Enhancing remineralisation using casein phosphopeptide complexes

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Submitted in total fulfilment of the requirements
of the degree of Doctor of Philosophy

December 2017

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University of Melbourne
ABSTRACT

Casein phosphopeptide (CPP) complexes have been shown to promote remineralisation of dental tissues affected by dental caries. However, remineralisation takes time and can be limited by the delivery and composition of the remineralisation agent. The aim of this thesis was to enhance remineralisation by CPP complexes through clinical and laboratory studies assessing various chemical changes to the remineralisation process.

Using an in vitro enamel remineralisation model, it was determined that intra-lesion serum albumin did not interfere with remineralisation by casein phosphopeptide stabilised amorphous calcium fluoride phosphate (CPP-ACFP). A high pH pre-treatment significantly increased remineralisation by CPP-ACFP. To expand on this finding, a cyclic in vitro remineralisation model tested intra-lesion pH modulation whereby enamel subsurface lesions were periodically exposed to CPP-ACFP (pH 5.5) and either sodium hypochlorite (pH 12.9), sodium hydroxide (pH 12.9) or distilled deionised water. Enamel subsurface lesions that had cyclic treatment with CPP-ACFP and sodium hydroxide were observed to have significantly higher remineralisation, displaying intra-lesion pH modulation enhanced remineralisation. Cyclic treatment with CPP-ACFP and sodium hypochlorite was observed to further demineralise and cause a surface precipitation due to a disadvantageous interaction of the treatment solutions. A second short-term cyclic in vitro remineralisation experiment revealed intra-lesion pH modulation with CPP-ACFP and sodium hydroxide was more effective than an equivalent exposure to CPP-ACFP alone.

The use of x-ray microtomography (XMT) to measure remineralisation by CPP-ACFP in vitro was assessed using conventional polychromatic and monochromatic synchrotron x-ray sources. These methods of analysis were compared with transverse microradiography (TMR) analysis to investigate the accuracy and practicality of each method. XMT analysis from both x-ray sources detected remineralisation in enamel lesions however the amount of remineralisation detected was significantly less than that detected by TMR. Due to a range of artefacts unique to the x-ray source and the devices used, it was determined that XMT analysis of remineralisation under the conditions used was less sensitive compared with TMR.
The remineralisation potential of a combined casein phosphopeptide stabilised amorphous calcium phosphate (CPP-ACP) and stannous fluoride (SnF$_2$) solution was tested *in vitro* and *in situ*. The combined CPP-ACP and SnF$_2$ solution showed significantly higher enamel remineralisation than all other treatments due to an increase in CPP complex stability and ion delivery. The interaction of a combined CPP-ACP and SnF$_2$ solution with surface dentine *in vitro* displayed an organic ‘nanocoating’ suggesting stannous ions mediated CPP cross-linking and ion release at the dentine surface.

A crossover clinical study was conducted on low caries-risk individuals to assess changes in the abundance of *Streptococcus sanguinis* in supragingival plaque following a two week intervention period chewing either CPP-ACP sugar-free gum, sugar-free gum or no gum. It was determined that chewing the CPP-ACP gum significantly increased the abundance of *S. sanguinis*, as well as other commensal, alkaline-producing microorganisms. This demonstrated chewing CPP-ACP gum exerted a prebiotic effect in supragingival plaque.

The promising results expounded in this thesis indicate modifications to the composition and delivery of CPP complexes have the potential to improve the rate and amount of remineralisation.
DECLARATION

This is to certify that:

i) The thesis comprises only my original work towards the degree of Doctor of Philosophy except where acknowledged.

ii) Due acknowledgement has been made in the text to all other material used.

iii) The thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Dr James Rohan Fernando
PREFACE

This thesis was completed with the support of the inaugural Nathan Cochrane Scholarship.
ACKNOWLEDGMENTS

I would like to extend sincere gratitude to my primary supervisor, Laureate Professor Eric Reynolds, for his unfaltering guidance and support. My academic career has been immeasurably enriched from his mentorship and wealth of knowledge. Additionally, I would like to extend gratitude to my secondary supervisor, Dr. Peiyan Shen, for his excellent knowledge and advice. I am grateful for the immense assistance and kindness of Dr. Yi Yuan, Dr. Glenn Walker and Mrs. Coralie Reynolds. I feel privileged to be part of the cariology group who have been welcoming friends and are a brilliant team.

To Professor David Manton, Professor Stuart Daspher, Mrs. Karen Escobar, Mr. William Singleton, Dr. Christina Sim, Dr. Tanya D’Cruze, Dr. Jacqueline Heath and Dr. Shaobing Fong, I am thankful for your wisdom and friendship. I would like to acknowledge the assistance and work of Mr. Geoff Adams, Ms. Kate Fletcher, Mrs. Gilda Pekin, Mrs. Eva Roden, Dr. Yu-Yen Chen, Dr. Catherine Butler, Dr. Helen Mitchell, Mrs. Brigitte Hoffman, Mr. Roger Curtain, Mr. David Stanton and the GC Corporation. Thank you to all other staff and students at the Oral Health Cooperative Research Centre and the Melbourne Dental School who have assisted me.

To the Camberwell boys and partners, friends from university, work and elsewhere, your friendship is greatly appreciated. Thank you to the staff and patients at Hastings Family Dental Care for supporting me throughout my degree.

The late Dr. Nathan Cochrane convinced me to begin a career in dental research. He is a constant inspiration and his presence is sorely missed.

Finally, I would like to extend gratitude to my family. Thank you to Ione, Andy, Aislinn and Skye for the holiday adventures in-between study. To my parents, Melanie and Robert Fernando, this thesis is dedicated to you. Your sacrifices, guidance and love have made this thesis and all my academic endeavours possible.
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<tr>
<td>AAS</td>
<td>Atomic absorption spectroscopy</td>
</tr>
<tr>
<td>AD</td>
<td>Arginine deiminase</td>
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<tr>
<td>AEP</td>
<td>Acquired enamel pellicle</td>
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<tr>
<td>ANCOVA</td>
<td>Analysis of covariance</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BHA</td>
<td>Butylated hydroxyanisole</td>
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<tr>
<td>BHT</td>
<td>Butylated hydroxytoluene</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>C</td>
<td>Carbon</td>
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<td>Cµ-CT</td>
<td>Conventional x-ray microtomography</td>
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<td>Ca</td>
<td>Calcium</td>
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<tr>
<td>CadAP</td>
<td>Calcium deficient carbonated apatite</td>
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<tr>
<td>CEJ</td>
<td>Cemento-enamel junction</td>
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<tr>
<td>CI</td>
<td>Chloride</td>
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<tr>
<td>CPITN</td>
<td>Community periodontal index of treatment needs</td>
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<td>CPP</td>
<td>Casein phosphopeptide</td>
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<td>CPP-ACP</td>
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<td>DDW</td>
<td>Distilled deionised water</td>
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<td>DEJ</td>
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<tr>
<td>DMFT</td>
<td>Decayed, missing, and filled teeth</td>
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<td>dmft</td>
<td>Decayed, missing, and filled deciduous teeth</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DS</td>
<td>Degree of saturation</td>
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<td>Energy dispersive x-ray spectroscopy</td>
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<tr>
<td>Ksp</td>
<td>Solubility product</td>
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<td>LAC</td>
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<td>Sodium fluoride</td>
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<tr>
<td>Pi</td>
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<td>PLM</td>
<td>Polarised light microscopy</td>
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<td>ppm</td>
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<tr>
<td>PRP</td>
<td>Proline rich protein</td>
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<td>PS-OCT</td>
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<td>QLF</td>
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POSTERS AND PRESENTATIONS FROM RESEARCH IN THIS THESIS


INTRODUCTION
1.1 Background
A recent review on the global prevalence of dental caries in permanent teeth placed the disease among 34% of the worldwide population [Kassebaum et al., 2017]. Despite advances in oral health prevention and education, this figure has remained relatively stable since 1990 and alarmingly indicates one third of the population requires treatment for a preventable disease.

The Australian Institute of Health and Welfare has predicted that within the next two decades the number of people aged 65 will rise by 91% and the number of people aged 85 or above will more than double [AIHW, 2012]. Advances in oral healthcare have increased the retention of teeth in the elderly although the prevalence and risk of dental disease in this age group is still relatively high. A recent study by Silva et al. [2014] observed the prevalence of coronal and root dental caries among a sample of dentate Australian nursing home residents to be 68% and 77%, respectively, indicating older Australians require improvements in caries prevention. Elderly patients consistently have a higher prevalence of root caries and gingival recession when compared to the rest of the population [Chalmers et al., 2002; Griffin et al., 2004; Kassab and Cohen, 2003]. Accordingly, the aging population will place a greater demand on dental health services to treat and prevent oral disease in the years to come.

Carious teeth often require restorative treatment at a financial and biological cost. A dental restoration condemns a tooth to the ‘restorative cycle’ whereby ongoing maintenance and occasionally invasive treatment are required as the restoration ages [Brantley et al., 1995]. Restorative dental treatment is avoidable through early interventions that prevent the initiation or reverse the progression of incipient caries lesions, thereby preserving natural tooth structure. As old restorations require periodic replacement (often with further loss of tooth structure), the early detection and treatment of caries is particularly significant to extend the lifetime and integrity of the tooth [Brantley et al., 1995; da Mata, 2011; Tan et al., 2010].

Since the 1960’s, research of tooth remineralisation has promoted advancements in non-invasive management of caries resulting in less restorative care and greater preservation
of natural tooth structure [Cochrane et al., 2010; Featherstone, 2009; Ten Cate, 2012]. The biochemical process of remineralisation is complex and it is impeded or enhanced by numerous factors. Casein phosphopeptide (CPP) complexes have been demonstrated to promote remineralisation of caries and have been formulated into products that allow topical application of the complexes at the tooth surface. The mechanism of action and remineralisation effect of CPPs have been well documented [Cochrane and Reynolds, 2012], although there remains scope to maximise their efficacy by improving their formulation and mode of delivery intra-orally. Advancing the knowledge of the caries process and remineralisation by therapeutic aids such as CPPs can potentially lead to treatments that are more effective in preserving natural tooth structure and preventing restorative treatment of caries.

1.2 Structure of enamel and dentine

Teeth consist of inner vascularised pulp tissue surrounded by a hard, vital tissue known as dentine. The coronal portion of the dentine is encased in highly mineralised tissue known as enamel and this tissue primarily interacts with the oral environment. In the root portion of the tooth, the dentine is covered by a thin layer of tissue called cementum which facilitates attachment of the tooth to the neighbouring alveolar bone through the periodontal ligament.

1.2.1 Biomineralisation

The dental hard tissues originate from a thickening of ectomesenchymal cells beneath the epithelial cells of the developing oral cavity. The ectomesenchymal cells condense to form the primary epithelial band which furthermore differentiates to form the vestibular lamina and the dental lamina. Individual ‘tooth germs’ develop from the dental lamina through the interaction of mesenchymal and epithelial cells and are further classified on their histological appearance as bud, cap and bell stage. By the bell stage, the dental lamina has detached and degenerated from the oral epithelium and a group of cells described as the enamel organ have differentiated into four distinct layers: the external enamel epithelium, the stellate reticulum, the stratum intermedium and the internal enamel epithelium. Beneath the internal enamel epithelium is the dental papilla, which contains densely packed mesenchymal cells [Berkovitz et al., 2002].
It is during the late bell stage that deposition of the dental hard tissues begins. Interactions between the epithelial and mesenchymal cells at the future dentino-enamel junction (DEJ) initiate dentine deposition, which precedes enamel deposition. The cells of the internal enamel epithelium differentiate to form pre-ameloblasts that induce the cells of the dental papilla to divide and differentiate into either odontoblasts or sub-odontoblastic cells. The odontoblasts begin to secrete the dentine matrix through a cellular process which elongates progressively following dentine deposition thereby demarcating the space of the presumptive dentine tubule. The dentine matrix consists of collagen type I and proteoglycan and it is secreted appositionally to the odontoblast cells towards the future DEJ. The collagen combines into helical structures containing three collagen molecules which organise into fibrils arranged perpendicular to the direction of the dentine tubule [Linde and Goldberg, 1993].

The organic matrix acts as a framework for the nucleation of dentine crystals. The collagen strands crosslink in a ‘quarter-staggered’ fashion leaving periodic gaps between the strands on the fibril surface; these gaps are referred to as ‘hole zones’. The odontoblast process secretes dentin phosphophoryn, a highly acidic phosphoprotein, which binds to the hole zones within the collagen matrix to create a binding motif for crystal nucleation. As calcium and phosphate ions are released by the odontoblast, they condense along the binding motif of the phosphophoryn to nucleate the formation of calcium deficient carbonated apatite (CadAP). Phosphophoryn acts as the template for crystal growth, regulating the size and direction of the crystal, which is parallel to the collagen matrix. As the dentine crystals elongate, it is thought that phosphophoryn continues to bind to growth sites along the crystal surface thereby limiting the size of the crystal [Boskey et al., 1990]. This sequence of organic matrix mediated crystal deposition continues until the full thickness of dentine is achieved [Linde and Goldberg, 1993].

Enamel deposition begins directly after dentine deposition has initiated. The cells of the internal enamel epithelium develop to become ameloblast cells that are then signalled by the presence of dentine to begin amelogenesis. The oldest and most mature ameloblasts will align at the sites of the future cusp tips whereas the youngest and most immature ameloblasts localise towards the cervical region of the future enamel. Like dentine
formation, an organic matrix is secreted prior to mineralisation. The ameloblast secretes the hydrophobic protein amelogenin and the highly acidic protein enamelin, as well as calcium and phosphate ions for crystal formation. The enamelin proteins assemble at the DEJ and initiate crystal nucleation while the amelogenins self-assemble into nanosized spherical complexes aligned in a helical arrangement around the growing crystal. The positioning of the amelogenin nanospheres ensures the crystal growth is only along the C-axis, resulting in an ordered pattern of crystal growth. As the enamel matures, the organic matrix is decreased through proteolytic degradation of the amelogenin thereby allowing crystal growth along the A- and B-axes. The resulting mature tissue is a highly structured and mineral dense tissue [Fincham et al., 1999].

Biomineralisation of the dental hard tissues relies on the organic molecules phosphophoryn, enamelin and amelogenin, and this is largely due to the chemical properties of the proteins and their interaction with calcium phosphates. The negatively charged residues of aspartate and phosphoserine present in phosphophoryn (and to a lesser extent enamelin) attract calcium ions in solution and subsequently phosphate molecules [Gu et al., 1998]. Isolated in solution, these proteins have been shown to inhibit crystal nucleation [Boskey et al., 1990; Bouropoulos and Moradian-Oldak, 2004], however when stabilised within an organic matrix they are effective crystal nucleators due to their ability to sequester large concentrations of calcium to a high spatial density and with a low surface energy. The conformation of the tethered proteins mimics the arrangement of electropositive and electronegative charges in a mineral lattice, thereby overcoming the activation energy for crystallisation. This reliance on organic molecules during odontogenesis ensures that deposition of mineral occurs under biological control (biomineralisation) at a specific location and phase of development [Mann, 2001].

1.2.2 Crystal structure and profile

Enamel and dentine crystals consist of CadAP surrounded by an organic matrix and water. For simplicity, enamel and dentine mineral is often equated to stoichiometric hydroxyapatite (HA) with the chemical formula $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. While similar in structure and solubility to HA, various ions such as sodium, magnesium, carbonate, potassium, chloride and fluoride substitute into the crystal lattice affecting the
crystallinity and increasing the solubility (except for fluoride which decreases the solubility). As a result, the solubility product (Ksp) of CadAP ($10^{-113}$) is higher than the Ksp of HA and fluorapatite (FA) ($10^{-117}$ and $10^{-121}$ respectively) [LeGeros, 1990].

Each unit in a crystal is called a crystallite. The crystals of enamel are approximately 68 nm in width, 26 nm in depth and upwards of 100nm in length. They are arranged into prisms which in cross section appear in a keyhole or fish-like shape with a ‘head’ and ‘tail’ region. Crystals are aligned to be parallel to the long axis of the prism in the head region, and gradually angle 65 – 70° to the long axis of the prism in the tail region. The head and the tail region are also termed the rod and interrod or the prismatic and interprismatic region respectively. It has been shown that due to the arrangement of crystals in the interrod region, it is slightly more porous than the rod region and thus has a higher water content and potential for ion transport. Though still porous, the densely packed crystals in enamel result in a highly mineralised tissue of 96 % inorganic matter, 1 % organic matter and 3 % water by weight [Berkovitz et al., 1992].

Dentine crystals also consist of CadAP, however there is a higher concentration of carbonate and magnesium ions substituted within the crystal lattice that renders it more reactive and therefore more soluble than enamel apatite crystals [LeGeros, 1990]. The crystals in dentine are also smaller in size than in enamel, though similar in structure having an elongated hexagonal conformation approximately 35 nm in width and 10 nm in depth. By weight, 70 % of dentine is mineral, 20 % is organic matter in the form of the organic matrix, and 10 % is water [Berkovitz et al., 1992]. The presence of magnesium appears to not only make the apatite more soluble, but also promotes the formation of magnesium substituted beta-tricalcium phosphate (β-TCP) within dentine tubules during caries-related dentine sclerosis [Daculsi et al., 1987].

1.3 Caries and dental hypersensitivity

1.3.1 Caries

1.3.1.1 Aetiology

Dental caries has been described as “the signs and symptoms of a localised chemical dissolution of the tooth surface caused by metabolic events taking place in the biofilm
(dental plaque) covering the affected area” [Fejerskov and Kidd, 2008]. The chemical dissolution of tooth structure is a consequence of an increase in acidity within the enamel fluid from the production of acid by adjacent bacteria through metabolic processes [Featherstone, 2004]. The different structure and chemical makeup of enamel and dentine affect their behaviour to acid attack and bacterial invasion.

1.3.1.1.1 The Dental Biofilm

The tooth surface has exposed calcium ions which attract and adsorb negatively charged acidic glycoproteins from saliva to form the acquired enamel pellicle (AEP). The AEP is up to 1 µm in thickness and through protein-protein interactions as well as calcium cross-linking it contains proline rich proteins (PRPs), statherin, histatins, mucins, amylase, lactoferrin, lysozyme, carbonic anhydrase and cystatins, proteins all derived from saliva [Hannig et al., 2005; Siqueira et al., 2012; Smith and Bowen, 2000]. The proteins within the AEP have a high number of amino acid residues containing negatively charged groups, such as carboxylate and sulphate, which enhance the overall negative charge of the tooth surface. Initially this net negative charge repels bacteria as they also have a net negative charge on their cell membrane; however, calcium and other cations cross-link these negative charges to allow bacterial adherence through specific binding to the adsorbed protein. This allows plaque to accumulate on the tooth surface [Loesche, 1986].

Although high variability exists between hosts and tooth sites, the initial colonisation of the tooth surface has been detected to be predominantly by Streptococcus spp., particularly Streptococcus sanguinis, Streptococcus mitis and Streptococcus oralis [Diaz et al., 2006; Nyvad and Kilian, 1990]. Other bacteria such as Actinomyces spp. and Fusobacterium nucleatum begin to gradually increase in numbers promoting greater diversity and adherence of bacteria within the plaque. Many of these species are commensal and are thought to be associated with health, particularly those bacteria that maintain a high pH from metabolism of arginine, such as S. sanguinis [Burne and Marquis, 2000].

1.3.1.1.2 Cariogenic plaque

The main microorganisms that have been associated with the development of carious lesions are the mutans streptococci (a term referring to several cariogenic species of
streptococci, particularly *Streptococcus mutans* and *Streptococcus sobrinus*) and lactobacilli [Marsh, 2010]. These species of bacteria are found in dental plaque from individuals without caries though appear to be present in higher numbers in plaque isolated from carious lesions [Takahashi and Nyvad, 2011]. While they have been shown to be particularly virulent with respect to dental caries, they cannot be defined as sole aetiological pathogens as they have also been shown to be absent in biofilms associated with carious lesions [Marsh, 2010].

Marsh [1994] described the ecological plaque hypothesis whereby a wide group of microorganisms present within the dental biofilm react and contribute in various amounts to a shift in ecological conditions resulting in the chemical dissolution of tooth apatite crystals. An important factor is the acidogenicity and acidurance of certain bacteria which may result in an increase in their colonisation within the biofilm due to the shift in conditions, particularly the mutans streptococci and lactobacilli. More recently, Marsh [2010] amended the description of the ecological plaque hypothesis to include the likelihood that certain non-mutans streptococci and *Actinomyces* species adapt to acidic conditions by developing a more acidogenic and aciduric phenotype. Strong evidence suggests a sub-group of bacteria previously thought not to contribute to the production of acid are capable of ‘acid-induced adaptation’ whereby acidurance and acidogenicity are increased by time dependent exposure to a low pH environment. This is thought to be due to a decrease in proton permeability through the cell membrane, a higher activity of the membrane proton pump, an intra-cellular increase in alkali due to arginine metabolism and a greater induction of stress proteins that protect nucleic acids and enzymes from being denatured by acid [Takahashi and Nyvad, 2011].

The shift to an acidic environment within the biofilm is primarily a result of the production of acid by bacterial fermentation of carbohydrates, especially through the glycolytic pathway. The glycolytic pathway within the cell utilises enzymes to convert glucose to pyruvate, resulting in the formation of the high-energy molecules adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide (NADH) [Bowden and Hamilton, 1998]. The enzyme lactate dehydrogenase then ferments pyruvate to lactic acid which is excreted from the cell and into the plaque fluid [Bowden and Hamilton,
Within the plaque fluid, the release of a hydrogen ion by lactic acid may be buffered by phosphate, bicarbonate or dissolution of calculus. Lactic acid may also diffuse into the saliva or the enamel fluid.

1.3.1.2 Enamel

1.3.1.2.1 Lesion progression

The mature biofilm on the enamel surface becomes a more acidic environment after production of lactic acid and other organic acids from the frequent bacterial fermentation of carbohydrates [Featherstone, 2004]. The lactic acid molecule has a neutral electrochemical charge that efficiently diffuses through the charged stern layer of the enamel surface into the inter-rod spaces of the enamel. These spaces are fluid filled and facilitate diffusion between the crystals so that a high influx of lactic acid results in a significant decrease in the pH level of the fluid surrounding the enamel crystals. As the pH of the enamel fluid drops, it becomes undersaturated with respect to CadAP to favour dissolution of the ions that make up the crystal [Featherstone, 2004]. Electron microscopy has revealed the dissolution can occur on the periphery of the crystal or through the centre of the crystal, causing a ‘central perforation’ of the crystal [Yanagisawa and Miike, 2003]. The initial dissolution occurs between the rod and inter-rod enamel regions where diffusion channels are most prevalent [Fejerskov et al., 2015].

The CadAP of human enamel readily dissolves under acid attack due to its relatively high Ksp. Fortunately, the presence of calcium and fluoride in the enamel fluid promote a reprecipitation of some of these dissolved components into new phases with improved crystallinity and acid resistance. These new phases, specifically FA (Ca_{10}(PO_{4})_{6}F_{2}) or fluorhydroxyapatite (FHA – Ca_{10}(PO_{4})_{6}(OH)F), decrease the solubility of the enamel crystals and prevent loss of these ions from the tooth unless a more extreme drop in pH occurs to decrease the degree of saturation (DS) with respect to these phases [Buzalaf et al., 2011].

On the enamel surface, calcium is stored in plaque, calculus or within bacterial cell walls [Driessens, 1981; Rose et al., 1993; Tenuta et al., 2006]. The frequent fermentation of carbohydrates by plaque bacteria drops the DS with respect to the calcium phases within
these reservoirs and increases the dissolved calcium in the plaque fluid. With a high frequency of acid production, these calcium reservoirs are gradually depleted and the calcium ions are lost to the saliva [Paes Leme et al., 2004]. This decrease in calcium in the plaque fluid creates a concentration gradient encouraging outward diffusion of calcium from the enamel fluid. Combined with the low DS with respect to the apatite phases in the enamel fluid, the result is a net loss of calcium, phosphate and fluoride ions from the enamel as they diffuse out into the plaque and saliva.

1.3.1.2.1.1 The surface zone

The early enamel carious lesion can be described as a demineralised subsurface region enclosed by a relatively mineralised surface zone. The surface zone varies in thickness, with two separate studies having measured it to be 35 – 130 µm [Cochrane et al., 2012a] and 10 – 160 µm [Meyer-Lueckel et al., 2007]. Multiple theories have been suggested to explain the existence of the surface layer; evidence indicates it most likely exists due to surface-absorbed salivary macromolecules inhibiting surface crystal dissolution, coupled with the high ion concentrations (especially that of fluoride) and pH of the surface enamel fluid promoting the reprecipitation of outward diffusing ions [Arends and Christoffersen, 1986; Robinson et al., 2000]. Theories suggesting the surface zone exists due to varying porosity or solubility caused by chemical gradients within the enamel are thought to be unlikely as HA pellets with homogenous porosity and atomic distribution have still formed subsurface lesions in vitro [Langdon et al., 1980].

The surface zone maintains a degree of porosity to allow transport of ions. Demineralised enamel pores have been measured to be up to 1 µm in diameter [Palamara et al., 1986]. Reports of bacteria detected within active and arrested uncavitated lesions have indicated that although bacteria enter the enamel through these pores, the bacteria count within an uncavitated lesion does not prevent consolidation of the lesion while the surface remains intact for plaque control [Brännström et al., 1980; Parolo and Maltz, 2006]. While an uncavitated lesion allows for greater plaque control, the highly mineralised surface zone is a hindrance to remineralisation as it impedes diffusion of external ions to deeper demineralised zones [Featherstone, 1977; Larsen and Pearce, 1992; Larsen and Fejerskov, 1989].
1.3.1.2.1.2 The subsurface zones

Along with the surface zone, the early enamel carious lesion has three distinct zones of demineralisation deeper to the surface. These zones vary in porosity and have been described in the literature based on their histological appearance. Directly below the surface layer is the ‘body of the lesion’ that has extensive mineral loss; it has a porosity of 25 – 50 % and accumulates significant amounts of exogenous organic matter [Shore et al., 2000]. The ‘positively birefringent zone’ (also known as the ‘dark zone’) exists deeper to the body of the lesion and has a porosity of 2 – 4 %. This zone is named for its appearance under a polarised light microscope after being imbibed by quinolone. It consists of numerous small pores as well as a few large pores. At the demineralisation front is the ‘translucent zone’ that typically has a porosity of 1 – 2 % and features a small number of larger pores. The larger but less abundant pores are thought to be a result of the dissolution of more soluble phases within the enamel, specifically those containing carbonate or magnesium [Robinson et al., 2000].

1.3.1.2.2 Microbiology

On the enamel surface, the biofilm is dynamic and changes in bacterial composition occur as the biofilm matures and as the environment changes. Different enamel sites, such as the fissures, approximal surfaces and gingival crevices differ in bacterial composition [Theilade and Theilade, 1985]. The bacterial composition is particularly sensitive to carbohydrate availability and the pH of the plaque fluid [Wade, 2013]. Most of the bacteria that colonise supragingival plaque have the ability to metabolise carbohydrates and produce lactic acid through the glycolytic pathway [Bowden and Hamilton, 1998]. Historical studies have pinpointed the high abundance of mutans streptococci at carious sites, especially fissure caries, as a significant factor in the microbiology of enamel caries [Loesche, 1986; Loesche and Straffon, 1979].

The main bacteria present in an enamel-colonised biofilm are grouped into non-mutans streptococi, Actinomyces, mutans streptococi, lactobacilli and Bifidobacterium [Marsh, 2010]. The bacteria shown to be the most acidoduric and acidogenic are mutans streptococci, lactobacilli and Bifidobacterium, though in caries-associated sites the
acidogenicity and acidurance of the non-mutans streptocci and *Actinomyces* is increased in emerging phenotypes [Marsh, 2010].

The virulence of enamel caries-associated bacteria is additionally enhanced in those species able to store excess carbohydrates as intracellular or extracellular polysaccharides allowing glycolysis to occur during periods of carbohydrate scarcity [Bowden and Hamilton, 1998]. The ability to transport and uptake a wide variety of carbohydrates despite pH and plaque fluid concentration fluctuations, as well as the ability to adhere to the enamel surface similarly increases the virulence of caries-associated bacteria [Fejerskov *et al.*, 2015; Takahashi and Nyvad, 2011].

1.3.1.3 Dentine

1.3.1.3.1 Lesion progression

The changes occurring in dentine during the carious process are dependent on surface activity, especially activity in the enamel. The dentine and dental pulp are vital tissues functioning together as the pulpo-dentinal organ that elicit responses to chemical and physical trauma. The gradual increase in porosity that occurs from caries attack allows more effective inward diffusion of acid and irritants towards the dentine and this initiates a response from the pulpo-dentinal organ. The odontoblast cells that line the pulpo-dentinal junction extend their odontoblastic processes through the dentine tubules. Receptors on the odontoblast sense chemical changes in the dentine, specifically drops in pH.

The initial response to acid diffusion from the overlying enamel is sclerosis of the dentine tubule. This area of sclerosis, often called the translucent zone, occurs by the secretion of ions by the odontoblastic process to initiate precipitation of non-apatitic phases believed to be β-TCP and whitlockite [Daculsi *et al.*, 1987]. Evidence from energy-dispersive x-ray spectroscopy examining areas of dentine sclerosis have confirmed these phases contain magnesium, are poorly organised and unlikely to have an organic precursor [Daculsi *et al.*, 1987]. The precipitation of whitlockite crystals within the dentine tubules is particularly significant as whitlockite has a lower solubility than CadAP at low pH and
acts as a physical barrier to acid diffusion during caries [Arnold et al., 2001; Arnold et al., 2003].

The pattern of dentine sclerosis is more extensive near the DEJ, and narrower near the pulp. The wide area near the DEJ is wider than the enamel demineralisation front, and this is thought to be a physiological defence mechanism that creates a wide barrier of acid resistant mineral. Once the enamel demineralisation has progressed to a greater extent, acid influx into the dentine causes demineralisation of the dentine crystals in a pattern similar to the sclerosis; this is sometimes referred to as the ‘lateral spread’ of demineralisation at the DEJ [Fejerskov et al., 2015]. It has been misinterpreted that the lateral spread occurs due to discontinuity between the enamel and dentine allowing greater spread of acid, however increasing evidence shows the width of the dentine demineralisation at the DEJ never extends beyond the width of the enamel demineralisation in the body of the lesion [Bjørndal, 2008]. The demineralised dentine is visualised clinically as having a brownish colour and this is a result of biochemical changes to the collagen within the organic matrix [Fejerskov et al., 2015].

