Conclusion

The results suggest that the controlled delivery of zinc ions from zinc-doped phosphate based glasses may have potential in oral applications due to the anti-bacterial effect of Zn-PBG demonstrated in the disc diffusion and liquid broth assay and the inhibition of demineralisation as shown in the CDFF model via TMR analysis. There is scope for further research in this area.

2.0 INTRODUCTION

Orthodontics and dento-facial orthopaedics is the specialised field of dentistry that involves the diagnosis, prevention, interception and correction of a malocclusion, in order to provide the patient with the ideal functional occlusion and aesthetics both dentally and facially as well as a stable dental occlusion (Proffit, 2000). Although orthodontic treatment can result in a healthy and attractive smile, it does have its limitations and potential risks. Successful treatment is achieved when the benefits of treatment outweigh the risks of orthodontic treatment. Some of the risks of orthodontic treatment include enamel decalcification (Chang *et al*., 1997), root shortening (Linge and Linge 1983), pain (Erdinc and Dincer, 2004), allergies (Rahilly and Price, 2014) and relapse (Little *et al*., 1981,1988). Dental caries is the dissolution of the enamel surface that occurs as a result of an imbalance of complex interactions between the oral environment, bacteria in dental plaque and the tooth substrate (Fejerskov and Kidd, 2008). The early stage of dental caries formation is also known as enamel as decalcification or demineralisation and is attributed to the accumulation of dental plaque on the tooth surface for a period of time (Edgar *et al*., 1987). Studies have shown that the type of dental appliance used can predispose a patient to a higher risk of developing enamel demineralisation with the early stage being termed as white spot lesions due to their frosty white or opaque appearance. The most common appliance used today is the fixed orthodontic appliance. The appliance is mainly made up of multiple brackets bonded onto individual teeth with a wire component and also auxillaries to help with controlling tooth movement. This complex design hinders thorough brushing and cleaning around the orthodontic brackets and encourages the adhesion of dental flora, which, may result in enamel demineralisation (Chatterjee and Kleinberg, 1979, O'Reilly and Featherstone, 1987). White spot lesions can become distinguishable around the brackets of fixed appliances within a month of bracket bonding, and it has been found that significant demineralisation can occur as early as 6 months after placement of fixed appliances (Ogaard, 2008, Lucchese and Gherlone, 2012). White spot lesions are possibly irreversible and can leave a permanent discolouration on the teeth affecting the smile (Chambers, 2013). These lesions can give rise to aesthetic concerns to the patient as they are frequently found on the buccal or outer surfaces of the teeth, around the brackets, particularly in the gingival area (Gorelick *et al*., 1982).

The bacterial flora of orthodontic patients has been studied to understand the causative factors of enamel demineralisation. The composition of the bacterial flora undergoes a swift change with levels of acid producing bacteria, such as *S.mutans* and *lactobacilli*, increasing considerably in patients with fixed appliances which occurs as soon as 4 months post insertion of the orthodontic appliance (Lundström and Krasse, 1987). Also, a

rapid increase in the volume of carbohydrates and a lower pH level of dental plaque after band placement has been found in orthodontic patients versus than that found in non-orthodontic patients (Chatterjee and Kleinberg, 1979). *S.mutans* is a component of dental plaque and is the principal bacteria involved in the demineralisation process (Tanzer *et al*., 2001).

Current measures involve fluoride therapy, which is the dental professions' main weapon to combat this problem. Prior to commencing orthodontic treatment, the level of oral hygiene has to be outstanding with a plaque index score of less than 15% with twice daily brushing, using a fluoridated toothpaste, to prevent damage to the teeth and supporting structures, and dietary instructions are provided as there are necessary dietary constraints that the patient is advised to adhere to (Gorelick *et al*., 1982). A common practice in orthodontics is for the patient to rinse with a 0.05% sodium fluoride (NaF) mouthwash on a daily basis at a separate time for brushing with fluoridated toothpaste. This acts as a supplemental measure to allow for the continuous delivery of fluoride to the teeth to inhibit demineralisation. Many studies using a variety of fluoride delivery systems such as mouthrinses, varnishes and toothpastes have shown that fluoride is effective in reducing white spot lesions (Marinho *et al*., 2016, Marinho *et al*., 2013, Benson *et al*., 2013, Sonesson *et al*., 2014, Walsh *et al*., 2010). Each method however does have its own limitations. Varnishes will inevitably result in increased appointment times and burden both the patient and clinician whilst mouthrinses and toothpastes rely entirely on patient compliance (Chambers *et al*., 2013).

The prevalence of enamel demineralisation is common, ranging from 2-96% and can occur if orthodontic patients consume foods or drinks containing excessive sugar and/or acid (eg, sweets, fizzy drinks, fruit juices and so forth) combined with poor oral hygiene practice and host factors such as reduced saliva flow and buffering capacity (Chang *et al*., 1997). Enamel opacities in patients with cemented bands has been associated with an increase in prevalence by 11.7% and the severity of the lesion which was measured by the opacity index, was significantly higher in the treated group despite daily toothbrushing with a fluoridated dentrifice (Mizrahi, 1982). As a result, the protection afforded by fluoride is not adequate for patients who have a high caries challenge and where compliance is the key issue. Therefore, despite the apparent benefits of fluoride in the inhibition of caries and the remineralisation process, the discovery of new anti-cariogenic agents and delivery systems for these agents are important for the prevention and treatment of enamel decalcification. This indicates a need for a low-level sustained release anti-bacterial and remineralising agent that provides long-term protection against white spot lesions, which are biocompatible with the oral environment and independent of patient compliance.

Zinc is a chemical element and an important trace element, which is found deposited within the human body (Lynch 2011). Zinc is also found intra-orally in plaque, saliva and enamel with very low baseline concentrations in the order of 0.05-0.1ppm in saliva and 15-30ppm in plaque (Lynch, 2011). The low baseline levels of zinc in saliva and plaque is sufficient to support the growth of certain types of oral microorganisms but concentrations that are higher than the background levels can become toxic to the oral bacteria (Burguera-Pascu *et al*., 2007). Zinc shows good oral substantivity, lasting up to 12 hours with as much as 15-40% being retained intra orally. Such an increased concentration of zinc can last for several hours in plaque and saliva after the use of mouthrinses and toothpastes (Lynch, 2011). The inhibition of the formation and metabolism of dental plaque by zinc salts has been well documented. Although the method of inhibition is currently ambiguous at best, studies suggest that zinc inhibits acid production and is associated with the adsorption of zinc on the bacterial cell wall (He *et al*., 2002). *S.mutans* is then incapable to obtain the essential nutrients upon which its survival and growth is dependent on due to this inhibition by zinc (Giertsen *et al*., 1987, Moermann and Muehlemann, 1983, Netuschil *et al*., 2003). Many studies have shown this anti-bacterial capacity of zinc and its ability to reduce the solubility of enamel. However, despite the potential that zinc shows as an anti-cariogenic agent, there seems to be limited evidence of zinc in reducing the levels of caries clinically using conventional methods of delivery. This inconsistency in the role of zinc has led to the application of zinc-doped phosphate-based glasses (Zn-PBGs) as an independent, controlled delivery agent, which may be effective in reducing white spot lesions.

Therefore the aims of this study are to prepare and evaluate Zn-PBGs as a controlled delivery agent, to assess the anti-bacterial effects on *S.mutans* and the anti-cariogenic potential of the Zn-PBGs using *in vitro* biological and non-biological models. The objectives of this study are:

1. To prepare novel anti bacterial zinc-doped phosphate based glasses using a melt quenching method

2. To assess glass degradation rate and ion release kinetics in water using weight loss method and inductively coupled plasma optical-emission spectrometry (ICP-OES)

3. To assess the anti bacterial effects of zinc-doped phosphate based glass on *Streptococcus mutans* via disc diffusion assays and liquid broth analyses

4. To evaluate the effect of zinc-doped phosphate based glasses on the remineralisation and demineralisation of bovine enamel using a pH cycling method

5. To assess the effect of zinc-doped phosphate-based glasses on bovine enamel samples via the constant depth fermentor model (CDFF) model

The null hypothesis for this study is that zinc-doped phosphate based glasses have no anti-bacterial effect on *S.mutans* and zinc-doped phosphate based glasses do not affect the demineralisation process of bovine enamel.

3.0 LITERATURE REVIEW

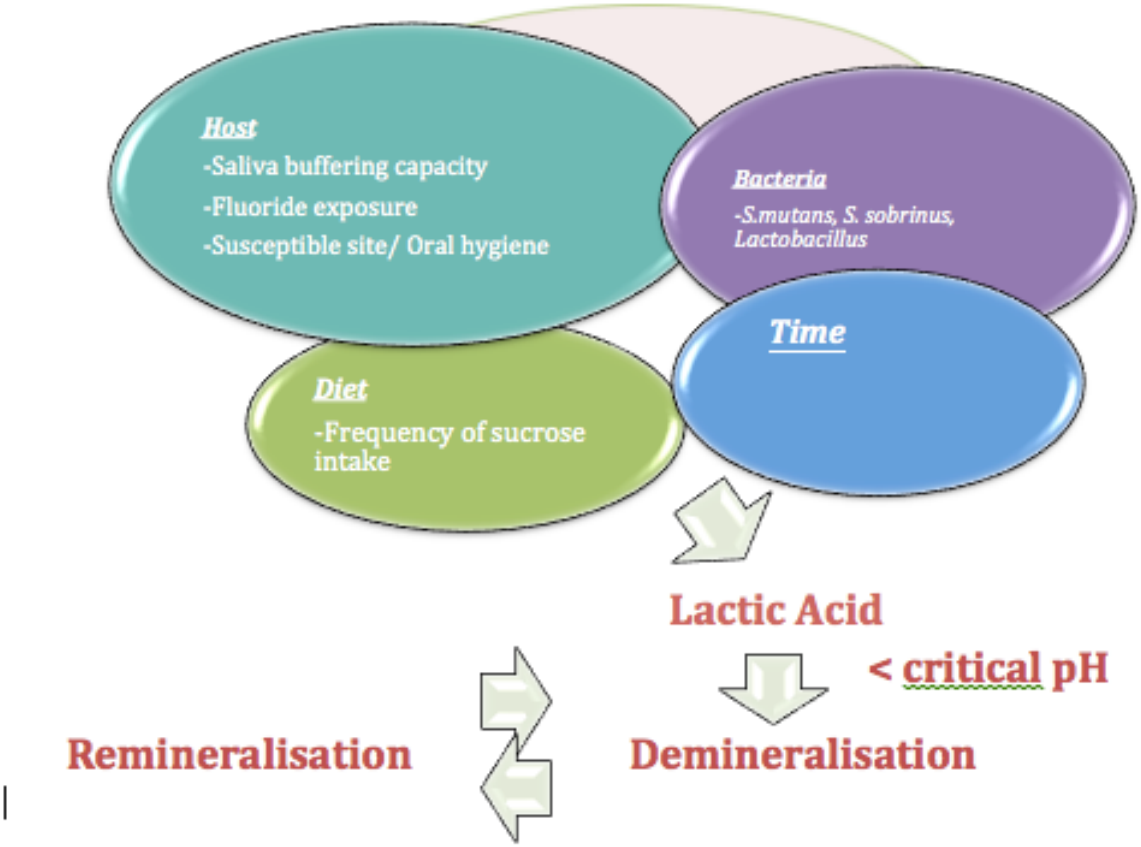
The following literature review provides a description of one of the salient problems faced by orthodontic practitioners during routine orthodontic care. The review is commenced by the introduction of the most prevalent disease faced by the population today with the ever-increasing intake of sucrose-laden foods shaping the nature and frequency of dental caries. This will be followed by a description of the iatrogenic effects of orthodontic treatment that lends itself to a patients' susceptibility to enamel demineralisation. This is followed by a look into the role of fluoride, which is the dental professionals main artillery in the battle against dental caries and the need for seeking novel agents will be described. The high prevalence of enamel decalcification is ultimately the driving force behind this research project, which is an investigation into Zn-PBG, which is being evaluated as a novel anti-bacterial and remineralising agent, and therefore its potential for application in routine orthodontic care.

3.1 Dental Caries

The most common pathology to affect dental enamel in most developed countries, is dental caries. Dental caries not only affects about 60-90% of schoolchildren but also a large number of adults, thus signifying a key public health dilemma (Petersen, 2016). Dental caries is the dissolution of the enamel and dentine that occurs as a result of an imbalance of complex interactions between bacteria in dental plaque, dental enamel, the oral environment and host factors (Stephan, 1942, Fejerskov and Kidd, 2008). The histological changes of a tooth affected by caries were first explained by Tomes in 1859 (Gottlieb *et al*., 1946). Historically, there were two theories, which were proposed to be the aetiological factor for dental caries. The acid theory proposed by Miller in 1885, states that caries is the dissolution of enamel and dentine as a result of the conversion of carbohydrate food stuff into acid by bacterial activity on the enamel surface, and that decalcification is followed by a proteolytic digestion of organic substance. In direct contrast to the acid theory was Gottlieb's proteolytic

theory, which suggests that proteolytic destruction of organic substances in the enamel is the key to caries formation (Tanz, 1949). Although the main factors involved in dental caries have been identified for almost 100 years, in recent decades the evidence has refined that the shift in ecology which leads to dental caries is influenced by host factors such as saliva flow and composition, fluoride exposure, oral hygiene habits as well as dietary consumption (Hunter, 1988, Fejerskov and Kidd, 2008). Frequent intake of carbohydrates provide the endogenous bacteria, mainly *S.mutans* with nutrients which undergo glycolysis to produce organic acids that result in the localised decalcification of susceptible tooth substrate (Bibby *et al*., 1942, Loesche *et al*., 1975). The acids cause the local pH to fall below a critical value which was found to be pH 5, resulting in dissolution of the dental hard tissue (Bibby *et al*., 1942, Kidd and Fejerskov, 2004, Featherstone, 2004). The acid diffusion, was shown to have a linear relationship with tooth solubility (Bibby *et al*., 1942) diffuses to reach susceptible sites on a crystal surface, which are areas of defects in the crystal lattice (Voegel and Frank, 1977, Featherstone *et al*., 1979) that are deficient of calcium (Likins *et al*., 1957). These crystalline defects or dislocations occur as a result of impurities or presence of other ions, such as carbonate ions, which are incorporated in the crystal lattice (Voegel and Frank, 1977, Hallsworth *et al*., 1979). Calcium and phosphate are then dissolved and transported into the surrounding aqueous medium amid the crystals (Featherstone *et al*., 1978). The continued process of dissolution and relocation of the calcium, phosphate and carbonate ions combined with a lack or remineralisation will eventually lead to the progression of decalcification and ultimately the formation of a dental cavity (Larsen, 1990).
Remineralisation is the healing process for subsurface non-cavitated carious lesions to prevent further progression (ten Cate and Featherstone, 1991). Remineralisation involves sources of calcium and phosphate either from topical dentrifices or saliva that diffuses into the tooth with the aid of fluoride, and deposits on existing crystalline remnants instead of the formation of new crystals (Featherstone, 2000, Koulourides and Reed, 1964). The rebuilt crystalline surface, is made of a veneer of well-formed mineral most likely analogous to fluorapatite due to the presence of fluoride. The remineralised crystal is much more resistant to acidic exposure than the original structure, which then reduces the critical pH value for enamel dissolution (Featherstone, 2008, Iljima and Koulourides, 1989). For the vast majority of people, the process of remineralisation and demineralisation occur concomitantly in the oral cavity. The development of dental caries is dependent on a balance between demineralisation and remineralisation and can revert between being progressive, static or reversible. This is illustrated in Figure 1. Therefore, any factor that can push this balance toward the proceeding of remineralisation can be utilised as a weapon in the battle against dental caries (Chen and Wang, 2010).

Figure 1: Demineralisation as a Multi-factorial and Dynamic Disease (Selwitz et al., 2007).



3.1.1 Enamel Demineralisation

The early stage of dental caries is termed as demineralisation and can occur due to poor oral hygiene and the accumulation of dental plaque on the tooth surface over a length of time alongside a combination of other factors (Edgar *et al*., 1978). The two chief types of enamel demineralisation are the incipient lesions and "surface-softened defect" which are the alternative terms used to describe white spot lesions (Koulourides *et al*., 1965). The initial clinical signs of demineralisation of the surface of enamel can be visualised intra- orally as a white spot lesion (WSL), which occurs due to the differing levels of mineral content within the lesion. This indicates the first stage of dental caries formation. One of the main features of an incipient carious lesion in the enamel is the area of the lesion is softer than the surrounding sound enamel. Upon drying with air, the white spot lesion appears increasingly whiter and takes on a frosty appearance. A cross-section of the white opaque spot reveals the surface layer to be relatively intact with a richer mineral content as opposed to the subsurface layers which are more porous, with reduced mineral content indicating that acid production by bacteria leads to a defect in the enamel surface (Arends and Christofferson,1986). Enamel demineralisation has been reported to be a significant problem during orthodontic treatment

as shown by several studies (Gorelick *et al*., 1982, Luchesse and Gherlone 2013). The prevalence of enamel demineralisation is common, and ranges from as low as 2% and can be as high as 96%. Enamel demineralisation can occur if orthodontic patients who have been fitted with appliances consume foods or drinks containing high levels of sugar and/or acid. These include carbonated drinks, confectionary and even energy drinks (Chang *et al*., 1997). Studies have shown that the type of dental appliance used can predispose a patient to a higher risk of developing enamel demineralisation with the early stage being termed as white spot lesions due to their frosty white or opaque appearance (Chatterjee and Kleinberg, 1979, O'Reilly and Featherstone, 1987). The most frequently used appliance today is the fixed orthodontic appliance as the demand for optimal dental aesthetics is on the rise. The appliance is mainly made up of multiple brackets bonded onto individual teeth with a wire component and auxillaries to assist with controlling tooth movements. This design is intricate and hinders thorough brushing and cleaning around the orthodontic brackets which can support the adhesion of oral bacteria, which, may result in enamel demineralisation (Chatterjee and Kleinberg, 1979, O'Reilly and Featherstone, 1987). This combined with frequent consumption of fermentable carbohydrates can result in the formation of white spot lesions which can become distinguishable around the brackets of fixed appliances within a month of bracket bonding, and it has been found that significant demineralisation can occur as early as 6 months after placement of fixed appliances (Ogaard, 2008, Lucchese and Gherlone, 2012).

The bacterial flora of orthodontic patients has been studied to understand the causative factors of enamel demineralisation. The composition of the bacterial flora undergoes a swift change with levels of acid producing bacteria, such as *S.mutans* and *lactobacilli*, increasing considerably in patients with fixed appliances which occurs as soon as 4 months post insertion of the orthodontic appliance (Lundström and Krasse, 1987). *S.mutans* is a constituent of dental plaque and is the main bacteria associated in the demineralisation process (Tanzer *et al*., 2001). Also, studies have shown that a rapid increase in the volume of carbohydrates occurs with the introduction of fixed orthodontic and that the pH level of dental plaque is lower than that found in non-orthodontic patients (Chatterjee and Kleinberg, 1979). White spot lesions can become evident around the brackets of fixed appliances within a month of bracket bonding, and it has been found that significant demineralisation can occur as soon as 6 months after placement of fixed appliances (Ogaard, 2008, Lucchese and Gherlone, 2012).

A clinical study found significantly more white spot lesions in orthodontically treated patients at 6 months (Tufecki *et al*., 2011). These lesions are usually found on the labial or buccal surfaces of the teeth, around the brackets, particularly in the gingival area and can

give rise to aesthetic concerns (Gorelick *et al*., 1982; Ogaard *et al*., 1988). The distribution of demineralisation between different types of teeth, for example the maxillary central incisors, maxillary lateral incisors and maxillary canines are equal indicating the equal susceptibility of the different types of teeth to demineralisation (Tufecki *et al*., 2011). The distribution and position of white spot lesions in locations that are visible upon smiling can cause aesthetic concerns for the patient. White spot lesions are potentially irreversible and treating these lesions can be challenging (Chambers *et al*., 2013). Dentistry today is focused on a preventive approach instead of an invasive restorative approach and white spot lesions are currently one of the greatest challenges that orthodontists face. In line with this current thinking, the need to discover an agent that can not only inhibit the causative bacteria but also promote remineralisation of dental hard tissues is imperative.

3.2 Role of Fluoride as an Anti-cariogenic Agent

The Grand Rapids water fluoridation study transformed dentistry by converting the profession to minimal intervention and prevention oriented as opposed to an invasive approach (Arnold *et al*., 1957). Fluoride therapy has since been the main caries preventive strategy since the introduction of water fluoridation schemes several decades ago (Arnold *et al*., 1957). Today, fluoride salts can be commonly found in drinking water, toothpastes and mouthrinses (Marthaler, 2004). The effectiveness of some topically applied fluorides such as toothpaste, varnish and rinses in preventing dental caries has been well established in systematic reviews of randomised controlled trials conducted in children. Also, regular use of fluoridated toothpaste is known to be effective in preventing caries in the permanent dentition (Walsh *et al*., 2010, Marinho *et al*., 2013, Benson *et al*., 2013, Marinho *et al*., 2016,). There are mainly two proposed modes of action of fluoride in the prevention of caries, which include the ability to inhibit demineralisation and enhance remineralisation and its anti-bacterial activity.

3.2.1 Fluoride in the Remineralisation and Demineralisation Process of Enamel

There have been many studies over the years to assess the effect of fluoride on remineralisation and demineralisation of enamel. This includes both *in vitro*, *in vivo* and *in situ* assessments. Studies, utilising pH cycling models, which consists of alternating periods of demineralisation and remineralisation in the laboratory daily to model the pH cycle intra-orally have shown the ability of fluoride to precipitate in pre-formed lesions as well as the inhibition of demineralisation. In a classical study using a pH cycling model, fluoride concentrations of 100 µmol/L present during the remineralisation phase caused lesion arrestment (ten Cate and Duijster 1982). Further *in vitro* studies albeit with varied designs have come to similar conclusions that fluoride tends to shift the mineral balance toward a less cariogenic condition (ten Cate, 1990). The dose response relationship of fluoride was reported to be evident whereby concentrations of 1mg/L or higher in the acid buffer reduced dissolution rates of the carbonated apatite and this was in proportion to the logarithm of the fluoride concentration in the buffer. The net mineral loss was found to be negatively correlated to the logarithm of the fluoride concentration (Featherstone *et al*., 1990). An in situ model whereby sound enamel blocks and blocks with pre-formed lesions were mounted onto an appliance and secured intra-orally showed significant differences in the remineralisation of the pre-formed lesion in the NaF groups as opposed to the placebo group. There was no statistical difference in demineralisation of sound enamel in all groups (Featherstone and Zero, 1992). These findings are supported by clinical data whereby the use of supervised regular use of fluoridated mouthrinse by children and adolescents, was associated with a large reduction in caries increment in permanent teeth (Marinho *et al*., 2016). In regards to fluoridated toothpaste, it was found that the use of toothpaste containing fluoride of at least 1,000-1250 parts per million (ppm) prevented the occurrence of caries represented by a DMFS prevention fraction of 23% that would have been observed without use of the toothpaste. This increased to 36% for higher concentrations of fluoride within the range of 2,400-2,800 ppm (Walsh *et al*., 2010). Professionally applied fluoride varnishes for preventing dental caries in children and adolescents were also assessed and the review found a DMFS prevented fraction of 43% which was significantly different from the placebo and no treatment groups (Marinho *et al*., 2013). It was suggested that there was a substantial caries-inhibiting effect of fluoride varnish in both permanent and primary teeth, however the quality of the evidence was evaluated as moderate, as it included mainly high risk of bias studies, with considerable heterogeneity (Marinho *et al*., 2013). This was supported by a review which found almost 70% reduction in enamel demineralisation following the

application of fluoride varnish at each orthodontic appointment (Benson *et al*., 2013). Apart from conventional methods of delivery, there have been efforts to incorporate fluoride into slow releasing devices, with the use of glass beads. A significant difference in the DMFS scores was found in the control versus high-risk children who were fitted with a fluoride-releasing glass device in their mouths. The mean salivary fluoride levels over two years was 0.11 ppm in the test group versus 0.03 ppm in the control group. However there was a 50% dropout rate that could not be analysed as the glass bead failed to be retained over the 2 year period (Toumba and Curzon, 2005). On the whole, there was insufficient evidence on slow release fluoride devices for the control of decay to determine a caries inhibiting effect (Chong *et al*., 2014). Collectively the evidence shows that fluoride has the ability to significantly reduce the caries experience, however, despite the promising effects of fluoride, caries is still a major health problem faced by both children and adults. As caries is a dynamic process, which involves *S.mutans* at the crux of the disease progression, to be an effective anti-cariogenic agent fluoride must play a role as an anti-bacterial agent.

3.2.2 Fluoride as an Anti-bacterial Agent

A possible mechanism of fluoride uptake in dental plaque was demonstrated using gel filtration methods to demonstrate ion exchanges between NaF and acidic groups meant to represent dental plaque. The materials that had been pre-equilibrated against calcium ions took up about 30 % of the available fluoride from the external fluid. It was proposed that calcium, which is freely available in saliva and bound to fixed acidic groups in dental plaque, markedly improves the retention of fluoride in dental plaque. Therefore, calcium-binding proteins in the pellicle as well as in the saliva, could be potential binders of fluoride (Rolla and Bowen, 1977). This experiment was conducted over 2 days but is a simplistic model to explain the relationship of fluoride uptake in dental plaque. Specifically in relation to fluoride uptake and *S.mutans*, the short-term kinetics was investigated from 20- to 22-h cultures using rapid filtration and centrifugation techniques. It was found that the uptake of fluoride was positively correlated with the magnitude of the pH gradient and this was statistically significant (Whitford *et al*., 1977). This experiment was conducted in the laboratory over a short time period of 4 hours. The inhibition of fluoridated mouthrinse on growth and acid produced by S*.mutans* was not found to be effective in a study on rats and the authors indicated that adaptation by *S.mutans* to fluoride may well occur causing the lack of clinical ineffectiveness (Van der Hoeven and Franken, 1984). More recently,varnished hydroxyapatite (HA) discs showed the diminishing effects of fluoride on the growth and acid

production of S.*mutans* as the age of biofilm increased which indicates that its action is not persistent over time (Chau *et al*., 2014). A longitudinal study looking at the effects of water fluoridation on bacterial growth in a localised population of children was carried out. All children were exposed to water fluoridation and comparisons of the frequencies of isolation of the different species of bacteria were drawn between children with caries and without caries. It was found that for *S.mutans*, fluoride reduced the initial glycolytic rate of the cells but after 2 hours there was no difference in the final amount of glucose uptake between the groups. Also, isolated strains of *S.mutans* were able to grow in the presence of fluoride over a range of pH that simulates plaque pH levels (Bowden *et al*., 1982). This study attempted to look into the clinical role of fluoride, however, the study had several drawbacks, which include a small sample size sampled from one town, and the study was prone to selection bias. More recent clinical trials have shown a reduction in the number of *S.mutans* with the use of fluoridated mouthrinse (Yoshihara *et al*., 2001, Kaneko *et al*., 2006). It should be noted that although there was a reduction in *S.mutans*, the difference in caries experience was not statistically significant between the groups (Kaneko *et al*., 2006). Salivary fluoride levels are known to be fairly low, around 0.02 ppm or less, and is dependent on factors such as the fluoride level in drinking water (Leverett *et al*., 1993) as well as the rate of clearance from the mouth due to salivary secretion and fluoride administrations or products (Naomova *et al*., 2014). The current advocated preventive measure such as toothpastes, mouthrinses and even varnish are unable to elevate this baseline concentration (Eakle *et al*., 2004), rendering the limited effect of fluoride on bacteria after using oral care products (Naomova *et al*., 2014). Therefore, the protection afforded by fluoride is insufficient for patients who are at a high risk of caries (Featherstone, 2004). Possibly due to these factors, an increase in caries-free population reached a plateau in the 1990s as depicted in Tables 1 and 2 below. Despite showing an overall decreasing prevalence over a 40-year period, there are still a majority of schoolchildren today and many adults, who have discernible dental decay. Many clinical studies have pointed out that fluoride therapy alone is not enough to overcome high caries challenges. There is a large body of evidence of fluoride as an anti-bacterial agent, however clinical trials have not been able to substantiate its' clinical effectiveness in reducing the caries experience. Therefore, despite the apparent benefits of fluoride in the inhibition of caries and the remineralisation process, the discovery of new anti-cariogenic agents and delivery systems for these agents are important and substantiated for the prevention and treatment of dental caries (Chen and Wang, 2010). This gives rise to the need for novel agents which are effective to address high caries challenges using methods of delivery that do not heavily rely on patient compliance, which is the basis of this project.

Table 1: Caries Prevalence in 5 year-old children in England and Wales (1973), UK (1983, 1993 and 2003) and England, Wales and Northern Ireland (2013), (Murray et al., 2015).

Caries Experience in 5-year old children in England and Wales					
	1973	1983	1993	2003	2013
% of decay	72	52	46	43	26
Mean dft	4.0	1.8	1.7	1.6	0.7
Mean dft in children with caries	5.5	3.5	3.7	3.5	2.8

Table 2: Caries Prevalence in1 5 year-old children in England and Wales (1973), UK (1983, 1993 and 2003) and England, Wales and Northern Ireland (2013), (Murray et al., 2015).

Caries Experience in 5-year old children in England and Wales					
	1973	1983	1993	2003	2013
% of decay	97	93	63	49	42
Mean DMFT	8.4	5.9	2.5	1.6	1.2
Mean DMFT in children with caries	8.7	6.3	4.0	3.2	2.9

3.2.3 Fluoride and White Spot Lesions (WSL)

Since there are a variety of mechanisms of fluoride delivery in patients who have orthodontic treatment, attempts have been made by researchers to shed light on the optimal mode of delivery. A study looking into the relationship of the use of fluoridated mouthwash and reduction of white spot lesions found that that use of a daily, fluoridated mouthwash led to a statistically significant 25% reduction in the number of patients with white spots However, only 13% of the 206 patients fully complied with the rinsing regime of once daily. There was a significant dose response relationship between compliance and white spots (Geiger *et al*., 1992). In a study looking at professionally applied varnish, children receiving orthodontic treatment with fixed appliances were randomly allocated to receive a fluoride varnish or a placebo varnish every sixth week during the treatment period. It was found that the incidence of WSL in the group having the professionally applied varnish was 7.4% whilst the incidence was much higher at 25.3% in the control group (Stecksen-Blicks *et al*., 2007).

This implies that patients will benefit clinically from routine application of a fluoride varnish at their orthodontic appointments (Chambers *et al*., 2013). The effectiveness of fluoride and its different delivery mechanisms (mouth rinse, toothpaste, varnish, intraoral fluoride-releasing glass bead device) in the prevention of WSL formation has been assessed as well. It was found that the professional application of a high concentration fluoride every 6 weeks reduces the incidence of WSL during orthodontic treatment by nearly 70%. This was moderate quality-evidence based on one well-designed clinical trial, but further well-designed studies are required to confirm this finding as only one out of the three included studies was determined to be at low risk of bias (Benson *et al*., 2013). The use of a high-fluoride toothpaste on a routine basis was found to significantly reduce the prevalence and incidence of white spot lesions in adolescents having fixed orthodontic appliances. A high-fluoride toothpaste should be used as an adjunctive supplementation of fluoride for patients who face a temporarily increased risk of demineralisation (Sonesson *et al*., 2014). This was a well-designed multi-centred randomised controlled trial with two parallel arms looking at the effectiveness of daily tooth brushing with a high-fluoride toothpaste. Many studies using a variety of fluoride delivery systems such as mouthrinses, varnishes and toothpastes have shown that fluoride is effective in reducing white spot lesions. Each method however does have its own limitations. Varnishes will undoubtedly result in longer sessions which burden both the patient and clinician whilst mouthrinses and toothpastes rely on patient compliance (Chambers *et al*., 2013). This indicates a need for a low-level sustained release anti-bacterial and remineralising agent that provides long-term protection against white spot lesions, which is independent of external factors that can compromise the effectiveness of delivery.

3.2.4 Biocompatibility of Fluoride

Currently fluoride is the most effective caries preventive strategy. Fluoride can either be delivered systemically or topically. Systemic sources include controlled addition to water, salt and milk through community prevention programmes. WHO has endorsed these community-based strategies since the late 1960s. Topical sources include incorporation into toothpaste, mouthwashes, varnishes, gels and restorative materials (Peterson, 2016). Topical sources can act systemically if ingested and absorbed by the gastrointestinal tract. Whilst inadequate fluoride exposure can lead to an increase in caries rates, excessive exposure to fluoride can result in adverse effects. Adverse effects of excessive exposure to fluoride include acute fluoride toxicity, skeletal fluorosis and dental fluorosis in young children with developing teeth due to ingestion of fluoride and rapid absorption by the gastrointestinal tract (Whitford and Pashley, 1984). The minimum dose that could cause toxic signs and symptoms, including death, has been set at 5mg/kg body weight. The lethal dose of fluoride

has been set at 15 mg/kg. The toxic effects of fluoride are mainly due to four different actions which include burning the tissues by forming hydrofluoric acid when exposed to moisture, impeding nerve function, cellular toxicity and impeding cardiac function by creating an electrolyte imbalance resulting in hyperkalemia (Martinez, 2011). General health effects of fluorosis in animals have shown to impair growth and reduce milk production. (Tao *et al*., 2006, Maylin *et al*., 1987). Experimental studies have shown that fluoride affects the release of pituitary lactotrophic hormone in mice leading to a decrease in the production of milk (Yuan *et al*. 1991). However, a prospective longitudinal study assessing the effects of fluoride on bone mineral density and bone mineral content in humans over a period of 15 years from birth to 15 years showed no statistically significant difference in bone scans between the groups comprising of lowest, intermediate and highest intake of fluoride. Sources of fluoride included community water system, dentifrices and other beverages as determined by questionnaire responses (Levy *et al*., 2014). The York review also found low evidence to suggest that fluoride could contribute to bony pathology such as hip fractures (Treasure *et al*., 2002). The studies in animal models need to be interpreted with caution as the levels of fluoride that the animals were exposed to were very high, up to 200mg/kg which, exceeds the lethal dose of fluoride in human beings. Currently the link between exposure to fluoride with any significant health effects is weak, however, this area will be kept under review as recommended by the Medical Research Council Working Group (MRC, 2002). Table 3 shows the recommended dosage of fluoride supplementation in individuals at high risk of caries according to the different ages. The amount of fluoride ingestion on a daily basis is subjective and difficult to monitor. Therefore an alternative agent to fluoride therapy, which does not raise health concerns, is worth exploring.