1.3.1.3.2 Microbiology

Upon microcavitation of the surface layer, bacteria gain access to the porous enamel and eventually infiltrate the dentine after progressive demineralisation. A wide variety of microbial species are present in carious dentine. Studies largely conclude that acidogenic gram-positive rods (especially Lactobacilli, Actinomyces and Propionibacterium spp.) predominate with less abundance of gram negative rods and gram-positive cocci being detected [Hahn et al., 1991; Hoshino, 1985; Martin et al., 2002; Munson et al., 2004]. The study by Martin et al. [2002] analysed carious dentine samples using real-time PCR to detect a high abundance of gram negative rods such as Prevotella spp. and Fusobacterium nucleatum alongside the predominantly gram-positive microflora. The authors observed teeth with pulpal inflammation could be positively associated with the detection of Micromonas micra and Porphyromonas endodontalis in carious dentine.

1.3.1.3.3 Root caries

The root surfaces of teeth can be subject to carious attack following gingival recession, which is typically a feature of increased age or previous periodontitis allowing the root
surface to be exposed and subsequently colonised by microflora [Banting, 2001; Griffin et al., 2004; Kassab and Cohen, 2003]. The root surface is naturally covered by a thin layer of cementum. However, in a tooth with root caries this layer of cementum is unable to resist plaque colonisation and acid production, and the resulting lesion progresses into the underlying dentine with dissolution of the mineral phase and destruction of the organic matrix as described in 1.3.1.3.1. Root caries has been shown to progress more rapidly in situ in individuals with a reduced unstimulated salivary flow [Bardow et al., 2003]. This type of hyposalivation is evident frequently among the elderly population due to polypharmacy [Silva et al., 2014]. Strong evidence suggests the hyposalivation, history of periodontal disease and gingival recession in the elderly population put them at higher risk of developing root caries than the rest of the population [Griffin et al., 2004; Kitamura et al., 1986].

The microflora associated with root caries is complex, but like most cariogenic biofilms there is a reduction in bacterial diversity when compared to the microflora colonising healthy tooth surfaces [Preza et al., 2008]. There have been multiple attempts to determine the predominant species associated with root caries and there are indications that it differs from the predominant species associated with coronal enamel caries. However, the bacteria isolated from plaque overlying root caries lesions or from carious root dentine are similarly believed to be aciduric and acidogenic. Reports have shown that S. mutans and lactobacilli abundance is increased in cariogenic plaque associated with root caries when compared to non-carious root surfaces [Bowden, 1990; Brailsford et al., 2001; Ellen et al., 1985; Preza et al., 2008]. Evidence suggests these species are neither sole aetiological agents nor the predominant species detected in root caries associated biofilms. Rather it appears likely a complex interplay between multiple species results in an acidogenic plaque that attacks the root surface to increase dentinal porosity and allow bacterial invasion. Much like coronal dentine caries, the presence of Actinomyces naeslundii and Actinomyces viscosus has been detected in root caries associated plaque [Bowden, 1990; Brailsford et al., 2001; Ellen et al., 1985], and additionally Veillonella spp. [Ellen et al., 1985; Preza et al., 2008]. Authors have suggested that while most of these species have been shown to colonise healthy root surfaces, a shift in the microenvironment that decreases the pH and increases the availability of carbohydrates
promotes phenotypes of various species capable of acidurance and acidogenicity. The ability of microorganisms to adapt to such changes within the biofilm parallels the changes that occur in cariogenic plaque associated with coronal caries, and demonstrates that the microbiology of root caries is not easily explained by the presence of a few specific pathogens.

1.3.2 Dentine hypersensitivity

Dentine hypersensitivity is a significant clinical problem that affects a wide range of the population. It is largely a symptom of exposed dentine that communicates with the oral environment, resulting from either the lack of overlying enamel or from gingival recession often in conjunction with periodontal disease [Goh et al., 2016]. Dentine hypersensitivity has been reported in the literature as having a varied prevalence among populations worldwide. Data have suggested a range of 4 – 74 % of the population experience the condition [Bartold, 2006]. A 2011 publication clinically assessing 12,692 patients attending private practices in Australia recorded approximately 9 % experiencing dental hypersensitivity with the majority (68 %) between the ages of 30 – 59 years old and the majority (60 %) being female [Amarasena et al., 2011]. The most common feature of dentine hypersensitivity is gingival recession caused by chronic periodontal disease. Between 72.5 – 98 % of patients who have experienced chronic periodontitis have been reported as having dentine hypersensitivity [Rees, 2000].

1.3.2.1 Hydrodynamic theory

Numerous theories have been postulated as to what causes dentine hypersensitivity, but the most widely accepted is the hydrodynamic theory as described by Brännström et al. [1967]. According to Brännström and colleagues, the movement of fluid within dentine tubules is responsible for activating A-fibre nerve cells within the pulp present at the dentino-pulp interface resulting in a hypersensitive response to stimuli. Triggers of dentine tubule fluid movement may be changes in temperature, osmotic pressure, air blasts or dental probing. Of particular note is that the outward flow of fluid appears to elicit a stronger pain response than inward fluid flow. As such cold sensations stimulate outward fluid movement and generally elicit a greater response than hot stimulation which promotes inward fluid flow [Addy, 2002]. The extent of the hypersensitivity is dependent on the hydraulic conductance of the dentine which increases as the patency or diameter
of the tubule increases and the length of the tubule to the pulp decreases [Fogel et al., 1988]. While the hydrodynamic theory explains the mechanism of dentine hypersensitivity, often dentine hypersensitivity is a symptom of an underlying disease process. Exposed or susceptibly communicable dentine can result from periodontitis, caries, abrasion, or dental erosion and can all show symptoms of dentine hypersensitivity due to patency of dentine tubules [West et al., 2013].

1.3.2.2 Gingival recession
A frequent finding of patients with dentine hypersensitivity is gingival recession. A review by Kassab and Cohen [2003] revealed evidence suggests more than 50% of the population has one of more sites in the mouth with gingival recession of at least 1 mm of more exposing the root surface of a tooth. The numerous aetiological factors of gingival recession are also discussed in that report, which mentions that among subjects with dentine hypersensitivity there appears to be a pattern of more gingival recession and hypersensitivity on the left side of the mouth. This was attributed to most people being right handed and having a higher likelihood of causing chronic toothbrush abrasion on the left side of the mouth resulting in gingival recession. Pathological factors such as periodontitis, anatomical factors and aging were also identified as potential causes of gingival recession, and evidence shows multiple aetiological factors are likely to be present. Grippo et al. [2004] discussed the influence occlusal load has on cervical enamel and dentine, with an indication that abfraction lesions may also contribute to gingival recession and cervical enamel breakdown subsequently causing dentine hypersensitivity. It has been suggested that abfraction lesions are not only caused by occlusal load but also an acidic component may play a role in enamel dissolution [Palamara et al., 2001].

1.3.2.3 Dentine hypersensitivity and mineralisation
A therapeutic goal for management of dentine hypersensitivity is the occlusion or narrowing of dentine tubules to prevent or reduce the movement of fluid through the tubules. Topical application of various agents has been shown to produce such an effect, typically with SEM imaging of dentine used as a tool to verify efficacy. Among those showing such an effect are products containing sodium fluoride, stannous fluoride, strontium chloride, strontium acetate, arginine/calcium carbonate, calcium sodium phosphosilicate, potassium oxalate, and casein phosphopeptide stabilised amorphous
calcium phosphate (CPP-ACP) [Arrais et al., 2003; Chen et al., 2015; Davies et al., 2011; Ling et al., 1997; Prati et al., 2002; Wang et al., 2010]. This type of management of dentine hypersensitivity involves delivery of ions to the dentine surface and crystal growth either on the dentine surface or within the dentine tubules. While strictly not remineralisation, this mechanism is very similar to remineralisation as it aims to mineralise and seal the superficial dentine from externally sourced bioavailable ions. Novel therapies are continually being formulated and tested to reduce the severity of dentine hypersensitivity through dentine tubule occlusion.

1.4 Remineralisation

1.4.1 Historical perspective

Remineralisation can be described as the repair of mineral deficient dental hard tissues by crystal growth with calcium phosphates. It has been observed for over a century, with early studies in 1912 observing a ‘rehardening’ of acid softened tooth enamel in vitro by saliva [Head, 1912]. Further experiments by Koulourides in the 1960’s showed softened enamel could be rehardened in vitro by exposure to solutions of dicalcium phosphate dihydrate at pH 6.8 – 7.3 as well as varying calcium phosphate fluoride solutions and human saliva [Koulourides et al., 1965; Koulourides and Pigman, 1960]. From these early observations, the phenomenon of remineralisation was investigated and demonstrated through countless studies. It is now understood that there is periodic demineralisation and remineralisation of the tooth surface that occurs at a subclinical level. When there is a net demineralisation by bacterial acid by-products caries progresses, and when there is a net remineralisation of mineral deficient tooth structure caries regresses. Under certain biochemical conditions, demineralisation of enamel and dentine can be repaired hence at this stage of the disease it is considered reversible to a certain extent [Featherstone, 2009].

1.4.2 Mechanism

1.4.2.1 Diffusion

Enamel and dentine are porous tissues, and within the body of a carious lesion the pore volume increases due to advanced demineralisation. Remineralisation occurs by the reaction of calcium, phosphate and fluoride ions in aqueous solution depositing in crystal voids of demineralised enamel and dentine. These ions must diffuse through the surface
layer to produce supersaturation of the lesion fluid for remineralisation to proceed. In carious enamel before cavitation occurs the surface layer is relatively mineralised and has a low pore volume. Diffusion of ions through tooth structure is limited by the porosity of the tissue, particularly the mineralised surface layer. The significance of the surface layer in regards to remineralisation has been discussed previously [Briner et al., 1974; Larsen and Fejerskov, 1989]. When an acid challenge occurs near the surface, CadAP is preferentially dissolved and replaced by FA or FHA using bioavailable fluoride resulting in a fluoride rich, low pore volume surface layer. An in vitro study by Larsen and Pearce proposed remineralisation of the deeper layers of the lesion will only occur after the surface layer has been removed or altered to facilitate ion diffusion [Larsen and Pearce, 1992]. Brudevold et al. [1982] showed that the rate of remineralisation of enamel decreases as enamel pore volume decreases.

Diffusion into the subsurface lesion is not only limited by the porosity of the enamel/dentine but the electrochemical charge of the ions present in plaque fluid and saliva and their ability to diffuse into the lesion without binding to superficial structures. The ions needed for mineralisation of HA/FA may be present as charged ions (Ca$^{2+}$, CaH$_2$PO$_4^+$, CaOH$^+$, CaF$^+$, CaPO$_4^-$, PO$_4^{3-}$, HPO$_4^{2-}$, H$_2$PO$_4^-$, F$^-$, OH$^-$), or may be incorporated into soluble ion pairs that have a neutral charge (CaHPO$_4^0$, H$_3$PO$_4^0$, HF$^0$) [Cochrane et al., 2008]. Enamel and dentine are charged tissues and the rate of diffusion through the tissues will be affected by the charge on the ion; there has been strong evidence that molecules with a neutral charge diffuse more readily through enamel [Featherstone, 1977; Reynolds, 1997]. Dissociation of neutral ion pairs within the lesion releases Ca$^{2+}$, PO$_4^{3-}$ and F$^-$ ions that are available for remineralisation, simultaneously maintaining a concentration gradient that drives further diffusion of these neutral ion pairs into the lesion. It has been suggested that the diffusion of calcium phosphate and fluoride ions through the relatively intact surface layer of enamel lesion (in particular the diffusion of the neutral ion pairs) is rate limiting for remineralisation [Cochrane et al., 2008].

1.4.2.2 Crystallisation

Upon diffusion into the enamel or dentine lesion fluid, the aqueous ions increase the DS for the mineral phase and so deposit into crystal voids to repair the damaged crystallites. For de novo crystallisation of apatite phases to occur, clusters of ions must aggregate in
an ordered spatial arrangement until they form a nucleus exceeding the ‘critical size’ for crystallisation; this is also known as crystal nucleation. The critical size of the nucleus is the (crystal specific) size where either crystallisation or termination of the cluster of ions will equally reduce the free energy within the system [De Yoreo and Vekilov, 2003]. Accordingly, an energy barrier exists and must be overcome to nucleate a crystal and result in a reduction of free energy. The surface molecules of the nucleus have a higher free energy than the molecules of the bulk (internal) phase. The difference in energy is called the interfacial free energy and it acts to destabilise the nucleus [De Yoreo and Vekilov, 2003]. When there has been sufficient adsorption of molecules, the size of the nucleus is increased beyond the critical size so that a drop in free energy occurs due to the increasing volume of the bulk phase. At this point the interfacial free energy becomes unimportant and the free energy of the system will only decrease after crystallisation [De Yoreo and Vekilov, 2003].

Nucleation can either be homogeneous or heterogeneous. Homogeneous nucleation occurs as spontaneous precipitation and proceeds without a surface or scaffold for the crystal to grow on [De Yoreo and Vekilov, 2003]. A higher DS is needed for homogeneous nucleation to occur; the interfacial free energy is large due to the presence of solvent surrounding all planes of the new phase and this will act against the formation of a crystal [Simmer and Fincham, 1995]. Heterogeneous nucleation occurs when crystals adsorb onto a pre-existing scaffold [De Yoreo and Vekilov, 2003]. A suitable example is a demineralised crystal in a carious lesion: the crystal surface acts as a nucleation site and bonds to the newly forming crystallite, reducing the interfacial free energy in the system. This type of nucleation occurs more readily. Remineralisation of carious tooth enamel or dentine can be considered heterogeneous nucleation of apatite crystals as the demineralised apatite crystals are used as templates for new crystal growth [Mann, 2001].

Crystallisation is also dependent on the concentration of ions in solution. For apatite crystals to nucleate, the lesion fluid must be supersaturated with respect to the solid phase. The DS with respect to a specific solid phase is calculated from the ion activity product (IP) of the dissolved constituents of the solid phase divided by the Ksp of the solid phase:

\[ DS = \left( \frac{IP}{Ksp} \right)^{1/n} \]
Where \( n \) = number of ions in a unit cell.

A DS > 1 means the solution is supersaturated and will favour growth of the solid phase, a DS = 1 means the solution is in equilibrium with the solid, and a DS < 1 means the solution is undersaturated and will favour dissolution of the solid phase. As mentioned in 1.2.2, the Ksp for CadAP, HA and FA is \( 10^{-113} \), \( 10^{-117} \) and \( 10^{-121} \) respectively and each unit cell contains 18 ions. As there are 10 calcium ions for every unit in stoichiometric HA, the activity of calcium has the most influence on its DS [Simmer and Fincham, 1995].

The kinetics of crystallisation is proportional to the DS; a higher DS will induce a higher rate of crystallisation [Aoba, 1997; Johnsson and Nancollas, 1992]. There has been evidence that the intra-lesion fluid of enamel is only marginally supersaturated with respect to apatite, and that supersaturation may only exist for a short period; this can be attributed to the rapid consumption of minerals into the crystal phase and a relatively small concentration gradient inducing diffusion of ions into the lesion [Larsen and Fejerskov, 1989].

1.4.2.3 Effect of pH

The amount of acidity or the pH level with the lesion fluid has numerous effects on the kinetics of remineralisation. The DS with respect to the various calcium phosphate phases is pH dependent. A sufficient drop in pH (such as in carious demineralisation) results in the lesion fluid being undersaturated with respect to the apatites and demineralisation occurs to compensate [Larsen, 1990]. Variation in the pH alters the activities of the different calcium and phosphate species present in solution and these ions and ion pairs have different electrical charges. As stated in 1.4.2.1, enamel and dentine are porous charged tissues and diffusion of molecules through the hard tissue depends largely on their ionic charge. The activity of neutral ion species has been positively correlated with rate of remineralisation at different pH as the change in acidity alters the ion activities of aqueous calcium phosphate species [Cochrane et al., 2008; Reynolds, 1997].

A simplified reaction shows eight hydrogen atoms (protons) are end products when stoichiometric HA is formed from \( \text{Ca}^{2+} \), \( \text{HPO}_4^{2-} \), and \( \text{H}_2\text{O} \):

\[
10 \text{Ca}^{2+} + 6 \text{HPO}_4^{2-} + 2 \text{H}_2\text{O} \leftrightarrow \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 + 8 \text{H}^+
\]
The production of protons in remineralisation reactions can induce a low pH environment in the lesion fluid thereby decreasing the rate of remineralisation by reducing saturation with respect to HA. An excess of protons increases the formation of protonated ion pairs (CaHPO$_4^0$, CaH$_2$PO$_4^+$, HPO$_4^{2-}$, H$_2$PO$_4^-$, H$_3$PO$_4^0$ and HF$^0$) and therefore reduces availability of free phosphate (PO$_4^{3-}$) and fluoride (F$^-$) ions for remineralisation.

1.4.2.4 Role of plaque and saliva

As remineralisation proceeds, there is a consumption of the aqueous minerals that lowers the IP; supersaturation of the lesion fluid will only be achieved either by diffusion of ions into the enamel fluid, or the reversal of the remineralisation reaction (demineralisation). The surrounding plaque and saliva are therefore important reservoirs of calcium, phosphate and fluoride to maintain supersaturation of the enamel fluid and prevent demineralisation.

Saliva is the main source of ions for remineralisation and it influences the ionic content of the plaque fluid [Matsuo and Lagerlöf, 1991]. Salivary proteins, particularly statherin and the PRPs, stabilise calcium and phosphate ions to prevent precipitation intra-orally while at the same time maintaining supersaturation with respect to calcium phosphate solid phases [Moreno et al., 1979]. Healthy saliva has been shown to be supersaturated with respect to HA when the pH is above 5.3 [Larsen and Pearce, 2003], and non-cariogenic plaque fluid has also been shown to be supersaturated with respect to the dental apatites [Margolis et al., 1988; Tatevossian and Gould, 1976; Tenuta et al., 2006]. Both mediums have high ionic concentrations that inherently promote remineralisation.

Dental plaque gives protection for microorganisms and stores carbohydrates and minerals such as calcium, phosphate and fluoride sourced from saliva or from within the tooth. It begins as the AEP which is a proteinacious film that develops on the tooth surface by the adherence of salivary glycoproteins [Yao et al., 2003]. The AEP protects the tooth surface from attack by bacterial acid by-products, prevents spontaneous precipitation of calcium phosphates, but also acts as a foundation for bacterial adherence allowing the formation of a biofilm or dental plaque [Fejerskov and Kidd, 2003]. A study by Tatevossian and Gould [1976] reported plaque fluid as having a calcium concentration of 6.5 mM ± 2.1, and inorganic phosphate concentration of 14.2 mM ± 3.1, maintaining a supersaturation
with respect to apatite. A similar study by Margolis et al. [1988] compared ion concentrations in plaque between caries free and caries susceptible individuals, and was able to show that both groups’ plaque fluid were supersaturated with respect to the dental apatites; the caries free and caries susceptible groups had a plaque fluid pH of 6.35 and 5.85 respectively, and the caries free group had a higher DS with respect to apatite.

Fluoride primarily enters the enamel fluid from the plaque when paired with a hydrogen atom (hydrofluoric acid – HF$^0$); this neutral molecule diffuses into the tooth and dissociates to H$^+$ and F$^-$. The fluoride content in plaque has been shown to be highly variable between communities due to the difference in water fluoridation [Tatevossian, 1990] and this is an important factor when considering the plaque fluid’s DS with respect to fluoride-containing apatites. An in vitro study by Lynch et al. [2006] concluded that the presence of fluoride was required to achieve net remineralisation with a solution mimicking calcium, phosphate and pH levels of plaque fluid; demineralisation was evident when enamel was exposed to these conditions without fluoride.

Salivary proteins inhibit precipitation of calcium phosphates in the oral environment, but have also been suggested to inhibit remineralisation. The study by Robinson et al. [1990] suggested that removal of intra-lesion protein before remineralisation by treatment with OCl$^-$ resulted in an increase in remineralisation. Based on this result, Robinson et al. [1990] claimed that intra-lesion protein was either impeding diffusion or blocking crystal nucleation sites. Salivary proteins statherin and acidic PRPs have been shown to adsorb to HA crystals, blocking sites for crystal growth whilst keeping the solution supersaturated with respect to HA and preventing nucleation of crystals [Moreno et al., 1979]. As well as salivary proteins, serum albumin has been suggested to be a remineralisation inhibitor [Robinson et al., 1998]. Localisation of albumin and amylase within demineralised carious enamel showed that they are found in relatively high quantities in areas of 10 – 20% demineralisation [Shore et al., 2000] and Garnett and Dieppe [1990] showed serum albumin inhibited HA crystal growth in a constant composition model. An in vitro remineralisation study by Fujikawa et al. [2008] concluded that although salivary macromolecules such as proteins may inhibit
remineralisation, they play an important role in preventing precipitation on the enamel surface especially in the presence of fluoride.

1.4.3 Analysis of remineralisation *ex vivo*

To assist with the development of remineralisation strategies, several analyses can be used to monitor regression of incipient carious lesions. *Ex vivo* assessment of remineralisation is generally a measurement of mineral growth within lesions, though some analyses assess changes in the visual appearance, hardness or morphology of lesions.

Transverse microradiography (TMR) is widely considered to be the gold standard for assessing remineralisation of carious lesions for *in vitro* and *in situ* studies [Arends and Ten Bosch, 1992; Ten Bosch and Angmar-Månsson, 1991]. The technique was first developed by Angmar et al. [1963] and it uses the densitometric data obtained from microradiographs of thin tooth sections (approximately 100 µm in thickness) to calculate mineral density [Ten Bosch and Angmar-Månsson, 1991]. The optical film transmission values within an area of interest are compared against an aluminium stepwedge to estimate an equivalent thickness of aluminium, and this is used to determine mineral density through an equation based on the linear attenuation coefficients (also called linear absorption coefficient) of aluminium, mineral, organic material and water [Ten Bosch and Angmar-Månsson, 1991]. The mineral density of lesions is assessed against adjacent sound enamel so that comparisons through trapezoidal integration of untreated and treated lesions are used to calculate remineralisation.

Microhardness testing involves assessing the size of indentations made by a particularly shaped object, usually a diamond, applied with a defined force into the tooth surface [Featherstone *et al.*, 1983]. Some authors have reported a linear relationship between enamel mineral content as assessed by TMR and cross-sectional microhardness values [Featherstone *et al.*, 1983; Kielbassa *et al.*, 1999]. As highly concentrated fluoride treatments promote remineralisation in the superficial layer of enamel which can inhibit remineralisation deeper in the lesion, microhardness values assessing the surface enamel hardness can misrepresent the extent of mineralisation present through the entire lesion.
Furthermore, the study by Buchalla et al. [2008] found only a weak linear relationship between cross-sectional hardness and TMR, and it was concluded that conversion of hardness values to mineral content was not accurate and therefore not recommended. A similar conclusion was reached by Magalhaes et al. [2009] who stated microhardness was not very accurate for estimating mineral content in lesions and should instead be used for insight about mechanical properties. Despite these significant limitations of microhardness assessment, some authors have used it to assess remineralisation [Hara et al., 2009; Zero et al., 2006].

Polarised light microscopy (PLM) has been suggested as an alternative to TMR for analysis of remineralisation [Ten Bosch and Angmar-Månsson, 1991]. Using the measurement of ‘retardation’ and sample thickness, the birefringence of the sample can be quantified to calculate mineral concentration with reasonable accuracy [Ten Bosch and Angmar-Månsson, 1991]. However, this technique relies on numerous assumptions and can be prone to error from the influence of porosities within the lesion [Theuns et al., 1993]. A more common use for PLM is the calculation of lesion depth, though this requires an assumption as to the orientation of enamel crystals within the sample section [Bajaj et al., 2016; Ten Bosch and Angmar-Månsson, 1991].

A relatively new technique for assessing remineralisation is optical coherence tomography (OCT). OCT is a non-invasive technique that utilised short coherence length light to construct three dimensional images of carious lesions [Feldchtein et al., 1998; Ko et al., 2005]. OCT is able to monitor intact lesions ex vivo and in vivo, with imaging of approximately 20 µm resolution and up to 2 mm depth from the tooth surface [Feldchtein et al., 1998]. Polarisation-sensitive optical coherence tomography (PS-OCT) uses polarised light to reduce the influence of the surface reflectivity, thereby giving more accurate detail about the lesion morphology and severity [Fried et al., 2002]. PS-OCT has been used frequently to assess lesion progression by assessing the lesion depth and morphology, however thus far it is not a widely accepted method for calculation of mineral concentration [Fried et al., 2002; Jones and Fried, 2006; Kang et al., 2012; Manesh et al., 2009].
The use of x-ray microtomography (XMT) to assess mineralisation of biological tissues was first described by Elliott et al. [1989]. The publication by Gao et al. [1993] used this technique to assess remineralisation of enamel lesions longitudinally. With the recent increase in commercially available XMT scanners for laboratory use, numerous studies have used this technique to calculate mineral density and quantify remineralisation of carious lesions [Kind et al., 2017; Kucuk et al., 2016; Lo et al., 2010]. Conventional/laboratory-based XMT scanners (Cµ-CT) have the advantage of non-destructively analysing samples, however they are costly and do not use monochromatic radiation which is desirable for image accuracy [Elliott et al., 1994]. To overcome this, synchrotron-sourced monochromatic radiation has been combined with XMT to assess mineralisation of biological tissues including teeth [Bonse and Busch, 1996; Dalstra et al., 2006a; Dalstra et al., 2006b; Dowker et al., 2004; Kinney et al., 2005; Lautensack et al., 2013; Prymak et al., 2005]. Comparisons of Cµ-CT to TMR for remineralisation assessment are limited and they suggest Cµ-CT is a suitable alternative [Hamba et al., 2012; Lo et al., 2010]. The study by Gao et al. [1993] utilised both scanning microradiography and Cµ-CT to analyse demineralised and remineralised lesions, however the measured mineral concentrations were obtained by microradiography and Cµ-CT was used to analyse the lesion and surface layer morphology.

1.4.4 Strategies for remineralisation

Numerous approaches to remineralisation have been suggested; the three main approaches of interest are:

i. Saliva

ii. Delivery of fluoride

iii. Delivery of calcium and phosphate (with/without fluoride)

1.4.4.1 Saliva

The study by Backer Dirks [1966] was one of the early studies to demonstrate the inherent remineralisation effect of saliva and remains one of the most significant studies to date. Longitudinal clinical diagnosis of caries was undertaken in the same group of children between the ages of 8 and 15 categorising tooth surfaces as either sound, uncavitated caries or cavitated caries. Without any intervention and without any fluoride exposure, approximately 50% of the uncavitated carious surfaces in the children at 8 years old were
deemed to be sound at 15 years old indicating the caries had regressed by saliva exposure alone. This not only signified the importance of remineralisation by saliva, but also its limitation and need for additional remineralisation aids.

Since the observations made by Backer Dirks [1966], the caries protective and remineralisation effect of saliva has been well documented. Saliva has an important biological role in keeping the oral environment hydrated and lubricated as well as allowing delivery of calcium, phosphate and fluoride ions without precipitation. Early evidence of in vitro remineralisation was shown to occur from exposure of demineralised lesions to saliva [Feagin et al., 1964]. The importance of saliva in promoting net remineralisation is often reflected in studies correlating caries risk/incidence and saliva quantity. Decreased quantity of saliva was shown to positively correlate with high caries experience in a review by Leone and Oppenheim [2001], while individuals with a high salivary buffering capacity and high salivary calcium and phosphate levels were found to have more protection against caries [Tenovuo, 1997] and patients with hyposalivation were found to be at higher risk of the disease [Spak et al., 1994]. Numerous randomised clinical trials have shown that chewing sugar-free gum reduces caries experience, and this has been attributed to increased production of saliva which promotes a net remineralisation of the dental hard tissues [Beiswanger et al., 1998; Machiulskiene et al., 2001; Szoke et al., 2001].

1.4.4.2 Fluoride
Since the early descriptions of ‘mottled teeth’ by Black [1916], there has been supporting evidence and widespread recognition that intra-oral fluoride exposure is not only effective in preventing caries progression but is important for remineralisation. Exposure to the highly reactive fluoride ion promotes the formation of fluoride-containing phases and prevents the loss of calcium and phosphate ions within the tooth by converting CadAP to either FA or FHA during acidic challenges [Lussi et al., 2012]. FA and FHA are more favoured to form than HA at low pH levels as their Ksp is lower than HA and the lesion fluid will tend to be supersaturated with respect to these apatites even with low concentrations of fluoride; the presence of the fluoride ion alongside calcium and phosphate ions acts as a driving force for crystallisation of fluoride-containing apatites [Aoba, 1997].
In the context of remineralisation, fluoride must diffuse into the tooth from the external plaque fluid and pair with extrinsic calcium and phosphate ions to drive crystal growth. Generally, the periodic high pH conditions at the lesion front promotes crystallisation in this zone, however when fluoride is present crystallisation is favoured at any site throughout the lesion; as a consequence the surface zone becomes more mineralised due to the influx of ions (especially fluoride) from the plaque fluid [Ten Cate, 1990]. The fluoride-rich mineralised surface zone reduces enamel pore volume thereby restricting ion diffusion to deeper layers of the lesions; in that respect high levels of fluoride is a hindrance to achieving maximum remineralisation of carious lesions [Cochrane et al., 2010]. Remineralisation by fluoride has been shown to occur at a pH as low as 4.8 at a concentration of 1 ppm fluoride, demonstrating low levels of fluoride still have a high propensity for crystallisation in acidic conditions [Lynch et al., 2006]. A review of the relevant literature found that a variety of clinical factors such as caries risk and intra-oral ecological variation affect the minimum level of fluoride required to induce remineralisation [Hellwig and Lussi, 2001]. Cochrane et al. [2010] commented that as calcium ions were important for remineralisation, novel remineralisation systems should ideally not only be focused on fluoride delivery but also calcium and phosphate ions, particularly for individuals with hyposalivation.

1.4.4.3 Delivery of calcium and phosphate

For any novel remineralisation agent to be of clinical relevance, they must have an additive effect to saliva and fluoride exposure. A review by Zero [2006] described an effective remineralisation agent as being:

1. Beneficial over and above fluoride
2. Beneficial in addition to the natural remineralisation properties of saliva
3. Efficacious despite salivary proteins and the AEP
4. Not favouring calculus formation

1.4.4.3.1 Bioactive glasses (Novamin™ and BioMin™)

Novamin™ has been marketed as a remineralisation agent and has been utilised in a dentifrice for treatment of dental hypersensitivity. The active ingredient is the patented bioglass ‘45S5’, and it is reported to release calcium and phosphate ions when interacting
with water [Burwell et al., 2009a]. *In vitro* studies have shown dentine tubule occlusion after exposure to the bioglass, thereby suggesting a clinical reduction in dental hypersensitivity is achievable [Burwell et al., 2009a; Wang et al., 2011]. There has also been evidence of a repair in enamel defects by Novamin™-containing products *in situ* as shown through surface roughness measurements by an optical profilometer [Burwell et al., 2009b]. This same study demonstrated *in vitro* remineralisation of bovine dentine root caries through microhardness testing and ‘healing’ of bovine enamel white-spot lesions using SEM images. A randomised clinical trial demonstrated a reduction in hypersensitivity in individuals when using a dentifrice containing Novamin™ as measured with a visual analogue scale [Pradeep and Sharma, 2010]. Despite evidence of dentine tubule occlusion and a reduction in hypersensitivity, further research needs to be done to assess the remineralisation potential of Novamin™ on carious human enamel and dentine.