Table 3*: American Dental Association Recommendation of Fluoride Supplementation for High Risk Individuals, (Rozier et al., 2010)*

	Water Fluoride Concentration (ppm)		
Age of child	***<0.3ppm***	***0.3-0.6ppm***	***>0.6ppm***
0-6 months	0	0	0
6 months – 3 years	0.25 mgF/day	0	0
3-6 years	0.5 mgF/day	0.25 mgF/day	0
6-16 years	1 mgF/day	0.5 mgF/day	0

3.3 Chlorhexidine as an Alternative to Fluoride

Chlorhexidine is composed of a bisbiguanide formulation with cationic properties (Sajjan *et al*., 2016) that interacts with the anionic enamel, proteins and glycoproteins of both saliva and plaque leading to its high intra-oral substantivity (Briner *et al*., 1994). The molecule is made up of two chlorophenyle rings and two biguanide groups linked by a central hexamethylene chain and are symmetrical. It is a strong base and the most common preparation is the digluconate salt because of its water solubility. Moreover, it is the most stable form as a salt. The use of chlorhexidine dates back to the 1940s when the search for an anti-viral agent was on going. It has since been established that chorhexidine plays a role as an anti-bacterial agent and not as an anti-viral agent (Sajjan *et al*., 2016). Chlorhexidine has shown the ability to decrease bacterial counts when used as a mouthrinse (Schiott *et al*., 1970). The role of 1% chlorhexidine gel as an anti-bacterial agent specifically targeting *S.mutans* in saliva was shown to reduce salivary counts in school children with some children showing the effects up to 6 months after treatment. The response to the chlorhexidine gel was subject to individual variation and there was a diminishing effect of the chlorhexidine with time (Maltz *et al*., 1981). The short-term inhibitory effect of chlorhexidine on *S.mutans* has been shown in many studies (Twetman *et al*., 1995, Madlena *et al*., 2000, Jenatschke *et al*., 2001). The concentration of chlorhexidine used, which ranged from 1% up

to 40% had no effect on the *S.mutans* counts or caries inhibition (Twetman *et al*., 1995, Madlena *et al*., 2000, Jenatschke *et al*., 2001). The effect of chlorhexidine in patients with fixed orthodontic appliance has been assessed in a number of studies. The chlorhexidine was prepared as a varnish and the majority of studies found a reduction in *S.mutans* over a 3-4 week period post application of the chlorhexidine varnish. However, the strength of the evidence is weak as the studies differed in terms of strength of chlorhexidine varnish used, variable regime of application, bacterial sampling methods and bacterial sampling times (Tang *et al*., 2016). The results indicate the short-term ability of chlorhexidine to inhibit bacteria and therefore the continual mode of action relies on the frequency of attendance so as to ensure frequency of application. However, the ability to reduce *S.mutans* does not automatically infer the ability to reduce caries as the effectiveness of chlorhexidine varnish for preventing caries is inconclusive (James *et al*., 2010). Going hand in hand with the apparent benefits of chlorhexidine, are certain side effects, which have been reported. This includes staining of the teeth, discolouration of the tongue and even mucosal desquamations with a 0.2% chlorhexidine digluconate mouthwash used over a period of 4 months (Flotra, 1971). Therefore, despite the apparent promise of chlorhexidine being an alternative to fluoride, the lack of substantive anti-cariogenic activity compounded by the problem of significant extrinsic staining of the teeth and tongue that can occur with long term use of chlorhexidine (Flotra *et al*., 1971, Van Stryndock *et al*., 2012), justifies the need for novel agents which have the potential not only as an anti-bacterial agent but also encompasses the ability to play a role in inhibiting the demineralisation of enamel. To fulfil this role, the novel agent must not be detrimental and must indeed be biocompatible with the oral environment to prevent the negation of its use.

3.4 Zinc as an Anti-bacterial Agent, Its Role in the Inhibition of Enamel Demineralisation and the Controlled Delivery of Zinc

Zinc is an essential trace element and is a natural constituent of the human body (Lynch, 2011). The distribution of zinc in the human body is within muscle tissue, bone and skin, which make up the majority of the 2g that is contained within the human system whilst the intra-oral environment contains relatively low baseline concentrations (Lynch, 2011). The role of zinc at this naturally low concentration intra-orally serves to sustain nutritional necessities of certain microorganisms and also to keep the enzymes functioning, which depend on traces of zinc, while elevated concentrations of this trace element of more than 32 µg/ml impairs growth of *S.mutans* and inhibits acid production (Moermann and Muehlemann, 1983).

3.4.1 Anti-bacterial Activity of Zinc

The mode of action of zinc is currently vague, but studies suggest that the adsorption of zinc on the bacterial cell wall impairs bacterial function and is correlated with the inhibition of acid production (Lemire *et al*., 2013, He *et al*., 2002, Brading *et al*., 2003, Finney *et al*, 2003, Phan *et al*, 2004). As zinc is a divalent metal ion, there are different mechanisms by which zinc can affect microorganisms, which include causing oxidative stress, damaging the cell membrane or cell DNA, or causing abnormality with protein function (Lemire *et al*., 2013). These divalent metal ions such as zinc may alter the function of the cells membrane and the enzymatic activity within the cell, possibly through the oxidation of thiol groups (Oppermann *et al*., 1980) thus impairing the production of acids during the glycolysis process (He *et al*., 2002, Brading *et al*., 2003, Finney *et al*, 2003 Phan *et al*, 2004, Hall *et al*., 2003). There have been many research studies looking into reducing dental plaque development through the incorporation of anti-bacterial agents into mouth rinses or toothpaste formulations so as to effect the inhibition of bacterial glycolysis and the subsequent production of acids (He *et al*., 2002, Brading *et al*., 2003, Finney *et al*., 2003, 2004, Hall *et al*., 2003). The zinc formulations showed a significant reduction in the viable counts of Gram negative and Gram positive bacteria, that included *S.mutans* which were cultivated using a CDFF model (Finney *et al*., 2003), and a significant reduction in pH levels when compared to the standard fluoridated toothpaste (He *et al*., 2002, Brading *et al*., 2003, Hall *et al*., 2003). It has been shown that when *S. mutans* is exposed to the presence of such metal ions, a bacteriostatic effect is demonstrated whereby *S.mutans* is unable to acquire the nutrients necessary for its growth

and reproduction (Phan *et al*, 2004).

As a successful anti-bacterial agent, not only must zinc display good oral substantivity, which is the ability to sustain concentrations at an effective level for a protracted length of time but it must also be able to inhibit the functioning of targeted microorganisms to inhibit growth of oral bacteria (Giertsen *et al*., 1987, Moermann and Muehlemann, 1983, Netuschil *et al*., 2003). The susbtantivity and retention times of zinc is essential since the anti-bacterial effect will only last while the active form of zinc is present at effective concentrations. Zinc shows the ability to maintain an effective concentration, indicating good oral substantivity, by being present, for many hours in plaque and saliva after use of oral dentifrices such as mouth rinses and toothpastes (Lynch 2011). Zinc is cleared from the saliva bi-modally, which results in a comparatively increased initial concentration post-application, falling quickly over 30 to 60 minutes. This is followed by the persistence of a low concentration over many hours, which is still elevated relative to baseline concentrations. This mode of clearance mirrors the faster removal of the loosely bound zinc ions whilst the more firmly bound zinc ions are cleared at a slower rate. Zinc mimics the effects of fluoride as repeated application demonstrates that a build-up effect happens in plaque (Lynch, 2011). Elevated levels of zinc were detected immediately post rinsing with zinc containing mouthwash (Afseth *et al*., 1983) and a decrease in *S.mutans* colonies was found both *in vitro* and *in vivo* (Harrap *et al*., 1983). A study looking into the substantivity of zinc salts used as rinsing solutions and their effect on the inhibition of *S. mutans* found that the inhibition of acid production by the bacteria was sustained. The study found that the corresponding pH values decreased as zinc concentrations increased which were measured from saliva samples collected 60 and 120 min after rinsing with a zinc mouthwash (Burguera-Pascu *et al*., 2007). However, the levels of zinc within the oral environment needs to be safe and the toxicity levels of zinc should be taken into account as some of the zinc containing formulation may be ingested during use. The recommended daily intake of zinc is 1 mg/day for men and 8 mg/day for women while toxicity occurs above the value of 27 g zinc/day (Plum *et al*., 2010).

3.4.2 Zinc as an Inhibitor of Enamel Demineralisation

Zinc has been shown to be capable of reducing enamel solubility almost as effectively as fluoride (Brudevold, 1963). In this study zinc was shown to be an active competitor with calcium to be incorporated into the hydroxyapatite lattice (Brudevold, 1963). An *in vitro* study assessing the effect of zinc on the crystal formation of calcium phosphate has shown that it can reduce crystal growth even at relatively low concentrations of 0.1mM/L and demonstrated the incorporation of zinc into the crystal structure at higher concentrations of 0.5mM/L to 2mM/L (Legeros *et al*., 1999). A study looking into the effect of zinc concentrations on the *in vitro* demineralisation of enamel during exposure to caries-simulating conditions found that zinc reduces enamel demineralisation (Mohammad *et al*., 2015) and possibly acts predominantly on enamel surfaces at calcium sites in the hydroxyapatite lattice (Brudevold, 1963). The effects of zinc and fluoride on the remineralisation of artificial carious lesions under simulated plaque-fluid conditions have been evaluated. Under static remineralising conditions simulating plaque fluid, zinc together with fluoride gave significantly greater remineralisation than did fluoride. This could be due to zinc maintaining a higher porosity level at the surface layer in the zinc/fluoride condition when compared with fluoride, enabling greater lesion-body remineralisation (Lynch *et al*., 2011). This is supported by an *in situ* trial whereby the concentration and distribution of zinc in remineralised enamel after gum chewing was investigated and the authors suggested that zinc is effectively incorporated into hydroxyapatite crystals through the normal processes of mineral deposition in the oral environment. It was proposed that the zinc substitution probably occurred at the calcium position in enamel hydroxyapatite (Matsunaga *et al*., 2009). These studies show that zinc has the potential to influence both demineralisation and remineralisation. Conversely, results obtained from a study that looked into the remineralisation of artificially created carious lesions in rats found that molar teeth that were exposed to water and food with 250 ppm zinc displayed a complete absence of caries limiting effects (McClure, 1948). Subsequently, the same finding was reported in in vitro pH-cycling studies, which involved both demineralisation and remineralisation investigations, have not detected the ability of zinc to influence remineralisation when delivered from fluoride toothpastes (ten Cate, 1993, Laucello *et al*., 2007). Although a reduction in enamel demineralisation in situ was found, it was surmised that this could not be ascribed to the direct interaction or incorporation of zinc within the enamel structure, and could possibly have been due to the anti-bacterial effects to a certain degree (ten Cate, 1993). Another clinical study investigated fluoridated tooth paste with the incorporation of zinc at three different concentrations of 1000, 1500 and 2500 ppm fluoride, with zinc demonstrating no caries inhibiting effect at all concentrations over a period of 3 years (Stephen *et al*., 1988). Despite

zinc showing potential as an anti-bacterial agent and enamel remineralising agent, there seems to be little evidence of zinc in reducing the levels of caries. This inconsistency in the role of zinc necessitates a novel method to be evaluated for its efficacy in inhibiting caries progression.

3.5 Phosphate-based Glass (PBG)

Phosphate glasses are a third generation biomaterial and is a class of optical glasses composed of metaphosphates of various metals (Brow, 2000). PBGs and the commonly used Bioactive glasses differ from each other in that PBGs use P2O5 as the network former and contain no silicon oxide (SiO2) (Ahmed *et al*., 2006). Melting together oxides of phosphorus, calcium, and sodium mainly produces the phosphate-based glasses. The phosphate (PO34-) tetrahedran make up the rudimentary building blocks of PBGs. Each (PO34-) tetrahedron can be attached to a maximum of three other (PO34-) tetrahedra as in phosphorus pentoxide (P2O5) (Bunker *et al*., 1984). The advantage mainly related with PBGs is that the body is able to eliminate and clear the glasses naturally from the system as part of the normal physiological process. This is due to the glasses being composed of elements naturally found within the body indicating their biocompatibility (Abou Neel, 2009). A manufacturing advantage is that they require comparatively low temperatures to melt compared to melt derived silica-based glasses (Ahmed *et al*., 2006). Phosphate glasses own distinctive dissolution properties in aqueous based fluids. The solubility of the glasses is influenced by the composition of the glass. For instance, the solubility of a ternary Na2O—CaO—P2O5 glass can be controlled by the concentration of calcium oxide (CaO). If the CaO concentration of such a glass is increased, the solubility or degradation of the glass will decrease and vice versa (Franks *et al*., 2000). The physical characteristics of phosphate glasses can be altered by the addition of dopants, which include a number of metal oxides (Abou Neel *et al*., 2009, Parsons *et al*., 2006) such as calcium oxide (CaO) and sodium oxide (Na2O) (Bunker *et al*., 1984). Modifying the glass constituents can alter the degradation rates ranging from several hours to several weeks. Furthermore, these glasses can be tailor made to produce a specific biological purpose and improve biocompatibility by adding in the specific dopants (Abou Neel *et al*., 2009). Anti-microbial glass systems including either copper, gallium or silver ions have been successfully synthesized for prospective applications in the management of oral infections (Valappil *et al*., 2007, Abou Neel *et al*., 2009, Valappil *et al*., 2014). Most PBGs are prepared by melt-quenching methods which is first made from a mixture of oxide precursors that is placed into furnace at temperatures of over 1000°C to facilitate a homogenous melt. The final composition of the glass determines the precise temperature used to achieve this. The variety of shapes, such as rods, plates

and discs are casted once an event melt of the glass mixture is attained, to form the final result. A fast cooling down period past the glass transition temperature follows this and in order to strengthen the glass, the glass is cooled down gradually to remove any stress remaining with the glass structure. PBGs provide an alternative platform for the existing methods of treating oral infections. This is due to their unique characteristics that allow for the inclusion of anti-bacterial metal ions such as zinc, copper, silver, and gallium, and allowance for the subsequent delivery of these ions to susceptible sites in a controlled manner (Abou Neel *et al*., 2009). A study looking into the inclusion of copper into phosphate based glasses in a constant depth film fermentor model (CDFF) found that the exposure of *Streptococcus sanguis* to the glasses resulted in a obvious reduction in viable counts of *Streptococcus sanguis* at 24 hours. The solubility of the glasses was found to be similar between artificial saliva and distilled water (Mulligan *et al*., 2003). Studies looking into the anti-bacterial effect of silver and gallium containing phosphate glasses found an effective bactericidal response (Ahmed *et al*., 2006, Valappil *et al*., 2008). These reports confirm the prospect of these glasses as a novel therapeutic agent for bacterial infections. Phosphate glasses with the incorporation of zinc have been assessed for its bulk and structural properties. The glasses with less than 5% zinc showed good surface wettability, hydrophilicity and surface reactivity (Abou Neel *et al*., 2008). Due to these desirable characteristics for cell adhesion, the current research was undertaken not only to assess the anti-bacterial effect against *S.mutans,* but also the ability for zinc to be incorporated into bovine enamel to promote remineralisation in an effort to reduce the incidence of white spot lesions.

The controlled release of PBGs has been assessed using cytotoxicity and animal implant studies. The element of controlled release of these glasses was initially examined on the L929 mouse fibroblast cell line. After duration of 1 week, the fibroblast cells stayed unaffected by the release of ions from the glasses. Succeeding this, the PBGs were implanted into sheep and, once more, there was an absence of cytotoxicity at the soft tissue implant locations and even some signs of formation of new bone (Burnie *et al*., 1981). PBGs doped with molybdenum ions showed no cytotoxic effect when tested on keratinocyte cell type with the optimal concentration being 5 mol% molybdenum (Lucacel *et al*., 2016). The controlled release feature of the glasses is provided for by the calcium incorporated into the glass. The solubility and dissolution rates of the glass can be altered by changing the concentration of calcium within the glasses (Abou Neel *et al*., 2009). The use of these glasses can be targeted towards susceptible sites within the mouth, with the purpose of a gradual and controlled release of anti-bacterial ions as the glass dissolves, which may be advantageous in the prophylactic inhibition of enamel demineralisation.

3.5.1 Glass Degradation and Ion Release Kinetics

Phosphate based glasses are degradable due to the presence of the P–O–P bonds which are easily hydrated and have the potential to be used as scaffold materials (Abou Neel *et al*., 2007). The degradation is dependent on the composition of the glass, which degrades into natural constituents that can be tolerated by the body (Abou Neel *et al*., 2008). Additionally, due to the fact that the glass degradation is dependent on the composition of the glasses, altering the glass composition can change the rate of degradation (Abou Neel *et al*., 2008). Therefore, a wide range of compositions with various degradation rates can be tailored, according to the objective of use. The solubility of phosphate glass doped with K_2O was shown to have a linear relationship when the weight loss per unit area over time was assessed (Knowles *et al*., 2001). A similar relationship of glass degradation as assessed by the weight loss of the glass over time was observed in silver-doped phosphate based glasses (Ahmed *et al*., 2006). The solubility of the glasses was affected by the concentration of CaO which, demonstrated an inverse relationship, as solubility decreased with increasing levels of CaO (Knowles *et al*., 2001, Ahmed *et al*., 2006). Na and Ca ion release mirrored the solubility of the glass whilst increasing the level of K_2O increased ion release of K (Knowles *et al*., 2001). This correlation of ion release with the degradation and solubility of the glasses was again found with silver and gallium-doped phosphate glasses (Valappil *et al*., 2007, Valappil *et al*., 2009). The controlled degradation and ion release kinetics of the phosphate glasses is a key feature of this novel method in an effort to maintain a low concentration at the site of delivery for its anti-bacterial and remineralisation effect.