BioMin™ (Biomin Technologies Ltd, London, United Kingdom) is another remineralisation agent that has very recently been made commercially available. The active ingredient is a bioactive glass that is claimed to slowly dissolve in water to deliver calcium phosphate and fluoride ions [Jones et al., 2016]. No laboratory or clinical evidence for the efficacy of BioMin™ is currently published although it is commercially available in a toothpaste.

1.4.4.3.2 Functionalised tricalcium phosphate (Clinpro™)
The manufacturers of Clinpro™ have developed a functionalised β-tricalcium phosphate (β-TCP) which is reported to be a precursor of HA [Karlinsey and Mackey, 2009]. The term functionalised refers to the ball-milling process used to couple β-TCP with organic and inorganic moieties such as carboxylic acid and sodium lauryl sulphate which is then utilised to deliver calcium and phosphate to repair tooth surfaces [Karlinsey and Mackey, 2009]. Numerous *in vitro* studies have claimed a repair or remineralisation effect and an uptake of fluoride in enamel when functionalised β-TCP was combined with fluoride in a dentifrice [Asaizumi et al., 2013; Asaizumi et al., 2014; Karlinsey and Mackey, 2009; Karlinsey et al., 2009a; Karlinsey et al., 2010a; Karlinsey et al., 2010b; Karlinsey et al., 2009b; Karlinsey et al., 2011b]. These studies were conducted predominantly on bovine enamel except for one which used human enamel [Karlinsey et al., 2011b]. The method
of remineralisation assessment within these studies was mainly surface microhardness [Karlinsey and Mackey, 2009; Karlinsey et al., 2009a; Karlinsey et al., 2010b; Karlinsey et al., 2009b] with two studies utilising micro CT [Asaizumi et al., 2013; Asaizumi et al., 2014] and one study TMR [Karlinsey et al., 2010a]. There has also been evidence to suggest functionalised β-TCP can occlude bovine dentine tubules in vitro through SEM analysis [Karlinsey et al., 2011a].

An in situ study testing a fluoride-containing β-TCP dentifrice against two positive controls showed a significant increase in microhardness with the test product, however no significant increase in remineralisation or decrease in lesion depth according to TMR [Mensinkai et al., 2012]. Similar results were seen in another in situ study comparing β-TCP/F in a mouthrinse against positive controls [Mathews et al., 2012]. An in situ study by Amaechi et al. [2012] did not show a difference in microhardness of treated enamel white spot lesions but observed a significant increase of remineralisation according to TMR. Another in situ study by Amaechi et al. [2010] was able to show a significant increase of remineralisation when comparing a β-TCP/F mouthrinse to a positive control according to TMR, however like the other in situ studies no change in lesion depth was noted. An in situ study by Shen et al. [2011] was unable to demonstrate any significant difference between the remineralisation effect of β-TCP/950 ppm F and 1000 ppm F using TMR; this is one of only two in situ studies on a β-TCP/F-containing product. Of these two studies, β-TCP-containing products were either statistically inferior to other remineralisation products according to TMR [Shen et al., 2011], or no significant difference was found between any intervention using PLM [Vanichvatana and Auychai, 2013]. Currently no clinical trials have been conducted to assess the remineralisation efficacy of β-TCP/F, and based on present evidence a conclusion cannot be made as to whether it can increase remineralisation or reduce carious lesion depth in human enamel and dentine when compared to fluoride application alone.

1.4.4.3.3 Nanosized HA (Remin Pro™)

Remin Pro™ (VOCO GmbH, Cuxhaven, Germany) is a relatively new remineralisation agent that delivers calcium phosphate in the form of nanosized HA together with fluoride. Limited evidence is available to show its clinical efficacy apart from one trial that displayed a remineralisation effect [Ebrahimi et al., 2017]. Three in vitro studies have
documented a positive remineralisation effect by the Remin Pro remineralisation agent, however a mechanism for this process is not well described [Attia and Kamel, 2016; Bajaj et al., 2016; Kamath et al., 2013]. Bajaj et al. [2016] showed that Remin Pro was effective for remineralising artificial enamel lesions in vitro using PLM for measurement of enamel lesion depth. Due to the variable angle of incision through the enamel and the fact that mineral content was not considered, this method of analysis may be viewed as being inferior to TMR. Further evidence is needed to confirm nanosized HA-containing products as an effective clinical choice for remineralisation of carious lesions.

1.4.4.3.4 Self-assembling peptide (CURODONT Repair™)

While strictly not delivering calcium and phosphate, the self-assembling peptide P11-4 is suggested to attract and nucleate calcium and phosphate within carious lesions to enhance remineralisation. It has been described as “a rationally designed small molecule that undergoes hierarchical self-assembly into fibrillary scaffolds in response to specific environmental triggers” [Kind et al., 2017]. These fibrillary scaffolds are thought to behave as an organic matrix within mineral deficient enamel promoting de novo crystal nucleation [Kirkham et al., 2007] and enhancing remineralisation by either saliva or another remineralisation aid; the P11-4 peptide is currently available as a professional dental product (CURODONT Repair™). In vitro remineralisation studies by Jablonski-Momeni and Heinzel-Gutenbrunner [2014] and Takahashi et al. [2016] demonstrated an improved remineralisation effect by pre-treating enamel samples with P11-4 before a remineralisation treatment. However, it was unclear as to whether this effect was due to the initial application of sodium hypochlorite and phosphoric acid that was given to only the P11-4 test samples before remineralisation. A clinical safety trial by Brunton et al. [2013] determined that P11-4 was safe to use in vivo and displayed an improvement in the appearance of white spot lesions over time despite no control sample population for comparison. Schmidlin et al. [2016] reported a significant remineralisation effect by P11-4 pre-treatment, though the statistical significance compared the surface microhardness of the demineralised lesion with the remineralised lesion from the same sample; with no statistical comparison made between P11-4 and the positive control group. The evidence advocating clinical use of P11-4 self-assembling peptide is still in its infancy and critical evaluation is lacking.
1.4.4.3.5 Casein phosphopeptide (CPP) complexes

The CPPs are a group of proteins present in milk and dairy products. It has been known for some time that CPPs sequester high quantities of calcium and phosphate in soluble complexes without precipitation [Reeves and Latour, 1958]. When early evidence indicated consumption of dairy had an anticariogenic effect [Mellanby and Coumoulos, 1944; Sprawson, 1932], further research determined the primary anticariogenic constituents of milk are the CPPs from their ability to increase bioavailability of calcium and phosphate ions and buffer capacity [Reynolds, 1987]. A process to isolate CPPs through a tryptic digestion was developed allowing their incorporation into therapeutic dental products for delivery of calcium and phosphate stabilised as complexes [Reynolds et al., 1994]. Commercially, CPP-ACP is available in a topical tooth crème (Tooth Mousse/MI Paste, GC Corporation), a sugar free chewing gum (Recaldent/Trident Xtra Care, Mondelez International), as well as a dental restorative material (Fuji VII GIC, GC Corporation). Casein phosphopeptide stabilised amorphous calcium fluoride phosphate (CPP-ACFP) has been incorporated into a topical tooth crème (Tooth Mousse Plus / MI Paste Plus, GC Corporation) and a professionally applied varnish (MI Varnish, GC Corporation).

1.5 Casein phosphopeptide complexes

As the focus of this thesis is enhancement of remineralisation using CPP complexes, a more detailed review of this remineralisation system is presented.

1.5.1 Structure

The structure of a CPP complex is proposed to be that of a cluster of mixed ions bound to peptides that prevent the further adsorption of ions needed to exceed the critical size for nucleation or phase transformation [Cross et al., 2007]. The major CPPs are $\alpha_{S1}(59-79)$, and $\beta(1-25)$ and the minor CPPs are $\alpha_{S2}(1-21)$ and $\alpha_{S2}(46-70)$ [Cross et al., 2007]. They are called phosphopeptides due to the inclusion of a O-phosphoseryl residue within the peptide chain. The amino acid sequences of these peptides are as follows [Cross et al., 2007]:
All the CPPs contain the negatively charged and highly acidic binding motif Ser(P)-Ser(P)-Ser(P)-Glu-Glu which binds to the surface calcium ions of a mixed cluster of calcium and phosphate ions (with or without fluoride). This motif, in addition to other acidic residues within the phosphopeptides, is responsible for the high calcium and phosphate binding capacity of CPP complexes [Cross et al., 2005]. The major CPPs αS1(59-79), and β(1-25) are capable of binding up to a maximum of 21 and 24 calcium ions and 14 and 16 phosphate ions respectively [Cross et al., 2005]. The clusters of ions within the complexes are an amorphous phase (ACP) [Reynolds, 1998], and this is evidenced by their inability to diffract x-rays when in a dried state [Cross et al., 2005]. Accordingly, the ions complexed by CPPs are identified as casein phosphopeptide stabilised amorphous calcium phosphate or amorphous calcium fluoride phosphate, otherwise known as CPP-ACP and CPP-ACFP [Reynolds, 1998].

In solution, CPP complexes bind ions in dynamic equilibrium with the surrounding water. The equilibrium is dependent on pH as this affects the charge of the amino acid residues within the phosphopeptides, which in turn affects the binding capacity of the complex.
It has been determined that the CPPs have a relatively weak affinity for calcium and phosphate ions, hence release of these ions from the complex is possible to maintain equilibrium and allow bioavailability of ions for remineralisation reactions [Meisel and Olieman, 1998; Park et al., 1998].

1.5.2 Mechanism of action

The CPPs strong capacity to bind and stabilise calcium phosphate and fluoride in solution confers an ability to maintain high ion concentrations and a supersaturation with respect to numerous solid calcium phosphate phases [Reynolds, 1997]. As their structure and properties are very similar to salivary statherin and PRPs, the CPPs are considered to be a salivary biomimetic [Cochrane and Reynolds, 2012].

For remineralisation to occur, the CPP complexes must first be delivered to the mineral deficient tooth surface whereupon interaction with the plaque and enamel fluids can proceed (see Figure 1.1). CPP-bound calcium, phosphate and fluoride ions are then released into solution from equilibrium driven release or pH dependent release as described in 1.5.1. As CPPs have an affinity to bind to other structures in the mouth such as apatite, the AEP, PRPs, mucin and bacteria [Huq et al., 2000; Huq et al., 2016; Reynolds et al., 2003; Rose, 2000a], conformational change of the peptide upon binding to these structures also drives ion release from CPP complexes. Finally, enzymatic breakdown of CPPs by host and bacteria derived peptidase and phosphatase can destabilise complexes and cause release of complexed ions into the plaque fluid [Reynolds et al., 2003; Reynolds and Riley, 1989]. Previous clinical studies have measured significantly higher ionic calcium and phosphate in plaque following treatment with CPP-ACP when compared to the negative control [Poureslami et al., 2016; Reynolds et al., 2003]. Upon release in the plaque fluid, diffusion through the porous enamel allows bioavailability of soluble calcium, phosphate and fluoride ions in the mineral deficient lesion. The CPP-ACP/ACFP complexes provide a reservoir for ions, create a concentration gradient for ion diffusion from the plaque into the enamel fluid, and promote remineralisation by preserving a supersaturation with respect to apatite in the enamel fluid.
In previous experiments, CPP-ACP complexes have been shown to bind to supragingival plaque [Reynolds et al., 2003; Rose, 2000a]. The method of CPP-binding in plaque was suggested to be from calcium cross-linking as well as hydrophobic and hydrogen bonding to the plaque matrix or bacterial cell walls. Reynolds et al. [2003] calculated that phosphopeptides had a half-life of approximately 125 minutes in plaque as the complexes are degraded by enzymatic breakdown. By being localised in plaque, CPP complexes not only release ions to promote their diffusion into subsurface lesions for remineralisation, but they also influence the plaque fluid pH through a buffering effect.

The CPPs contain several amino acids in their sequence such as phosphoseryl, histidyl and glutamyl that possess R groups capable of buffering acid between pH 5 and 7 [Reynolds, 1987; Swaisgood, 2003]. Phosphate ions incorporated in the complex (as PO$_4^{3-}$ and HPO$_4^{2-}$) can be driven by equilibrium dependent release to buffer acid [Reynolds, 1987]. Additionally, it has been shown that bacteria are able to metabolise cleaved arginyl, asparaginyl, and glutaminyl from CPPs by consuming H$^+$ and producing ammonia as an end product thereby increasing the pH [Reynolds and Riley, 1989]. The buffering capability of CPPs is therefore a significant anticariogenic attribute as it inhibits bacterial acid-mediated demineralisation, and may also support alkaline producing bacteria.

Previous clinical studies have demonstrated treatment with CPP-ACP/ACFP has produced an increase in plaque fluid pH and inhibited acid-producing bacteria [Caruana et al., 2009; Heshmat et al., 2014; Karabekiroğlu et al., 2017; Marchisio et al., 2010;
Evidence has displayed CPPs interact with specific bacteria to alter their binding properties, consequently affecting the composition of the plaque. It has been proposed that CPPs competitively bind calcium to inhibit calcium bridging between bacteria cell walls [Rose, 2000a], or bind to the surface of bacteria to result in an electrostatic repulsion between cells [Neeser et al., 1994]. Rahiotis et al. [2008] demonstrated that biofilm formation in the presence of CPP-ACP was significantly delayed, and this was consistent with alteration of bacterial adherence by the CPPs. Hence, the favourable effect of CPP complexes on plaque formation and bacterial composition provides an additional mechanism whereby caries progression is inhibited.

Enamel surface-bound CPPs have been suggested to inhibit demineralisation of enamel apatite crystals. CPPs and CPP-ACP both bind to HA in the presence of saliva, and this has displayed an inhibition in the rate of HA demineralisation [Reynolds et al., 1982]. By binding to apatite crystal faces where active demineralisation occurs, CPPs provide a physical barrier whereby calcium and phosphate ions are protected from the hydration layer and are more resistant to leave the crystal lattice through acid demineralisation [Reynolds et al., 1982]. This imparts another anticariogenic mechanism of CPP complexes, all of which combine to result in a multifaceted anticariogenic remineralisation complex.

1.5.3 Evidence of efficacy

There are over 400 published studies assessing the efficacy of CPP-ACP/ACFP in vitro, in situ and in vivo and a complete analysis of these studies is beyond the scope of this review.

Of the published in vitro studies, of particular note are those by Reynolds [1997] and Cochrane et al. [2008] who demonstrated remineralisation of artificial enamel lesions by CPP-ACP and CPP-ACFP respectively at varied concentrations over a wide pH range. The relative supersaturation with respect to solid calcium phosphate phases was reported for all the test solutions and demonstrated the ability of CPP-ACP and CPP-ACFP complexes to maintain a high DS with respect to apatite in both acidic and alkaline
conditions. These findings supported further clinical investigation and revealed the significance of pH with regards to remineralisation using CPP-ACP/ACFP complexes.

The *in situ* method of remineralisation analysis described by Shen *et al.* [2001] has been used in multiple studies to demonstrate various forms of CPP-ACP and CPP-ACFP significantly increased remineralisation of artificial caries when compared to negative and positive controls [Cai *et al.*, 2007; Cai *et al.*, 2003; Cai *et al.*, 2009; Cochrane *et al.*, 2012b; Iijima *et al.*, 2004; Manton *et al.*, 2008; Reynolds *et al.*, 2008; Reynolds *et al.*, 2003; Shen *et al.*, 2011; Srinivasan *et al.*, 2010; Walker *et al.*, 2006; Walker *et al.*, 2010]. Using TMR, these studies demonstrated the remineralisation efficacy of CPP-ACP/ACFP complexes complemented that of saliva, resulting in even distribution of crystal growth throughout the lesion body.

In addition to these studies, a wealth of clinical evidence has been published assessing the remineralising effect of CPP-ACP/ACFP contained in commercially available chewing gum and tooth crème.

1.5.3.1 CPP-ACP chewing gum

The publication by Morgan *et al.* [2008] reported the results of a clinical trial assessing the effect of CPP-ACP chewing gum on regression of posterior approximal caries. The trial involved 2,720 participants and was conducted over 24 months. Participants were between 11 and 14 years old, and were allocated to two intervention groups to chew either a sugar-free chewing gum or a CPP-ACP sugar-free chewing gum. Both groups chewed their allocated gum for 10 minutes three times daily for the duration of the study. At baseline and after 24 months, bitewing radiographs were taken and assessed for posterior caries diagnosis by a single examiner who was blinded to both the participants and intervention. Participants were also blinded as to which gum they received. At the conclusion of the study it was evident that chewing the CPP-ACP gum had significantly reduced progression and enhanced regression of posterior approximal caries relative to the control sugar-free chewing gum.

Prestes *et al.* [2013] used a crossover *in situ* study design to test the protective effect CPP-ACP gum has on dental erosion. Microhardness was used as an assessment of
surface hardness recovery of eroded bovine enamel tooth surfaces after intra-oral exposure while participants were chewing CPP-ACP gum, sugar-free gum or no gum. It was found that chewing the CPP-ACP significantly increased the microhardness recovery of eroded enamel compared to chewing the sugar-free gum and no gum. A similar study by de Alencar et al. [2014] also found that surface hardness recovery was significantly enhanced by CPP-ACP gum compared to the sugar-free gum and chewing no gum. Both short-term *in situ* studies advocated the use of CPP-ACP gum to enhance salivary repair of dental erosion by increasing mineralisation of mineral deficient tooth surfaces.

1.5.3.2 CPP-ACP/ACFP tooth crème

Increasing clinical evidence has demonstrated CPP-ACP/ACFP tooth crème (Tooth Mousse, Tooth Mousse Plus, MI Paste, MI Paste Plus, GC Corporation) imparts a significant remineralisation effect on incipient carious lesions when used in conjunction with regular oral hygiene.

A clinical trial by Fredrick et al. [2013] compared the use of CPP-ACP or CPP-ACFP tooth crèmes with a sodium fluoride mouthrinse in 45 participants with occlusal white spot lesions over 30 days. All interventions were used twice daily in addition to regular tooth brushing and monitored using visual assessment and laser fluorescence. It was found that use of either CPP-ACP or CPP-ACFP significantly remineralised occlusal white spot lesions when compared to use of the sodium fluoride mouthrinse. Yazıcıoğlu and Ulukapı [2014] used visual assessment, bitewing radiographs and laser fluorescence to conclude use of CPP-ACP tooth crème by participants significantly reduced progression of incipient caries and promoted a remineralisation effect when compared to the control. Güçlü et al. [2016] reported the results of a clinical trial that assessed twice daily use of CPP-ACP tooth crème in children aged 8 – 15 years old in comparison to other treatments. Over the 12 week intervention period, visual assessment and laser fluorescence found that regular application of fluoride varnish had no added benefit though CPP-ACP tooth crème was found to significantly remineralise white spot lesions compared to all other treatments.

Numerous studies have tested the remineralisation effect of CPP-ACP tooth crème for treatment of white spot lesions associated with orthodontic treatment. Bailey et al. [2009]
conducted a clinical trial on participants following orthodontic treatment that showed twice daily application of CPP-ACP tooth crème significant regressed early smooth surface caries when compared to use of a placebo paste. All subjects brushed their teeth twice daily with fluoride toothpaste and were assessed using ICDAS II criteria [Ismail et al., 2007] every 4 weeks for a total of 12 weeks. Robertson et al. [2011] reported a similar effect on participants undertaking orthodontic treatment following once daily application of CPP-ACFP tooth crème in a study of similar duration. ICDAS II criteria and a negative control placebo paste were used to show a significant preventive and remineralisation effect of CPP-ACFP on white spot lesions. An in situ crossover study by Garry et al. [2017] demonstrated that during orthodontic treatment, twice daily application of CPP-ACP tooth crème after tooth brushing with fluoride toothpaste significantly remineralised lesions when compared to twice daily tooth brushing with fluoride toothpaste alone.

The results of these studies demonstrated tooth crèmes containing CPP-ACP/ACFP have the potential to prevent caries progression and promote remineralisation in vivo, and support their recommendation for use by high caries-risk patients, particularly those undertaking orthodontic treatment.

1.5.3.3 Evidence of other positive health effects

The majority of studies assessing the effect of CPP-ACP/ACFP-containing products on plaque and saliva bacterial composition, pH and buffering capacity have concluded that CPP use has a positive effect on all of these variables in regards to oral health [Alexandrino et al., 2017; Caruana et al., 2009; Chandak et al., 2016; Emamieh et al., 2015; Fadl et al., 2016; Heshmat et al., 2014; Karabekİroğlu et al., 2017; Marchisio et al., 2010; Ozdas et al., 2015; Peric et al., 2015; Poureslami et al., 2016; Pukallus et al., 2013].

The outcomes measured in many of these publications observed effects that can indirectly enhance remineralisation, such as a high available calcium and phosphate in plaque and saliva [Poureslami et al., 2016] and an increase in saliva or plaque pH/buffering capacity [Caruana et al., 2009; Heshmat et al., 2014; Karabekİroğlu et al., 2017; Marchisio et al., 2010; Ozdas et al., 2015; Peric et al., 2015]. The effect of CPP-ACP/ACFP on saliva and
plaque bacterial composition has been observed to reduce the abundance of acid-producing mutans streptococci typically associated with caries [Chandak et al., 2016; Emamieh et al., 2015; Fadl et al., 2016; Karabekiroglu et al., 2017; Pukallus et al., 2013].

As described in 1.5.2, the buffering ability of CPPs allows a relatively higher pH to be maintained in the plaque fluid, thus inhibiting mutans streptococci that prefer an acidic environment.

In addition to these effects, use of CPP-ACP/ACFP has been shown clinically to provide relief of dentine hypersensitivity. A recent study by Alexandrino et al. [2017] demonstrated significantly reduced hypersensitivity in individuals applying CPP-ACFP tooth crème directly after bleaching teeth when compared to individuals applying a toothpaste containing bioglass 45S5 (Novamin Repair and Protect). This was attributed to a remineralisation effect of the CPP-ACFP tooth crème reducing the porosity of enamel post-bleaching. Mahesuti et al. [2014] reported regular use of CPP-ACP tooth crème for 2 weeks significantly decreased dentine hypersensitivity compared to a placebo paste. The participants in the clinical trial were observed to have a significant reduction in dentine hypersensitivity 60 days after treatment with CPP-ACP.

1.5.3.4 Systematic reviews

A systematic review with meta-analysis by Yengopal and Mickenautsch [2009] concluded that there was sufficient clinical evidence that CPP-ACP-containing products provided a short-term remineralisation effect and long-term caries-preventing effect compared to saliva and fluoride application alone.

More recently, another systematic review of clinical trials by Kecik [2017] concluded that products containing CPP-ACP/ACFP were able to prevent caries, induce remineralisation and reduce dentine hypersensitivity. The systematic review by Lopatiene et al. [2016] advocated use of CPP-ACP/ACFP-containing products to prevent and remineralise white spot lesions during and after orthodontic treatment, suggesting they were significantly more effective than fluoride treatments based on current literature. Ekambaram et al. [2017] published another review of calcium and phosphate based remineralisation systems and it was determined that CPP-ACP remineralisation agents were superior to all other calcium and phosphate remineralisation systems.
1.5.4 Future research

CPP-ACP/ACFP complexes have been shown to be the most effective remineralisation system available for clinical treatment of incipient caries. Despite their efficacy, complete remineralisation of incipient lesions is not always possible due to various challenges. Hence, further research of CPP complexes and exploration of these challenges is advocated to improve remineralisation efficacy. Furthermore, additional research is required to fully understand the anticariogenic mechanism of CPP-ACP/ACFP, in particular its effect on the bacterial composition of supragingival plaque.

1.6 Aims

The overall aim of this thesis was to enhance the remineralisation efficacy of CPP complexes and to better understand their mechanism of action. Specifically, this included:

1. Assessing the effect of intra-lesion serum albumin on remineralisation by CPP-ACFP.
2. Assessing the effect of intra-lesion pH modulation on remineralisation by CPP-ACFP.
3. Assessing the suitability of XMT for measurement of remineralisation by CPP-ACFP.
4. Assessing the effect of stannous fluoride incorporation by CPP complexes on remineralisation.
5. Assessing the effect of CPP-ACP chewing gum on *S. sanguinis* levels in supragingival plaque.
2

GENERAL MATERIALS AND METHODS
2.1 Preparation of remineralisation solutions

2.1.1 CPP-ACP/CPP-ACFP

A commercial powder of CPP-ACP (Recaldent™, Cadbury Enterprises Pte Ltd, VIC, Australia) was used for CPP-ACP solution preparations. The required amount of powder was weighed and dissolved in distilled deionised water (DDW) (Milli Q, VIC, Australia) using a magnetic stirrer. After all CPP-ACP powder was visualised to be suspended in solution, a pH probe was used to measure the pH of the solution at room temperature; 1M HCl solution or NaOH pellets were used to adjust the pH to the desired level while stirring. The preparation of CPP-ACFP solutions was done similarly with a laboratory prepared CPP-ACFP powder (Oral Health CRC, The University of Melbourne) to the desired percentage concentration.

2.1.2 CPP-ACP and stannous fluoride

The CPP-ACP solution was first prepared with the desired weight of powder for the required volume. Using a standard solution of 2200 parts per million (ppm) of fluoride as stannous fluoride, the calculated volume required of the standard was slowly pipetted into the CPP-ACP solution, taking care to wait until the pH settled between each drop in order to prevent precipitation within the solution.

2.2 Tooth preparation

2.2.1 Enamel lesion preparation

For the in vitro and in situ experiments using demineralised enamel blocks, extracted human third molars were obtained from private dental practices with any extracted soft tissues removed before the teeth were sterilized by exposure to a dose of 4.1 kGy of gamma radiation. Sound relatively planar buccal and lingual surfaces free of cracking, staining and fluorosis (as viewed under a dissecting microscope) were selected and thoroughly rinsed with DDW. The outer enamel surface was polished wet to a mirror finish using Sof-Lex™ discs (3M, MN, USA) on a slow speed contra-angle dental handpiece. Each polished surface was cut from the tooth as an approximately $8 \times 4$ mm block, using a water-cooled diamond blade saw and the whole block was then covered with acid-resistant nail varnish except for two (occlusal and gingival) mesiodistal windows (approximately $1 \times 7$ mm each) separated from each other by about 1 mm. The
blocks were then treated to create lesions in the enamel windows by suspending each block in 40 mL of unagitated demineralisation buffer, consisting of 80 mL/L Noverite K-702 polyacrylate solution (Lubrizol Corporation, OH, USA), 500 mg/L HA (Bio-Gel® HTP, Bio Rad Laboratories, CL, USA), and 0.1 mol/L lactic acid (Ajax Chemicals, NSW, Australia) pH 4.8, for 4 days at 37°C. This is a modified version of the protocol described by White [1987]. A change of solution was made after 2 days at which time the blocks were removed from the solution, rinsed thoroughly with DDW, blotted dry and placed into fresh demineralisation buffer. The blocks were then rinsed and dried after four days of demineralisation. This demineralisation procedure produced consistent subsurface lesions of approximately 100 µm depths with intact surface layers, as evaluated by microradiography of sections of the artificial lesions.

2.2.2 Dentine disc preparation

Extracted human third molars donated by private practices were inspected for defects as described in 2.2.1. A horizontal section was cut through the tooth approximately halfway up the crown using a water-cooled diamond blade saw. A parallel section was then cut approximately 1 mm below the first section, creating a dentine disc with enamel periphery. Discs were inspected under a dissecting microscope to ensure they were free from defects and the central area free of enamel. The discs were then polished using Sof-Lex™ polishing discs to ensure a smooth planar surface on the coronal aspect of the disc. The whole disc was attached to the lid of a plastic tube with dental sticky wax with the polished side facing up. Acid-resistant nail varnish was used to cover the entire enamel periphery leaving a circular window on the central dentine (approximately 5 mm in diameter). These discs were used for the experimental procedures assessing the effect of various remineralisation solutions on surface dentine.

2.3 Transverse Microradiography (TMR)

2.3.1 Embedding

In experiments assessing mineral content by TMR, the control and experimental half-blocks were paired following remineralisation. Each half-block had any remaining wax removed following remineralisation and was dried with triplex air. Paladur methacrylate powder (Heraeus Kulzer, Germany) was placed in the base of a plastic mould with a few
drops of Paladur methacrylate liquid (Heraeus Kulzer, Germany). The corresponding control and experimental enamel half-blocks were placed in the resin with the midline incision upon the base of the mould and the lesions facing inwards towards each other. Cold curing methacrylate resin was added to cover the enamel half-blocks and this was allowed to set at room temperature in a fume hood overnight. A black marker was used to indicate the experimental half-block on the plastic mould.

2.3.2 Sectioning

The embedded blocks were removed from the plastic moulds and a marker was placed on the side of the resin with the experimental half-block. These resin blocks were then sectioned with a water-cooled diamond blade saw to a rectangular shape with a cut corner on the side which contained the experimental half-block. The rectangular blocks were mounted with green dental wax and sections approximately 300 µm thick were cut from these rectangular blocks perpendicular to the lesion surface using an internal annulus saw microtome (Leica SP1600, Leica Microsystems, Germany). Three to four sections from each enamel block were cut and stored in glass microscope slides on tissue denoting the order with which each was sectioned, the first section being closest to the midline between control and experimental half-blocks.

2.3.3 Lapping

The sections for each block were cleaned of any wax and attached with clear nail varnish to a metal cylinder and left overnight to allow the varnish to set. The sections were then lapped down to between 90 – 120 µm using a RotoPol/RotoForce lapping instrument with 1200 grit lapping papers (Struers, Denmark) and water. A digital micrometer (Nikon, Japan) was used to measure the thickness of each section. The lapped sections were then removed from the lapping instrument using a razor, rinsed in DDW, blotted dry and stored on the labelled tissue between glass slides as before.

2.3.4 Microradiography

The sections were radiographed along with an aluminium stepwedge of 7 x 37.5 µm thick increments using Microchrome High Resolution glass plates (3 x 3 x 0.06 in., Microchrome, Tech Inc., CA, USA) and copper Kα radiation at 20 kV, 30 mA for eight
minutes. Each glass plate was developed in Microchrome Developer D-5C (Microchrome Tech Inc., CA, USA) for five minutes, placed into glacial acetic acid stop bath (Kodak, Coburg, Australia) for thirty seconds and then fixed in Microchrome Fixer F-4C (Microchrome Tech Inc., CA, USA) for five minutes.

2.3.5 Image analysis

The glass plates with the radiographic images of the lesions were viewed via transmitted light through a Leica DM 5500B microscope (Leica, Germany). A ProgRes® MF scan digital camera (Jenoptik, Jena, Germany) was used to acquire the images using Image-Pro Plus version 7.0 imaging software on a SciTech Imaging Workstation (SciTech, VIC, Australia). Images of the lesions and the adjacent sound enamel were scanned using the program’s line luminance function to give readings in grey values between 0 and 65000. Areas free of artifacts or cracks were selected for analysis. Each scan comprised 200 readings taken from the tooth surface to sound enamel; the start and end of the lesion were defined as the points where the mineral density was 20 % and 95 % that of the sound enamel. Six scans of the lesion and adjacent sound enamel were taken to reduce the standard error to below 2.5.

The stepwedge image on each slide was scanned and the average grey value of each step was plotted against its known aluminium thickness. The grey values of the enamel were within the linear segment of the aluminium stepwedge curve and were converted into values of equivalent aluminium thickness using linear regression. Utilising the section thickness values, the volume % mineral (vol%min) data was computed using the equation of Angmar et al. [1963] and the linear absorption coefficients of aluminium, organic matter plus water and apatite mineral (131.5, 11.3, and 260.5 respectively).

The volume % mineral profile of each enamel block’s demineralised and remineralised lesion was compared with the median adjacent sound enamel volume % mineral profile of the same section. The difference between the densitometric profile of the demineralised lesion and the median sound enamel, and of the remineralised lesion and the median sound enamel ($\Delta Z_d$ and $\Delta Z_r$ respectively) were calculated by trapezoidal integration.
Percent remineralisation ($%R$) was calculated using the % change in $\Delta Z$ values:

$$%
R = \frac{\Delta Zd - \Delta Zr}{\Delta Zd} \times 100$$

2.4 Reverse phase high performance liquid chromatography (RP-HPLC)

RP-HPLC was conducted on a Hewlett Packard Series 1100 automated system with manual injector, dual pumps, variable multi-wavelength detector and data processing software. A 5 µm reverse phased C18 column with a 300 Å pore size column was used with measurements of 4.6 mm x 250 mm (Vydac, Alltech Associates, NSW, Australia). The injector system allowed 50 – 500 µL to be injected and the multi-wavelength detector was set at either 214 or 555 nm. Samples were run through the system at 25 °C.

2.5 Scanning electron microscopy (SEM)

Examination of samples using a Scanning Electron Microscope (SEM) was done using a FEI QUANTA SEM at the Bio21 Advanced Microscopy Facility (VIC, Australia). For imaging under high vacuum, poor conducting samples were sputter-coated with gold approximately 2 nm in thickness. Energy dispersive x-ray spectroscopy (EDS) was performed on uncoated samples under low vacuum for elemental analysis.

2.6 In situ remineralisation

2.6.1 Intra-oral appliance

The appliance used for all subjects in the in situ remineralisation study has been described previously by Shen et al. [2001]. The appliances for the maxillary arch were custom-made from acrylic covering the posterior two thirds of the hard palate and clasping four posterior teeth for retention. On the palatal acrylic adjacent to the posterior teeth a recess was made and demineralised enamel blocks attached using dental sticky wax.
THE EFFECT OF BOVINE SERUM ALBUMIN ON THE
REMINERALISATION OF ENAMEL SUBSURFACE LESIONS *IN VITRO*
3.1 Introduction

When treating early uncavitated carious lesions, contemporary dental techniques aim to arrest the disease process through disruption of the biofilm and repair the mineral deficient enamel through remineralisation. The process of remineralisation involves the direct deposition of apatite crystals on the demineralised enamel crystal template from soluble ions sourced external to the tooth [Arends and Ten Cate, 1981]. Although the intrinsic organic matrix is essential for crystal nucleation and regulation for biomineralisation of enamel [Margolis et al., 2014], the presence of exogenous organic material such as albumin within partially demineralised mature enamel has been speculated to impede diffusion of ions or block crystal growth sites thereby inhibiting remineralisation [Robinson et al., 1990; Robinson et al., 1998].

Human serum albumin (HSA) is an abundant circulatory protein that contributes to the transport and metabolism of ligands within the body [He and Carter, 1992]. It has been shown to be present in saliva and gingival crevicular fluid particularly in individuals with inflammation of the periodontium [Henskens et al., 1993]. The properties of HSA allow it to adsorb to HA crystals, especially at high concentrations and at low pH [Hlady and Furedimilhofer, 1979]. Enamel carious lesions have been shown to contain higher levels of organic material such as albumin than adjacent sound enamel, with the greatest concentration of albumin apparent in the enamel zone of 10 – 20 % mineral loss [Robinson et al., 1998; Shore et al., 2000].

While the properties of albumin and its interaction with HA have been previously investigated, its effect on the remineralisation of enamel subsurface lesions is unknown. Using an in vitro remineralisation model, the effect of bovine serum albumin (BSA) on remineralisation of artificially created subsurface lesions was investigated. BSA was chosen as an inexpensive alternative to HSA as it shares high amino acid sequence similarity and chemical behaviour [Kragh-Hansen, 1981].

3.2 Objectives

1) To investigate uptake of BSA into artificial enamel subsurface lesions in vitro using immunofluorescence and confocal microscopy.
2) To assess the effect of BSA uptake on remineralisation of artificial enamel subsurface lesions in vitro.

3) To assess the effect of a deproteinising pre-treatment, sodium hypochlorite (NaOCl), on the remineralisation of artificial enamel subsurface lesions with BSA uptake in vitro.

3.3 Study methods

3.3.1 Enamel block preparation

Sixty human enamel blocks were sectioned and demineralised (as described in 2.2.1) to contain artificial lesions approximately 100 μm deep.

3.3.2 Localisation of albumin in enamel subsurface lesions

Ten of the enamel blocks with subsurface lesions had a groove cut into their undersurfaces (the opposite side of the outer enamel surface) through the dentine and just into the adjacent enamel with a small dental bur on a slow speed dental handpiece. The groove extended along the width of the block approximately half-way along the lesion to facilitate manually splitting of the enamel block near the midline of the lesion.

To localise BSA in enamel subsurface lesions, five enamel blocks were immersed for two days at 37 °C in a solution of BSA conjugated to a fluorophore (Alexa Fluor® 555, orange-red # A34786; Life Technologies Australia Pty Ltd., VIC, Australia) diluted to 1 mg/mL in 100 mM HEPES buffer at pH 4.7 (the isoelectric point of albumin to maximize enamel uptake [Van Der Linden et al., 1987]). The purity of the BSA-fluorophore conjugate was confirmed using high performance liquid chromatography (HPLC). Separately, five enamel blocks were immersed in a solution of the same concentration of Alexa Fluor® 555 alone at pH 4.7 for two days at 37°C. Only the surfaces of the lesions were exposed to the solutions. After two days of exposure the enamel blocks were briefly wiped with a cotton bud moistened in DDW, dried and manually fractured into two halves.

The resulting fractured surfaces of the half blocks were examined with an LSM510 confocal laser scanning microscope with an inverted stage (Zeiss, Germany). The
fluorophore was excited using the He-Ne laser at 543 nm and images of the subsurface lesions were obtained. Albumin was identified by the presence of red fluorescent staining associated with the fluorophore.

3.3.3 Effect of NaOCl on BSA inside enamel subsurface lesions

To determine the effect of NaOCl on the BSA-fluorophore conjugate, a sample of the BSA-fluorophore conjugate solution was incubated for two minutes in a 1:5 dilution with 134 mM NaOCl (1 % w/v, pH 12.9, Endosure Hypochlor, Dentalife Pty Ltd, VIC, Australia) then immediately analysed with HPLC. To determine the effect of NaOCl on albumin inside the subsurface lesions, ten enamel blocks were exposed to the BSA-fluorophore conjugate as described in 3.3.2 and were subsequently immersed in a 134 mM NaOCl solution for two minutes. The lesions were again manually fractured into halves and the exposed fractured surfaces were visualized with confocal microscopy as described in 3.3.2.

3.3.4 Assessing the effect of BSA and NaOCl treatment on subsequent remineralisation of enamel subsurface lesions in vitro.

To assess whether the BSA competitively bound to crystal nucleation sites within the enamel lesion thereby inhibiting remineralisation, the effect of NaOCl solution on remineralisation of lesions containing albumin was investigated. Thirty enamel blocks with subsurface lesions were cut into control and experimental half-blocks using a water-cooled diamond saw. The control half-blocks were stored in a humidified container until processed with their matching experimental half-blocks for TMR. Each experimental half-block was randomly allocated to one of three pre-treatments (n = 10):

(i) Immersion in 100 mM HEPES buffer (pH 4.7) alone for two days at 37 °C.
(ii) Immersion in 100 mM HEPES buffer (pH 4.7) with 1 mg/mL BSA for two days at 37 °C.
(iii) Immersion in 100 mM HEPES buffer (pH 4.7) with 1 mg/mL BSA for two days at 37 °C and then immersion in 500 µL of 134 mM NaOCl (pH 12.9) agitated with a vortex for two minutes at room temperature.

After the pre-treatments, the experimental half-blocks were immersed in 2 mL of a 1 % (w/v) CPP-ACFP solution (pH 5.5) for ten days at 37 °C (unagitated) with a change of solution every two days. After ten days of remineralisation in the CPP-ACFP solution,
each experimental half-block was removed from the solution, washed in DDW, and matched with its corresponding control half-block.

3.3.5 Assessing the effect of a high pH pre-treatment (NaOH) on remineralisation of enamel subsurface lesions in vitro.

Upon analysis of the results of the first remineralisation experiment, it was decided to investigate whether the mechanism of action of the NaOCl pre-treatment was primarily due to a high pH effect as opposed to an oxidative effect. Sodium hydroxide (NaOH) was chosen as an alternative high pH pre-treatment to test before remineralisation. Ten experimental half-blocks were treated by immersion in 100 mM HEPES buffer (pH 4.7) with 1 mg/mL BSA for two days at 37 °C and were then immersed in 500 µL of 134 mM NaOH (pH 12.9) agitated with a vortex for two minutes at room temperature. Following this pre-treatment, the enamel half blocks were remineralised with 1 % (w/v) CPP-ACFP as described in 3.3.4.

3.3.6 TMR

Following remineralisation, each experimental enamel half-block from the experiments described in 3.3.4 and 3.3.5 was paired with its control half-block and analysed using TMR (as described in 2.3.1) to calculate values for lesion depth after demineralisation (LDd), lesion depth after remineralisation (LDr), integrated mineral loss after demineralisation (ΔZd), integrated mineral loss after remineralisation (ΔZr) and percent remineralisation (%R).

3.3.7 Data analysis

Values obtained from TMR in the first remineralisation experiment (3.3.4) were statistically analysed using a one-way ANOVA with Tukey post hoc multiple comparison tests (Minitab Version 16, Pennsylvania, USA). The values obtained from the TMR of the second remineralisation experiment (3.3.5) were calculated and subsequently compared to the first experiment using a one-way ANOVA. For all statistical tests, the significance level was set at α = 0.05.
3.3.8 Hypotheses

The null hypotheses tested were:

1) The amount of albumin in enamel subsurface lesions after subsequent exposure to a NaOCl solution is not different to that in enamel subsurface lesions not exposed to the NaOCl solution.

2) Percent remineralisation of enamel subsurface lesions exposed to a 1 % (w/v) CPP-ACFP solution in vitro is not different if pre-treated with HEPES buffer alone, HEPES and BSA, or HEPES, BSA and NaOCl.

3) Percent remineralisation of enamel subsurface lesions exposed to 1 % (w/v) CPP-ACFP solution in vitro is not different if pre-treated with HEPES, BSA and NaOCl or pre-treated with HEPES, BSA and NaOH.

3.4 Results

3.4.1 Localisation of BSA in enamel subsurface lesions

Confocal microscopy clearly demonstrated fluorescent staining in the entire body of the test enamel subsurface lesions exposed to the BSA-fluorophore conjugate for two days, as well as intense fluorescent staining associated with some parts of the enamel surface (Figure 3.1, left). In contrast, fluorescent staining was only observed on the surface of the control enamel lesions exposed to the fluorophore alone; this staining was associated with the acid-resistant nail varnish (Figure 3.1, right). The fluorophore had greater penetration of the lesion when conjugated to BSA; this demonstrated that BSA was capable of diffusing through the porous enamel.

3.4.2 Effect of NaOCl on BSA inside enamel subsurface lesions

The BSA-fluorophore conjugate diluted in DDW showed a chromatogram with a peak at 40.8 minutes (Figure 3.3). When the conjugate complex was diluted in NaOCl, this peak disappeared and it appeared that NaOCl had rapidly oxidised and degraded the complex including the BSA as only BSA peptides remained (Figure 3.4). This demonstrated NaOCl was an effective agent to use to remove BSA from the enamel subsurface lesions. Using confocal microscopy, it was found that enamel subsurface lesions immersed in NaOCl following exposure to the BSA-fluorophore solution (Figure 3.2) contained much
less red fluorescent stain than lesions not treated with the NaOCl solution (Figures 3.1, left).

**Figure 3.1:** Confocal images of enamel subsurface lesions exposed to BSA-fluorophore conjugate showing red fluorescence throughout the lesion body (left) and exposed to fluorophore without BSA showing red fluorescence associated with nail varnish on the surface of the lesion (right).

**Figure 3.2:** Confocal image of enamel subsurface lesion exposed to BSA-fluorophore conjugate and subsequent immersion in NaOCl showing minimal fluorescence.
Figure 3.3: Chromatogram of the BSA-fluorophore conjugate diluted 1:5 in DDW; upper trace = 214 nm absorbance, lower trace = 555 nm absorbance.

Figure 3.4: Chromatogram of the BSA-fluorophore conjugate diluted 1:5 in NaOCl; upper trace = 214 nm absorbance, lower trace = 555 nm absorbance.
Table 3.1: Comparison of enamel subsurface lesion parameters assessing the effect of BSA and NaOCl pre-treatment on remineralisation (3.3.4).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LDd (µm)</th>
<th>LDr (µm)</th>
<th>ΔZd (vol% min.µm)</th>
<th>ΔZr (vol% min.µm)</th>
<th>%Remin</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES</td>
<td>109.2 ± 11.9</td>
<td>106.2 ± 12.8</td>
<td>3316.6 ± 601.5</td>
<td>2153.2 ± 417.9</td>
<td>34.8 ± 6.9</td>
</tr>
<tr>
<td>HEPES + BSA</td>
<td>111.7 ± 12.9</td>
<td>112.7 ± 10.7</td>
<td>3201.8 ± 845.4</td>
<td>2169.0 ± 477.0</td>
<td>31.4 ± 6.0</td>
</tr>
<tr>
<td>HEPES + BSA + NaOCl</td>
<td>106.7 ± 16.8</td>
<td>96.3 ± 21.4</td>
<td>2986.5 ± 725.4</td>
<td>1629.3 ± 446.9</td>
<td>45.5 ± 5.5</td>
</tr>
</tbody>
</table>

| p-value§               | NS > 0.05  | NS > 0.05  | NS > 0.05         | < 0.05            | < 0.001   |

1LDd = lesion depth after demineralisation, 2LDr = lesion depth after remineralisation, 3ΔZd = integrated mineral loss prior to remineralisation, 4ΔZr = integrated mineral loss after remineralisation, 5%R = percent remineralisation ((ΔZd - ΔZr)/ΔZd)*100 %. Displayed as mean ± standard deviation. § 1-way ANOVA (α = 0.05); NS = not significant. Differences between treatments were tested using Tukey post hoc multiple comparison tests: Values similarly marked are significantly different ab (p < 0.05), c (p < 0.01) d (p < 0.001).

Table 3.2: Enamel subsurface lesion parameters assessing the effect of BSA and NaOH pre-treatment on remineralisation (3.3.5).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LDd (µm)</th>
<th>LDr (µm)</th>
<th>ΔZd (vol% min.µm)</th>
<th>ΔZr (vol% min.µm)</th>
<th>%Remin</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES + BSA + NaOH</td>
<td>121.4 ± 14.0</td>
<td>106.5 ± 18.0</td>
<td>3385.5 ± 642.0</td>
<td>1926.2 ± 403.3</td>
<td>43.1 ± 4.7</td>
</tr>
</tbody>
</table>

1-5As described for Table 3.1. Displayed as mean ± standard deviation. * % Remineralisation for HEPES + BSA + NaOH pre-treatment showed no significant difference to HEPES + BSA + NaOCl pre-treatment from experiment 3.3.4 though was significantly different to both the HEPES and HEPES + BSA pre-treatment (p < 0.02) using Tukey post hoc multiple comparison tests.
3.4.3 Influence of intra-lesion BSA, NaOCl and NaOH pre-treatment on remineralisation of enamel subsurface lesions

The results for lesion depth, mineral content and percent remineralisation within the first remineralisation experiment (3.3.4) are summarised in Table 3.1. The mean lesion depths of the demineralised lesions were similar for all treatment groups (p > 0.05).

Percent remineralisation of lesions pre-treated with HEPES and BSA (31.4 ± 6.01 %) was not significantly different to the percent remineralisation of lesions pre-treated with HEPES alone (34.8 ± 6.9 %). The percent remineralisation of lesions pre-treated with HEPES, BSA and NaOCl (45.5 ± 5.5 %) was significantly higher than for lesions pre-treated with HEPES and BSA (p < 0.001) but was also significantly higher than lesions only pre-treated with HEPES alone (p < 0.01).

The results from the second experiment which tested the NaOH pre-treatment are shown in Table 3.2. Percent remineralisation for lesions pre-treated with NaOH after exposure to HEPES and BSA in the second remineralisation experiment (43.1 ± 4.7 %) showed no significant difference to those that had been pre-treated with the HEPES, BSA and NaOCl combination in the first remineralisation experiment. The percent remineralisation of these lesions pre-treated with NaOH was also seen to be significantly higher than the lesions pre-treated with HEPES and BSA or HEPES alone in the first experiment (p < 0.02). This suggested that pre-treatment with either NaOCl or NaOH was having an equal and positive effect on remineralisation.

3.5 Discussion

3.5.1 Localisation of BSA in enamel subsurface lesions

It was evident that BSA diffused into the body of subsurface lesions during the two day exposure. This was consistent with previous literature demonstrating BSA infiltrated enamel lesions [Van Der Linden et al., 1987]. As the molecular weight of the highly globular fluorescent-albumin complex was relatively small at 67.71 kDa (66.46 kDa for BSA and 1.25 kDa for fluorophore), diffusion of the fluorescent-albumin complex into the porous subsurface lesions was expected. Van Der Linden et al. [1987] used autoradiography to show $^{14}$Ca-labeled BSA suspended in a HEPES buffer solution.
penetrated artificially demineralised bovine enamel subsurface lesions in vitro to a depth of up to 120 µm at pH 4.7 and 6.8. In that study, BSA penetration into the lesion was greater when the pH was 4.7 as opposed to 6.8; this was attributed to pH 4.7 being the isoelectric point of BSA to allow the protein to diffuse into the lesion having minimal interaction with the charged enamel surface. This was in agreement with the present study which showed lesion infiltration of the BSA-fluorophore conjugate occurred at pH 4.7. It would be expected that BSA would be present both free in the lesion and adsorbed onto HA crystals with the equilibrium determined by the affinity of BSA for the HA crystal surface [Hlady and Furedimilhofer, 1979; Wassell et al., 1995].

3.5.2 Effect of NaOCl on BSA inside enamel subsurface lesions

It appeared that when lesions were treated with NaOCl following exposure to the BSA-fluorophore conjugate, the NaOCl solution diffused into the lesion and broke down the BSA (and the fluorophore). The chromatograms from the HPLC analysis revealed the NaOCl was effective in degrading the fluorophore and the BSA when it was incubated with the conjugate solution (see Figures 3.3 and 3.4). This was expected and suggested minimal intact BSA was present in the enamel subsurface lesions following the two minute exposure to the NaOCl solution. These results are consistent with Robinson et al. [1990] who utilised NaOCl as a non-specific proteolytic solution to solubilise and degrade organic matter within natural carious lesions.

3.5.3 Influence of intra-lesion BSA, NaOCl and NaOH pre-treatment on remineralisation of enamel subsurface lesions

The effect of exogenous protein within enamel has been debated, with some evidence indicating a positive effect in terms of caries prevention and inhibition of demineralisation. Protein has been shown to inhibit dissolution of HA in vitro [Reynolds et al., 1982] and albumin added to a demineralising buffer was shown to inhibit demineralisation and potentially limit lesion progression in enamel in vitro [Arends et al., 1986; Van Der Linden et al., 1987]. Bibby [1971] suggested organic material may replace dissolved enamel mineral inside carious lesions and subsequently increase caries resistance.
Exogenous proteins which have infiltrated carious enamel lesions have also been hypothesised to inhibit remineralisation by binding to partially-dissolved HA crystals or occupying enamel pores. For example, deproteination of enamel lesions using NaOCl has been shown to enhance uptake of calcium in natural carious lesions in vitro [Robinson et al., 1990]; the authors suggested pores within the surface zone may be partially occluded by salivary proteins thereby inhibiting diffusion of ions to deeper parts of the lesion and restricting remineralisation to the outer surface of the lesion. As remineralisation of the surface zone proceeds, diffusion of ions to the body of the lesion is increasingly impeded.

Salivary proteins have been reported to both bind to and inhibit nucleation of HA crystals in vitro using constant composition/seeded growth models [Johnsson et al., 1991; Moreno et al., 1979]. Similar experiments have been conducted assessing the effect of serum albumin on HA crystal growth that revealed a comparable inhibition of growth [Garnett and Dieppe, 1990; Gilman and Hukins, 1994b, a]. However, it should be noted that these experiments were conducted over a relatively short period (1 – 3 hours) and in two of the studies the data were produced from a single experiment without repetition [Gilman and Hukins, 1994b, a]. These same two studies used crushed HA powder as seeds for crystal growth, thereby not standardising the available surface area for crystal nucleation between experiments. The variable surface morphology of the HA powdered seeds may have encouraged protein aggregation and would not have mimicked the demineralised yet well-defined spatial arrangement of crystals within an enamel subsurface lesion. Additionally, these constant composition or seeded growth models did not factor the variable pH of enamel lesion fluid which affects the binding ability of albumin, as well as the calcium-binding capacity of dissolved albumin which would have decreased the DS with respect to HA and discouraged crystal growth.

The results of the current study showed that infusing a subsurface lesion with BSA had little influence on subsurface remineralisation under the conditions studied. This suggested that BSA was not impeding ion diffusion through the enamel pores or blocking nucleation sites for crystal growth. BSA binds to HA primarily through carboxyl groups that are attracted to calcium on the crystal surface. However, free phosphate ions are known to have 20 times greater affinity for HA than the carboxyl groups of proteins, and
preferentially bind to the exposed calcium of HA crystals to displace bound proteins [Bernardi and Kawasaki, 1968; Wassell et al., 1995]. While the BSA may have slightly reduced the bioavailable calcium within the lesion by its ability to bind free calcium, it has little affinity for phosphate ions and its weak bond to HA crystals would have been unlikely to significantly block crystal growth sites and impede remineralisation explaining the insignificant effect observed in this study. As pH levels rise HSA has been demonstrated to have a reduced affinity to HA due to charge repulsion [Hlady and Furedimilhofer, 1979]. Remineralisation in vivo typically occurs at higher pH values than the isoelectric point of HSA, hence these results taken together suggest that HSA in natural caries lesions may have little impact on remineralisation.

The current study presented evidence that pre-treatment with a solution of high pH such as 134 mM NaOCl or 134 mM NaOH increased remineralisation. The higher remineralisation that was observed following pre-treatment with NaOCl or NaOH compared with lesions exposed to HEPES alone indicated that removal of BSA inside the subsurface lesion was not responsible for the increased remineralisation but rather the increase was associated with another mechanism. NaOCl is a strong oxidising agent that degrades and solubilises proteins; chlorine within the hypochlorite ion (OCl⁻) behaves as Cl⁺, disrupting carbon double bonds, amide bonds, amino groups and thiol groups and reducing to Cl⁻ [Fukuzaki, 2006]. In comparison, NaOH is known to decrease protein aggregation and increase protein solubility without an oxidising effect. NaOH has been used to remove protein from enamel however its deproteinising effect is a slow process requiring up to 4 days exposure [Eimar et al., 2012]. The current experiment exposed the enamel lesions to NaOH for 2 minutes which would have had a minor deproteinising effect unlikely to be equivalent to NaOCl over such a short time frame. Regardless of whether any removal of BSA occurred during the 2 minutes exposure time, both the NaOH and NaOCl pre-treatments produced higher remineralisation than the enamel lesions exposed to HEPES alone. This suggested that a common characteristic of the NaOCl and NaOH pre-treatments was enhancing remineralisation, and the high pH of both solutions was the most logical explanation. It is worth noting that the lesions were prepared by exposure to a demineralisation buffer with a pH of 4.8. The intra-lesion pH before commencement of remineralisation by the pH 5.5 CPP-ACFP solution therefore
would be low. Hence the short pre-treatment with NaOCl or NaOH (at pH 12.9) would have substantially increased the intra-lesion pH.

It has been reported previously that in vitro treatment of enamel subsurface lesions with casein phosphopeptide remineralisation solutions are most effective at a pH of 5.5 [Cochrane et al., 2008]. This was associated with the activity of the neutral ion pairs CaHPO$_4^0$ and HF$^0$ being highest at pH 5.5, which maximised diffusion of calcium, phosphate and fluoride deep into the lesion without binding to superficial enamel crystals. Once diffused into the lesion, the ion pairs dissociate to maintain equilibrium of calcium, phosphate and fluoride ions in the lesion fluid and react to form apatite phases such as HA (Ca$_{10}$(PO$_4$)$_6$(OH)$_2$) or FHA (Ca$_{10}$(PO$_4$)$_6$(OH)F). The formation of these phases follows these reactions:

\[
10 \text{Ca}^{2+} + 6 \text{HPO}_4^{2-} + 2 \text{H}_2\text{O} \leftrightarrow \text{Ca}_{10}((\text{PO}_4)_6(\text{OH})_2) + 8 \text{H}^+ \quad [1]
\]

\[
10 \text{Ca}^{2+} + 6 \text{HPO}_4^{2-} + \text{F}^- + \text{H}_2\text{O} \leftrightarrow \text{Ca}_{10}((\text{PO}_4)_6(\text{OH})\text{F}) + 7 \text{H}^+ \quad [2]
\]

The effect of the NaOCl or NaOH pre-treatment favoured an increase in the rate of these reactions. The pre-treatment of the lesions with these solutions would have raised the intra-lesion pH, increasing the supersaturation with respect to apatite phases [Larsen, 1975], and accordingly increasing the rate of reactions [1] and [2]. Consequently, the free calcium, phosphate and fluoride ions in the lesion fluid would have decreased, promoting diffusion to replace these ions in the lesion fluid once exposed to the CPP-ACFP solution. Both reactions [1] and [2] produce hydrogen ions; however, the hydroxide ions within the lesion provided by the pre-treatment solutions would have acted as a buffer thereby additionally favouring a forward shift in both reactions. Hence it can be postulated that although diffusion of ions into the lesion from a CPP-ACFP solution occurs most efficiently at a slightly acidic pH, the remineralisation reactions [1] and [2] occur more readily in the lesion at a higher intra-lesion pH.

Results from the current study demonstrated BSA can penetrate enamel lesions without significantly inhibiting remineralisation and can be effectively broken down and removed by NaOCl treatment. Enhanced remineralisation of subsurface lesions following pre-
treatment with NaOCl in this study was consistent with the results reported by Robinson 
et al. [1990] who found that calcium uptake increased when subsurface lesions were pre-
treated with NaOCl for four hours at 20°C. However, the current study demonstrated that
the mechanism of increased remineralisation after NaOCl pre-treatment was more closely
associated with the high pH of the NaOCl solution. This was evident in samples
remineralised after pre-treatment with a solution of equivalent pH (NaOH). Application
of high pH pre-treatment solutions such as NaOCl or NaOH may significantly enhance
remineralisation of early enamel caries lesions by remineralising agents such as CPP-
ACFP by increasing intra-lesion pH to drive remineralisation.

3.6 Conclusions
1. Artificially-created enamel subsurface lesions immersed in a fluorescent-labeled
BSA solution at pH 4.7 demonstrated significant penetration of BSA into the lesion body.

2. Application of 134 mM (1 % w/v) NaOCl to lesions following BSA uptake
indicated loss of fluorescence in the lesion; this result together with the HPLC analysis
demonstrated almost complete degradation of the BSA-fluorophore conjugate (including
the BSA) by the NaOCl treatment.

3. Remineralisation of lesions pre-treated with BSA and HEPES was not
significantly different to lesions pre-treated with HEPES alone (p > 0.05); this suggested
the presence of BSA within the lesion had little effect on remineralisation under the
conditions studied.

4. Remineralisation of lesions pre-treated with 134 mM NaOCl or 134 mM NaOH
at an equivalent pH of 12.9 after exposure to the HEPES and BSA solution was
significantly greater than remineralisation of lesions exposed to the HEPES and BSA
solution alone (p < 0.001) and HEPES alone (p < 0.02). This suggested the high pH of
the NaOCl and NaOH solutions had a positive effect on remineralisation by raising the
intra-lesion pH.
THE EFFECT OF HYPOCHLORITE AND SODIUM HYDROXIDE ON THE REMINERALISATION OF ENAMEL SUBSURFACE LESIONS BY CPP-ACFP IN AN IN VITRO CYCLE MODEL
4.1 Introduction

The diffusion of ions through the relatively intact surface layer of a carious lesion is the rate limiting step for remineralisation. It is affected by the pH and ionic concentrations of the plaque and enamel fluid as well as the presence of organic material [Cochrane et al., 2008; Rose, 2000b; Zahradnik et al., 1976]. Diffusion of ions from a CPP-ACFP remineralisation solution has been demonstrated to be most efficient at a pH of 5.5 [Cochrane et al., 2008]. As the DS with respect to apatite is increased with a rise in pH [Larsen, 1975], and as previous evidence has shown that a high pH pre-treatment enhances remineralisation of enamel subsurface lesions by CPP-ACFP (see Chapter 3), an in vitro cyclic remineralisation model was tested to assess the effect of cyclic intra-lesion pH modulation on remineralisation with CPP-ACFP.