3.5.2 Evaluating Ion Release

The inductively coupled plasma emission spectrometry (ICP-OES) is a highly precise technique (Olesik, 1991) that allows quantification of inorganic analytes by transforming the analytes present in a solution to generate free atoms (Fassel and Kniseley, 1974). The solution sample is introduced into the centre of the ICP, which is made of partially ionized gas, usually argon and is under 1% ionized in the plasma. A quartz torch is used to produce the argon gas using a 1-2.5-kW radiofrequency electromagnetic power supply. The temperature in the ICP ranges from between 5000 to 9000°C which results in the ions entering a state of excitement. A light beam is then centered onto the slit of a monochromator or polychromator to trace emissions from the sample solution. The signal that is traced relies on the quantity of analyte ions and the degree of excitation, which produces a characteristic wavelength. Due to the high state of excitation, intense emissions are produced from many lines at the same time allowing for multiple analysis to be performed

(Olesik, 1991). The free atoms that are generated are the detected by atomic absorption, emission, or fluorescence spectroscopic techniques to form elemental concentrations (Fassel and Kniseley, 1974).

3.6 Evaluating the Effects of Zinc on Bacteria

3.6.1 Disc Diffusion Assays

Disc diffusion assay is the approved technique employed in numerous clinical microbiology laboratories for routine antimicrobial susceptibility testing. Currently, there are several recognised and approved standards for bacteria and yeasts testing, which have been published by the Clinical and Laboratory Standards Institute (CLSI) (Wayne, 2012). Agar disc-diffusion testing was first developed in 1940 and is recommended for certain bacterial pathogens like streptococci, *Haemophilus influenzae*, *Haemophilus parainfluenzae*, *Neisseria gonorrhoeae* and *Neisseria meningitis,* which can be tested for susceptibility to an anti-bacterial agent. However, not all bacteria can be tested precisely in this manner (Balouiri *et al*., 2016). Despite the fact that this method does not accurately test all fastidious or slow growing bacteria, the disc test has been standardised for testing streptococci. The disc diffusion test is carried out through the use of specialised media, the correct incubation temperatures, and specific zone size interpretive measures (Wayne, 2012). In this recognized procedure, agar plates are inoculated with a standardised inoculum of the test microorganism followed by the placement of filter paper discs on the agar surface that contain the test compound at a suitable concentration. The incubation process of the Petri dishes is then carried out under appropriate conditions. Usually, the antimicrobial agent diffuses into the agar and inhibits the development and growth of the test microorganism which, then result in zones of inhibition which are devoid of bacterial growth that surround the discs. The diameters of these zones of growth inhibition are measured, as they are associated with the sensitivity of the isolate and with the diffusion rate of the disc through the BHI medium. The diameters of the zone of inhibition of each disc is analysed and interpreted by measuring around each of the glass or antibiotic discs (Balouri *et al*., 2016). This is measured to the closest millimeter using a ruler and compared with controls (Wayne, 2012). There are many advantages of disc diffusion assay compared to other anti-bacterial evaluation techniques which include that it is inexpensive, the ease of conduct and the ability to test large numbers of microorganisms and antimicrobial agents, as well as the uncomplicated interpretation of the results provided. In addition, there is good correlation between *the in vitro* assessments and the *in vivo* investigations (Balouiri *et al*., 2016).

3.6.2 Liquid Broth Analyses

The macrobroth or tube-dilution method was one of the first antimicrobial susceptibility testing techniques that were developed. This method involved the preparation of antibiotics in a two-fold dilution (eg, 1, 2, 4, 8, and 16 µg/mL). This was carried out in a liquid growth medium dispensed in test tubes. The antibiotic-containing tubes were inoculated with a standardised bacterial suspension of $1–5\times10^5$CFU/mL which were then incubated overnight at 35°C. Visible bacterial growth as shown by turbidity was examined. There were errors in precision of this method due to the fact that the practice of manually preparing serial dilutions of the antibiotics was carried out. The advantage of this technique was the generation of a quantifiable or measurable result. With the advent of the disposable plastic microdilution trays, this has made broth dilution testing both practical and accepted (Jorgensen and Ferraro, 2009) and the choice method in this study. The broth dilution method using microdilution methods is a technique that can be used to assess the susceptibility of either a single isolate or a small number of isolates to an anti-bacterial agent. Dilutions and inoculations are prepared in a similar manner as described for the determination of MIC. The cell suspension of the isolate tested is placed into 5mls of the control and test solutions followed by serial microdlilution and subsequently cultured onto BHI plates to assess viable bacteria counts (expressed as $\log_{10}$ CFU). The results may show bacteriostasis only which would be expressed by colony numbers that are similar, a decrease in the number of colonies which represents a limited or gradual bactericidal action or no colonies present which would mean the whole inoculum has been killed (Balouiri *et al*., 2016).

3.6.3 Biofilm Assay- Constant Depth Film Fermentor (CDFF) Model

A frequently desirable technique of growing microorganisms is to grow them in such a way so as to replicate their physiological growth environment *in vivo*, which is carried out by developing them as biofilms in the laboratory. Several in vitro biofilm models have been utilised to simulate dental plaque (Wimpenny *et al*., 1999) and amongst the different methods, the most representative of these is the constant-depth film fermenter (CDFF) (Kinniment *et al*., 1996 and Peters and Wimpenny, 1988). The operating parameters of the CDFF are complex and permit the research of the growth of bacterial communities in a simulated oral ecosystem. Investigations over a long time frame can be carried out with alternating remineralisation and demineralisation cycles and this model is mainly suited to investigating the different biofilms which occur in the oral cavity, due to the ability to control

environmental factors such as the substratum, nutrient source, and gas flow can be altered. The rotating turntable with recessed discs of hydroxyapatite (HA) or discs of bovine enamel forms the distinctive aspect of a CDFF, into which a large number of biofilms can be grown on. The turntable is the platform onto which nutrient medium is dripped onto and fixed scraper blades work to spread substrata over the turntable while at the same time the blades also serve to remove surplus cellular material, to preserve the biofilms at a constant depth (Hope and Wilson, 2006, Valappil *et al*., 2009). The CDFF model has been used to investigate biofilm structure and bacterial vitality in supragingival and subgingival micrososms (Hope and Wilson 2006), to study the efficacy of antimicrobials on biofilm (Kinniment *et al*., 1996, Pratten *et al*., 2000), the remineralisation and demineralisation of dentine by single species model biofilm (Deng and ten Cate, 2004) and the effects of gallium-doped phosphate glasses on the viability of biofilms of *Pseudomonas aeruginosa* and *S.mutans* (Valappil *et al*., 2009, Valappil *et al*., 2014). This model, which accomplishes many of the ideal requirements of a biological caries model, was used to support biofilm growth of *S.mutans* on bovine enamel discs which were subjected to the effects of the Zn-PBGs.

3.7 Evaluating the Effects of Zinc-doped Phosphate-based Glass on Enamel mineralisation

3.7.1 pH Cycling

One of the aims of this study is to assess the ability of Zn-PBGs to be incorporated into bovine enamel to inhibit demineralisation and possibly to remineralise bovine enamel in the presence of dissolving acids. A method, which has proven useful to assess a caries preventive agents' impact on the pattern of demineralisation and remineralisation of enamel, is the pH cycling system (Buzalaf, 2010). The pH cycling system was first described in 1986 (Featherstone *et al*., 1986). This approach involves mirroring the pH depressions in the oral environment *in vitro* (White, 1995*).* The oral environment is affected by fermentable carbohydrate intake, whereby the pH levels of plaque demonstrate a steep drop after ingestion of refined sugars, which eventually recovers to physiological levels. The frequency of consumption in a day results in a series of 30-minute reduced pH levels mingled with normal physiological levels, (ten Cate, 1990). The demineralisation and remineralisation process within enamel is pH dependent as assessed by the calcium and phosphate concentration in the buffer solution in an in vitro assessment. The repetitive nature of this cycle within the oral environment leads to the cyclical dissolution and remineralisation of tooth mineral (Theuns *et al*, 1985). Since this is the basis to caries development, this model has been used to determine the remineralisation effect of fluoride (Buzalaf, 2010). Therefore samples are exposed to daily intervals of demineralising and remineralising solutions for a

set amount of time to achieve the cyclical nature of pH depressions experienced in the oral cavity (ten Cate, 1990). Many studies using the *in vitro* pH cycling method have shown the positive effect of fluoride on the remineralisation process of enamel, which has formed the basis of clinical trials that have followed (Viera *et al*., 2005, Carvalho *et al*., 2010, Danelon *et al*., 2017). A study looking into the effects of different concentrations of iron on sound enamel demineralisation and on the remineralisation of artificial enamel caries using the pH cycling method has also been carried out (Alves *et al*., 2011). In this study, the aim is to utilise the pH cycling method to determine the effect of zinc-doped phosphate-based glasses on the demineralisation and remineralisation of bovine enamel followed by progression to a biological caries model to investigate the anti-caries effect. Although bovine enamel has a similar microstructure when compared to human enamel, it is not necessarily a like-for-like replacement as shown in many studies. However, bovine enamel does confer many advantages over human enamel which include a, a larger surface area, more uniform enamel thickness and ease of obtaining the enamel (Schile *et al*., 2000, Fonseca *et al*., 2008).

3.7.2 Non-contact Surface Profilometry (NCSP)

According to the British Standard (BS 1134, 1972), the surface contour or roughness of materials can be assessed by profilometry to quantify the surface characteristics and enamel surface loss. The apparatus is made up of a stylus with a sapphire or diamond-tip fitted onto a probe, which in turn is attached on a pick-up arm. The contact stylus tracing appears to damage the surface of enamel as shown by a study (Heurich *et al*., 2010) and there are comparable differences to the surface texture measurements when compared to a non-contact surface profilometer. The non- contact stylus is navigated across the surface to scan the surface at an even pace and provides a trace for the surface (Whitehead *et al*., 1997). A fourier transform profilometry is then applied to the surface scan (Su and Chen, 2001) to give a three-dimensional surface roughness value (Bashaiwoldu *et al*., 2003). This method of assessment has been used previously to assess the effect of PBG doped with gallium on bovine enamel samples post pH cycling (Valappil *et al*., 2014). The non-contact surface profilometry avoids the limitations of enamel damage and is a suitable method to evaluate the changes in surface texture (Whitehead *et al*., 1997), and can be utilised to assess enamel demineralisation, which occurs in the surface layers of enamel up to the depth of 50μm (O' Reilly and Featherstone, 1987).

3.7.3 Transverse Microradiography (TMR)

TMR is an analytical technique and is the gold standard used to quantify changes in the mineral content of enamel during caries lesion formation (Lippert and Lynch, 2014) and is also utilised for quantifying changes in mineral content of early carious lesions (Cochrane *et al*., 2012). TMR utilises an approximately unicoloured X-ray beam to determine the mass reduction of the sample, which is translated to volume percent mineral (vol%min) (Cochrane *et al*., 2014). An *in vitro* investigation into the usage of TMR to quantify changes in the mineral content of enamel found that TMR was a valuable and satisfactory method for the measurement of initial mineral loss when compared with measurements by profilometry (Hall *et al*., 1997). The software was progressed further leading to a TMR scanning system, which features a high speed and low presence of errors (de Josselin de Jong and ten Bosch 1985) resulting in an extremely efficient system for the assessment of the bovine enamel. TMR was reliably used to quantify mineral loss in an *in vitro* study looking at erosive lesions (Amaechi *et al*., 1998) and has been used as a validation tool for newly developed diagnostic instruments such as the quantitative light-induced fluorescence (QLF) (Pretty *et al*., 2004) and Diagnodent methods for quantifying incipient lesions adjacent to orthodontic brackets (AlJehani *et al*., 2004).

4.0 Research Question

In view of the high prevalence of dental caries and enamel demineralisation during orthodontic treatment, the need for a novel agent which can be highly effective on its own or even in conjunction with the use of fluoride to reduce the incidence of such dental pathology is imperative. Although zinc has displayed anti-bacterial properties, a reliable link has yet to be established between the anti-bacterial property and a reduction in caries (Lynch, 2011). Given zinc's potential as an anti-bacterial agent and its ability to affect both demineralisation and remineralisation of enamel, an apparent contradiction exists clinically and it was felt imperative to investigate the potential of zinc as an effective anti-bacterial agent and also its role in preventing demineralisation through a controlled method of delivery that is essential to the nature and progression of enamel demineralisation while effectively relieving the practitioners' reliance on patient compliance in disease prevention.

5.0 Study Aims

Therefore the aim of the study is to prepare zinc-doped phosphate-based glasses (Zn-PBGs) and evaluate its anti-bacterial effect on the growth of *S. mutans* NCTC10449 and also the role of Zn-PBGs in the demineralisation and remineralisation process of bovine enamel, *in vitro,* using biological and non-biological models.

6.0 Study Objectives

The objectives of this study are:

1. To prepare novel anti bacterial zinc-doped phosphate based glasses using a melt quenching method

2. To assess glass degradation rate and ion release kinetics in water using weight loss method and inductively coupled plasma optical-emission spectrometry (ICP-OES)

3. To assess the anti bacterial effects of zinc-doped phosphate based glass on *Streptococcus mutans* via disc diffusion assays and liquid broth analyses

4. To evaluate the effect of zinc-doped phosphate based glasses on the remineralisation and demineralisation of bovine enamel using a pH cycling method

5. To assess the effect of zinc-doped phosphate-based glasses on bovine enamel samples via the constant depth fermentor model (CDFF) model

The null hypothesis for this study is that zinc-doped phosphate based glasses have no anti-bacterial effect on *S.mutans* and zinc-doped phosphate based glasses do not affect the demineralisation process of bovine enamel.

7.0 Study Design[1]

This was a laboratory-based project and experiments were conducted using *in vitro* models. Zinc-doped phosphate-based glasses were prepared using a conventional melt quenching technique. Zinc-doped phosphate based glasses (Zn-PBGs) were used as a medium for release of the zinc whilst zinc free glass discs (C-PBG) were used as a control. The calcium concentrations in the zinc-doped phosphate based glasses were altered from 11mol%, 12mol% and 13mol%. This was done to control the dissolution of the glasses and the rate of degradation. Glass characteristics and biocompatibility were assessed through pH analysis, ion release kinetics and degradation rates were assessed using a weight loss method.

Anti- bacterial assessments were conducted through disc diffusion assays and liquid broth analyses. Zinc doped phosphate-based glasses (Zn-PBG) were investigated for its ability to inhibit the growth of the bacterial species of interest, *S. mutans* using the disc diffusion methodology (Wayne, 2012). BHI plates were used to revive the culture of *S. mutans,* from the freezer. The overnight growth colonies from those plates were used to prepare standardised culture and testing on fresh Iso-sensitest agar plate (IST; Oxoid). The standardised cultures of *S.mutans* were then diluted in phosphate-buffered saline (PBS) at an optical density (ODS) of 0.05 prior to being spread onto the IST agar, after which zinc doped phosphate-based glasses with the differing levels of calcium (C_{11}, $C_{12,}$ C_{13}) and the control glasses (C-PBG) were placed onto the IST. All IST plates were incubated anaerobically overnight at 37°C. The anti-bacterial agent diffused into the agar and inhibited germination and growth of the *S.mutans* and the diameters of inhibition growth zones were measured (Balouiri *et al*., 2016). The diameter of the zone that formed is associated to the sensitivity of the isolate and to the diffusion rate of the disc through the BHI medium. The diameters of the zone of inhibition of each disc was then analysed and interpreted by measuring around each of the glass or antibiotic disc. This was measured to the nearest millimeter using a ruler and compared with controls. The broth dilution method is an accepted procedure for evaluating individual isolates or a small number of isolates (Jorgensen and Ferraro, 2009). Overnight cultures of *S.mutans* from the freezer which were revived on BHI agar plates were inoculated into 10 ml of nutrient broth and were incubated overnight at 37 °C with 200 rpm agitation in an orbital shaker (Stuart Scientific, UK). The overnight cultures were then used to inoculate 5 mL volume of phosphate buffered saline (PBS; Sigma) to a standardised optical density. The cell suspension of the isolate tested were placed into 5ml of the control and test solutions followed by serial micro-dlilution and was subsequently cultured onto BHI plates where the assessment of viable bacteria counts (expressed as

log_{10} CFU) was carried out. The results may show a bacteriostatic effect with similar number of colonies, a reduction in colony formation, which implies an incomplete or slow bactericidal activity or an absence of any growth of colonies if the whole inoculum has been killed (Balouiri *et al*., 2016). A biofilm assay utilising a constant depth film fermentor model (CDFF) was conducted which allows for the investigation of the growth of *S.mutans* in the oral ecosystem. The CDFF model, which replicates the physical environment intra-orally, had a rotating turntable, which supported the formation of the *S.mutans* biofilm onto recessed discs of bovine enamel. The turnatable received the dripped nutrient medium whilst the fixed scraper blades acted to spread over the substrata which maintained the biofilm at an even depth whilst simultaneously removing the surplus cellular material (Hope and Wilson, 2006, Valappil *et al*., 2009). TMR analysis of the bovine enamel samples was carried out.

The pH cycling method as described by Alves in 2011 was employed with some modifications. Bovine enamel discs were used to evaluate the effects of zinc-doped phosphate-based glass on the demineralisation and remineralisation process of bovine enamel. This involved replicating the pH depressions in the oral environment *in vitro* (White, 1995*),* which occurs following the ingestion of fermentable carbohydrates. The bovine enamel blocks were individually subjected to five pH cycles during 7 days, at 37 °C by being immersed in demineralising and remineralising solutions.The effect of zinc exposure on bovine enamel was measured and analysed through non-contact surface profilometry to quantify surface roughness and TMR to quantify mineral loss.

8.0 Materials and Methods

8.1 Preparation of Zinc-doped phosphate-based Glass Discs

Phosphate based glasses were produced using a melt quenching technique. The different component of the glasses were weighed out individually and vortexed together. This includes sodium hydroxyl (phosphate NAH_2P0_4) (Sigma ≥ 99%) in a powder form, diphosphorus pentoxide powder (P2O5) (Sigma-Aldrich = 99%) and calcium carbonate powder (CaCO3) (Sigma-Aldrich; MW: 100.09 g/mol). For the preparation of zinc-containing phosphate based glasses, zinc oxide powder ($Zn_2O_{3)}$ (Sigma-Aldrich, 99.99%) was added into the mixture. The compositions of each glass are depicted in Table I below. Once the required amount of each reagent was weighed and vortexed, the combination was then added to a Fused Quartz crucible 100mL (Fisher Scientific UK Ltd). The heat resistant crucible was then placed in a pre-heated furnace at 1100°C for 1 hour. The molten glass was subsequently poured into graphite moulds, which were preheated to 350°C. The glass

samples were then cooled to room temperature and the resulting glass rods cut into 5 x 2 mm with 5mm representing the diameter of the disc and 2mm being representative of the thickness of the glass disc. A low speed rotary diamond saw (ISOMET, Buehler Ltd, UK), was used to cut the discs to dimension and ethanol was used as a lubricant. All discs were individually weighed and sterilised in a drying oven (Memmert) that applies dry heat at 140°C for 1.5 hours.

Table 4: Composition of the different types of Phosphate-based glasses which are made up of Calcium (Ca), Sodium (Na) and Phosphate (P) for the control glass C-PBG and the addition of Zinc (zn) concentrations for the zinc doped phosphate based glass (Zn-PBG)

Glass Discs	Ca	Na	P	Zn
C-PBG	16	39	45	-
C11	11	41	45	3
C12	12	40	45	3
C13	13	39	45	3

8.2 Glass Degradation Analysis

Zinc-doped phosphate-based glass discs (5 mm diameter and 2 mm thickness) with different contents of calcium (C_{11}, $C_{12,}$ C_{13}) and control discs (C-PBG) were placed in plastic containers and filled with 50 ml of deionised water (pH 7±0.1). At various time points (6, 12, 24, 30, 48 and 54 hours) the glass discs were removed from their individual containers, and the samples were blotted dry using paper towels with the aim of removing any excess moisture prior to determining the weight. The glass discs were then individually weighed and a fresh solution of deionised water was then prepared. All the glass discs were placed into the solution. The rate of weight loss of the glasses was calculated by measuring the initial weight (M_0) of each sample and the weight at time t (M_t) to give a weight loss per unit area. Therefore, weight loss=$(M_0–M_t)/A$, where A is the surface area (mm^2). The measurements were carried out in triplicate.

8.3 pH Measurements

The pH measurements of the water that the glass discs were placed in was taken at each time point (6, 12, 24, 30, 48 and 54h) using a Hanna Instruments pH 211 Microprocessor pH meter (BDH, UK) with an attached glass combination pH electrode (BDH, UK). The pH electrode was calibrated using pH calibration standards. Both dissolution studies and standards for ion release study were prepared using high purity water with a start pH of 7 ± 0.1 using 0.1M ammonium hydroxide (Sigma Aldrich, Gillingham, UK).

8.4 Ion Release Kinetics

Ion release studies were conducted at the same time as pH measurements, and the solution was analysed for using ICP-OES (inductively coupled plasma optical-emission spectrometry). Calcium, sodium, zinc and phosphate ions released from all glass compositions and remaining in solution at time points (6, 12, 24, 30, 48 and 54h) was measured using ICP-OES. The measurements were done with A Spectro Ciros CCD Spectrometer (Spectro Analytical Instruments GmbH, Kleve, Germany), which was calibrated across concentrations in an estimated range of 0.0–40 ppm by dilutions of 1,000-ppm element standards (Sigma) in dH_2O. All samples obtained were exposed to an initial broad-spectrum analysis and diluted in dH_2O so as to result in an elemental composition within the estimated concentration range. The resulting elemental distribution charts from the broad-spectrum analysis were used to confirm the lack of residues from the both the purification and separation processes. The sample analysis was conducted under standard operating conditions (power: 1,400 W; coolant flow rate: 12 l · min $^{-1}$; auxiliary flow rate: 1 l · min $^{-1}$; nebuliser flow rate: 1 l · min $^{-1}$) with a side-on plasma detection system providing minimum detection limits for zinc, calcium and phosphorus of 50, 0.5 and 50 ppb, respectively. Regular acidic washouts periods (35% HCl), which were timed to happen approximately every 4–6 sample runs. This was carried out as a predictive measure of the relatively high calcium content within the samples so as to avoid possible signal weakening from residual build-up inside the nebuliser unit. Data was collected with specialist software provided by the manufacturer (Smart Analyser of Spectro Smart Studio, version 2.11.0631, Spectro Analytical Instruments GmbH, Kleve, Germany).

8.5 Antibacterial Analyses of Zinc-doped Phosphate-based Glass Discs

8.5.1 Growth of S.mutans

S. mutans NCTC 10449 strain was grown on brain heart infusion agar (BHI; Fluka) in an anaerobic (N_2:CO_2:H_2, 80:10:10) environmental chamber (Don Whitley MG1000; Don Whitley Scientific, Shipley, UK) at 37°C.

8.5.2 Disc Diffusion Assay

Zinc doped phosphate-based glasses (Zn-PBG) were investigated for its ability to inhibit the growth of the bacterial species of interest, *S. mutans* using the disc diffusion methodology (Wayne, 2012). BHI plates were used to revive the culture of *S. mutans,* from the freezer. The overnight growth colony from those plates was used to prepare standardised culture and was tested on fresh Iso-sensitest agar plate (IST; Oxoid). The standardised cultures of *S.mutans* was diluted in phosphate-buffered saline (PBS) at an optical density (ODS) of 0.05 prior to being spread onto the IST, after which zinc doped phosphate-based glasses with the differing levels of calcium (C_{11}, $C_{12,}$ C_{13}) and the control glasses (C-PBG) were placed onto the IST. All IST plates were incubated anaerobically overnight at 37°C prior to determination of results. All test were conducted in triplicate to allow for the calculation of means and standard deviations. The discs were examined at day 2 after anaerobic incubation of the IST plates. The zones of growth inhibition around each of the PBG discs were measured to the nearest millimeter using a ruler and compared with controls. The diameter of the zone is related to the rate of diffusion of the disc through the IST agar and the susceptibility of the *S.mutans* to the disc. The illustrations of the discs on the IST plate and the associated zones of inhibition can be found in Figure 2.

Figure 2: Schematic Representation of Glass Discs and Zones of Inhibition

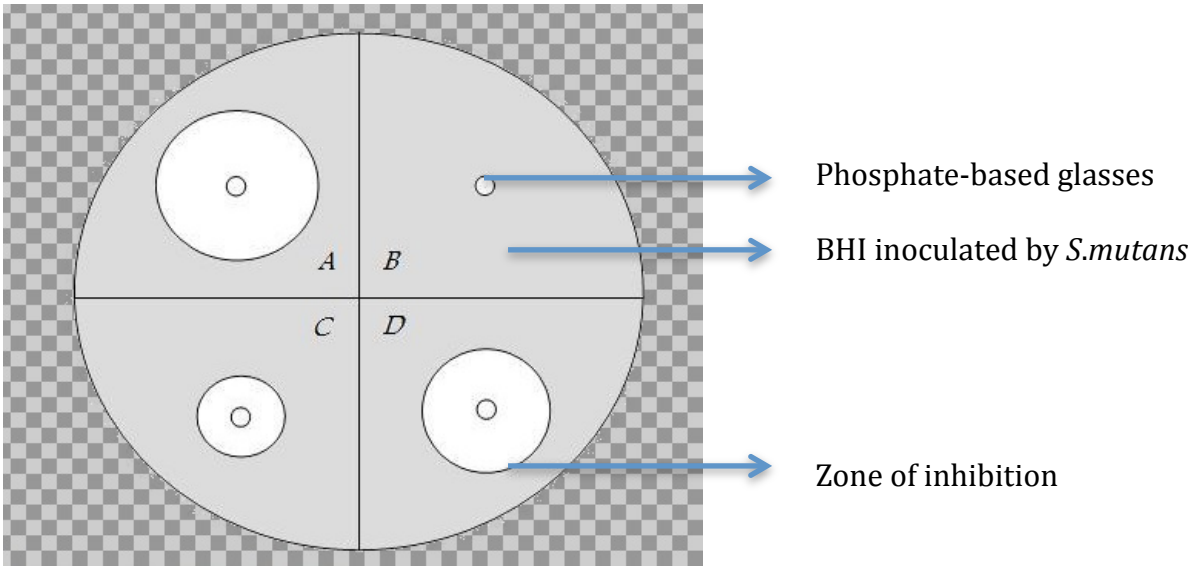


Figure 2 illustrates the disc diffusion plate that was set up. Each glass type (C-PBG and Zn-PBG with the different levels of calcium, C11, C12 and C13) which is represented by the inner circle was placed in each quadrant which is represented by the letter A, B, C and D. The outer circle represents the effective zones of inhibition by each glass type.

8.5.3 Liquid Broth Analyses

Overnight cultures of *S.mutans* which were grown on BHI agar plates were inoculated into 10 mL of nutrient broth and incubated overnight at 37 °C with 200 rpm agitation in an orbital shaker (Stuart Scientific, UK). The overnight cultures were then used to inoculate 5 mL volume of phosphate buffered saline (PBS; Sigma) to a standardised optical density (ODS) of 0.03 at a wavelength of 600 nm (OD_{600}). Zinc doped phosphate-based glasses (Zn-PBG) discs of 5 mm diameter and 2 mm thickness were added to each tube of phosphate buffered saline solution containing 5mls of solution. While the zinc-free control disc (C-PBG) was used as a control. 500ml of 0.2% diluted chlorhexidine (100 ml diluted in 900 ml of distilled water) and 500mls of 0.05% sodium fluoride solutions were also used as controls. The diluted chlorhexidine and sodium fluoride solutions were each placed in 4.5mls of phosphate buffered solution. This was done with the exception of one tube, which was used to assess the normal growth of *S.mutans*. The tubes were then incubated at 37 °C. At various time intervals (2, 4, 6 and 24 hours) serial dilutions of the suspensions were carried out in 90µl phosphate buffered saline solution (PBS; Sigma, Gillingham, UK). Using a pipette, 20µl volumes of each serial dilution were then spread onto BHI plates. The plates were then incubated anaerobically at 37°C for 48 hours. All test were conducted in triplicate to allow for

the calculation of means and standard deviations. For each type of disc, viable counts (colony forming units; CFUs) were assessed per ml and a log conversion was carried out. The process that was carried out during the liquid broth analysis is illustrated in Figure 3 as below.

Figure 3: Schematic Representation of Liquid Broth Analysis

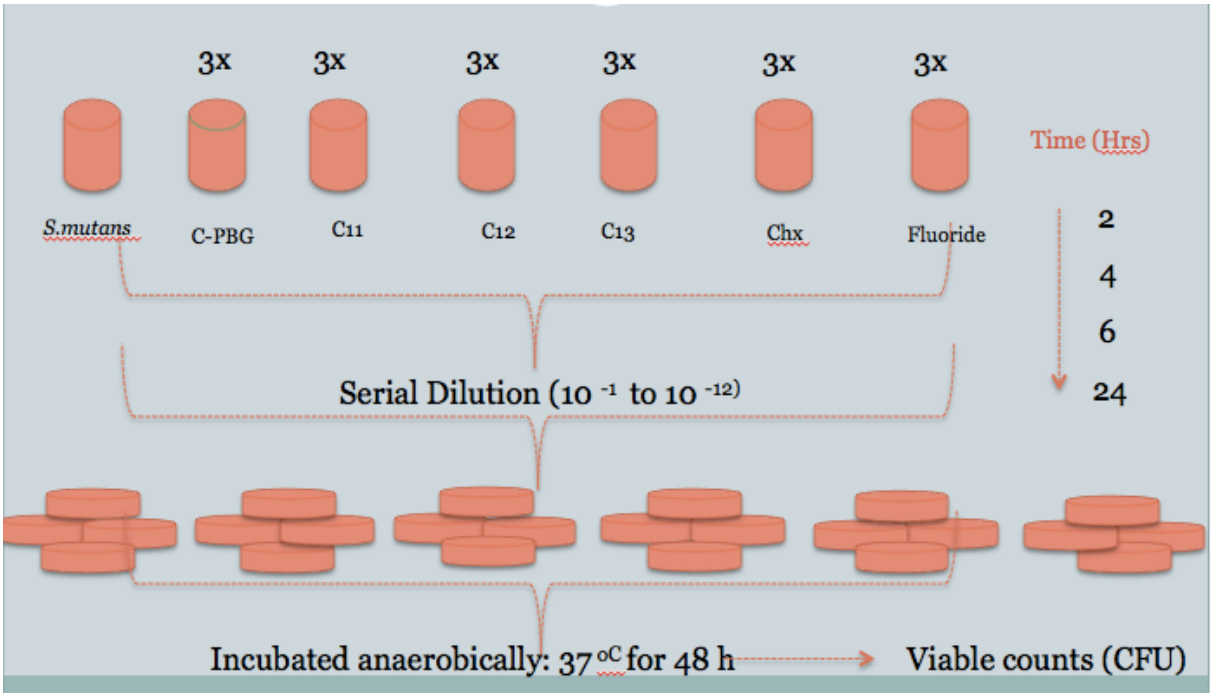


Figure 3 shows the tubes containing the 5mLs of phosphate buffered solution and the seven different conditions (S.mutans, C-PBG, ZnC11, ZnC12, ZnC13, Chlorhexidine (Chx) and Fluoride). Each condition was replicated three times. Serial dilutions (from 10^{-1} to 10^{-12}*) were carried out at each time point (2, 4, 6 and 24 hours) over a period of 24 hours. Each dilution was spread onto BHI plates followed by anaerobic incubation at 37°C for 48 hours. This was followed by viable bacterial counts (CFU).*

8.6 Biofilm Assay

8.6.1 Constant Depth Film Fermentor (CDFF) Model

A CDFF (CDFF; Cardiff University, Cardiff, UK) was used for the production of biofilms (Valappil *et al*., 2013). The CDFF contains a stainless steel turntable, which can hold up to 15 polytetrafluoroethylene (PTFE) pans. Each of the PTFE pan holds up to 5 PTFE plugs. Bovine enamel discs, 5 mm in diameter, were placed on 15 pans and each plug was recessed to a depth of 200 μm. The PTFE pans were inserted so that they were even with the turntable. A cylindrical glass vessel and two stainless steel end plates encase the turntable. Two 0.2 μm high-efficiency particulate air filters (Fisher Scientific, UK) was attached to the air inlet port at the top plate. The top plate also contains three media inlet ports. Incoming medium of STGM will be dripped onto the rotating turntable and will be distributed over the PTFE pans by two scraper blades. In addition to this, the scraper blades functioned to maintain the biofilms on the discs at the required depth, equal to the depth of the recess. The bottom plate contains a medium outlet port. The CDFF was sterilised in a hot air oven, using a temperature of 160 °C for 1 hour. The turntable was rotated at a speed of 3 rpm. In order to get pellicle formation on the bovine enamel discs before the run started, a 24-hour culture of *S. mutans* in BHI broth, 10 ml (Oxoid Ltd, Basingstoke, UK) was inoculated into 0.5 l of STGM and circulated through the which was kept in an incubator at 37°C. This was maintained for the entire duration of the experiment. After the pellicle formation, which was after 8 hours, the fermentor was fed from an 8-litre medium reservoir of STGM, with the waste flowing into an effluent bottle. The STGM was delivered at a rate of 0.5 ml · min^{-1} (to mimic the resting-saliva flow rate of healthy individuals (Dawes, 1996) using a peristaltic pump (Watson and Marlow, Falmouth, UK). A modification of the CDFF run was made with sucrose pulses of eight, 30 minute period of sucrose (2%) being pumped in per day to simulate sucrose intake in an individual. 5 conditions were set up for the CDFF model run. Zinc-doped phosphate glasses with 11mol% calcium (C11) and the control glass (C-PBG) were dissolved in 4ml of deionised water whilst the 0.2% diluted chlorhexidine solution (50μL of 0.2% diluted in 49.5 ml deionised water) and a 0.05% sodium fluoride (NaF) mouthwash in 4ml deionised water were set up. At the 5^{th} day and 12^{th} day, the pans were removed aseptically from the CDFF. 3 bovine enamel discs were removed and the excess biofilm formation was removed by dipping in deionised water. The remaining bovine enamel discs were dipped in 10 minutes in each condition of ZnC11, C-PBG, NaF and Chlorhexidine. The excess biofilm was then removed with deionised water and stored in air-tight tubes. All experiments were conducted in triplicate.

The bovine enamel discs were then sectioned using the diamond precision saw (Well

Diamond Wire Saw SA, Le Locle, Switzerland). The resulting sections were mounted lengthwise on brass anvils, fixed in place with nail varnish (MaxFactor Nailfinity; Proctor and Gamble, UK) and polished on a diamond impregnated grinding disc (15-μm particles; Buehler, Lake Bluff, Ill., USA) on one side to a thickness of 150 μm. Once this was achieved the nail varnish was removed using acetone, turned over and remounted using the same nail varnish (MaxFactor Nailfinity; Proctor and Gamble, UK). This was left to dry and again the diamond impregnated grinding disc (15-μm particles; Buehler, Lake Bluff, Ill., USA) was used to polish the bovine enamel samples to a final thickness of 80 μm. The polished samples were then mounted on a plastic template along with an aluminium step wedge. Microradiographs of the templates were taken on Kodak type 1A high-resolution plates (Kodak, Rochester, N.Y., USA) exposed to a CuKα X-ray source operating at 10 mA and 30 kV. The exposure time was 25 minutes, while the distance from source to template was 57 cm. The microradiographs were then examined microscopically (Leica, Germany). An image of the central, homogeneous portion of each sample will be optically centred, typically capturing an area of 300 μm of the enamel surface using a computerised image capturing system (TMR 2000 V2.0.27.16, Inspektor Research Systems BV Amsterdam, The Netherlands).). The resultant images will then be analysed using the software and ΔZ will represent the product of the depth of detectable mineral loss (lesion depth) relative to sound enamel (units; vol% · μm; positive values indicate an increase in mineral content).