The intra-lesion pH modulation was designed to increase the rate of remineralisation by alternately encouraging ion diffusion into the lesion at low pH and driving remineralisation within the lesion at high pH. As discussed in 3.5.3, remineralisation causes an increase in the intra-lesion concentration of H⁺ and acidic phosphate ion species which decreases the DS with respect to apatite. By periodically raising the intra-lesion pH it was hypothesised that the level of supersaturation with respect to apatite would be restored, accelerating remineralisation and facilitating diffusion of ions upon subsequent exposure to CPP-ACFP. The high pH solutions tested were NaOCl and NaOH at an equivalent pH of 12.9. To ensure that any result observed was not due to simply a washing out effect, DDW was used as a third treatment between remineralisation periods.

4.2 Objectives

1) To assess the effect of cyclic exposure to NaOCl on the remineralisation of artificial enamel subsurface lesions in vitro by CPP-ACFP.

2) To assess the effect of cyclic exposure to NaOH on the remineralisation of artificial enamel subsurface lesions in vitro by CPP-ACFP.

3) To assess the effect of cyclic intra-lesion pH modulation on the remineralisation of artificial enamel subsurface lesions in vitro by CPP-ACFP.
4) To increase the rate of remineralisation by CPP-ACFP using cyclic intra-lesion pH modulation.

4.3 Study methods

4.3.1 Enamel block preparation

Forty human enamel blocks were demineralised as described in 2.2.1 and halved using a water-cooled diamond edge saw. One half of the block was used as the control half-block and the other as the experimental half-block.

4.3.2 Remineralisation cycling

4.3.2.1 Cyclic pH modulation

Thirty of the experimental half-blocks with subsurface lesions were randomly allocated into 3 groups (n = 10). Each group of experimental half-blocks was mounted with dental sticky wax to the lid of a sealed jar to allow the lesions of each of the half-blocks to be immersed in 2 mL solution as listed below. The three groups of enamel blocks were exposed to one of the following solutions for 10 minutes:

(i) DDW
(ii) 134 mM (1 % (w/v)) NaOCl (pH 12.9)
(iii) 134 mM NaOH (pH 12.9)

Afterwards, each experimental half-block was briefly washed with DDW before 50 minutes exposure to 1 % (w/v) CPP-ACFP solution at pH 5.5. The half-blocks were then rinsed briefly with DDW before restarting this one hour cycle. A total of 105 cycles were completed with seven cycles per day over fifteen non-consecutive days. Between experimental periods the half-blocks were suspended in air above DDW in a sealed jar. All solutions were maintained at 37 °C and changed daily.

4.3.2.2 Short-term remineralisation with cyclic pH modulation

Five of the experimental half-blocks were allocated to a short-term cycle model with treatment solutions of higher concentrations. The half-blocks were mounted as described in 4.3.2.1 and exposed to 300 mM NaOH (pH 12.9) for 2 minutes followed by 10 % (w/v) CPP-ACFP at pH 5.0 for 10 minutes. This 12 minute cycle was repeated 20 times so that the half-blocks were exposed to the remineralisation solution for a total of 200 minutes.
and an overall treatment time of 4 hours. The experimental half-blocks were briefly rinsed with DDW and blotted dry between treatments. An additional five experimental half-blocks were mounted as described in 4.3.2.1 and exposed to 10 % CPP-ACFP at pH 5.0 for 200 minutes continuously (without any cycling) as a control group. All experimental half-blocks were briefly rinsed with DDW and blotted dry at the end of the experimental period.

4.3.2.3 Sectioning and microradiography

Each experimental half-block was paired with its corresponding demineralised control half-block and analysed using TMR as described in 2.3.1. Lesion depth after demineralisation (LDd), lesion depth after remineralisation (LDr), integrated mineral loss after demineralisation (ΔZd), integrated mineral loss after remineralisation (ΔZr) and percent remineralisation (%R) were calculated.

4.3.2.4 Data analysis

The values calculated from the TMR analysis in the first remineralisation experiment (4.3.2.1) were statistically compared between groups using a one-way ANOVA with post hoc multiple comparison tests [Sokal and Rohlf, 1969]. The values obtained from treatment groups in the second remineralisation experiment (4.3.2.2) were compared using a two sample t-test. All statistical tests were performed with Minitab statistical software (Version 16, Pennsylvania, USA). Using the values obtained in 4.3.2.3, the rate of remineralisation was calculated for the short-term remineralisation cycle and expressed as moles of apatite per meters squared per second (mol apatite/m²/s). Although it is recognised that the rate of remineralisation is unlikely to be linear [Gao et al., 1993], for the purposes of comparisons the rate of remineralisation was calculated with the assumption that it occurred at a constant rate over the experimental period.

4.3.2.5 Scanning electron microscopy – energy-dispersive x-ray spectroscopy (SEM-EDS)

Selected microradiography slides embedded with samples from the NaOCl treatment group of the first remineralisation experiment (4.3.2.1) were dehydrated and examined in an SEM at 15 kV under low vacuum to assess elemental composition using SEM-EDS (FEI, Quanta, USA - Bio21 Advanced Microscopy Facility, VIC, Australia).
4.3.2.6 Hypotheses

The null hypotheses tested were:

1) Percent remineralisation of lesions periodically exposed to CPP-ACFP and either DDW, NaOCl or NaOH in a 105 hour in vitro remineralisation cycle were not different.

2) Percent remineralisation of lesions periodically exposed to CPP-ACFP and NaOH in a 4 hour in vitro remineralisation cycle were not different to the control group.

4.4 Results

4.4.1 Remineralisation with cyclic pH modulation

Table 4.1 shows the values calculated from TMR for each treatment group in the first remineralisation experiment (4.3.2.1). No significant differences were found in demineralised lesion depths between treatment groups. All groups were significantly different in percent remineralisation of enamel subsurface lesions (p < 0.001). The percent remineralisation of the NaOH (pH modulation only) treatment group (43.8 ± 6.9 %) was significantly higher than that for the DDW group (28.2 ± 5.8 %, p < 0.001) and that for the NaOCl group (0.8 ± 11.0 %, p < 0.001).

Surface level remineralisation, demineralisation at the advancing lesion front and a surface layer precipitation was evident in the NaOCl treated samples, with the overall remineralisation being only 0.8 % with a high variability. Figure 4.1 shows a distinct surface precipitation layer weakly bonded above a lesion in the NaOCl group, with demarcated radiolucencies around the periphery of the lesion (advancing front) indicating further demineralisation had occurred. Photographs taken of treated enamel half-blocks before processing indicated that this precipitate was present on all lesions in the NaOCl group (see Figure 4.2). To accurately represent the changes within the lesion, the mineral profile and lesion depths in the NaOCl group were calculated from the enamel surface, not from the surface of the precipitation. Not all lesions in the NaOCl group showed a distinct radiolucency around the base of the lesion, though it was clear that demineralisation had occurred in the majority of lesions in the group.
Due to the unexpected result of the surface precipitate forming in the NaOCl treatment group, an energy-dispersive x-ray spectroscopy (SEM-EDS) analysis was done investigating the composition and structure of the precipitate. Elemental weight percentage from the SEM-EDS analysis is shown in Table 4.3. The calcium phosphate and fluoride ratio (Ca : PO₄ : F) of the surface precipitate was calculated to be 10 : 6.5 : 1.36. This indicated a fluoridated apatite precipitated on the surface of the lesions. Figure 4.3 shows a representative image that was used for the elemental analysis.
### Table 4.1: Comparison of enamel subsurface lesion parameters before and after remineralisation following different cyclic treatments over 15 days (4.3.2.1).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LDd (μm)</th>
<th>LDr (μm)</th>
<th>ΔZd (vol%min.μm)</th>
<th>ΔZr (vol%min.μm)</th>
<th>ΔZd-ΔZr (vol%min.μm)</th>
<th>%Remin&lt;sup&gt;6&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDW</td>
<td>112.0 ± 18.1</td>
<td>110.2 ± 22.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2923.5 ± 903.3</td>
<td>2100.5 ± 678.8&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>823.0 ± 299.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>28.2 ± 5.8&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>NaOCl</td>
<td>103.3 ± 14.0</td>
<td>108.7 ± 13.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2523.9 ± 420.4</td>
<td>2491.9 ± 427.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>32.1 ± 282.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.8 ± 11.0&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>NaOH</td>
<td>111.4 ± 12.6</td>
<td>88.2 ± 8.5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2980.0 ± 638.6</td>
<td>1668.5 ± 392.5&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>1311.5 ± 354.9&lt;sup&gt;e&lt;/sup&gt;</td>
<td>43.8 ± 6.9&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>p-value</td>
<td>NS &gt; 0.05</td>
<td>&lt; 0.001</td>
<td>NS &gt; 0.05</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

1<sup>LDd</sup> = lesion depth after demineralisation, 2<sup>LDr</sup> = lesion depth after remineralisation, 3<sup>ΔZd</sup> = integrated mineral loss prior to remineralisation, 4<sup>ΔZr</sup> = integrated mineral loss after remineralisation, 5<sup>ΔZd-ΔZr</sup> = gain in mineral content after remineralisation; 6<sup>%R</sup> = percent remineralisation ((ΔZd-ΔZr)/ΔZd)*100 %). Displayed as mean ± standard deviation. § 1-way ANOVA (α = 0.05) NS = not significant. Differences between treatments were tested using Tukey HSD post hoc multiple comparison tests: abcdedef Values similarly marked are significantly different (p < 0.001).

### Table 4.2: Comparison of enamel subsurface lesion parameters before and after remineralisation following a 4 hour treatment cycle (10 % CPP-ACFP / 300 mM NaOH) or 200 minute remineralisation (10 % CPP-ACFP) (4.3.2.2).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LDd (μm)&lt;sup&gt;1&lt;/sup&gt;</th>
<th>LDr (μm)&lt;sup&gt;2&lt;/sup&gt;</th>
<th>ΔZd (vol%min.μm)&lt;sup&gt;3&lt;/sup&gt;</th>
<th>ΔZr (vol%min.μm)&lt;sup&gt;4&lt;/sup&gt;</th>
<th>ΔZd-ΔZr (vol%min.μm)&lt;sup&gt;5&lt;/sup&gt;</th>
<th>%Remin&lt;sup&gt;6&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPP-ACFP / NaOH cycle</td>
<td>138.5 ± 14.6</td>
<td>124.7 ± 12.6</td>
<td>3685.6 ± 659.3</td>
<td>2837.5 ± 542.0</td>
<td>848.1 ± 197.5</td>
<td>23.1 ± 3.4</td>
</tr>
<tr>
<td>CPP-ACFP</td>
<td>122.7 ± 17.2</td>
<td>114.2 ± 13.1</td>
<td>3328.6 ± 403.7</td>
<td>3265.0 ± 389.8</td>
<td>63.6 ± 46.7</td>
<td>1.9 ± 1.3</td>
</tr>
<tr>
<td>p-value</td>
<td>NS &gt; 0.05</td>
<td>NS &gt; 0.05</td>
<td>NS &gt; 0.05</td>
<td>NS &gt; 0.05</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

1<sup>LDd</sup> = lesion depth after demineralisation, 2<sup>LDr</sup> = lesion depth after remineralisation, 3<sup>ΔZd</sup> = integrated mineral loss prior to remineralisation, 4<sup>ΔZr</sup> = integrated mineral loss after remineralisation, 5<sup>ΔZd-ΔZr</sup> = gain in mineral content after remineralisation; 6<sup>%R</sup> = percent remineralisation ((ΔZd-ΔZr)/ΔZd)*100 %. Displayed as mean ± standard deviation. § Two sample t-test (α = 0.05) NS = not significant. As described in Table 4.1. Displayed as mean ± standard deviation. § Two sample t-test (α = 0.05) NS = not significant.
**Figure 4.1:** Microradiography image of an enamel subsurface lesion from the NaOCl group (4.3.2.1). The surface precipitate (A) and deep demineralisation (B) are indicated.

**Figure 4.2:** Experimental (left) and demineralised (right) enamel half-blocks showing the precipitation growth on the experimental half-block from the NaOCl group (4.3.2.1).
Table 4.3: Elemental weight percentage of the surface precipitate from the NaOCl group (4.3.2.1). Analysis using SEM-EDS was conducted on the highlighted region in Figure 4.3.

<table>
<thead>
<tr>
<th>Element</th>
<th>C</th>
<th>O</th>
<th>F</th>
<th>Na</th>
<th>Mg</th>
<th>P</th>
<th>Cl</th>
<th>Ca</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt%</td>
<td>17.8</td>
<td>38.0</td>
<td>1.8</td>
<td>0.6</td>
<td>0.3</td>
<td>13.9</td>
<td>0.2</td>
<td>27.6</td>
</tr>
</tbody>
</table>

Figure 4.3: SEM image of a microradiography slide with a sample from the NaOCl group. The area used for SEM-EDS analysis (the precipitate) is indicated in the box. The upper third of the image above the box is enamel.

4.4.2 Short-term remineralisation with cyclic pH modulation

The values from the TMR analysis for the short-term remineralisation cycle and the remineralisation control (4.3.2.2) are shown in Table 4.2. After the 4 hour cycle treatment
a mean of 23.1% remineralisation was observed, significantly higher than the control group that was observed to have a mean of 1.9% remineralisation after 200 minutes. The level of remineralisation in 4 hours through pH cycling was similar to that achieved after 105 hours by cyclic exposure to CPP-ACFP and DDW (see Tables 4.1 and 4.2). Assuming a linear rate of remineralisation occurred, the rate of mineral deposition during the cyclic remineralisation was calculated to be $2.2 \times 10^{-6}$ mol apatite/m$^2$/s.

### 4.5 Discussion

#### 4.5.1 Cyclic pH modulation

Previous authors have presented evidence to suggest changes to the mineral content of the enamel surface can increase diffusion and bioavailability of ions for remineralisation whether it be through a pre-treatment such as acid etching [Al-Khateeb et al., 2000; Flaitz and Hicks, 1993], or by increasing the acidity of the remineralisation solution [Flaitz and Hicks, 1996; Yamazaki and Margolis, 2008]. By increasing the acidity of the remineralisation solution, Yamazaki and Margolis [2008] hypothesised a low pH in the surface enamel would cause an undersaturation and dissolution of Ca$_2$AP, increasing porosity and allowing diffusion of ions deeper into the lesion where a supersaturation of less soluble apatites such as FA would be favoured to crystallise. Robinson et al. [1990] similarly attempted to increase the porosity of the surface enamel for remineralisation by removing absorbed organic matter with a NaOCl solution, inadvertently increasing the lesion pH. This was also found to increase calcium uptake in the underlying demineralised enamel. The results of the current study support the theory that an acidic remineralisation solution is effective in promoting diffusion of ions into an enamel subsurface lesion, but also that cyclic pH modulation is very effective in increasing the rate of remineralisation. This was particularly evident in the remineralisation cycling between NaOH and CPP-ACFP for the 4 hour cycle remineralisation experiment. This effect can be attributed to two aspects of the remineralisation process that are influenced by pH: the delivery (diffusion) of external calcium phosphate and fluoride ions into the lesion from the external solution and the DS with respect to HA, FHA and FA in the lesion fluid.

Within the plaque and enamel fluid, dissolved calcium, phosphate and fluoride are present in equilibrium as various ions or ion pairs; calcium exists as $\text{Ca}^{2+}$, $\text{CaPO}_4^-$, $\text{CaHPO}_4^0$, $\text{CaF}_2$, $\text{CaF}_2^-$, $\text{F}^-$.
CaH₂PO₄⁺, CaOH⁺ and CaF⁺, phosphate additionally as PO₄³⁻, HPO₄²⁻, H₂PO₄⁻, H₃PO₄⁰, and fluoride additionally as F⁻ and HF⁰. The pH of the fluid surrounding the enamel influences the concentration of each of these ion species which in turn affects the ability of the ions to diffuse into a carious subsurface lesion [Featherstone et al., 1981]. The study by Cochrane et al. [2008] positively correlated the activity of the neutral ion pairs with the rate of remineralisation in an *in vitro* model. This study demonstrated that diffusion of calcium phosphate and fluoride ions through a surface layer into a mineral deficient enamel lesion was most efficient when there was a high activity of neutral ion pairs such as CaHPO₄⁰ and HF⁰ in the remineralisation solution that were able to pass through the enamel lesion surface layer without reacting with the electrochemically charged enamel crystal faces near the surface [Cochrane et al., 2008]. The highest rate of remineralisation with CPP-ACFP observed by Cochrane et al. [2008] occurred at a slightly acidic pH of 5.5, and the same pH level was used in this study as this is the optimal pH for the remineralisation solution to produce neutral ion pairs to drive diffusion.

In the context of dental caries and erosion, acidic environments are generally thought to be detrimental to the dental hard tissues. The CadAP of human enamel and dentine decreases in DS as the surrounding fluid pH decreases. The phases that are promoted during remineralisation reactions are HA, FHA and FA that similarly decrease in DS under acidic conditions. While the rate of ion diffusion through the porosities at the enamel lesion surface layer may be maximised at a slightly acidic pH of 5.5, in the body of the lesion at the site of crystal growth a higher pH of the lesion fluid increases the DS with respect to these phases and favours an increase in ion deposition into crystal voids, increasing the rate of remineralisation. This was shown to be the case in the previous study (see Chapter 3) and the current study as cyclic NaOH exposures increased the intra-lesion pH between CPP-ACFP exposures and accelerated mineral deposition, more so than the cyclic DDW exposure which would have had little effect on intra-lesion pH.

Apart from changing the DS within the lesion with respect to apatite, another expected effect of treatment with NaOH would be an ultimate reduction in the concentration of calcium, phosphate, and fluoride ions within the lesion due to enhanced remineralisation.
Increasing the supersaturation of the enamel fluid with a high pH would have promoted ion deposition to the point where the majority of the intra-lesion calcium, phosphate and fluoride ions would have been consumed by reaction into the mineral phase. Upon subsequent exposure to the low pH remineralisation solution, the low ionic content of the enamel intra-lesion fluid would have helped to drive diffusion of ions into the lesion thus maximising the bioavailability of these ions for further remineralisation upon the next high pH cycle. As stated in 3.5, the process of remineralisation with HA and FHA follows reactions:

\[
10 \text{Ca}^{2+} + 6 \text{HPO}_4^{2-} + 2 \text{H}_2\text{O} \leftrightarrow \text{Ca}_{10}((\text{PO}_4)_6(\text{OH})_2 + 8 \text{H}^+ \quad [1]
\]

\[
10 \text{Ca}^{2+} + 6 \text{HPO}_4^{2-} + \text{F}^- + \text{H}_2\text{O} \leftrightarrow \text{Ca}_{10}((\text{PO}_4)_6(\text{OH})\text{F} + 7 \text{H}^+ \quad [2]
\]

Following exposure to NaOH, a high concentration of OH\(^-\) was likely to be present within the lesion. The excess H\(^+\) produced during reactions [1] and [2] would have been buffered by OH\(^-\) and the equilibrium shifted to the right, thereby further promoting the formation of HA and FHA.

Based on the results from the first remineralisation experiment (4.3.2.1), the null hypothesis that percent remineralisation of lesions periodically exposed to CPP-ACFP and either DDW, NaOCl or NaOH in a 105 hour in vitro remineralisation cycle were not different was rejected, and this was primarily because of pH modulation. An unexpected consequence of NaOCl treatment was also observed to reject this hypothesis, and this is discussed in 4.5.2.

4.5.2 NaOCl treatment

The oxidising effect of NaOCl negated the most advantageous attribute of CPPs, which is to stabilise high concentrations of calcium phosphate and fluoride in solution and deliver these soluble bundles into a subsurface demineralised lesion for remineralisation. Following treatment with the NaOCl solution, hypochlorite ions (OCl\(^-\)) remained in the lesion and appeared to diffuse out of the lesion as hypochlorous acid (HOCl\(^0\)) when exposed to the low pH CPP-ACFP remineralisation solution. This resulted in breakdown of the CPPs and destabilization of the CPP-ACFP complexes causing precipitation of FA
on the enamel surface. Figure 4.4 shows the activity of HOCl\(^0\) as a function of pH. When the pH drops to 5.5, nearly all OCl\(^-\) is converted to HOCl\(^0\) [Fukuzaki, 2006]. HOCl\(^0\) is a strong oxidising agent, but it is also a weak acid with a neutral charge. As the current study involved the enamel blocks being subjected to a total of 87.5 hours immersion in the remineralisation solution, it was likely that such a prolonged period of exposure to HOCl\(^0\) caused further demineralisation within the lesion and this was evident from the microradiographic images (see Figure 4.1, B). The neutrality and weak acidity of HOCl\(^0\) allowed it to diffuse deep into the lesion and dissociate to release H\(^+\) at the advancing front of the lesion to cause demineralisation.

![Figure 4.4: Distribution of HOCl\(^0\) as a function of pH (in aqueous solution; A and B represent the absence and presence of 100 mM NaCl respectively) [Fukuzaki, 2006].](image)

As detected from SEM-EDS analysis of the precipitate, the calcium, phosphorous and fluoride ratio indicated a fluoridated apatite-like phase had crystallised on the enamel surface. This was expected as the ratio of ions in the CPP-ACFP solution is designed to promote fluoridated apatite formation in remineralisation [Cross et al., 2004]. The low levels of sodium (0.6 %) and chlorine (0.2 %) incorporated in the precipitate can be
explained by the sodium and chlorine in the NaOCl solution and chlorine in the hydrochloric acid used to adjust the pH during preparation of the CPP-ACFP solution. The relatively high percentage of carbon (17.8 %) suggested that fragments (degradation products) of CPPs were also present within the precipitate.

The pH of the fluid on the surface enamel was cycled between 5.5 and 12.9 during the experiment. Within this pH range, particularly if the CPPs were degraded, it was highly likely that the free, unstabilised calcium phosphate and fluoride ions were supersaturated with respect to fluoridated apatite and precipitated and/or heterogeneously nucleated on the enamel surface.

4.5.3 The effect of short-term remineralisation with cyclic pH modulation

The short-term remineralisation cycle was conducted to assess whether the rate of remineralisation could be increased by cyclic pH modulation using NaOH, negating the requirement for prolonged exposures to remineralisation solutions. As the remineralisation observed after the 4 hour remineralisation treatment cycle was significantly higher than that observed after the 200 minute CPP-ACFP treatment, the null hypothesis was rejected. Previous in vitro enamel remineralisation studies using laboratory formulated CPP-ACP/CPP-ACFP solutions have been conducted over longer periods of time where 10 days has been commonly used [Cao et al., 2013; Cochrane et al., 2008; Reynolds, 1997] as well as 30 days [Mayne et al., 2011]. The highest average remineralisation of enamel subsurface lesions with similar initial lesion depth to the current study was seen by Reynolds [1997] with an average remineralisation of 63.9 ± 20.1 % over 10 days using 1 % CPP-ACP at pH 7.0. The highest rate of in vitro remineralisation using CPP-ACFP was observed by Cochrane et al. [2008] with an average of 57.7 ± 8.4 % remineralisation after 10 days with 2 % CPP-ACFP at pH 5.5. The mean rate of remineralisation in that study was calculated to be 7.3 x 10^{-8} mol apatite/m^2/s. In comparison, the short-term remineralisation cycle in the current study had a remineralisation rate of 2.2 x 10^{-6} mol apatite/m^2/s, two orders of magnitude faster. This demonstrated a considerable enhancement in the rate of remineralisation of enamel subsurface lesions by CPP-ACFP in vitro compared with all previous studies.
The concentration of the remineralisation solution in the short-term experiment (10% CPP-ACFP) was higher than that used *in vitro* by Cochrane *et al.* [2008] and Reynolds [1997], and is the same concentration of CPP-ACFP used in a commercially available topical tooth crème (Tooth Mousse Plus, GC Corporation, Japan). While the increase in the rate of remineralisation during the short-term cycle experiment may be partly attributed to the high CPP-ACFP concentration, the comparison with the 200 minute control experiment that used 10% CPP-ACFP alone showed intra-lesion pH modulation was the most significant factor accelerating the rate of remineralisation. The high rate of remineralisation over the short time frame suggested that activity within the lesion mainly occurred during the initial few minutes of exposure to the treatment solutions, and was enhanced after changing solutions due to the influx of ions down a concentration gradient and the intra-lesion pH modulation maximising ion diffusion and the DS with respect to apatite.

4.5.4 Clinical relevance

For the dental patient, the same challenges of increasing bioavailability of calcium phosphate and fluoride and increasing the DS with respect to apatite within the lesion fluid apply for regression of uncavitated carious lesions. To approach these challenges, modification of the intra-lesion pH level was found to have a significant influence in the context of *in vitro* remineralisation. A method to modulate the pH within the lesion with NaOH successfully increased the rate of remineralisation using CPP-ACFP, however additional challenges would arise if this method were to be translated *in vivo*. High pH solutions such as the NaOH solution used in the short-term remineralisation experiment (4.3.2.2) can be toxic to the mucosa [Vancura *et al.*, 1980]. Other toxic materials such as NaOCl and hydrofluoric acid are frequently used in clinical dentistry though with isolation using rubber dam. This may be an option to permit use of solutions with high pH levels without causing soft tissue damage. However, the time used in the short-term cycle experiment (4 hours) is still too long to be clinically relevant and further work is required to develop a method that could be translated for use *in vivo*.

The tested *in vitro* remineralisation model showed that changes to the pH of the fluid surrounding the enamel lesion can enhance remineralisation with CPP-ACFP.
Fluctuations in plaque fluid pH are known to occur in vivo due to by-products of bacterial metabolism (i.e. lactic acid, ammonia), dietary influences and variation in saliva flow and components [Abelson and Mandel, 1981]. While these pH fluctuations may facilitate remineralisation of early enamel caries by saliva, there is scope for further research to develop a practical clinical protocol augmenting intra-lesion pH to accelerate the rate of remineralisation, particularly using CPP complexes.

4.6 Conclusions
1. Cyclic exposure of enamel subsurface lesions to NaOCl and CPP-ACFP solutions in vitro resulted in a disadvantageous interaction of the solutions. From this interaction, calcium phosphate and fluoride ions were destabilised causing a surface precipitation. Further demineralisation of the subsurface lesion was apparent which was attributed to the presence of HOCl⁰ penetration deep into the lesion.

2. Cyclic exposure of enamel subsurface lesions to NaOH and CPP-ACFP solutions in vitro resulted in an enhancement of remineralisation. This was significantly more than that produced by exposure to DDW and CPP-ACFP (p < 0.001). This effect was due to pH modulation of the lesion fluid which increased ion diffusion through the lesion and supersaturation of the lesion fluid with respect to FA/FHA.

3. A decrease in interval time and increase in solution concentrations produced a high rate of remineralisation by cyclic exposure of enamel subsurface lesions to NaOH and CPP-ACFP solutions in vitro. A similar percent remineralisation was observed after 4 hours using this method as the 105 hour cyclic exposure to DDW and CPP-ACFP.
THE USE OF X-RAY MICROTOMOGRAPHY TO ASSESS REMINERALISATION OF ENAMEL BY CPP-ACFP
5.1 Introduction

For decades the preferred method for assessing remineralisation of the dental hard tissues has been TMR, however this method is destructive to the sample and technique sensitive [Angmar et al., 1963; Arends and Ten Bosch, 1992]. Analysis of dentine remineralisation using microradiography is particularly difficult as dentine becomes brittle in thin sections and is prone to shrinkage or fracture during x-ray exposure time [Ruben and Arends, 1993].

X-ray microtomography (XMT) has been increasingly used as a tool for measuring the mineral content in dental hard tissues [Swain and Xue, 2009]. The technique involves rotation of the sample slowly around a central axis with sequential radiographic images being recorded at each rotation step. These images are subsequently compiled by software reconstruction to give a three-dimensional representation of the sample. Grey levels are assigned to volume units (voxels) corresponding to the x-ray attenuation of the sample material and are utilised to calculate mineral density. By using XMT, the lesion volume in its entirety can be assessed for changes in mineral content as opposed to transverse sections that may only reveal a snapshot of the mineral profile.

The incident x-rays in XMT may be polychromatic such as in benchtop/conventional x-ray microtomography (Cµ-CT), or sourced from the monochromatic beam of a synchrotron. Previous comparisons between Cµ-CT and TMR for analysis of remineralisation are limited, however authors have suggested Cµ-CT is a suitable alternative to TMR despite having artefacts such as beam hardening and a low signal to noise ratio [Clementino-Luedemann and Kunzelmann, 2006; Hamba et al., 2012; Lo et al., 2010; Swain and Xue, 2009]. As synchrotron radiation computed tomography (SR-CT) utilises the high intensity, highly collimated monochromatic source of a synchrotron, beam hardening is eliminated and noise levels are decreased in captured images [Dalstra et al., 2006b; Kazakia et al., 2008].

Assessment of both Cµ-CT and SR-CT for measurement of mineral density changes in an in vitro enamel remineralisation experiment was conducted to compare the accuracy and practicality of each analysis for future experimentation. A novel approach to
segmenting lesion volumes was adopted to utilise volumetric data of the XMT images. To compare the results against an accepted method for remineralisation analysis, the samples were additionally processed and assessed using TMR.

5.2 Objective

The objective of this study was to compare Cµ-CT, SR-CT and TMR analysis of enamel subsurface lesion remineralisation by CPP-ACFP in vitro.

5.3 Study methods

5.3.1 Tooth preparation

Eight extracted human third molars were sectioned into enamel blocks, painted with nail varnish and demineralised to produce artificial lesions approximately 1 mm x 7 mm (as described in 2.2.1).

5.3.2 Remineralisation

The demineralised enamel blocks were attached with dental sticky wax to the lid of a sealed jar to allow the demineralised enamel window to be immersed in solution. Half the lesion was covered by dental sticky wax so that only the other half of the lesion contacted the remineralisation solution. Each block was exposed to 2% (w/v) CPP-ACFP at pH 5.5 for eight days with a change of solution every two days. After eight days, the enamel blocks were removed from the remineralisation solution, rinsed thoroughly with DDW and blotted dry. All dental sticky wax and nail varnish was removed and a marker was placed to differentiate the border between the demineralised and remineralised lesion halves.

5.3.3 Cµ-CT

A Bruker Skyscan 1172 desktop x-ray microtomography machine (Bruker Skyscan, Kontich, Belgium) was used to scan enamel blocks for mineral density. In preparation for scanning, each block was attached to light cured composite resin (Gradia Direct X, GC Australasia) and mounted in dental putty (Hydrospeed Putty Hard, Itena, France) in a plastic lid. Blocks were hydrated with DDW and wrapped in paraffin film (Parafilm M, Bemis, USA) during scanning time to prevent dehydration.
A pixel size of 4 µm, tube voltage of 100 kV and current of 100 mA with an aluminium and copper filter were applied for all samples. At the commencement of each scan a fresh flat field image was acquired to minimise artefacts and improve contrast in the projection images. Three raw projection images were averaged at each increment of angle to improve the signal to noise ratio. Two HA calibration phantoms of density 0.25 and 0.75 g/cm³ (Bruker Skyscan, Kontich, Belgium) were similarly scanned using these settings to calibrate attenuation with mineral density. Images were reconstructed to 8-bit bitmap images using NRecon (Bruker Skyscan, Kontich, Belgium) with a Gaussian smoothing value of 4, beam hardening correction value of 62 % and ring artefact correction set at maximum (20).