8.7 Assessing the Impact on Remineralisation and Demineralisation of Bovine Enamel

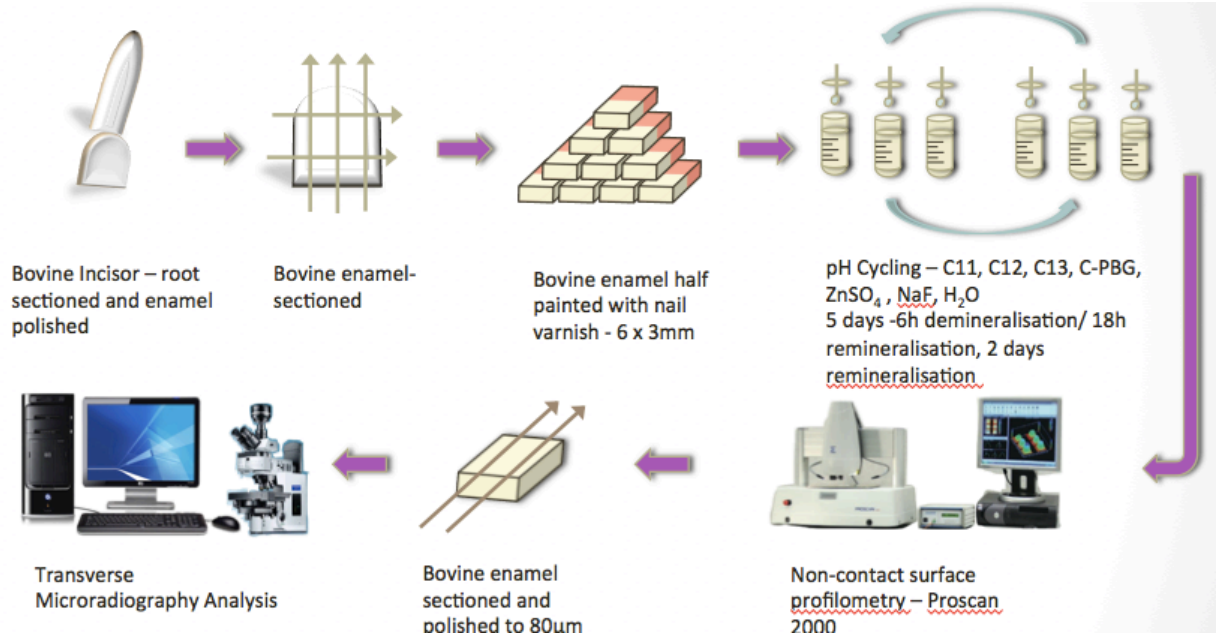
8.7.1 Bovine Enamel Preparation

Twenty-four blocks were prepared from sound bovine permanent incisors previously stored in 2% formaldehyde solution (pH7.0). The age of the cattle from which the bovine incisors were collected from did not exceed 36 months. The central portions of the labial surfaces which represents the most level part of the tooth was sequentially abraded to a depth of approximately 0.5 mm, using wet 120- grit paper followed by 240- grit and finally 1,000-grit carborundum paper (Rhynowet Whiteline Sheets, INDASA UK). Once polished, the roots of the incisors were first sectioned and the crown mounted onto the precision diamond saw apparatus. The blocks were then cut to approximately 6 × 2 mm using a precision diamond wire saw (Well Diamond Wire Saw SA, Le Locle, Switzerland). One half of the enamel blocks were painted with red nail varnish (MaxFactor Nailfinity; Proctor and Gamble, UK) to leave the other half of the block of approximately 3 × 2 mm exposed. After drying at room temperature, the blocks were then mounted in 50 ml of Sterilin disposable

containers (Sterilin Ltd, Newport, UK) using GreenStick impression compound (SpofaDental a.s, Kerr, Czech Republic).

8.7.2 pH Cycling

Figure 4: Schematic Representation of the pH cycling method



The pH cycling method as described by Alves in 2011 was carried out with slight modifications. This method was used to assess the effect of Zn-PBG on enamel remineralisation and demineralisation. Enamel blocks were allocated to one of 5 conditions (Zn- PBG, C-PBG, no glass, NaF and water). The bovine enamel blocks were individually subjected to a 7-day pH cycle, at 37 °C. The enamel blocks were first exposed to 5 days of cyclic exposure to an acidic challenge by being immersed in demineralising solutions (2.0 mM $CaCl_2$, 2.0 mM KH_2PO_4, 0.04 ppm F-form NaF and 0.75 mM acetic acid adjusted to pH 4.7 with 1.0 M NaOH; 6 h demineralisation/ 18 h remineralisation), followed by 2 days in remineralising solution (1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4, 150 mM KCl, 0.05 ppm F-form NaF and 20 mM HEPES adjusted to a pH of 7.0 with 1.0 M NaOH) (Alves *et al*., 2011). The blocks were washed in deionised water at each change of solution and treatments. After the 5th day, the blocks were kept in fresh remineralising solution for 48 h (6th–7th day). In the $ZnS0_4$ condition the bovine enamel samples were placed into 231µg of $ZnS0_4$ for 5 minutes

at each solution change whilst in the water and NaF conditions, the samples were immersed in water or 228 ppm NaF solution for 5 minutes at each solution change.

8.8 Surface and Mineralisation Analyses

8.8.1 Non-contact Surface Profilometry (NCSP)

The enamel blocks were scanned and analysed prior to pH cycling using non-contact surface profilometry (NCSP; Proscan 2000; Scantron Industrial Products Ltd, Taunton, UK), to form the baseline data. The pH-cycled samples were subsequently scanned and analysed with the nail varnish intact and then with removal of the nail varnish using acetone for comparison. NCSP data was obtained using a S5/03 scan head (resolution: 0.01 µm; measuring range: 0.3 mm, with a spot size of 4 µm) at a sensor rate of 300 Hz with the averaging (Kalman) filter set at 1 iteration. Each scan was carried out to include both the exposed and unexposed enamel surface. The enamel blocks were scanned in both, the x and y directions (step size: 0.01; steps x = 250 and steps y = 100, reversed for y direction), All measurements were performed in triplicate per enamel block, and the mean surface roughness R*a* was calculated. The images and data were recorded and stored on a computer hard drive in PRS file format and analysed using software provided by the manufacturer (Proscan 2000, version 2.1.1.15)

8.8.2 Transverse Microradiography (TMR)

TMR is the analytical technique to quantify changes in the mineral content of enamel during caries lesion formation (Lippert and Lynch, 2014) and is the gold standard technique for quantifying changes in mineral content of early carious lesions (Cochrane *et al*., 2012). TMR utilises an X-ray beam that is monochromatic to determine the mass decrease of the enamel sample, which is then translated to volume percent mineral (vol% min) (Cochrane *et al*., 2014). Several thin slices were cut from each block using a diamond precision saw (Well Diamond Wire Saw SA, Le Locle, Switzerland). This was done in a length-wise fashion for the pH cycled samples,that included both, the exposed and unexposed areas of the enamel blocks, to allow for the unexposed area to act as a control on each slice of bovine enamel. The sections were mounted lengthwise on brass anvils, fixed in place with nail varnish (MaxFactor Nailfinity; Proctor and Gamble, UK) and polished on a diamond impregnated grinding disc (15-µm particles; Buehler, Lake Bluff, Ill., USA) on one side to a thickness of 150 µm. Once this was achieved the nail varnish was removed using acetone, turned over

and remounted using the same nail varnish (MaxFactor Nailfinity; Proctor and Gamble, UK). This was left to dry and again the diamond impregnated grinding disc (15-µm particles; Buehler, Lake Bluff, Ill., USA) was used to polish the bovine enamel samples to a final thickness of 80 µm. The polished samples were then mounted on a plastic template along with an aluminium step wedge. The templates were then exposed to CuKα X-ray source operating at 10 mA and 30 kV and microradiographs of the templates were taken on Kodak type 1A high-resolution plates (Kodak, Rochester, N.Y., USA). The exposure time was 25 minutes, while the distance from source to template was 57cm. The microradiographs were then examined microscopically (Leica, Germany). An image of the central, homogeneous portion of each sample was optically centred, to classically capture an area of 300 µm of the enamel surface using a computerised image capturing system (TMR 2000 V2.0.27.16, Inspektor Research Systems BV Amsterdam, The Netherlands). In order to compare the effect of exposure (exposed enamel section) with respect to a baseline sample (unexposed section) with as little variation as possible, the equivalent sample sets will then be captured. Parameters of integrated mineral loss (ΔZ) will be assessed using a computerised image analysis system (TMR2006 v 3.0.0.17; Inspektor Research BV Systems, Amsterdam, The Netherlands). ΔZ will represent the depth of detectable mineral loss (lesion depth) relative to sound enamel (units; vol% · µm; positive values indicate an increase in mineral content).

9.0 Statistical Analyses

Statistical analyses were done using Paired t-tests, one-way analysis of variance (ANOVA) and Tukey Kramer multiple comparisons post-hoc test. Paired t-test was used to assess mineral loss in bovine enamel samples placed in the constant depth film fermentor (CDFF) model. Paired t-tests were also used to assess baseline and treated pH cycled bovine enamel samples. The ANOVA was initially carried out and when there was a difference in the means between groups, this was followed by Tukey Kramer multiple comparisons post hoc tests to assess in which groups the difference lay. Tukey Kramer multiple comparison tests were carried out for pH analyses, weight loss during glass degradation study, zones of inhibition from the disc diffusion assay and viable colony forming units (CFU) which was transformed logarithmically, from the liquid broth assay reflecting anti-bacterial analysis. The Pearson Correlation was used to examine the linear relationship between ion release and weight loss for glass degradation. P values <0.05 is was considered statistically significant. Statistical analyses were conducted using the Prism GraphPad Software (San Diego, California, USA).

10.0 RESULTS

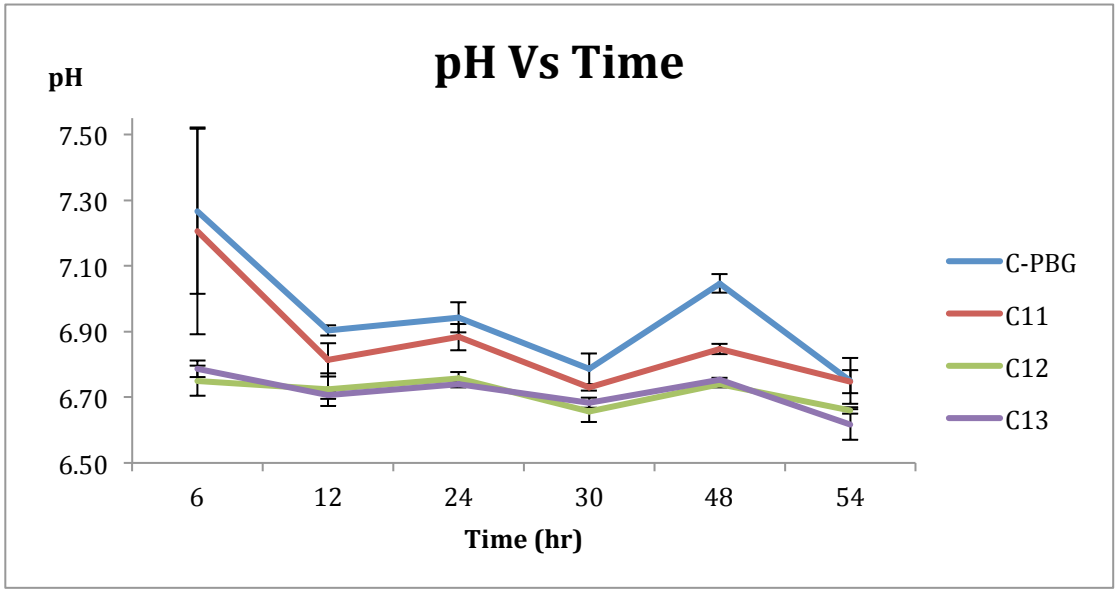
10.1 Glass Characteristics

In this study glass characteristics was determined by pH analysis, glass degradation as determined by the PBG solubility, which was calculated using weight loss method and ion release kinetics. The pH range needs to be compatible and not detrimental in the oral environment whilst controlled weight loss and ion release kinetics of Zn-PBG is essential in ensuring a low level, sustained delivery of ions at a susceptible site.

10.1.1 pH Analysis

pH analyses which was carried out in water over a 54 hour period is shown in figure 5. The pH values showed a statistically significant difference ($p < 0.05$) between C12 and C13 compared to C-PBG. There was no significant ($p > 0.05$) difference between C11and C-PBG. The average pH values over time were 6.95 ± 0.19 for C-PBG, 6.87 ± 0.17 for C11, 6.71 ±0.04 for C12 and 6.71 ± 0.06 for C13.

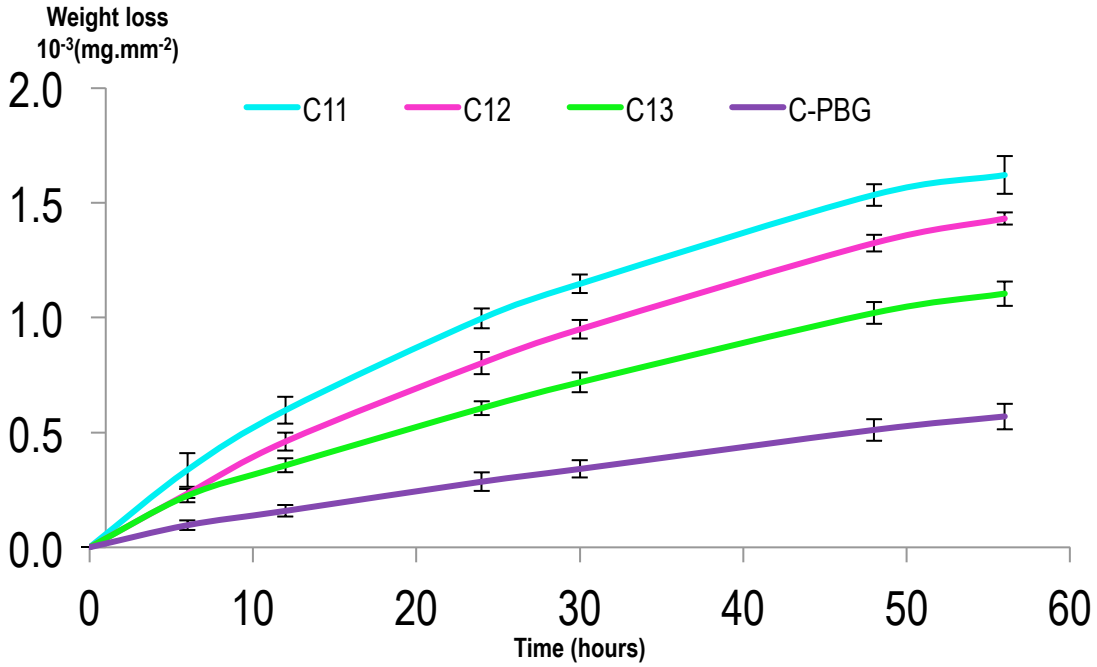
Figure 5: pH fluctuations as a function of time



10.1.2 Weight Loss

The weight loss of the PBGs over time is shown in Figure 6. The weight loss of the glasses showed an almost linear relationship. Degradation rates of the Zn-PBGs were significantly ($p<0.05$) different, 28.94µg mm^{-2} h^{-1} for C11, 25.56µg mm^{-2} h^{-1} for C12 and 19.72µg mm^{-2} h^{-1} for C13 compared with 0.17µg mm^{-2} h^{-1} for C-PBG. C11 showed the highest amount of cumulative ion release and C-PBG showed the lowest amount of ion release, which relates to the solubility of the glasses. The results show that the solubility of the glasses is dictated by the calcium concentration whereby increasing the calcium concentration reduces the solubility of the glasses.

Figure 6: Dissolution rates as determined by weight loss of the C-PBG, C11, C12 and C13 as a function of time

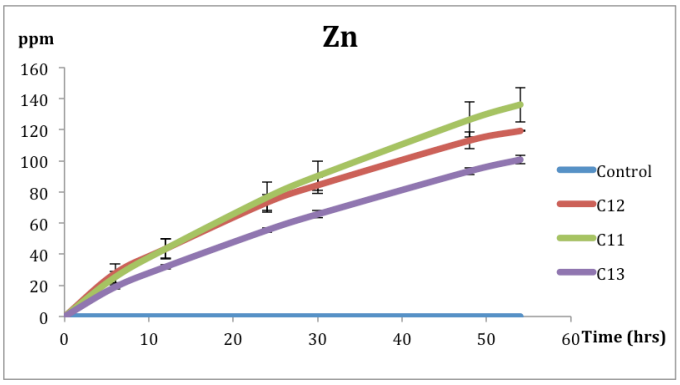


10.1.3 Ion Release

Ion release for Zn, Ca, P and Na and the correlation between ion release of the glasses and time is shown in Figure 7. Zn, Ca, P and Na ion release rates were decreased as the calcium levels were increased in the glasses, demonstrating an inverse relationship.