5.3.4 Synchrotron radiation computed tomography

An application to use the Australian Synchrotron (Clayton, VIC, Australia) was submitted and approved for 48 hours beam time at the Medical and Imaging beamline utilising the computed tomography apparatus (Reference No. AS163/IMBL/11429). Due to the limited beam time, it was necessary for the remineralisation procedure to be complete before using the synchrotron facility. Therefore a longitudinal analysis of the same volume of enamel before and after remineralisation was not possible, and instead the Cµ-CT and SR-CT analyses compared the experimental and control lesion halves. This also allowed for comparison with TMR which analysed the experimental and control half-blocks concurrently.

Enamel blocks were mounted as for Cµ-CT and scanned at an energy of 45 KeV with a 5.75 µm pixel size. X-ray attenuation was measured using the Ruby detector (Australian Synchrotron, Clayton, VIC, Australia). This pixel size was the highest resolution available for the sample size. A higher resolution scan of approximately 1 µm was attempted using the Diamond detector, however progressive darkening of the detector lens rendered these datasets unusable. The two HA calibration phantoms of density 0.25 and 0.75 g/cm³ were scanned for mineral density calculations using the same settings. Images were reconstructed on site by technical staff and converted to 8-bit bitmap images. Synchrotron staff assisted with scanning setup and software reconstruction to reduce phase contrast and ring artefact.
5.3.5 Transverse microradiography (TMR)

Following Cµ-CT and SR-CT scanning, enamel blocks were sectioned into remineralised and demineralised (experimental and control) halves and processed for TMR as described in 2.3.1.

5.3.6 Remineralisation analysis

The reconstructed 8-bit images of each sample from the Cµ-CT and SR-CT scans were resized using CTAn software (Bruker Skyscan, Kontich, Belgium) to include only the volume of enamel required, being the enamel lesion and the surrounding sound enamel (see Figure 5.1, image A). This volume image stack was further resized (halved) to separate the portion of enamel containing the demineralised lesion and the portion of enamel containing the remineralised lesion, retaining the adjacent sound enamel in each image stack. After calibrating the software for mineral density using the datasets of the HA phantoms, a ‘task-list’ was created through the custom processing function to segment the lesion from the surrounding enamel. This involved trial and error to test the upper and lower limits of the lesion grey level threshold (unique to each sample) and the addition of morphological operations to define the total and specific volume of the demineralised and remineralised lesion as a binarised/monochrome image stack (see Figure 5.1, image B). This monochrome image stack was reloaded as a binary mask upon the 8-bit image stack to demarcate a volume of interest (VOI), either the demineralised lesion or the remineralised lesion (see Figure 5.1, image C). This selection was used to calculate the average mineral density (g/cm³) within the volume of the remineralised or demineralised lesions based on the grey values within the VOI. The image stack was again binarised to segment the surrounding sound enamel to calculate its average mineral density (g/cm³), and this value was defined as 100 % volume mineral (vol%min) specific to each sample. The difference between the mineral density of the demineralised lesion and median sound enamel was calculated and converted to vol%min, and the difference between the mineral density of the remineralised lesion and median sound enamel was calculated and converted to vol%min to represent $\Delta Z_d$ and $\Delta Z_r$ respectively. For each sample, these values were used to assess relative remineralisation, or % R:

\[
% R = \frac{\Delta Z_d - \Delta Z_r}{\Delta Z_d} \times 100
\]
This method of calculating remineralisation was identical for datasets obtained from SR-CT and Cµ-CT.

Values for ΔZd, ΔZr and % R were obtained from TMR as described in 2.3.1.5. To maintain the same unit of measurement (vol%min) for ΔZd and ΔZr, the values in vol%min.µm obtained by TMR were divided by the corresponding lesion depth in µm. A repeated measure analysis of variance was used with Tukey comparison tests to compare measurements obtained from SR-CT, Cµ-CT and TMR analysis. Method of analysis and sample were used as fixed and random factors respectively (Minitab Version 16, Pennsylvania, USA).
Figure 5.1: Reconstructed slice from a SR-CT dataset of an enamel lesion showing (A) original image (B) segmented region of interest demarcating the demineralised lesion in white (C) the original image after reloading (B) as a VOI in red (CTAn).
5.4 Results

The results from the remineralisation analysis are shown in the Table 5.1. There was no significant difference in average % R calculated by either SR-CT (20.55 ± 6.27 %) or Cµ-CT (20.77 ± 7.12 %), however the average % R calculated by TMR (49.29 ± 3.23 %) was significantly higher than either of the CT analyses (p < 0.00001). Values for ΔZr were significantly different between all methods (p < 0.001), while only the SR-CT analysis was significantly different to both the Cµ-CT and TMR analysis for ΔZd (p < 0.0001 and p < 0.02 respectively).

Table 5.1: Percent remineralisation of lesions as calculated from remineralisation analysis by Cµ-CT, SR-CT and TMR.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>ΔZd (vol%min)1</th>
<th>ΔZr (vol%min)2</th>
<th>ΔZd-ΔZr (vol%min)3</th>
<th>%Remin4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cµ-CT</td>
<td>29.14 ± 3.15a</td>
<td>23.16 ± 3.19ab</td>
<td>5.98 ± 2.64a</td>
<td>20.77 ± 7.12a</td>
</tr>
<tr>
<td>SR-CT</td>
<td>22.80 ± 1.97ab</td>
<td>18.18 ± 2.37ac</td>
<td>4.63 ± 1.49b</td>
<td>20.55 ± 6.27b</td>
</tr>
<tr>
<td>TMR</td>
<td>26.60 ± 1.98b</td>
<td>13.44 ± 0.58bc</td>
<td>13.16 ± 1.78ab</td>
<td>49.29 ± 3.23ab</td>
</tr>
<tr>
<td>p-value§</td>
<td>&lt; 0.02</td>
<td>&lt; 0.001</td>
<td>&lt; 0.00001</td>
<td>&lt; 0.00001</td>
</tr>
</tbody>
</table>

1ΔZd = integrated mineral loss prior to remineralisation, 2ΔZr = integrated mineral loss after remineralisation, 3ΔZd-ΔZr = gain in mineral content after remineralisation; 4%Remin = percent remineralisation ((ΔZd-ΔZr/ΔZd)*100 %). Displayed as mean ± standard deviation. §T-test (α = 0.05) NS not significant. Differences between treatments were tested using Tukey HSD post hoc multiple comparison tests. abcValues in the same column similarly marked are significantly different.

Figure 5.2: Reconstructed SR-CT images from the same sample showing (A) the demineralised enamel lesion and (B) the remineralised enamel lesion.
5.5 Discussion

SR-CT and Cµ-CT imaging was used successfully to detect changes in mineral content in enamel subsurface lesions. However, the average % R observed from these analyses was considerably lower than that observed by TMR (49.29 ± 3.23 %). Although the SR-CT and Cµ-CT analyses showed an agreement, the accuracy of the TMR analysis could be confirmed as it closely corresponded with the % R published in a similar *in vitro* remineralisation study by Cochrane *et al.* [2008] (57.7 ± 8.4 %). The difference in remineralisation protocol between Cochrane *et al.* [2008] and the current study is that the enamel subsurface lesions were exposed to a 2 % CPP-ACFP solution at pH 5.5 for 10 days (as opposed to 8 days in the current study). A logical explanation for the inaccuracy of the SR-CT and Cµ-CT analyses is that they were heavily influenced by poor image quality and artefacts not present in TMR.

Lesion segmentation has been used previously to assess remineralisation with Cµ-CT images obtained from a Bruker Skyscan 1172 scanner. The study by Kind *et al.* [2017] counted the number of voxels within the lesion after thresholding to specific grey levels, and used this number to assess remineralisation as a ratio of voxels present pre- and post-treatment. Kucuk *et al.* [2016] segmented lesions and analysed mineral density using the same software and method as described in the current study, and additionally measured the lesion volume, depth and area. Remineralisation was not significantly different between treatment groups after 30 days treatment *in vitro* and all treatments displayed a similar amount of remineralisation to that observed from Cµ-CT analysis in the current study (which included a 10 % CPP-ACP tooth crème treatment). As Cµ-CT considerably underestimated remineralisation when compared to TMR in the current study, it is possible that the mineral density changes reported by Kucuk *et al.* [2016] were also an underestimation.

A challenge for remineralisation analysis with SR-CT and Cµ-CT is demarcation of the lesion borders to define a VOI in which the imaging software can assess changes in mineral. An advantage of the lesion segmentation method in the current study was that the VOI closely matched the apparent lesion borders and the surface enamel on the x-ray image. However, the accuracy of the lesion borders and surface enamel was dependent
on the image resolution and the image quality. Despite using the highest possible resolution to allow the entire sample to be visible within the respective scanning field of view, the XMT from both sources had resolutions lower than that of TMR [Ten Bosch and Angmar-Månsson, 1991] and as a result showed less defined lesion borders, particularly in the Cµ-CT images where a high amount of noise was evident. Apart from the resolution, segmentation and assessment of remineralisation of the lesion in the current study was influenced by the image quality. Various factors contributed to the quality of the images captured by SR-CT and Cµ-CT and they are discussed hereafter.

5.5.1 Calculating enamel mineral density from x-ray attenuation

In the context of Cµ-CT and SR-CT, x-ray attenuation is directed by two factors: the photoelectric effect and the Compton effect [Zou et al., 2011]. At lower energies, the incident x-ray photon excites a core electron within the absorbing atom, completely transferring its energy. The excitation of this electron can result in a small amount of energy being emitted, however this amount is unlikely to impact on the image signal. This transfer of energy is termed the photoelectric effect and it is the predominant attenuation that occurs when the x-ray energy is less than 25 KeV [Ritman, 2004]. When the x-ray energy exceeds 50 KeV, the incident x-ray photon transfers part of its energy to the absorbing atom to excite and eject an outer electron. Consequently, there is a scattering and loss of energy of the original photon. This scattering of the x-ray is termed the Compton effect, or Compton scattering [Ritman, 2004]. Lower energy is often more effective to achieve contrast between mediums due to the influence of the photoelectric effect, however higher energy may be required to penetrate through dense samples. An appropriate x-ray energy is generally selected to utilise both the Compton and photoelectric effect, allowing suitable contrast of the sample image [Ritman, 2004]. The x-ray energies used for the Cµ-CT and SR-CT scans in the current study were selected according to this principal, though only the SR-CT had a monochromatic beam whereas the Cµ-CT had a spectrum of x-ray energies at and below 100 KeV.

The software chosen for the remineralisation analysis of the Cµ-CT and SR-CT datasets, CTAn, calibrated attenuation to HA density on the assumption that the x-ray attenuation, corresponding grey values and HA density had a linear relationship [Zou et al., 2009].
This required calculation of the linear attenuation coefficient (also referred to as LAC or μ), which is the measurement of the fraction of x-ray energy that is absorbed or scattered through a specific volume of a material, in this case HA [Elliott et al., 1998]. To correlate attenuation of the HA phantoms with the enamel samples, it was necessary to assume the LAC of the organic content of enamel was the same as the epoxy resin filler in the HA phantoms, and that the mineral phase within enamel was stoichiometric HA. In actuality, the mineral phase of natural enamel is closer to CadAP [LeGeros, 1990], while the remineralised lesions in the current study were likely to additionally contain fluoridated apatites, such as FA or FHA, meaning the x-ray attenuation by the enamel was unlikely to be linear. The consequence of equating the LAC of the enamel samples to that of the HA phantoms was therefore a slightly inaccurate calculation of mineral density (though the same may be said for mineral analysis using TMR where assumptions of the enamel/dentine mineral profile are also made [Ten Bosch and Angmar-Månsson, 1991]).

The HA phantoms used for mineral density calculations in the current study were a mix of HA and epoxy resin. They had a density of 0.25 and 0.75 g/cm³, much lower than the density range reported for sound enamel, 2.57 – 3.00 g/cm³ [Angmar et al., 1963; Clementino-Luedemann and Kunzelmann, 2006; Huang et al., 2007]. Previous authors have questioned the homogeneity of such phantoms, suggesting non-linear x-ray attenuation between phantoms gives rise to poor software calibration for mineral density calculations [Zou et al., 2011]. The study by Huang et al. [2007] utilised five HA phantoms of 1.52, 1.63, 1.85, 2.08 and 3.14 g/cm³ density to plot a calibration curve within the range of enamel thereby increasing the accuracy of enamel mineral density measurements. While it was possible there were inconsistencies in ‘absolute’ mineral density calculations in the current study due to the aforementioned assumptions and the HA phantoms chosen for software calibration, these reasons alone were unlikely to significantly affect the calculation of relative mineral change (% R) from the Cµ-CT and SR-CT datasets. Rather, it was the difficulty in accurately recording grey values corresponding to mineral density that resulted in calculations for % R considerably lower than that observed by TMR.
5.5.2 Noise

Accuracy of grey values from x-ray attenuation is increased by having a high signal to noise ratio (SNR). A high SNR can be achieved by increasing the scanning resolution and minimising software beam hardening correction [Van de Casteele et al., 2004]. The SNR can also be increased by appropriate denoising during reconstruction or increasing frame averaging during scanning, though the latter increases scanning time [Neves et al., 2010]. The study by Hamba et al. [2012] scanned teeth with frame averaging set at twelve frames per rotation step albeit at a relatively low resolution of 12.5 μm. As increasing both the frame averaging and resolution increases scanning time, a compromise was made for the Cµ-CT analysis in the current study to have a practical scanning time with a suitable SNR and high resolution. Accordingly, the Cµ-CT scans averaged three frames per rotation step resulting in a scanning time of approximately 6 hours per sample. During reconstruction, the images were denoised using a Gaussian smoothing kernel which reduced though did not eliminate noise (see Figure 5.3 image B). Shahmoradi et al. [2016] described an alternate denoising method termed BM3D denoising that showed an improvement in the diagnostic value of images when compared to images smoothed using the Gaussian method. Despite improving the SNR for Cµ-CT images of teeth, Shahmoradi et al. [2016] commented that TMR still had less noise and would likely remain the gold standard for mineral analysis until further advancements can be made to reduce the noise of Cµ-CT images.

5.5.3 Beam hardening and phase contrast

Imaging the surface zone is especially important when assessing remineralisation in vitro. The artificial carious lesions produced by the modified White method [White, 1987] are approximately 100 μm depth with a relatively mineral dense surface zone superficial to the subsurface demineralised zone. This follows the pattern of early enamel caries where a combination of internal reprecipitation, the presence of fluoride and surface organic molecules maintain a relatively intact enamel surface overlying the demineralised lesion [Arends and Christoffersen, 1986]. Both Cµ-CT and SR-CT were found to be problematic for accurately imaging the surface zone of lesions though for different reasons.
A significant disadvantage of Cµ-CT imaging is the artefact produced by surface absorption of low energy x-rays from the polychromatic source upon contact with a high-density medium. This artefact is termed beam hardening, and it creates a false higher density measurement by the x-ray camera within the superficial regions of the higher density medium (ie. enamel). The high-energy x-rays of the beam are able to pass through the enamel despite some attenuation (predominantly Compton scattering), and the camera registers the respective interaction of these x-rays within the sample. Beam hardening occurs as the low energy x-rays are absorbed in the surface zone of the enamel, thereby not penetrating the sample and registering a false reading of higher density when compared to the bulk of the sample. Accuracy of mineral density measurements in radiographic analysis is maximised by the entire sample volume being measured by its interaction with exactly the same x-ray energy. As lower energy x-rays do not interact with the deeper tissues of the sample, there is an unequal recording of attenuation between the superficial and deeper zones thereby creating the discrepancy and artefact of surface zone mineral density. To compensate for this artefact, beam filtration and software correction are used. Beam filtration using a combination of aluminium and copper filters pre-hardens the beam reducing the artefact [Hamba et al., 2012; Meganck et al., 2009], and this was done in the current study for the Cµ-CT scans. In addition to filtration, the reconstruction software was adjusted to reduce the beam hardening artefact in the reconstructed datasets. Zou et al. [2011] described this method of software correction in detail as a utilisation of the Lambert-Beer law to adjust polynomial correction values resulting in improved absorption values. Van de Casteele et al. [2002] suggested a bimodal energy model whereby linearisation of attenuation can be moderated instead of the polynomial fit model. Ultimately software beam hardening correction allows for better approximation of a material’s x-ray attenuation however these enhancements at the border between different mediums results in poor differentiation for quantitative measurements [Van de Casteele et al., 2004], and this likely contributed to inaccuracies in the current study where the first 100 µm of surface enamel was being analysed.

In NRecon (Bruker Skyscan, Kontich, Belgium) the ‘beam hardening correction’ feature allowed adjustment between 0 and 100 % software correction of the artefact. Essentially, this was a polynomial fit model. An observed flaw in this method was that the beam
hardening correction percentage was set according to the visual interpretation of the software user. The image preview window allowed the user to visually estimate the likely surface grey value, which was further complicated by the fact that surface enamel has a higher mineral density than the subsurface enamel in demineralised and remineralised lesions [Arends and Christoffersen, 1986]. In essence, the software correction of beam hardening reduced the grey level or attenuation value of the surface enamel, resulting in values for $\Delta Z_d$ and $\Delta Z_r$ that were much closer together. This reason alone may explain why the mean percent remineralisation measured was $20.77 \pm 7.12\%$ for the $\mu$-CT scans, considerably lower compared to in vitro studies of similar duration measuring remineralisation by CPP-ACP/ACFP and assessed using TMR [Cochrane et al., 2008; Reynolds, 1997]. Beam hardening and its method of correction therefore poses difficulty in achieving an accurate mineral density measurement when conducting remineralisation studies with $\mu$-CT analysis.

SR-CT utilises a specific x-ray energy as it is from a monochromatic source. In that respect the entirety of the samples in the current study were subjected to a single x-ray energy and the measurement of attenuation within the sample was more standardised than $\mu$-CT. However, the borders between mediums of different density were affected by another signal produced by phase contrast. In both $\mu$-CT and SR-CT, the signal produced by the interaction of the beam with the sample is a mixture of the absorption signal and the phase contrast signal, though usually the phase contrast signal is negligible. At higher resolutions the influence of the phase contrast signal is no longer negligible and interferes with the final projection image to produce an artefact where different phases (materials of different density) contact. The artefact is caused by small angle refraction of the x-ray beam as it enters a new medium [De Witte et al., 2009]. While for the purposes of the current study the phase contrast signal was an artefact, the signal itself can be used to image lower density materials such as organic matter using $\mu$-CT [Bonse and Busch, 1996].

The current study observed phase contrast signal as most noticeable in the SR-CT images and software reduction of the artefact was done in the reconstruction stage. The software correction was minimised to limit the unwanted effect of altering the enamel surface layer
grey level, much like beam hardening correction in Cµ-CT image reconstruction. Despite software correction, the final reconstructed images still appeared to display a higher attenuation in the sample surface due to the phase contrast signal (see Figure 5.3 image A). Alteration of the enamel surface layer grey layer was unavoidable due to correction of the phase contrast signal and this was a disadvantage of the SR-CT method which likely resulted in inaccurate ΔZd and ΔZr measurements that affected the % R calculation.

5.5.4 Ring artefact

A common feature of both SR-CT and Cµ-CT images is ring artefact which is caused by non-uniformities in the incident x-ray beam or its detection. These non-uniformities in the incident x-ray beam can arise from variation of the source (scintillator), the pixels of the camera or any stationary object within the field of view [Zou et al., 2009]. To minimise these variations in the camera and any interference from the flat field, ‘flat field correction’ was applied before scanning with Cµ-CT. This allowed the scanning software to recognise any inhomogeneity of the camera pixels when no sample was in the field of view. Unfortunately, ring artefacts can persist despite flat field correction and any non-uniformity in detection or stationary object in the image frame becomes circumferentially ‘stained’ as the sample rotates around a central axis, hence the term ‘ring’ artefact. For the Cµ-CT scans in this study, reconstruction using NRecon allowed for adjustment of ‘Ring Artefact Correction’ with a value set between 0 and 20 affecting the degree of correction. All Cµ-CT scans within the study were reconstructed with a Ring Artefact Correction value of 20 which greatly reduced the observed ring artefact, though did not completely remove it. Similarly, the SR-CT scans were reconstructed with software ring artefact correction though complete elimination of the artefact was not possible. Both the SR-CT and Cµ-CT scans displayed ring artefacts within the sample volume to some degree (see Figure 5.3), and this reduced the accuracy of the attenuation measured at certain depths and consequently affected the mineral density calculation within the lesion. This was yet another disadvantage of studying the mineral content of teeth with either SR-CT or Cµ-CT as compared with TMR. Davis et al. [2010] described a novel Cµ-CT scanner (the MuCat 2) which implemented time delay integration and a sliding camera to effectively eliminate the influence of any non-uniformity between camera pixels that caused ring artefacts. However, while images produced by the MuCat 2 had no ring

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artefact, this method of scanning increased the scanning time considerably. Perfecting this technology to have little effect on scanning time may significantly benefit the practicality of Cµ-CT.

**Figure 5.3:** Reconstructed images from SR-CT (A) and Cµ-CT (B) showing ring artefact. The red dot approximates the axis of rotation. Of note are the radiopaque surface layer in image A caused by the phase contrast signal and the low SNR in image B.
Application of computer tomography for tooth mineral studies

Numerous authors have published studies outlining Cµ-CT measurement of human enamel and dentine mineral density [Anderson et al., 1996; Clementino-Luedemann and Kunzelmann, 2006; Cochrane et al., 2012a; Dowker et al., 2003; Gao et al., 1993; Hamba et al., 2011; Hamba et al., 2012; Huang et al., 2007; Koji et al., 2012; Kucuk et al., 2016; Willmott et al., 2007; Wong et al., 2004]. As in the current study, the authors made the assumption that the predominant mineral phase of enamel and dentine was HA and that there was a linear association between x-ray attenuation, grey values and mineral density. In the majority of these studies, the same artefacts for Cµ-CT were encountered as the current study and it can be expected the assumptions and artefacts caused a degree of error in mineral density calculations.

Assessment of enamel mineral concentration from x-ray attenuation was suggested to be most accurate using a monochromatic source such as a synchrotron [Elliott et al., 1998], though this was not observed with images provided by the Australian Synchrotron. Few studies have reported the use of synchrotron radiation to study tooth mineral content [Dowker et al., 2004; Kinney et al., 2005; Lautensack et al., 2013; Prymak et al., 2005], and this is likely due to the difficulty accessing synchrotron facilities. The studies by Dowker et al. [2004] and Lautensack et al. [2013] used a higher resolution than the current study and were able to demonstrate much clearer images more suited to mineral analysis, with Lautensack et al. [2013] publishing images with minimal phase contrast signal by adjusting the sample to detector distance. The resolution of the current study was intermediate to that used in previous studies, though the anticipated benefits of using a monochromatic beam as compared to the polychromatic Cµ-CT were not apparent. The Australian Synchrotron was not able to image samples at a higher resolution than 5.75 µm, nor was the software correction of the phase contrast signal sufficient to remove the artefact without corrupting the surface volume grey values. The images from the Australian Synchrotron had an equivalent diagnostic value to that of the Cµ-CT scans, though coupled with an appropriate micro-CT setup such as the BAMline synchrotron facility (Helmholtz Zentrum für Materialien und Energie, Berlin, Germany) SR-CT has
been shown to image tooth samples with high resolution and of excellent diagnostic value for mineral density calculations [Lautensack et al., 2013].

Only two studies thus far have compared TMR to Cµ-CT with respect to tooth remineralisation/demineralisation analysis [Hamba et al., 2012; Lo et al., 2010] and both concluded that Cµ-CT is a suitable method for this type of analysis. Interestingly, the study by Lo et al. [2010] asserted this by comparing two separate measurements (the change in LAC after remineralisation using Cµ-CT and the change in lesion depth after remineralisation using TMR) to assess the relationship between the two techniques. Hamba et al. [2012] correlated measurements of mineral change and lesion depth from Cµ-CT with TMR to support the contention that Cµ-CT is a valid alternative to TMR. However it should be noted that the use of a correlation coefficient to compare the same quantity calculated by different methods does not assess accuracy of the measurements [Bland and Altman, 1986].

5.5.6 Concluding remarks

Due to the noise and artefacts encountered in both SR-CT and Cµ-CT, it is still recommended to use TMR where possible for tooth remineralisation analysis ex vivo, particularly as the surface layer x-ray attenuation is of utmost importance. The TMR analysis in the current study observed a considerably higher percent remineralisation when compared to the XMT analyses and this emphasized TMR’s sensitivity to mineral changes within the first 100 µm of surface enamel. In addition to the artefacts encountered as described in this text, the high cost of equipment and the limited access to either Cµ-CT or SR-CT facilities is an obvious disadvantage of these methods. It should be mentioned that new Cµ-CT technology is constantly being developed, and that the results of the current study can only comment on analysis of remineralisation using the Bruker Skyscan 1172 scanner, software and HA phantoms (Bruker Skyscan, Kontich, Belgium). Although TMR can be technique sensitive and difficult to assess longitudinal mineral change or dentine mineral content, previous authors have developed protocols to overcome these challenges [Damen et al., 1997; Ruben and Arends, 1993; Ten Cate, 2001]. However, if intact samples are required for longitudinal analysis of mineral change, XMT is a promising technique [Dowker et al., 2003; Koji et al., 2012]. In the
field of dental research, it appears XMT is best used for analysing the three-dimensional morphology of teeth and lesions such as caries or developmental dental defects, and with an appropriate setup mineral density calculations can be made to maintain intact samples [Cochrane et al., 2012a; Dowker et al., 2003; Farah et al., 2010; Huang et al., 2007; Koji et al., 2012; Shahmoradi and Swain, 2016].

5.6 Conclusions

1. Cµ-CT and SR-CT datasets were used to measure the percent remineralisation of artificial enamel subsurface lesions by CPP-ACFP in vitro as 20.77 ± 7.12 % and 20.55 ± 6.27 % respectively, though they were less sensitive to mineral changes than TMR which measured percent remineralisation of the same lesions as 49.29 ± 3.23 %.

2. There was no significant difference in mean percent remineralisation as measured by the Cµ-CT and SR-CT analyses indicating they were equivalent in diagnostic value.

3. Both Cµ-CT and SR-CT images contained artefacts. Software correction reduced these artefacts though they contributed to a degree of error in mineral density calculations.

4. TMR is currently more suited for assessment of tooth remineralisation than Cµ-CT and SR-CT using the conditions and devices tested.
REMINERALISATION OF MINERAL DEFICIENT ENAMEL AND DENTINE USING CPP-ACP AND STANNOUS FLUORIDE
6.1 Introduction
There has been a recent resurgence in the use of products containing stannous fluoride (SnF₂) due to strong in vitro evidence suggesting they are effective in inhibiting acid-induced demineralisation of enamel and dentine [Ganss et al., 2010; Schlüeter et al., 2009a; Schlüeter et al., 2009b]. Faller and Eversole [2014] demonstrated that a Sn/F containing surface layer on SnF₂ treated enamel specimens appeared to prevent exposure of calcium on the enamel surface and protect the bulk of the underlying tooth structure by increasing its resistance to demineralisation. In addition, increasing evidence suggests the antibacterial effect of SnF₂ has the potential to aid the prevention of caries and periodontal disease [Cheng et al., 2017; Fernandez et al., 2016].

Casein phosphopeptides (CPPs) have been shown to sequester calcium, phosphate and fluoride ions allowing a high bioavailability of soluble ions for remineralisation of mineral deficient tooth structure [Cochrane and Reynolds, 2012]. The binding motif within all the CPPs (Ser(P)-Ser(P)-Ser(P)-Glu-Glu) strongly attracts calcium ions and subsequently phosphate and fluoride ions to form soluble complexes of amorphous ion clusters [Cross et al., 2007]. To assess the hypothesis that CPP-ACP would similarly bind stannous ions, thereby allowing an additional anticariogenic component to be delivered to mineral deficient lesions, in vitro and in situ enamel remineralisation experiments were designed to test the combination of CPP-ACP and SnF₂ as a potential treatment solution for dental caries. Additionally, the interaction of the combination of CPP-ACP and SnF₂ with the surface of dentine was investigated using electron microscopy.

6.2 Objectives
The objectives of this study were to:
− Assess the efficacy of a combined SnF₂ and CPP-ACP remineralisation treatment on enamel subsurface lesions using in vitro and in situ caries models.
− Assess the surface interaction of a combined SnF₂ and CPP-ACP solution with dentine through SEM imaging and SEM-EDS.

6.3 Study methods
The University of Melbourne Human Research Ethics Committee approved the research conducted in this study (Nos. 1136623 and 1441572).
6.3.1 Preparation of remineralisation solutions

The remineralisation solutions were prepared with a commercial CPP-ACP preparation (Recaldent™, Cadbury Enterprises Pte Ltd, VIC, Australia) and pH adjusted as required (described in 2.1.2).

For the *in vitro* enamel remineralisation experiment, the following solutions were formulated at pH 5.6:

i. 0.4 % w/v CPP-ACP + 220 ppm F as SnF$_2$ + 70 ppm F as NaF

ii. 0.4 % w/v CPP-ACP + 290 ppm F as NaF

For the *in situ* enamel remineralisation experiment, the following solutions were formulated at pH 4.0:

iii. 0.4 % w/v CPP-ACP + 220 ppm F as SnF$_2$ + 70 ppm F as NaF

iv. 0.4 % w/v CPP-ACP + 290 ppm F as NaF

v. 0.4 % w/v CPP-ACP

vi. 220 ppm F as SnF$_2$ + 70 ppm F as NaF

vii. 290 ppm F as NaF

For the *in vitro* dentine experiment, the following solutions were formulated without pH adjustment (final pH indicated in parentheses):

viii. 5 % w/v CPP-ACP (pH 7.9)

ix. 500 ppm F as SnF$_2$ (pH 3.2)

x. 5 % w/v CPP-ACP + 500 ppm F as SnF$_2$ (pH 7.2)

A pH of 4.0 was used for the *in situ* enamel remineralisation solutions as the SnF$_2$ solution alone (solution vi) was not stable in water with a pH higher than 4.0. When preparing solutions for the *in vitro* experiment, the addition of SnF$_2$, NaF and CPP-ACP to the desired concentration yielded an unadjusted pH of 5.6; accordingly the CPP-ACP + NaF *in vitro* solution was adjusted to this pH to allow comparisons at the same pH value. It is estimated that toothpaste is diluted 1:4 in saliva during toothbrushing [Duke and Forward, 1982]. Therefore, the concentrations of fluoride and CPP-ACP in the fluoride-containing *in vitro* and *in situ* remineralisation solutions were 290 ppm and 0.4 % (w/v), respectively,
to simulate a 1:4 dilution of toothpaste containing 1450 ppm F or 2 % (w/v) CPP-ACP. A higher concentration of CPP-ACP and fluoride was used for the in vitro dentine treatment solutions to simulate placing a commercial CPP-ACP containing product (GC Tooth Mousse) directly on an exposed sensitive tooth root in vivo. All solutions prepared were stable at room temperature.

6.3.2 Enamel remineralisation protocol

6.3.2.1 In vitro remineralisation model
Twenty four human third molars were sectioned into enamel blocks and demineralised to produce artificial carious lesions as described in 2.2.1. Enamel blocks were halved into an experimental half-block and control half-block. One group of experimental half-blocks (n = 12) was suspended in the CPP-ACP + SnF₂ + NaF remineralisation solution (solution i) and another group of experimental half-blocks (n = 12) was suspended in the CPP-ACP + NaF remineralisation solution (solution ii). The blocks were suspended for 10 days at 37 °C with a change of solution every 48 hours and were subsequently paired with their control half-block and analysed for mineral content change using transverse microradiography (TMR).

6.3.2.2 In situ remineralisation model
The in situ study had a randomised, controlled, double-blind cross-over study design. Artificially demineralised carious lesions (as described in 2.2.1) were created on enamel blocks sectioned from human third molars and the blocks were each cut into experimental and control half-blocks as in the in vitro remineralisation model. Experimental half-blocks were mounted with wax to intra-oral appliances as described previously by Shen et al. [2011]. Eight healthy subjects with an average age of 43 ± 11 years old (4 males and 4 females) participated. It was calculated that a minimum of 8 participants was required to provide the required statistical power (90 %, p < 0.05) based on previous publications using a similar cross-over in situ model [Cai et al., 2009; Walker et al., 2010].

Participants were randomly allocated to rinse with 5 mL of one of five solutions (solutions iii – vii, pH 4.0) for 1 minute, four times each day for 14 consecutive days (treatment
period). Participants rinsed at 10:00am, 11:30am, 2:00pm and 3:30pm and were instructed to continue their normal dietary regime and were given a toothbrush and sodium fluoride toothpaste to brush their teeth with twice a day. Intra-oral appliances were removed during eating or oral hygiene procedures. Participants were also instructed to clean their intra-oral appliances with a toothbrush and a fluoride-free denture paste that was supplied to them, taking care to avoid the attached enamel half-blocks. At the conclusion of the 14 days, participants rested from the study for one week (washout period) then they began another treatment with a randomly assigned treatment solution. This was repeated until participants had rinsed with all five solutions. Intra-oral appliances were kept in a sealed humidified container whenever not in the mouth. During the treatment periods subjects maintained a diary recording each rinse and duration of rinse. After each treatment period, participants returned their appliance and diary to the investigators and new enamel half-blocks were attached for the next treatment period. In addition, each experimental half-block was paired with its control half-block for analysis of mineral change using TMR.

6.3.2.3 TMR
Enamel blocks from the in vitro and in situ remineralisation experiments were assessed for percentage remineralisation using TMR as described in 2.3.1.

6.3.2.4 Analysis of ion concentrations in the remineralisation solutions
The total and free calcium, tin, phosphate and fluoride concentrations were calculated from the 0.4 % CPP-ACP + SnF$_2$ + NaF solutions at both pH 4.0 and 5.6 (solutions i – iv) and the 0.4 % CPP-ACP solution at pH 4.0 (solution v). The total ion concentration was measured by diluting 1 mL of solution with 19 mL of 1M HNO$_3$ and left for 24 hours before being centrifuged at 1000g for 15 minutes at room temperature. The supernatant was analysed for calcium and tin using atomic absorption spectroscopy (AAS), as well as phosphate and fluoride using ion chromatography. The ‘free’ ion concentration (not stabilised within CPP complexes) was calculated by filtering a sample of solution in a centrificon using a 1000 MWCO filter (Pall Corporation, USA) and the resulting filtrate was centrifuged at 3000g for 60 minutes at room temperature. The supernatant was then analysed for ion concentration using AAS and ion chromatography as described for the total ion concentration measurements.
6.3.2.5 SEM-EDS

Scanning electron microscope energy-dispersive x-ray spectroscopy (SEM-EDS) analysis was conducted on TMR sections of lesions treated *in vitro* by CPP-ACP + SnF₂ + NaF (solution i) to assess the distribution of elements within the lesion following remineralisation. The samples were examined at 10 kV under low vacuum using a solid state diode backscatter electron detector in a FEI Quanta FEG 200 SEM operating at 10 kV with an energy-dispersive spectrometer at the Bio21 Advanced Microscopy Facility (VIC, Australia). Characteristic x-rays from areas of interest were then detected using an energy dispersive x-ray spectrometer and microanalysis software (AZtec Microanalysis Suite Ver 3.1, Oxford Instruments).

6.3.2.6 Electron probe micro-analysis (EPMA)

TMR sections from the CPP-ACP + SnF₂ + NaF, CPP-ACP + NaF and SnF₂ *in situ* treatments (solutions iii, iv and vi) were chosen for elemental analysis using EPMA. The tooth sections within the slides were embedded in epoxy resin (Epofix; Struers, Denmark) on a specimen holder. The embedded enamel sections were initially polished using 2,400 grit abrasive paper, then were polished using 3 and 1 µm diamond polishing pastes until finally optical smoothness was achieved with a 0.25 µm aluminium oxide polishing paste. Samples and standards were coated with a 20 nm layer of carbon using a Dynavac 300 coater. The EPMA was conducted using parameters as described previously [Cochrane *et al.*, 2014].

6.3.2.7 Statistical analysis

Initial lesion depth (LDd), lesion depth change (LDd-LDr), initial mineral content (ΔZd), mineral content change (ΔZd-ΔZr), and percent remineralisation (% R) were measured from analysis of the demineralised and remineralised lesion mineral profiles from the TMR images (see 2.3.1). Means and standard deviations for each parameter for each treatment were calculated. For the *in vitro* data, a two sample t-test was used to measure differences in lesion parameters (LDd, LDd-LDr, ΔZd, ΔZd-ΔZr and %R) between the two treatment groups. For the *in situ* data, the subject was the unit of analysis and the same lesion parameters were compared across the five treatments using analysis of covariance (ANCOVA). Data were analysed for normality using Q-Q plots and the Shapiro-Wilk test and homogeneity of variance was tested using Levene’s test [Sokal and
Rohlf, 1969]. Post hoc pairwise differences between treatments were performed on the estimated marginal means using the Sidak adjustment for multiple comparisons. The statistical significance was set at p < 0.05. SPSS software version 22 (IBM Corp. NY, USA) was used for all statistical tests.

6.3.3 Dentine surface treatment

6.3.3.1 Dentine Disc Preparation
Twenty extracted human third molars were sectioned into 20 x 1 mm discs as described in Chapter 2. To remove the smear layer, the discs were exposed to 15 % EDTA for 2 minutes [Wang et al., 2011] after which the discs were rinsed thoroughly with DDW for 5 seconds and blotted dry. The discs were placed into a sealed humidified environment before exposure to experimental solutions.

6.3.3.2 Exposure to Experimental Solutions
Discs were randomly allocated into four treatment groups (a-d) with group (a) having 2 discs and groups (b-d) having 6 discs each. Treatment solutions viii-x were used for treatment groups (b), (c) and (d). The groups of discs were exposed to (a) no treatment, or 10 mL of (b) 5 % CPP-ACP, (c) 500 ppm F as SnF₂, or (d) 5 % CPP-ACP with 500 ppm F as SnF₂ for 20 minutes before being removed, immersed in DDW for 5 seconds and stored in a humidified environment.

6.3.3.3 SEM
One disc from group (a) and four discs from groups (b-d) were desiccated silica gel for 72 hours. Following dehydration, samples were mounted on sample holders, gold sputter-coated (2 nm) and examined with an Everhart-Thornley detector in a FEI Quanta FEG 200 SEM at 10 kV under high vacuum at the Bio21 Advanced Microscopy Facility (VIC, Australia).

6.3.3.4 SEM-EDS
The remaining discs (one disc from group (a) and two discs from groups (b-d)) were mounted to epoxy resin (Epofix, Struers, Denmark) prior to exposure to 15 % EDTA and the experimental solutions. Following exposure to the solutions, the discs were desiccated
using silica gel for 72 hours and examined with an energy dispersive x-ray spectrometer as described for the enamel samples.

6.4 Results

6.4.1 Enamel remineralisation

6.4.1.1 In vitro model

The in vitro remineralisation model demonstrated the combined CPP-ACP + SnF₂ + NaF solution was more effective remineralising enamel subsurface lesions in vitro than the CPP-ACP + NaF despite both solutions having the same CPP-ACP and fluoride concentration. The mean percentage remineralisation of the CPP-ACP + SnF₂ + NaF solution calculated from TMR was 32 % greater than mean percentage remineralisation by the CPP-ACP + NaF solution (see Table 6.1).

SEM-EDS analysis of the CPP-ACP + SnF₂ + NaF treated enamel blocks revealed the remineralised lesions had a calcium to phosphorous ratio consistent with HA with traces of fluoride suggesting FA and FHA were also present (see Table 6.3). The lesions showed an increase in carbon, fluoride and tin when compared to sound enamel. On the surface of the enamel was a mineralised surface layer approximately 2 µm thick; this layer was found to have a high amount of carbon (26.5 %) as well as tin (1.3 %) (see Fig 6.1). The ratio of Ca:P:O in this layer was consistent with apatite.

6.4.1.2 In situ model

The TMR results of the in situ remineralisation experiment are shown in Table 6.2. The CPP-ACP + SnF₂ + NaF mouthrinse produced the greatest mean remineralisation in the enamel subsurface lesions (30.6 ± 1.6 %) and was significantly higher compared with all other treatments. The CPP-ACP and CPP-ACP + NaF mouthrinses produced 13.4 ± 1.0 % and 24.6 ± 2.1 % remineralisation respectively, which were significantly different to the other three treatments. The NaF and SnF₂ mouthrinses were not statistically different to each other in terms of percentage remineralisation and produced the lowest mean remineralisation (10.8 ± 0.8 % for both). The significantly greater % remineralisation following exposure to the CPP-ACP + SnF₂ + NaF treatment over CPP-ACP + NaF in
situ was in agreement with the in vitro remineralisation model and suggested the combination of CPP-ACP and SnF₂ had a synergistic effect.

The total, free and CPP-bound ion concentrations of the CPP-ACP + SnF₂ + NaF solutions at pH 4.0 and 5.6 are shown in Table 6.4. AAS analysis demonstrated that the majority of stannous ions were incorporated into the CPP complexes in the CPP-ACP + SnF₂ + NaF solution; 90.8 % of stannous ions were stabilised by the CPPs at pH 4.0, and 99.3 % were stabilised at pH 5.6. The ion concentrations for the CPP-ACP + NaF and CPP-ACP solutions are shown in Table 6.5 and Table 6.6 respectively. The calcium to CPP ratio was calculated for all the in situ solutions and plotted against % remineralisation (see Figure 6.2). A higher calcium to CPP ratio was observed in the CPP-ACP + NaF solution (7.7) compared with the CPP-ACP solution (2.0), while the CPP-ACP + SnF₂ + NaF solution showed an even higher calcium to CPP ratio (9.4). The calcium to CPP ratio is used as a surrogate for CPP complex stability and it was shown to be positively correlated to the percent remineralisation produced by the treatment solutions in situ (R = 0.99).

A high fluoride weight percentage was measured throughout the CPP-ACP + SnF₂ + NaF treated lesions (see Fig 6.3 A & B); using EPMA approximately twice as much fluoride was measured in these lesions as compared to the SnF₂ alone treated lesions. The fluoride content present in the CPP-ACP + SnF₂ + NaF treated lesions was also shown to be higher than the CPP-ACP + NaF treated lesions; this demonstrated that at the same fluoride concentration the combination of CPP-ACP and SnF₂ was promoting more fluoride deposition through the depth of the lesion than CPP-ACP combined with NaF.

According to the EPMA measurements, the tin content of the CPP-ACP + SnF₂ + NaF treated lesions was also higher than the lesions treated with SnF₂ alone (See Fig 6.3 C). For both of these treatments, the tin was mainly concentrated in the outer 10 μm of the lesion, with the maximum tin apparent 5 μm from the enamel surface and minimal tin present beyond 15 μm. At the peak concentration 5 μm from the enamel surface, approximately twice as much tin was measured in the lesions treated with CPP-ACP + SnF₂ + NaF as the lesions treated with SnF₂ alone.
Table 6.1: Comparison of enamel subsurface lesion parameters before and after remineralisation *in vitro* (pH 5.6) as measured by TMR.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LDd (µm)</th>
<th>ΔLD (µm)</th>
<th>ΔZd (vol%min.µm)</th>
<th>ΔZd-ΔZr (vol%min.µm)</th>
<th>%Remin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPP-ACP + NaF</td>
<td>124.6 ± 18.6</td>
<td>23.5 ± 12.8</td>
<td>3587.0 ± 923.9</td>
<td>1259.8 ± 370.8</td>
<td>35.0 ± 5.4</td>
</tr>
<tr>
<td>CPP-ACP + SnF2 + NaF</td>
<td>127.4 ± 20.5</td>
<td>30.2 ± 17.6</td>
<td>3784.6 ± 1398.7</td>
<td>1757.1 ± 757.8</td>
<td>46.1 ± 5.8</td>
</tr>
</tbody>
</table>

p-value$^8$ NS > 0.05 NS > 0.05 NS > 0.05 < 0.05 < 0.0001

Table 6.2: Comparison of enamel subsurface lesion parameters before and after remineralisation *in situ* (pH 4.0) as measured by TMR.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LDd (µm)</th>
<th>ΔLD (µm)</th>
<th>ΔZd (vol%min.µm)</th>
<th>ΔZd-ΔZr (vol%min.µm)</th>
<th>%Remin</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaF</td>
<td>103.6 ± 10.9</td>
<td>2.0 ± 3.5$^{abc}$</td>
<td>2728.9 ± 578.8</td>
<td>291.4 ± 48.6$^{abc}$</td>
<td>10.8 ± 0.8$^{abc}$</td>
</tr>
<tr>
<td>SnF2 + NaF</td>
<td>97.6 ± 6.9</td>
<td>5.0 ± 3.8$^{def}$</td>
<td>2273.3 ± 294.5</td>
<td>245.4 ± 43.4$^{def}$</td>
<td>10.8 ± 0.8$^{def}$</td>
</tr>
<tr>
<td>CPP-ACP</td>
<td>105.8 ± 6.9</td>
<td>10.8 ± 1.2$^a$</td>
<td>2729.2 ± 427.6</td>
<td>367.2 ± 68.0$^{adgh}$</td>
<td>13.4 ± 1.0$^{adgh}$</td>
</tr>
<tr>
<td>CPP-ACP + NaF</td>
<td>103.4 ± 8.1</td>
<td>12.5 ± 7.0$^{bd}$</td>
<td>2742.4 ± 490.1</td>
<td>670.2 ± 102.8$^{begi}$</td>
<td>24.6 ± 2.1$^{begi}$</td>
</tr>
<tr>
<td>CPP-ACP + SnF2 + NaF</td>
<td>104.3 ± 6.3</td>
<td>15.0 ± 2.9$^{ce}$</td>
<td>2527.5 ± 449.1</td>
<td>776.6 ± 159.9$^{efhi}$</td>
<td>30.6 ± 1.6$^{efhi}$</td>
</tr>
</tbody>
</table>

p-value$^8$ NS > 0.05 < 0.0001 NS > 0.05 < 0.0001 < 0.0001

For Tables 6.1 & 6.2: $^1$LDd = lesion depth after demineralisation, $^2$ΔLD = reduction in lesion depth after remineralisation, $^3$ΔZd = integrated mineral loss prior to remineralisation, $^4$ΔZd-ΔZr = gain in mineral content after remineralisation; $^5$%R = percent remineralisation ((ΔZd-ΔZr/ΔZd)*100%). Displayed as mean ± standard deviation. § ANCOVA ($\alpha = 0.05$) NS not significant. Differences between means were measured using post hoc multiple comparison tests on the marginal means using a Sidak adjustment. $^{abcdefghi}$ Values in the same column similarly marked are significantly different (p < 0.05).
Table 6.3: Elemental composition of enamel remineralised by CPP-ACP + SnF₂ + NaF (solution i.) as detected by SEM-EDS. Expressed as mole fraction (weight percentage in parentheses). ND = not detected.

<table>
<thead>
<tr>
<th></th>
<th>Ca</th>
<th>P</th>
<th>C</th>
<th>O</th>
<th>F</th>
<th>Sn</th>
<th>Na</th>
<th>Cl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Remineralised</td>
<td>0.141</td>
<td>0.089</td>
<td>0.286</td>
<td>0.476</td>
<td>0.004</td>
<td>ND</td>
<td>0.003</td>
<td>0.002</td>
</tr>
<tr>
<td>lesion</td>
<td>(28.7 %)</td>
<td>(14.0 %)</td>
<td>(17.5 %)</td>
<td>(38.7 %)</td>
<td>(0.3 %)*</td>
<td></td>
<td>(0.4 %)</td>
<td>(0.4 %)</td>
</tr>
<tr>
<td>Surface layer</td>
<td>0.094</td>
<td>0.059</td>
<td>0.394</td>
<td>0.442</td>
<td>0.005</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>(21.2 %)</td>
<td>(10.3 %)</td>
<td>(26.5 %)</td>
<td>(39.6 %)</td>
<td>(0.5 %)*</td>
<td>(1.2 %)</td>
<td>(0.3 %)</td>
<td>(0.3 %)</td>
</tr>
</tbody>
</table>

Figure 6.1: SEM image of CPP-ACP + SnF₂ + NaF treated enamel; the coloured map represents element distribution in the surface layer.

*Surface enamel typically has a fluoride concentration of 1000 ppm, approximately 0.03 % (w/w) [Angmar et al., 1963; Fejerskov et al., 1994].
Table 6.4: Ion concentrations of calcium, phosphorus, tin and fluoride of the 0.4 % CPP-ACP + SnF₂ + NaF solution at pH 5.6 and 4.0 (solutions i and iii) as measured using AAS and ion chromatography. Data are displayed as mean ± standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>Ca mM</th>
<th>Pi mM</th>
<th>Sn mM</th>
<th>F mM [ppm]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total</strong></td>
<td>5.6</td>
<td>15.29 ± 0.17</td>
<td>10.88 ± 0.11</td>
<td>5.58 ± 0.14</td>
<td>15.39 ± 0.09 [292.5 ± 1.7]</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>16.25 ± 0.16</td>
<td>11.62 ± 0.22</td>
<td>5.67 ± 0.12</td>
<td>15.24 ± 0.63 [289.5 ± 12.0]</td>
</tr>
<tr>
<td><strong>CPP-stabilised</strong></td>
<td>5.6</td>
<td>15.25 (99.7 %)</td>
<td>10.52 (96.7 %)</td>
<td>5.55 (99.3 %)</td>
<td>6.33 (41.1 %) [120.2]</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>12.53 (77.1 %)</td>
<td>9.50 (81.8 %)</td>
<td>5.15 (90.8 %)</td>
<td>8.64 (56.7 %) [164.2]</td>
</tr>
<tr>
<td><strong>Free</strong></td>
<td>5.6</td>
<td>0.04 ± 0.00 (0.3 %)</td>
<td>0.36 ± 0.01 (3.3 %)</td>
<td>0.037 ± 0.00 (0.7 %)</td>
<td>9.07 ± 0.14 (58.9 %) [172.3 ± 2.7]</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>3.72 ± 0.17 (22.9 %)</td>
<td>2.12 ± 0.10 (18.2 %)</td>
<td>0.52 ± 0.01 (9.2 %)</td>
<td>6.59 ± 0.06 (43.3 %) [125.3 ± 1.1]</td>
</tr>
</tbody>
</table>

*CPP-stabilised stannous was measured to have a concentration of approximately 8 mole per mole of phosphopeptide in the CPP-ACP + SnF₂ + NaF solution at pH 5.6.*
Table 6.5: Ion concentrations of calcium, phosphorus and fluoride of the 0.4 % CPP-ACP + NaF solution at pH 5.6 and 4.0 (solutions ii. and iv.) as measured using AAS and ion chromatography. Data are displayed as mean ± standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>Ca mM</th>
<th>Pi mM</th>
<th>F mM [ppm]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total</strong></td>
<td>5.6</td>
<td>14.56 ± 0.05</td>
<td>11.11 ± 0.17</td>
<td>15.0 ± 0.33 [285.0 ± 6.3]</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>15.13 ± 0.37</td>
<td>11.16 ± 0.20</td>
<td>14.8 ± 0.77 [281.3 ± 14.5]</td>
</tr>
<tr>
<td><strong>CPP-stabilised</strong></td>
<td>5.6</td>
<td>14.53 (99.8 %)</td>
<td>7.20 (64.8 %)</td>
<td>4.0 (26.8 %) [76.4]</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>10.30 (68.1 %)</td>
<td>2.89 (25.9 %)</td>
<td>12.2 (82.7 %) [232.7]</td>
</tr>
<tr>
<td><strong>Free</strong></td>
<td>5.6</td>
<td>0.03 ± 0.00 (0.2 %)</td>
<td>3.91 ± 0.18 (35.2 %)</td>
<td>11.0 ± 0.72 (73.2 %) [208.6 ± 14.0]</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>4.82 ± 0.20 (31.9 %)</td>
<td>8.27 ± 0.26 (74.1 %)</td>
<td>2.6 ± 0.02 (17.3 %) [48.6 ± 1.2]</td>
</tr>
</tbody>
</table>

Table 6.6: Ion concentrations of calcium and phosphorus of the 0.4 % CPP-ACP solution at pH 4.0 (solution v.) as measured using AAS and ion chromatography. Data are displayed as mean ± standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>Ca mM</th>
<th>Pi mM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total</strong></td>
<td>4.0</td>
<td>15.00 ± 0.33</td>
<td>11.17 ± 0.42</td>
</tr>
<tr>
<td><strong>CPP-stabilised</strong></td>
<td>4.0</td>
<td>2.72 (18.1 %)</td>
<td>2.45 (21.9 %)</td>
</tr>
<tr>
<td><strong>Free</strong></td>
<td>4.0</td>
<td>12.28 ± 0.38 (81.9 %)</td>
<td>8.72 ± 0.02 (78.1 %)</td>
</tr>
</tbody>
</table>
Figure 6.2: CPP-bound calcium to CPP ratio (based on treatment) compared against percent remineralisation. Calculated from ion chromatography data (assuming 1.33 mM CPP in each solution).
Figure 6.3: EPMA analysis of atomic weight percentage within enamel subsurface lesions. A = fluoride weight % comparison between CPP-ACP + SnF₂ + NaF and CPP-ACP + NaF treated lesions, B = fluoride weight % comparison between CPP-ACP + SnF₂ + NaF and SnF₂ treated lesions, C = stannous weight % comparison between CPP-ACP + SnF₂ + NaF and SnF₂ treated lesions.
6.4.2 Dentine surface treatment

6.4.2.1 SEM

Representative images from each group are shown in Figures 6.4 – 6.7. The control dentine from group (a) showed the dentine surface had no precipitation and had patent tubules (see Figure 6.4). Similarly, the dentine treated by CPP-ACP from group (b) did not appear to show any surface precipitation and appeared to be relatively unchanged by the treatment (see Figure 6.5). In contrast, the SnF2 treated dentine from group (c) displayed globular deposits on the dentine surface and within the dentine tubules (see Figure 6.6). The combined SnF2 and CPP-ACP treated dentine in group (d) displayed areas with a visible ‘nanocoating’ of cross-linked nanofilaments covering the dentine (including the tubules) accompanied by electron dense spheres. These spheres ranged from nanosized up to 2 µm in diameter and were consistently found in areas where the grey nanocoating was visible (see Figure 6.7).

6.4.2.2 SEM-EDS

The elemental analysis (weight percentage) of dentine from the control group (a) revealed the main elements present were oxygen (38.2 %), calcium (28.4 %), carbon (18.0 %), and phosphorus (12.9 %). Trace amounts of sodium and magnesium were detected (1.6 % and 0.9 %, respectively). This was consistent with the organic and inorganic components of sound dentine previously reported [LeGeros, 1990; Miller et al., 1993].

Dentine treated with CPP-ACP had an atomic composition closely matching the control dentine suggesting minimal surface change during the experimental period. The analysis of the surface deposits visible on the dentine treated by SnF2 (group (c)) revealed it contained on average 15.8 % tin and 2.0 % fluoride; approximately two stannous ions for every fluoride ion after taking into account atomic weight. Other elements present on the surface layer were oxygen (35.3 %), carbon (20.5 %), calcium (16.2 %) and phosphorous (10.3 %).

Areas of dentine showing an effect of the treatment were chosen from the CPP-ACP + SnF2 group for SEM-EDS analysis. An area displaying the surface nanocoating and spherical particles (see Figure 6.8) revealed the electron dense spheres were rich in
oxygen and carbon (37.1% and 31.0%, respectively) as well as calcium (18.1%), phosphorous (10.6%), tin (1.4%), sodium (0.9%), magnesium (0.5%) and fluoride (0.5%). The nanocoating was similar in composition although relatively higher in carbon content (46.8% carbon, 29.9% oxygen, 14.4% calcium, 8.2% phosphorous and 0.7% magnesium), and it did not contain detectable levels of fluoride or tin. The molar fractions for these elements are displayed in Table 6.7.
**Figure 6.4:** SEM images of untreated dentine. Right image = magnified image of dentine tubule.
Figure 6.5: SEM images of dentine treated by CPP-ACP (solution viii.). Right image = magnified image of dentine tubule.
Figure 6.6: SEM images of dentine treated by SnF$_2$ (solution ix.). Right image = magnified image of dentine tubule.
Figure 6.7: SEM images of dentine treated by CPP-ACP + SnF$_2$ (solution x.). Right image = magnified image of dentine tubule.
Table 6.7: Elemental composition of sound dentine and dentine treated by CPP-ACP, SnF$_2$, or CPP-ACP + SnF$_2$ expressed as mole fraction (weight percentage in parentheses) as detected by SEM-EDS. ND = not detected.

<table>
<thead>
<tr>
<th></th>
<th>Ca</th>
<th>P</th>
<th>C</th>
<th>O</th>
<th>F</th>
<th>Sn</th>
<th>Mg</th>
<th>Na</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sound dentine</strong></td>
<td>0.139 (28.4%)</td>
<td>0.081 (12.9%)</td>
<td>0.293 (18.0%)</td>
<td>0.467 (38.2%)</td>
<td>ND</td>
<td>ND</td>
<td>0.007 (0.9%)</td>
<td>0.014 (1.6%)</td>
</tr>
<tr>
<td><strong>CPP-ACP dentine</strong></td>
<td>0.117 (24.7%)</td>
<td>0.082 (13.4%)</td>
<td>0.282 (17.8%)</td>
<td>0.507 (42.6%)</td>
<td>ND</td>
<td>ND</td>
<td>0.006 (0.8%)</td>
<td>0.006 (0.7%)</td>
</tr>
<tr>
<td><strong>SnF$_2$ (precipitation)</strong></td>
<td>0.083 (16.2%)</td>
<td>0.068 (10.3%)</td>
<td>0.349 (20.5%)</td>
<td>0.451 (35.3%)</td>
<td>0.022 (2.0%)</td>
<td>0.027 (15.8%)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>CPP-ACP + SnF$_2$ (sphere)</strong></td>
<td>0.078 (18.1%)</td>
<td>0.059 (10.6%)</td>
<td>0.446 (37.1%)</td>
<td>0.400 (31.0%)</td>
<td>0.004 (0.5%)</td>
<td>0.002 (1.4%)</td>
<td>0.004 (0.5%)</td>
<td>0.007 (0.9%)</td>
</tr>
<tr>
<td><strong>CPP-ACP + SnF$_2$ (nanocoating)</strong></td>
<td>0.056 (14.4%)</td>
<td>0.041 (8.2%)</td>
<td>0.607 (46.8%)</td>
<td>0.291 (29.9%)</td>
<td>ND</td>
<td>ND</td>
<td>0.004 (0.7%)</td>
<td>ND</td>
</tr>
</tbody>
</table>
Figure 6.8: Representative image for SEM-EDS analysis for dentine treated by CPP-ACP + SnF₂ (solution x.) showing electron dense spheres of submicron size over the nanocoating.

6.5 Discussion

6.5.1 Enamel experiments

It was evident from both the *in vitro* and *in situ* enamel experiments that the addition of SnF₂ to CPP-ACP had a synergistic effect on remineralisation of mineral deficient subsurface lesions. This was apparent as the CPP-ACP solutions with added SnF₂ had a higher percent remineralisation *in vitro* and *in situ* when compared to the CPP-ACP + NaF, CPP-ACP or SnF₂ solutions alone. The proposed mechanism for this synergy is the ability of CPPs to bind and release calcium phosphate and fluoride ions within the enamel lesions being improved by the presence of stannous ions.
The mechanism for the increased efficacy of the CPP-ACP + SnF₂ + NaF treatment solutions was expounded by the ion analysis (Table 6.4) where approximately 90% of the stannous ions were bound to the CPP complexes at pH 4.0, while at pH 5.6 approximately 99% of the stannous ions were bound to the complexes. The addition of SnF₂ to CPP-ACP appeared to increase the binding capacity of CPPs to stabilise larger soluble complexes; this was apparent upon calculation of the calcium to CPP molar ratio of the various solutions. As there was a concentration of 1.33 mM of CPP in the treatment solutions, at pH 4.0 the CPP-ACP solution had a calcium to CPP ratio of 2.0, while the calcium to CPP ratio for the CPP-ACP + NaF solution was 7.7. The addition of SnF₂ resulted in a calcium to CPP ratio of 9.4, further increasing the calcium binding ability of the CPP complexes. The ratio of calcium to CPP was an important marker of CPP efficacy as it strongly correlated with percent remineralisation (Figure 6.2). While it has been documented that the addition of fluoride to CPP complexes increases their stability, calcium binding capacity, and remineralisation efficacy [Cochrane et al., 2008; Cross et al., 2004], the current experiment presented evidence that tin acts in a similar manner. Accordingly, the increased stability and calcium binding capacity of the tin-containing CPP complexes allowed greater amounts of calcium, phosphate and fluoride to be delivered to the mineral deficient enamel, increasing the percent remineralisation when compared with other treatments.