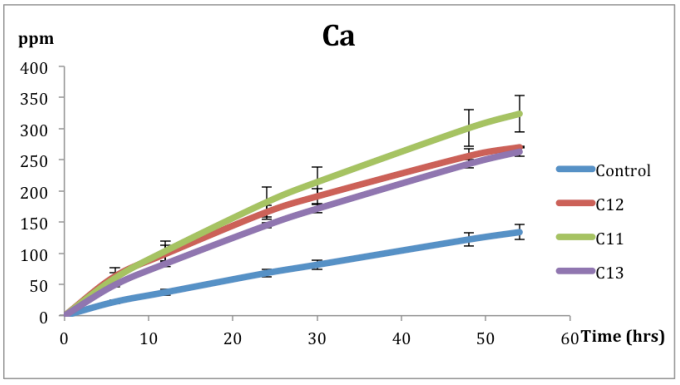
a) Zinc (Zn) Ion release as a function of time

Figure 7: ICP-OES analyses showing cumulative ion release of zinc (a), calcium (b), phosphorus (c) and sodium (d) as a function of time for C-PBG, C11, C12 and C13



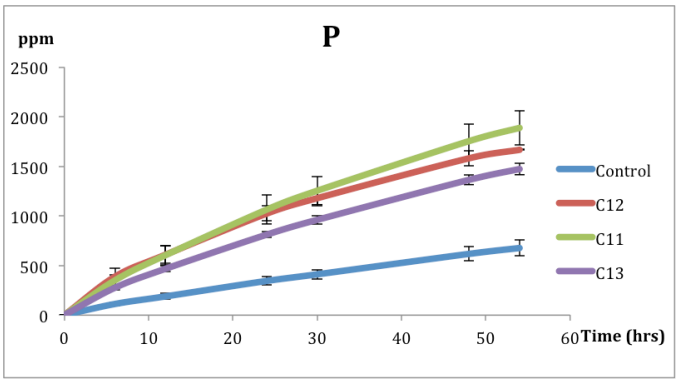
Zinc ion release was decreased as calcium concentration increased in the glasses. Zinc ion release for C11 (r=0.998), C12 (r=0.998), and C13 (r=1.00) showed a strong correlation ($p < 0.05$) with the weight loss of the glasses.

b) Calcium (Ca) Ion release as a function of time



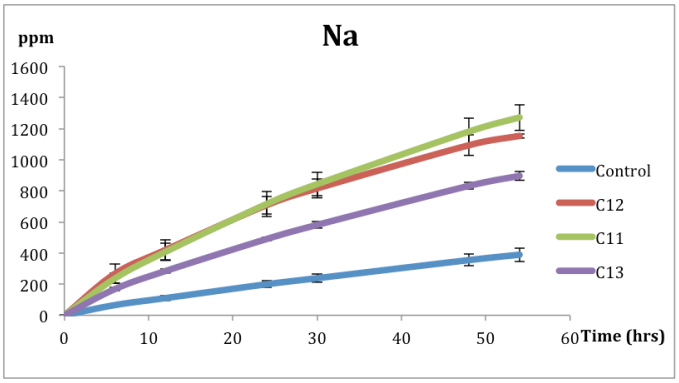
Calcium ion release was decreased as calcium concentration increased in the glasses. Calcium ion release for C-PBG (r=0.895), C11 (r=0.998), C12 (r=0.998), and C13 (r=1.00) showed a strong correlation ($p < 0.05$) with the weight loss of the glasses.

c) Phosphate (P) Ion release as a function of time



Phosphate ion release was decreased as calcium concentration increased in the glasses. Phosphate ion release for C-PBG (r=0.895), C11 (r=0.998), C12 (r=0.998), and C13 (r=1.00) showed a strong correlation ($p < 0.05$) with the weight loss of the glasses.

d) Sodium (Na) Ion release as a function of time



Sodium ion release was decreased as calcium concentration increased in the glasses. Sodium ion release for C-PBG (r=0.895), C11 (r=0.998), C12 (r=0.998), and C13 (r=1.00) showed a strong correlation ($p < 0.05$) with the weight loss of the glasses.

10.2 Anti-bacterial Assessments

10.2.1 Disc Diffusion

The disc diffusion method was used to assess the anti-bacterial activity of the PBGs. The zones of inhibition from the disc diffusion assay are shown in Figure 8. The zones of inhibition as shown in Figure 8 demonstrate the measured zone of inhibition of C11, C12 and C13 after subtracting the zone of inhibition of C-PBG. The zones of inhibition were found to decrease (from 18± 1.7, 16± 1.6 to 15.0 ± 1.1 mm) as the calcium concentration in the glasses increased from 11, 12 and 13mol% of calcium. Zones of inhibition were significantly increased for C11, C12 and C13 ($p < 0.01$) when compared with C-PBG. The trend for zones of inhibition showed the highest inhibition for C11 followed by C12 and lastly C13. C-PBG showed some inhibition, which could be due to the release of Ca ions.

Figure 8: Zones of inhibition of C-PBG, C11, C12 and C13 on BHI plate

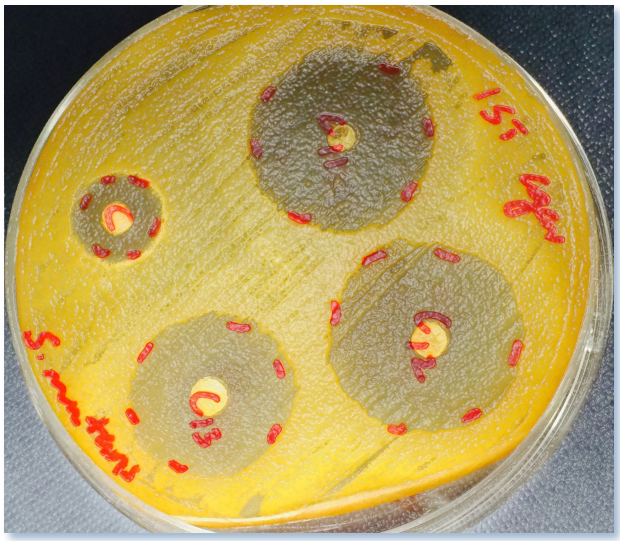
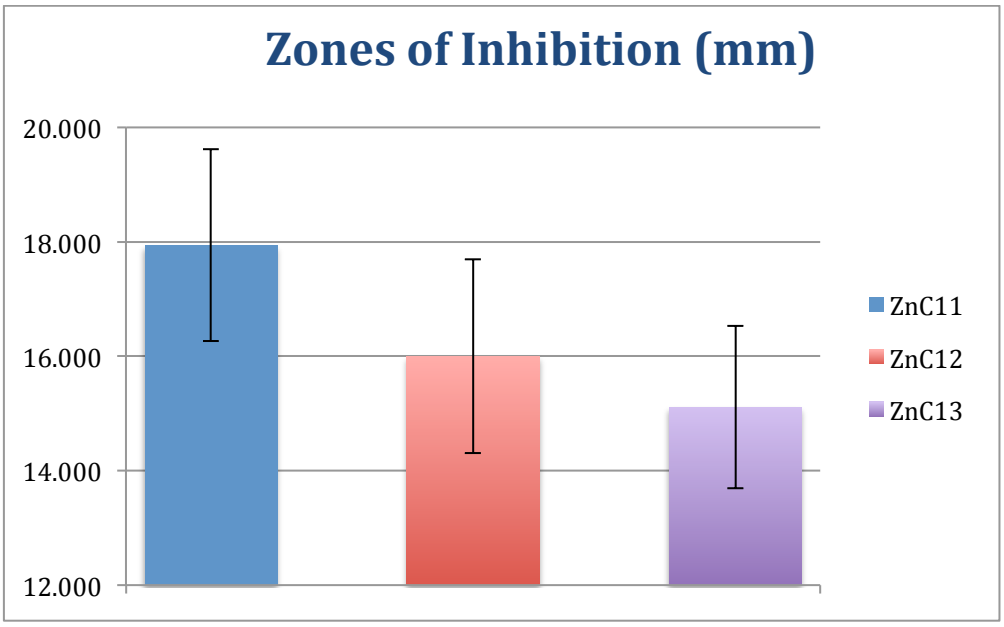


Figure 9: Zones of inhibition of C11, C12 and C13

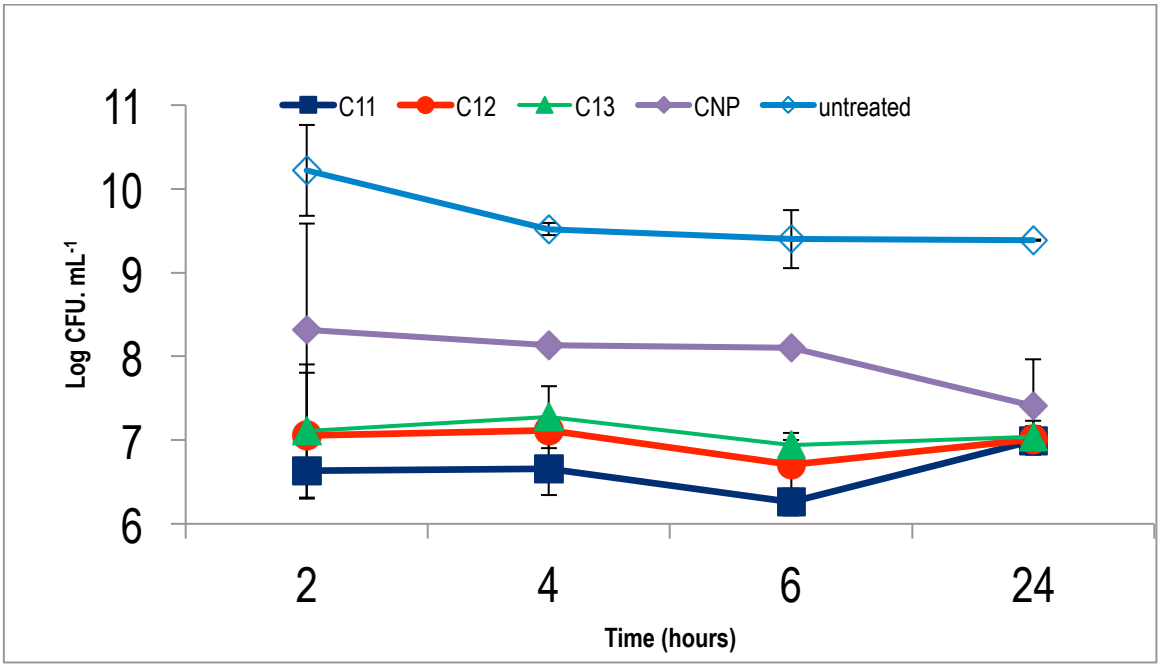


Zones of inhibition were found to decrease (from 18± 1.7, 16± 1.6 to 15.0 ± 1.1 mm) as the calcium concentration in the glasses increased from 11, 12 and 13mol% of calcium. Zones of inhibition were significantly increased for C11, C12 and C13 ($p < 0.01$) when compared to C-PBG.

10.2.2 Liquid Broth Analysis

Liquid broth analysis was also carried out to assess the anti-bacterial activity of the PBGs. The liquid broth assay was carried out where the viable cell counts in the liquid broth assay was taken and is depicted in Figure 10. The viable cell counts were found to increase as the calcium concentration in the glasses increased from 11, 12 and 13mol% of calcium. Viable cell counts in the C-PBG group were significantly increased ($p< 0.01$) when compared to groups C11, C12 and C13.

Figure 10: The log_{10} number of colony forming units of S. mutans at 2, 4, 6 and 24 h after being exposed to; C-PBG, C11, C12, C13 and untreated sample

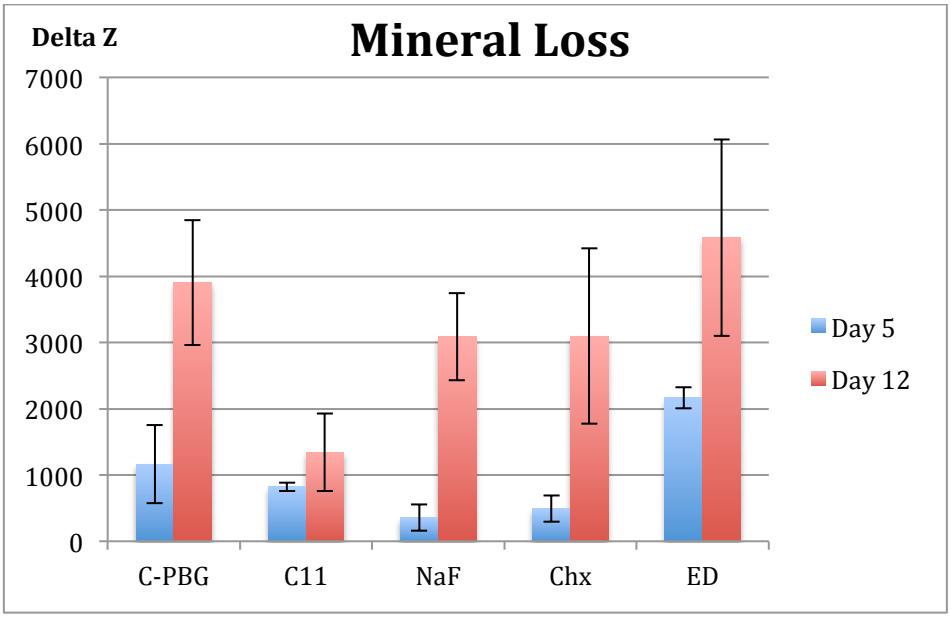


Viable cells in the liquid broth assay were found to increase as the calcium concentration in the glasses increased from 11, 12 and 13 mol% of calcium. Viable cell counts in the untreated and C-PBG group were significantly increased ($p < 0.01$) when compared to groups C11, C12 and C13.

10.2.3 Constant Depth Film Fermentor (CDFF)

C11 showed the most optimal trends for pH analysis, weight loss, ion release kinetics and anti-bacterial analyses, which dictated its use for the CDFF model. TMR analyses to evaluate the average mineral loss (delta z) of the bovine enamel samples at day 5 and day 12 were carried out, and are demonstrated in Figure 11. The CDFF model showed statistically significant mineral loss ($p < 0.05$) in the bovine enamel samples that were exposed to C-PBG, NaF, Chlorhexidine and water at day 12 compared to day 5. There was no statistically significant difference in mineral loss ($p > 0.05$) in the bovine enamel samples that were exposed to C11 between day 5 and day 12.

Figure 11: TMR analyses of average mineral loss (delta z) of bovine enamel samples exposed to C-PBG, C11, NaF, Chx, and simply Enamel Disc (ED) between Day 5 and Day 12 in the CDFF model



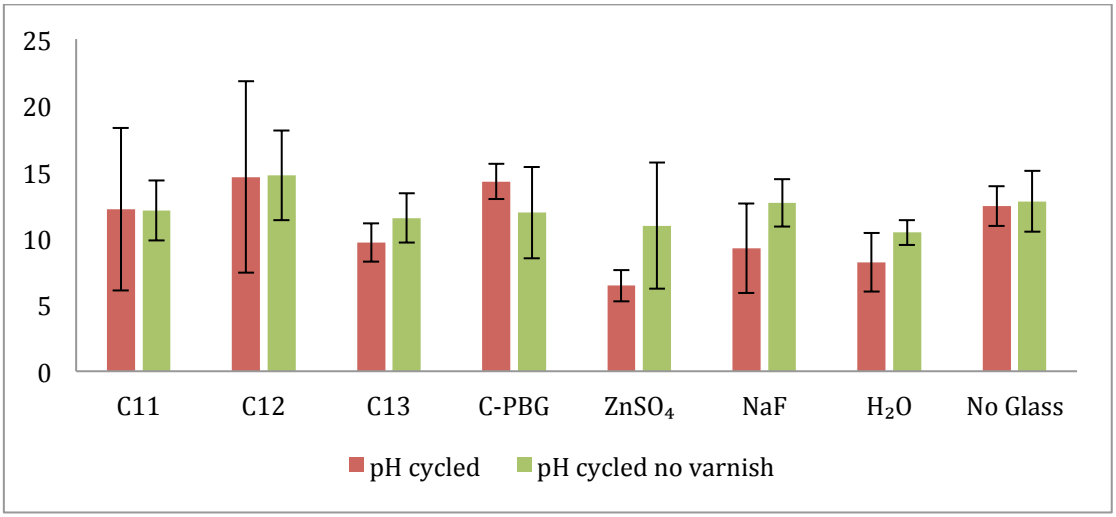
There was statistically significant mineral loss ($p < 0.05$) in the bovine enamel samples that were exposed to C-PBG, NaF, Chx and ED at day 12 compared to day 5. There was no statistically significant difference in mineral loss ($p > 0.05$) in the bovine enamel samples that were exposed to C11 between day 5 and day 12.

10.3 Remineralisation and Demineralisation

10.3.1 pH cycling

To be clinically useful in reducing white spot lesions, the glass discs have to demonstrate either the ability to inhibit demineralisation or the ability to remineralise enamel, alongside displaying anti-bacterial activity. This was assessed through the pH cycling method. NCSP scans were undertaken to indicate surface roughness post pH cycling, which is show in Figure 12. NCSP analyses revealed no significant differences in surface roughness between C11, C12, C13, C-PBG, $ZnS0_4$, NaF and H_20.

Figure 12: Non-Contact Surface Profilometry (NCSP); (Proscan 2000 Scantron Industrial Products Ltd, Taunton, UK) to indicate surface roughness (ISO Ra) of bovine enamel samples

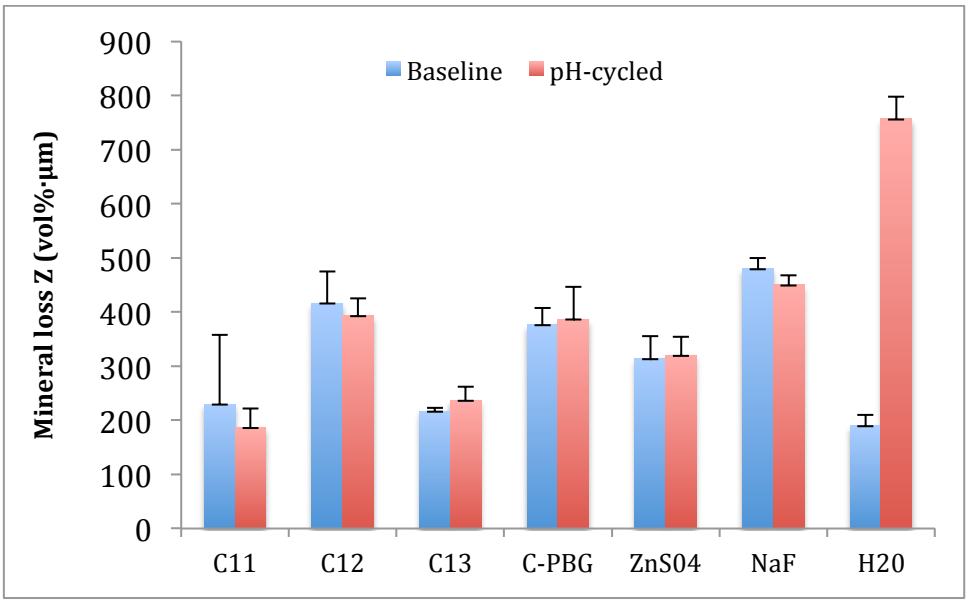


Non-contact surface profilometry analyses revealed no significant differences in surface roughness between C11, C12, C13, C-PBG, $ZnS0_4$, NaF and H_20.

10.3.2 Transverse Microradiography (TMR)

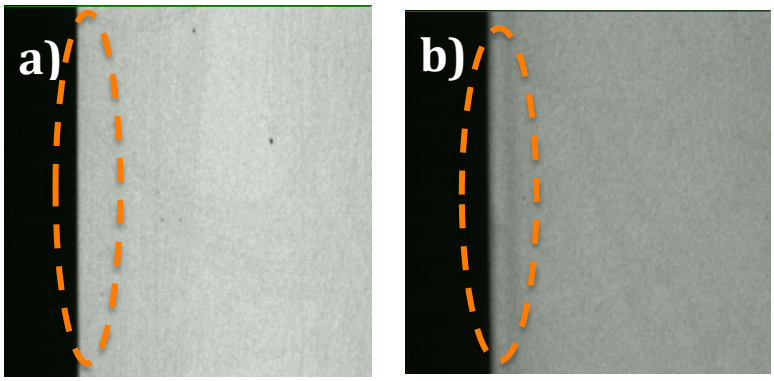
TMR is an analytical technique to quantify changes in the mineral content of enamel during caries lesion formation and is the gold standard technique for quantifying such changes in mineral content of early carious lesions. TMR was carried out for the pH cycled bovine enamel samples. TMR analyses in figure 13 showed significant ($p<0.05$) mineral loss in the a) pH-cycled H_20 group when compared to b) pH-cycled C11, C12, C13, C-PBG, $ZnS0_4$ and NaF.

Figure 13: TMR analyses of average mineral loss(delta z) between baseline and pH-cycled bovine enamel samples exposed to C11, C12, C13, C-PBG, $ZnS0_4$, NaF and H_20



Transverse micro-radiography (TMR) analyses showed significant ($p<0.05$) mineral loss in the a) pH-cycled H_20 group when compared to b) pH-cycled C11, C12, C13, C-PBG, $ZnS0_4$ and NaF.

Figure 14: TMR images of pH-cycled bovine enamel exposed to C11 (a) and H_20 (b)



The grey shadowing of the subsurface lesion produced in the water group with an intact enamel surface is shown by the circular red highlight in image b) whilst image a) shows an intact surface of the bovine enamel with the absence of any form of grey shadowing. This type of lesion is characteristic of the pH cycling protocol used and mirrors enamel demineralisation that occurs intra-orally.

11.0 Discussion

This was a lab-based experimental study that aimed to assess the anti-bacterial effect of zinc-doped phosphate based glasses on the growth of *S.mutans* and the role of zinc in the demineralisation and remineralisation process of enamel. The anti-bacterial activity of the glasses was shown through the anti-bacterial analyses whilst the glasses also demonstrated the ability to inhibit demineralisation when bovine enamel samples were exposed to a biofilm in the CDFF model.

11.1 Glass characteristics

11.1.1 pH Analysis

pH analyses showed a statistically significant difference between C12 and C13 compared to C-PBG. There was no significant difference between C11and C-PBG. Despite the statistical significance, the pH values for all the glasses ranged between pH 6.62 and pH 7.27, which is a narrow range clinically and is in keeping with the average pH value of un-stimulated saliva, which is approximately 6.8. Upon stimulation this increases up to 7.8 and is subject to individual variation. The critical pH of enamel dissolution ranges between 5.5-5.7 (Loke *et al*., 2016) and is below the pH range of the glasses. On the whole, the glasses have an acceptable pH range which would be compatible and not detrimental in the oral environment. The pH value is also an important parameter in influencing the quality of the delivery system as shown by glasses incorporated with dexamethasone which demonstrated increasing dissolution rate as the pH value increased from pH 4.3 to pH 8.6 at 37°C (Hum and Boccaccini, 2012). The quality of delivery of the glasses which is pH dependent needs to be taken into account as pH changes can occur locally or at pathological sites within the body as would happen in an individual facing a high caries challenge. The insertion of fixed orthodontic appliances induces a quick increase in the amount of carbohydrates, which has the potential of lowering the pH levels of plaque, which was found in orthodontic patients (Chatterjee and Kleinberg, 1979). This is ascribed to the rapid shift in the composition of the bacterial flora in plaque following the placement of orthodontic appliances, which specifically involves the levels of acidogenic bacteria, such as *S. mutans* and lactobacilli, increasing significantly in orthodontic patients and also a rise in the levels of carbohydrates (Lundström and Krasse, 1987, Chatterjee and Kleinberg, 1979). Therefore the Zn-PBG need to be able to dissolve at a controlled rate at a lower pH level to be able to perform effectively at these sites. The release of Na and P ions from the glasses can have an effect on the pH locally (Abou Neel *et al*, 2009). The ion release profile of Na and P tends to be higher than Ca (Knowles *et al*., 2001, Valappil *et al*., 2008) and is also shown in this study. Glass

compositions with higher Na mol% released more Na ions into water with C11 releasing the highest amount. An initial increase in pH is associated with the high initial release of Na and P ions (Abou Neel *et al*., 2009), which, was not reflected in this study. Therefore the performance of the zinc doped phosphate-based glasses at different pH levels needs to be evaluated.

11.1.2 Glass Degradation: Weight loss, Ion release

C11 showed the highest amount of cumulative ion release and C-PBG showed the lowest amount of ion release, which relates to the solubility of the glasses. The results show that the solubility of the glasses is dictated by the calcium concentration whereby increasing the calcium concentration reduces the solubility of the glasses. This is in agreement with a study, which looked at solubility and ion release in a glass system. The researchers found altering the levels of calcium oxide affected the solubility of the glasses in an inverse manner. Zinc ion release was decreased as calcium concentration increased in the glasses, which correlated strongly with the weight losses of glasses Similarly, Na and P ions showed a strong correlation with weight loss of the glasses (Knowles *et al*., 2001). Glass degradation in this study was 28.94µg mm^{-2} h^{-1} for C11, 25.56µg mm^{-2} h^{-1} for C12 and 19.72µg mm^{-2} h^{-1} for C13 compared with C-PBG, which was 10.17µg mm^{-2} h^{-1}. Degradation rates of PBGs in this study were lower compared to other studies with doped PBGs. The dissolution rates of PBGs doped with 0, 1, 3, and 5 mol % Ga_2O_3, were 41.70µg mm^{-2} h^{-1}, 23.60µg mm^{-2} h^{-1}, 7.30µg mm^{-2} h^{-1}, and 3.70µg mm^{-2} h^{-1}. The Ca levels were fixed at 16 mol% and the Na levels altered accordingly to the concentration of doped Ga_2O_3 (Valappil *et al*., 2008). This was also reflected in a different study which showed higher degradation rates of Cu doped PBGs (Mulligan *et al*., 2003). In other studies however, the rate of degradation of doped PBGs have shown a lesser rate of degradation than what was found in this study. Silver-doped PBGs showed a degradation rate of 1.22µg mm^{-2} h^{-1}, 0.41µg mm^{-2} h^{-1} and 0.42µg mm^{-2} h^{-1} for Ag10, Ag15 and Ag20 each (Valappil *et al*., 2007). This lower degradation rate was also shown in K_20-doped PBGs (Knowles *et al*., 2001). The dopant used in the glasses can have an effect on the degradation rates and can account for the variability in the results seen. One study used a glass composition with 50% P (Valappil *et al*., 2007) while the other studies used 45% P, which can also affect the rate of glass degradation (Knowles *et al*., 2001, Valappil *et al*., 2008). There is scope to improve the degradation rates of the glasses in this study and therefore the controlled delivery of ions to susceptible sites. Reducing the concentration of calcium within the glass and increasing the concentration of Na ions can achieve this. Overall, the degradation of the glasses as depicted by the weight loss and ion release shows the potential for these glasses as controlled delivery agents intra-orally and

the zinc release rates in this study is well below the toxicity levels and therefore safe to be implemented.

11.2 Anti-bacterial Assessments

11.2.1 Disc Diffusion – Zones of inhibition; Broth Analyses – Viable cell counts

The trend for zones of inhibition showed the highest inhibition for C11 followed by C12 and lastly C13. There was some anti-bacterial activity demonstrated by the C-PBG disc which, could be possibly due to the release of Ca ions as Ca ions have shown to disrupt the membrane of *S.aureus* (Xie and Yang, 2016). The diameter of the zone of inhibition is related to the susceptibility of the isolate and to the diffusion rate of the disc through the BHI medium (Wayne, 2012). This established the anti-bacterial ability of the Zn-PBGs, which then paved the way for an investigation into viable cell counts. The anti-bacterial effect was supported in the liquid broth assay where the viable cell counts in the liquid broth assay were found to increase as the calcium concentration in the glasses increased from 11, 12 and 13mol% of calcium. Viable cell counts in the C-PBG group were significantly increased ($p < 0.01$) when compared to groups C11, C12 and C13**.** The anti-bacterial activity of zinc has been shown in other oral treatment modalities (He *et al*., 2002, Brading *et al*., 2003, Finney *et al*., 2003, Hall *et al*., 2003) while this study depicts the anti-bacterial effect of a novel method of delivery using Zn-PBGs with zinc as the active agent.

11.3 Remineralisation and Demineralisation

11.3.1 pH cycling- Non-contact surface profilometry, Transverse microradiography

In order to be an effective caries-inhibiting agent, the glass discs have to also demonstrate either the ability to inhibit demineralisation or the ability to remineralise enamel. This was assessed through the pH cycling method. NCSP analyses revealed no significant differences in surface roughness between C11, C12, C13, C-PBG, $ZnS0_4$, NaF and H_20. This is to be expected as the protocol of pH cycling used was alternating between remineralising solutions and demineralising solutions, which would create subsurface lesions as compared to a continuous or progressive protocol, which would create an erosive-like lesion (Buzalaf *et al*., 2010). The alternation between demineralisation and remineralisation was carried out over the progressive protocol to mimic the pH depressions and rises that would be seen intra-orally. In order to assess the action of the glasses on mineral content, the mineral loss was assessed using TMR. The results showed significant ($p<0.05$) mineral loss in the pH-cycled H_20 group when compared to pH-cycled C11, C12, C13, C-PBG, $ZnS0_4$ and NaF. The Zn-PBGs did not show a bigger effect when compared to the controls. This could be due to a few factors, which include the fact that fluoride was present in both the demineralising solution and the remineralising solution, which could have exerted an effect on the enamel. Fluoride alongside calcium and phosphate are incorporated into the solution as the ions play an important role in preserving the surface structure of the enamel in order to simulate the progression of a carious lesion (Theuns *et al*., 1984). A different approach to the solutions is the utilisation of lactic acid preparation to represent the acid produced by *S.mutans*. Also the concentration of zinc that was incorporated in the glasses was low at 3mol% and may not be at an effective concentration. The zinc ion release rate per hour was 2.5 ppm, 2.2 ppm and 1.9 ppm for C11, C12 and C13 respectively. The optimal concentration for zinc to be incorporated into hydroxyapatite ranges between 0.1–36ppm before saturation of the binding site takes place (Mohammed *et al*., 2015). The cumulative ion release over a period of 56 hours in this study does exceed the optimal concentration that has been reported on. However, in this study the ion release was assessed in water and not under acidic conditions with a lower pH, which could have an effect. A further investigation of zinc incorporated into the glasses at varying concentrations would be useful to understand the optimal concentration of zinc ions for the inhibition of enamel demineralisation. Apart from this, one half of the enamel blocks was painted with red nail varnish and the other half was left exposed so that the enamel blocks acted as its' own control. The quality or quantity of enamel across the block may vary and could influence the quantification of mineral loss.

Perhaps an alternative way of assessing the role of the glasses on mineral loss could be on enamel blocks with pre-formed lesions, which may be able to clearly demarcate the baseline and treated groups.

11.3.2 Constant Depth Film Fermentor (CDFF)

For the CDFF run, it was decided to only use zinc-doped phosphate based glass with 11%mol calcium as there were no statistically significant differences between the different concentrations of calcium for glass characteristics and anti-bacterial analyses. However, C11 showed the most optimal trends for pH analysis, weight loss, ion release kinetics and anti-bacterial analyses, which dictated its use for the CDFF run. The CDFF model showed statistically significant mineral loss in the bovine enamel samples that were exposed to C-PBG, NaF, Chx and ED at day 12 compared to day 5. There was no statistically significant difference in mineral loss in the bovine enamel samples that were exposed to C11 between day 5 and day 12. The CDFF model is the most representative amongst other methods to simulate dental plaque that is found in the oral cavity (Kinniment *et al*., 1996, Peters and Wimpenny, 1988). To further represent the oral environment and white spot lesion or caries formation, a modification to the CDFF model was carried out with sucrose pulses of eight, 30 minute period of sucrose (2%) being pumped in per day to simulate sucrose intake in an individual. The frequency of sucrose consumption has shown to be associated with a significantly higher mineral loss in subjects who used either fluoridated or non-fluoridated toothpaste (Duggal *et al*., 2001). The effect of sucrose pulsing has been assessed previously in an *in vitro* biofilm model, which found a significant difference in mineral loss between the 50mM group, 10mM and the control group (Hodgson *et al*., 2001). Therefore a well-designed CDFF model with sucrose pulsing is a sensitive method of evaluating demineralisation by *S.mutans* (Hodgson *et al*., 2001). A single species of *S.mutans* was used in this study to reduce the variation and the difficulty of replicating the oral biofilm and to allow for a reliable model to be carried out (Deng *et al*., 2004). The results from the CDFF run shows the potential of zinc in inhibiting demineralisation, which is supported by (Legeros *et al*., 1999, Mohammad *et al*., 2015) and contradicts *in vitro* pH cycling studies by (ten Cate, 1993, Laucello *et al*., 2007). Zinc actively competes with Ca within the hydroxyapatite lattice at phosphate binding sites. Several factors have been proposed for determining the incorporation of zinc into hydroxyapatite crystal. These include site preferences, such as substitution mechanisms, equalization of bond valence and spatial accommodation (Tang *et al*., 2009), which could account for the inhibition of demineralisation seen in in the bovine enamel samples exposed to C11.

11.4 Limitations of the study

This study was a lab based study and the experiment results cannot be extrapolated directly into a clinical setting. Bovine enamel was used over human enamel due to the advantages *in vitro*. The mechanical properties of the glasses were not assessed in this study. A previous study reported the clinical use of fluoride releasing glass devices that had a problem with the retention rates of the devices (Toumba and Curzon, 2005), therefore an assessment into the mechanical properties which include assessments of fracture toughness and hardness of the zinc doped phosphate-based glasses is imperative prior to their use *in-vivo* depending on the mode of use and delivery method. Also in this study the protocol did not include assessing the composition of the bovine enamel post-exposure to the glasses and the controls. Such an assessment would allow for the analysis of the incorporation of zinc into the bovine enamel microstructure.

12.0 Conclusion

This was a first step to look into the possibility of Zn-PBG as a novel method in caries prevention especially in high-risk individuals. The results of this study show that Zn-PBGs exhibit controlled degradation and release of ions in water and do have the potential as an anti-bacterial agent specifically against *S.mutans* as was demonstrated in the anti-bacterial assessments. Zn-PBGs also have possible protective effects on bovine enamel by inhibition of demineralisation by *S.mutans*. The results from this study warrant further investigation into the controlled delivery of zinc ions from Zn-PBGs, which may have potential in oral applications as an anti-cariogenic agent.

13.0 Regulatory Issues

13.1 Indemnity

The University of Liverpool holds Indemnity and insurance cover with Marsh UK LTD, for this research.

13.2 Sponsor

This study is sponsored by The Ministry of Health Malaysia and is undertaken by the University of Liverpool.

13.3 Funding

The costs of this research is covered by University of Liverpool as part of the DDSc course.

13.4 Audits

This research study may be subject to inspection and audit by the University of Liverpool under their remit as supervisors and other regulatory bodies.

14. Study Management

The day to day management of the study will be coordinated through the Chief Investigator.

15. End of study

The study will end once the data is analysed.

16. Archiving

All appropriate documentation will be stored for a minimum of 10 years after the completion of the study, unless otherwise directed by the funder/sponsor/regulatory bodies.

17. Publication Policy

The results from this *in vitro* research will be reported at relevant local and regional presentations. Publication of the results will also be sought in a peer-reviewed journal.

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