As calculated from the SEM-EDS data, the remineralised lesion and the surface layer formed from the CPP-ACP + SnF₂ + NaF in vitro treatment showed calcium to phosphate ratios of 1.58 and 1.59, respectively. This was close to the published value of 1.62 for enamel [Simmer and Fincham, 1995] and indicated an apatite-like mineral phase was present in the surface layer after treatment with CPP-ACP + SnF₂ + NaF. The detection of carbon within the surface layer (26.5 %) suggested an organic component was bound to the mineral phase, most likely CPPs upon release of ions into the enamel fluid. This was in agreement with previous evidence that confirmed CPPs have a high affinity for apatite and may regulate apatite growth [Huq et al., 2000]. Taking into account the oxygen paired with phosphorous as phosphate in the surface layer, and the theoretical oxygen as hydroxide in stoichiometric HA, the resulting carbon to oxygen ratio of the surface layer was 2.10; this finding is in agreement with the surface layer observed in the
dentine experiment (discussed in 6.5.2). Small amounts of tin and fluoride were detected in the enamel surface layer (1.2 % and 0.5 % respectively) implying these ions were either lattice substitutions within the apatite or incorporated as other less organised acid-resistant mineral phases dispersed between the apatite crystals.

The hypothesis of a stannous fluoride ‘barrier’ surface layer providing protection against acid erosion has been described by Faller and Eversole [2014]. Previous in vitro evidence has shown enamel when treated with a stannous fluoride solution promotes a tin and fluoride containing surface layer thought to inhibit acid demineralisation of the underlying hard tissue [Schlueter et al., 2009b]. The thickness of the surface layer observed by Schlueter et al. [2009b] was reported to be approximately 500 nm and with an amorphous appearance which is a similar finding to the current study where a stannous-rich surface layer was observed.

6.5.2 Dentine experiment

Dentine treated with CPP-ACP alone appeared to be relatively unchanged and the EDS results were consistent with this interpretation. As the pH of the treatment solution was 7.9 and the dentine surface was not mineral deficient, there was little driving force for remineralisation within the short time frame. Calcium and phosphate released by the CPPs at the dentine surface would be unlikely to produce surface precipitation and this was corroborated by the lack of visible surface deposits.

The appearance of surface dentine treated with the SnF₂ solution was similar to that seen in previous studies assessing its effect on surface dentine [Ellingsen and Rolla, 1987; Wang et al., 2015]. Ellingsen and Rolla [1987] hypothesised the surface mineral present on dentine after treatment with SnF₂ was likely to be a mixed phosphate-coated calcium fluoride (CaF₂) deposit (that acts as a fluoride reservoir) and a stannous phosphate derivative. Other authors have suggested Sn(OH)₂, Sn₂(PO₄)OH, Ca(SnF₃)₂, Sn₃F₃PO₄, Sn₂(OH)PO₄, Sn₃F₃PO₄, or SnHPO₄ may precipitate on the surface of dentine treated with SnF₂ [Ganss et al., 2010]. As the interaction of SnF₂ with dentine is complex and not fully understood, it was difficult to extrapolate the SEM-EDS observations to speculate which of these tin-containing phases may be have been present on the dentine surface and in
what ratio. The SEM-EDS detected mole ratio of tin to fluoride was 1.26; this suggested more tin-containing phases were present on the surface than fluoride-containing phases and that additional fluoride was likely to have been dissolved in solution.

The hydrodynamic radius of CPP-ACP complexes has been shown to be approximately 2 nm, with a slightly larger radius observed when fluoride is incorporated as CPP-ACFP [Cross et al., 2007; Cross et al., 2004]. The spherical particles observed following the CPP-ACP + SnF$_2$ treatment varied in size. In some instances they were nanosized or up to 2 µm in diameter (see Figure 6.8). The phenomenon observed on the dentine surface after this treatment and the structures of the spherical particles and nanocoating can be explained by the elemental composition obtained from SEM-EDS. The high carbon and oxygen percentage suggested the spherical particles had an organic component accompanying the bundles of calcium, phosphate, fluoride and stannous ions. Under the experimental conditions, this organic structure was consistent with CPPs bound together or cross-linked by the electron dense atom, tin. Using one of the major peptides present in the commercial CPP-ACP preparation as representative of the peptide content, it can be shown that the measured carbon to oxygen ratio of the sphere was very close to that of CPPs. The major CPP β(1-25) has 106 carbon atoms, 59 oxygen atoms and 4 phosphorous atoms per peptide resulting in a carbon to oxygen ratio of 1.80 [Cross et al., 2005]. Assuming the total carbon fraction of the sphere was from β(1-25) and using the mole fractions shown in Table 6.7, the relative phosphorous present in the peptide (0.017 phosphorous) can be subtracted from the total mole fraction of phosphorous observed (0.059), and the remaining fraction can be assumed to be phosphorous present as phosphate within the CPP complex (0.042). The corresponding mole fraction of oxygen as phosphate within the CPP complex (0.168) can be subtracted from the total mole fraction of oxygen observed to estimate the remaining oxygen as contained within the peptide (0.232). The carbon to oxygen ratio of the organic component can then be calculated as 1.92, very close to the known ratio of 1.80 for β(1-25). Additionally, the calcium to CPP ratio was determined to be 18.5 and approximately two peptides were present per stannous ion. As CPP-ACFP complexes typically contain 15 calcium ions per peptide [Cross et al., 2004], the finding from the current study was consistent with the
incorporation of the stannous ion increasing the calcium binding capacity of CPPs, which was consistent with the ion analysis in the enamel experiment.

The electron dense nanocoating of cross-linked nanofilaments observed covering the dentine in the SEM images following treatment with CPP-ACP + SnF₂ was especially high in carbon (46.8%). Remarkably, the carbon to oxygen ratio of this layer (2.09) was nearly identical to that of the organic component in the surface layer of the CPP-ACP + SnF₂ + NaF treated enamel lesions (2.10). While this was higher than the theoretical ratio for β(1-25), a cross-linking of CPPs on the surface through the well characterised chemically induced β-elimination of serine phosphate in casein would have resulted in some loss of oxygen explaining the higher carbon to oxygen ratio [Reynolds et al., 1994; Wang et al., 2014]. This was highly suggestive of a nanosized network of cross-linked CPPs being present on both the enamel and dentine samples following treatment with stannous-containing CPP solutions.

To explain the surface interaction of the CPP-ACP + SnF₂ solution in the dentine experiment, a mechanism is proposed. Figure 6.9 illustrates this mechanism whereby large numbers of CPP-ACFP complexes are cross-linked by stannous ions to form large spherical particles up to 2 µm in diameter as visible in the SEM images. Upon contact with dentine the negatively charged residues of the CPPs became increasingly attracted to the exposed positively charged apatite crystal faces on the dentine surface. In addition, the stannous ions are attracted to the dentine phosphate and protein, promoting complexes to separate and attach to the dentine while releasing calcium, phosphate and fluoride ions contained within the complexes. This release of ions reveals more residues on the CPPs, particularly the CPP binding motif Ser(P)-Ser(P)-Ser(P)-Glu-Glu, which is strongly attracted to the apatite crystals of dentine by the formation of lower, free-energy polydentate structures [Huq et al., 2000]. The CPP complexes accordingly changed conformation and cross-link on the dentine surface to form an organic nanocoating, thereby releasing their payload of calcium, phosphate and fluoride to the surrounding solution and dentine fluid. The cross-linking of the CPPs on the dentine/enamel surface would also be facilitated by stannous-catalysed β-elimination of phosphoseryl residues to form reactive dehydroalanine residues that can engage in Michael addition reactions with
nucleophiles on other CPP residues to produce covalently cross-linked CPP filaments, which would help explain their presence on the CPP-ACFP/Sn-treated surfaces. This proposed mechanism explains the high electron conductivity of the spheres was due to the intrinsic calcium and stannous ions associated with the CPPs, but also the nanocoating was an indirect result of the stannous ion delivering and promoting release of high quantities of CPP-stabilised calcium phosphate and fluoride at the tooth surface.

Figure 6.9: Diagram illustrating the proposed mechanism for Sn$^{2+}$ mediated release of Ca$^{2+}$/PO$_4^{3-}$/F$^-$ from bundled CPP complexes and subsequent CPP nanocoating formation on the dentine surface.

The observed outcome after treatment with CPP-ACP + SnF$_2$ should be viewed within the context of the experimental conditions. However, the proposed mechanism of surface interaction with dentine may give an insight into the interaction of stannous containing
CPP-ACFP complexes with mineral deficient enamel in the \textit{in vitro} and \textit{in situ} experiments. The stannous ions appeared to bestow an added stability to the complexes as evidenced by their higher percent remineralisation of enamel; these complexes were capable of stabilising greater amounts of calcium, phosphate and fluoride in solution which were then made bioavailable upon conformational release by the CPP complexes. The SEM-EDS analysis of enamel and dentine treated by stannous-containing CPP solutions revealed the organic component of the enamel and dentine surface layers was nearly identical, suggesting a similar interaction occurred in both experiments whereby a nanocoating of cross-linked CPPs was bound to the surface after releasing bioavailable calcium, phosphate, fluoride and stannous ions. In summary, the combination of CPP-ACP and SnF$_2$ appears to have the potential to increase the anticariogenic properties of either component alone and may lead to more favourable clinical outcomes when applied therapeutically.

\section*{6.6 Conclusions}

The combination of CPP-ACP and SnF$_2$ was shown to enhance the potential therapeutic ability of both agents on the dental hard tissues \textit{in vitro} and \textit{in situ}.

\begin{itemize}
\item Significantly higher enamel remineralisation was observed \textit{in vitro} by the combined CPP-ACP + SnF$_2$ + NaF solution compared to the CPP-ACP + NaF solution at pH 5.6 ($p < 0.00001$).
\item A synergistic effect upon enamel remineralisation by CPP-ACP and SnF$_2$ was observed \textit{in situ} by the combined CPP-ACP + SnF$_2$ + NaF solution which showed significantly higher remineralisation than the CPP-ACP + NaF, CPP-ACP, NaF and SnF$_2$ solutions at pH 4.0 ($p < 0.00001$).
\item A stannous and fluoride containing apatite surface ‘barrier’ layer was observed in lesions treated by CPP-ACP + SnF$_2$ + NaF \textit{in vitro}.
\item The addition of SnF$_2$ to CPP-ACP appeared to increase the complex stability, ion binding capacity and ion delivery at the tooth surface.
\item The combined CPP-ACP and SnF$_2$ solution displayed a unique interaction with dentine; it was theorised stannous ions aggregated CPP complexes and promoted ion release and cross-linking of CPPs upon contact with the dentine surface. The proposed mechanism for this stannous mediated ion release gave insight into the
formation of a carbon-based nanocoating and a carbon-rich surface layer formed in the enamel experiments.
THE PREBIOTIC EFFECT OF CPP-ACP SUGAR-FREE CHEWING GUM
7.1 Introduction

Dental caries progresses when there is an imbalance between demineralisation and remineralisation of the tooth resulting in a net loss of ions and an increase in tooth porosity [Featherstone, 2004]. A clinical goal of caries management is to modify the oral environment to favour remineralisation during or after acid challenges. This not only involves increasing bioavailable calcium, phosphate and fluoride to the tooth surface, but also the promotion of commensal microorganisms that colonise and maintain plaque with a neutral pH. These favourable commensal microorganisms are the non-mutans streptococci and other health-related species as opposed to the mutans streptococci, lactobacilli and other species associated with carious lesions [Marsh, 2010].

The primary anticariogenic effect of CPPs is the ability to deliver bioavailable calcium, phosphate and fluoride ions to mineral deficient carious lesions to promote remineralisation [Cochrane and Reynolds, 2012]. Recent clinical evidence suggests CPPs can also reduce the number of mutans streptococci in dental biofilms thereby limiting its acid production and demineralisation potential [Chandak et al., 2016; Emamieh et al., 2015; Pukallus et al., 2013]. This has also been reflected in studies demonstrating an increased plaque pH and buffering capacity following the use of CPP-containing products [Caruana et al., 2009; Heshmat et al., 2014; Peric et al., 2015]. *Streptococcus sanguinis* is known to be a biomarker of dental health having been shown to possess an inverse relationship with *Streptococcus mutans* correlating to caries status [Caufield et al., 2000; Corby et al., 2005; Giacaman et al., 2015; Loesche and Straffon, 1979]. Due to the evidence suggesting therapeutic use of CPP-containing products reduces mutans streptococci levels, it was hypothesised that a CPP-containing product would accordingly favour an increase in abundance of *S. sanguinis* in dental plaque. To test this hypothesis, a randomised double-blind crossover clinical study was conducted assessing the effect of chewing a CPP-ACP gum with plaque composition used as an assessment of treatment effect.
7.2 Objective
The objective of this study was to compare the abundance of *S. sanguinis* and accompanying microorganisms in supragingival dental plaque obtained from healthy subjects following regular use of CPP-ACP sugar-free chewing gum, sugar-free chewing gum or no gum chewing.

7.3 Study methods

7.3.1 Subject recruitment
Twenty healthy participants were recruited for this clinical study according to selection criteria and a clinical protocol approved by the University of Melbourne Human Research Ethics Committee (Application number 1441865). Inclusion criteria for participants included having at least 20 natural teeth, a stimulated whole salivary flow rate above or equal to 1.0 mL/min, and an unstimulated whole salivary flow rate above or equal to 0.2 mL/min. Participants were screened clinically by a qualified dentist and excluded from the study for any of the following reasons:

- Allergy to milk protein or any ingredient in the experimental chewing gums
- Pregnancy or lactation
- Treatment with antibiotics or anti-inflammatory medications in the previous month prior to starting the study
- Medical conditions requiring antibiotic prophylaxis prior to invasive dental procedures such as extractions or periodontal probing
- Wearing dentures or orthodontic appliances
- Dental veneers or more than one incisor with a crown
- Oral pathology including periodontitis or tumours of the soft or hard dental tissues
- Participation in another clinical study

During clinical screening of participants, the presence of periodontal disease was determined by assessing 10 preselected teeth according to the Community Periodontal Index of Treatment Needs (CPITN) utilising a World Health Organisation periodontal probe [Cutress *et al.*, 1987]. Any subjects with a CPITN score of 3 or above were deemed to have periodontal disease and unable to participate in the study.
7.3.2 Clinical protocol

The study had a randomised, double-blind, crossover study design. Participants were randomly allocated to one of three treatment periods. One week prior to commencing the first treatment period, participants were instructed to use a specific toothbrush with a sodium fluoride toothpaste supplied to them and advised not to use any interdental cleaning aids such as floss during the entirety of the study. Following the first treatment period, participants had a 14 day washout period after which they commenced another treatment period in randomised order; this was followed by another 14 day washout period and the final treatment period so that participants completed all three treatment periods. Participants were instructed not to use any oral hygiene measures (including mouthrinse) during the treatment periods. The three treatment periods (A, B and C) were 14 days in duration:

Treatment period A: Abstain from oral hygiene and chew two pellets of gum for 20 minutes six times a day each day (sugar-free gum containing 1 % CPP-ACP).
Treatment period B: Abstain from oral hygiene and chew two pellets of gum for 20 minutes six times a day each day (sugar-free gum).
Treatment period C: Abstain from oral hygiene.

For periods requiring participants to chew gum, a log-sheet was given to record time and duration of chewing; participants were advised to leave no less than 50 minutes between chewing times. Participants were blinded as to which gum they were chewing during treatment periods A and B, and the dental examiner was blinded as to which treatment period the participants were completing. The ingredients of both gums are outlined in Table 7.1.
**Table 7.1**: Ingredients of chewing gum used in treatment periods A and B. Ingredients highlighted in grey were less than 2% of final composition.

<table>
<thead>
<tr>
<th>INGREDIENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sorbitol</td>
</tr>
<tr>
<td>Gum base</td>
</tr>
<tr>
<td>Glycerin</td>
</tr>
<tr>
<td>Mannitol</td>
</tr>
<tr>
<td>Natural and artificial flavouring</td>
</tr>
<tr>
<td>Xylitol</td>
</tr>
<tr>
<td>Acesulfame potassium</td>
</tr>
<tr>
<td>Aspartame</td>
</tr>
<tr>
<td>BHA</td>
</tr>
<tr>
<td>BHT</td>
</tr>
<tr>
<td>Green colour</td>
</tr>
<tr>
<td>Soy lethicin</td>
</tr>
<tr>
<td>Acetylated monoglycerides</td>
</tr>
<tr>
<td>Monoglycerides</td>
</tr>
<tr>
<td>CPP-ACP (treatment A only)</td>
</tr>
</tbody>
</table>

At the commencement of each treatment period participants received a scale and clean using an ultrasonic scaler and a polish with pumice paste. At the end of each treatment period participants were seen for a similar appointment however prior to the scale and clean supragingival plaque was collected from the upper right first and second molars using a sterile sickle scaler; these plaque collections were pooled for both teeth, labelled and frozen until DNA processing. Participants received a sodium fluoride mouthrinse at the conclusion of the final appointment. Before each clinical session participants were screened for any adverse events or change in medical history.

7.3.3 Adverse event

One of the twenty participants was unable to complete the third and final treatment leg due to an unrelated adverse event. The data collected were therefore from the remaining nineteen participants.

7.3.4 DNA Processing

The plaque samples taken from the nineteen participants at the end of each treatment period were analysed using 16S ribosomal RNA gene analysis (57 samples). Genomic DNA was extracted from the plaque and quantitation was attained using a Qubit dsDNA High Sensitivity Assay kit (ThermoFisher, VIC, Australia). A template of 5 ng DNA was
used for the PCR reaction to amplify the V4 variable region of the 16S ribosomal RNA gene and individually barcode the PCR product of each sample. The barcoded DNA was sequenced using Torrent Suite™ Software and an Ion Torrent Personal Genome machine (ThermoFisher, VIC, Australia). The resulting sequence of the 16S ribosomal RNA gene was then analysed against both the premium curated MicroSEQ™ ID 16S rRNA reference database and the curated Greengenes database to identify the bacteria present in each sample down to the genus or species level.

7.3.5 Statistical analysis

Descriptive statistics (mean and standard deviation) were calculated for the bacteria relative abundance, and the data were analysed using a linear mixed modelling approach [Verbeke and Molenberghs, 2000]. Treatment group (A, B or C) was included in the models as a fixed effects term and participant was included as a random effect term. Post hoc comparisons of treatment differences were performed on the marginal means using the Sidak adjustment for multiple comparisons. Modelling assumptions were checked using residual and normal probability plots. Complementary log-log transformations of the relative abundance data were used prior to analysis. P values less than 0.05 were regarded as being statistically significant. All analyses were conducted using SPSS (version 22; SPSS Inc., IL, USA) statistical software.

7.4 Results

The proportion of plaque bacteria identified as *S. sanguinis* following the no gum treatment period was 2.6%; this was shown to increase to 3.6% after chewing the sugar-free gum, and 5.5% after chewing the CPP-ACP gum. While there was a trend for an increase in *S. sanguinis* abundance following periods of chewing gum, only the CPP-ACP gum treatment period showed a significant (p < 0.01) increase in *S. sanguinis* when compared to the no gum treatment period. The mean abundance and 95% confidence interval of *S. sanguinis* based on treatment period is represented in Figure 7.1.
Figure 7.1: Mean abundance and 95% confidence interval of *S. sanguinis* according to treatment period. A significant difference between the CPP-ACP gum and no gum treatment periods was observed (p = 0.003).

In addition to *S. sanguinis*, over 300 different bacterial taxa were identified from the 57 samples. Some species were so closely related that they were unable to be differentiated with the current analysis. Of the taxa identified, the 40 most abundant taxa accounted for approximately 80% of the total bacteria in the plaque (see Table 7.2). The most abundant taxa were members of the *Corynebacterium*, *Streptococcus* and *Actinomyces* genera. Major pathogenic species associated with dental caries such as *Streptococcus mutans*, *Lactobacillus casei* and *Bifidobacteria* were not commonly detected in the plaque samples.

Comparison of the supragingival plaque composition across treatment periods revealed significant differences between chewing gum (either CPP-ACP gum or the sugar-free gum) and not chewing gum (see Table 7.2). There were statistically significant changes in the abundances of the following bacterial taxa between the chewing and non-chewing periods: *Actinomyces massiliensis*; *Corynebacterium durum*; *Lautropia* genus; *Leptotrichia sp./wadei*; *Leptotrichia shahii*; *Rothia dentocariosa*; *Streptococcus*
gordonii/mitis. Bacteria of the Actinomyces genus decreased in abundance during the sugar-free gum period compared to the non-chewing period. Lautropia mirabilis/sp., Leptotrichia buccalis and the Propionibacterium genus significantly decreased after the CPP-ACP chewing gum period compared to the non-chewing period.

Table 7.2: Composition of supragingival plaque following treatment periods A (1% CPP-ACP gum), B (sugar-free gum) or C (no gum). The results are presented as averages of the percentage of the total bacteria in supragingival plaque of the 40 most abundant bacterial taxa in the 19 participants. Some genus level classifications are also presented to demonstrate broad changes in supragingival plaque composition. Taxa in bold font were significantly different.

<table>
<thead>
<tr>
<th>Genus Level</th>
<th>Abundance (%)</th>
<th>Significance</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>CPP-ACP Gum</td>
<td>Sugar-free Gum</td>
</tr>
<tr>
<td>Actinobaculum</td>
<td>0.6 (1.1)</td>
<td>0.6 (0.8)</td>
</tr>
<tr>
<td>Actinomyces</td>
<td>5.0 (4.2)</td>
<td>3.0 (2.7) a</td>
</tr>
<tr>
<td>Corynebacterium</td>
<td>3.1 (3.8)</td>
<td>2.7 (2.9)</td>
</tr>
<tr>
<td>Eubacterium</td>
<td>1.1 (1.0)</td>
<td>1.0 (1.1)</td>
</tr>
<tr>
<td>Fusobacterium</td>
<td>0.7 (0.8)</td>
<td>1.0 (1.1)</td>
</tr>
<tr>
<td>Kingella / Neisseria</td>
<td>0.8 (0.8)</td>
<td>0.8 (0.9)</td>
</tr>
<tr>
<td>Lautropia</td>
<td>0.6 (1.2) a</td>
<td>0.2 (0.4) b</td>
</tr>
<tr>
<td>Leptotrichia</td>
<td>2.4 (1.3)</td>
<td>3.4 (3.2)</td>
</tr>
<tr>
<td>Neisseria</td>
<td>1.0 (1.6)</td>
<td>0.7 (1.0)</td>
</tr>
<tr>
<td>Porphyromonas</td>
<td>2.9 (1.9)</td>
<td>3.4 (2.1)</td>
</tr>
<tr>
<td>Propionibacterium</td>
<td>1.5 (2.8) a</td>
<td>1.4 (1.9)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Species Level</th>
<th>Abundance (%)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abiotrophia defective</td>
<td>1.1 (1.0)</td>
<td>1.3 (2.0)</td>
</tr>
<tr>
<td>Actinomyces dentalis</td>
<td>1.6 (1.9)</td>
<td>1.5 (1.6)</td>
</tr>
<tr>
<td>Actinomyces georgiae</td>
<td>0.6 (0.6)</td>
<td>0.9 (1.1)</td>
</tr>
<tr>
<td>Actinomyces johnsonii/naeslundii/oris/sp.</td>
<td>0.8 (1.1)</td>
<td>1.0 (1.6)</td>
</tr>
<tr>
<td>Actinomyces massiliensis</td>
<td>0.5 (0.5) a</td>
<td>0.5 (0.5) b</td>
</tr>
<tr>
<td>Actinomyces naeslundii</td>
<td>6.9 (5.3)</td>
<td>5.5 (4.9)</td>
</tr>
<tr>
<td>Actinomyces odontolyticus</td>
<td>1.2 (1.2)</td>
<td>1.3 (1.6)</td>
</tr>
<tr>
<td>Aggregatibacter segnis</td>
<td>0.7 (0.7)</td>
<td>0.8 (0.6)</td>
</tr>
<tr>
<td>Capnocytophaga granulosa</td>
<td>1.3 (1.2)</td>
<td>1.5 (1.3)</td>
</tr>
<tr>
<td>Corynebacterium durum</td>
<td>2.7 (2.2) a</td>
<td>2.7 (1.9) b</td>
</tr>
<tr>
<td>Corynebacterium matruchatti</td>
<td>10.3 (7.7)</td>
<td>10.8 (6.1)</td>
</tr>
<tr>
<td>Abundance (%)</td>
<td>CPP-ACP Gum</td>
<td>Sugar-free Gum</td>
</tr>
<tr>
<td>---------------</td>
<td>-------------</td>
<td>----------------</td>
</tr>
<tr>
<td><strong>Fusobacterium canifelinum</strong></td>
<td>0.8 (0.7)</td>
<td>0.9 (0.8)</td>
</tr>
<tr>
<td><strong>Gemella haemolysans/morbillorum/sanguinis</strong></td>
<td>0.7 (0.7)</td>
<td>0.8 (0.7)</td>
</tr>
<tr>
<td><strong>Kingella denitrificans / Neisseria elongata</strong></td>
<td>0.8 (1.1)</td>
<td>0.5 (0.5)</td>
</tr>
<tr>
<td><strong>Lautropia mirabilis / Lautropia sp.</strong></td>
<td>0.4 (0.5) a</td>
<td>0.4 (0.5)</td>
</tr>
<tr>
<td><strong>Leptotrichia buccalis</strong></td>
<td>0.7 (0.8) a</td>
<td>1.6 (2.4)</td>
</tr>
<tr>
<td><strong>Leptotrichia hofstadii</strong></td>
<td>0.8 (0.9)</td>
<td>0.9 (1.0)</td>
</tr>
<tr>
<td><strong>Leptotrichia hongkongensis</strong></td>
<td>0.9 (1.3)</td>
<td>0.4 (0.5)</td>
</tr>
<tr>
<td><strong>Leptotrichia shahii</strong></td>
<td>0.3 (0.5) a</td>
<td>0.2 (0.3) b</td>
</tr>
<tr>
<td><strong>Leptotrichia sp/wadei</strong></td>
<td>0.1 (0.1) a</td>
<td>0.1 (0.1) b</td>
</tr>
<tr>
<td><strong>Morococcus cerebrosus/cinerea/flava/macacae mucosa/sp.</strong></td>
<td>0.9 (1.1)</td>
<td>1.0 (1.5)</td>
</tr>
<tr>
<td><strong>Prevotella nigrescens</strong></td>
<td>0.5 (1.1)</td>
<td>0.4 (0.7)</td>
</tr>
<tr>
<td><strong>Rothia aeria</strong></td>
<td>2.5 (3.3)</td>
<td>3.6 (5.9)</td>
</tr>
<tr>
<td><strong>Rothia dentocariosa</strong></td>
<td>5.9 (7.0) a</td>
<td>6.6 (6.0) b</td>
</tr>
<tr>
<td><strong>Streptococcus cristas/oligofermentans/sinensis</strong></td>
<td>2.2 (1.6)</td>
<td>2.3 (2.7)</td>
</tr>
<tr>
<td><strong>Streptococcus gordoni/mitis</strong></td>
<td>3.4 (3.4)</td>
<td>4.9 (6.5) a</td>
</tr>
<tr>
<td><strong>Streptococcus infantis/mitis/oralis/tigurinus</strong></td>
<td>2.3 (1.7)</td>
<td>2.1 (1.3)</td>
</tr>
<tr>
<td><strong>Streptococcus sanguinis</strong></td>
<td>5.5 (4.3) a</td>
<td>3.6 (2.7)</td>
</tr>
<tr>
<td><strong>TM7-3</strong></td>
<td>1.0 (1.1)</td>
<td>0.8 (0.9)</td>
</tr>
</tbody>
</table>

a,b Significant differences to the No Gum Treatment.

7.5 Discussion

The analysis of the supragingival plaque samples revealed the bacterial composition among all individuals belonged to taxa typical in oral health [Zaura et al., 2009]. The predominant phyla were Firmicutes and Actinobacteria and this was expected across all treatment groups for dentally healthy individuals. No mutans streptococci or lactobacilli were among the 40 most abundant species detected from any individuals, unsurprisingly for a sample population with very low caries risk.

The CPP-ACP chewing gum significantly increased the proportion of *S. sanguinis* compared to the non-chewing group, a species that has been consistently associated with dental health and low caries incidence [Agnello et al., 2017; Becker et al., 2002; Giacaman et al., 2015; Stingu et al., 2008]. While the sugar-free chewing gum also
appeared to increase the abundance of *S. sanguinis*, it was not significantly different to the non-chewing treatment period. *S. sanguinis* is a well characterised commensal microorganism that is an early coloniser of the dental hard tissues [Caufield et al., 2000]. Multiple studies have suggested *S. sanguinis* possesses an antagonistic relationship with *S. mutans*, promoting a delay or inhibition of *S. mutans* colonisation largely due to its production of H₂O₂ [Caufield et al., 2000; Ge et al., 2008; Giacaman et al., 2015; Kreth et al., 2005; Kreth et al., 2008]. Clinical studies demonstrating a decrease in plaque *S. mutans* following use of CPP-ACP-containing products hypothesised CPP-ACP induced a buffering effect and interfered with cell binding to impede *S. mutans* colonisation [Emamieh et al., 2015; Pukallus et al., 2013]. However, the CPP-ACP associated increase in *S. sanguinis* in the current study suggested increased *S. sanguinis* antagonism may have also contributed to decreased *S. mutans* levels observed in previous studies.

*S. sanguinis* is also known to metabolise arginine through expression of the enzyme arginine deiminase (AD) which produces alkali to inhibit drops in plaque pH [Ferro et al., 1983]. There has been cumulative evidence that bacterial alkali production through AD is an important mechanism maintaining oral biofilm homeostasis in healthy individuals, translating to a lower risk of developing caries [Burne and Marquis, 2000; Gordan et al., 2010; Liu et al., 2012; Nascimento et al., 2009; Nascimento et al., 2013]. Arginine occurs naturally in saliva in its free form at a concentration of approximately 50 µM and is additionally present within numerous salivary proteins/peptides [Burne and Marquis, 2000; Van Wuyckhuyse et al., 1995]. AD expression occurs when bacteria are exposed to arginine and low pH; as the bacterium generates adenosine triphosphate, the resulting products citrulline and ammonia are released into the surrounding plaque fluid [Liu et al., 2012]. One of the major CPP β(1-25) contains N- and C- terminal arginine which can be released from the peptide through enzymatic hydrolysis (peptidase) and catabolised through the AD pathway by oral bacteria to produce ammonia [Cross et al., 2005; Reynolds and Riley, 1989]. Reynolds et al. [2003] have shown that chewing CPP-ACP gum results in the incorporation of CPP-ACP into supragingival plaque; the CPP-ACP could still be detected in plaque 3 hours after chewing one piece of gum. It therefore would be expected that chewing the CPP-ACP gum in the current study would have
loaded the plaque with CPP. This may have promoted AD expression and production of ammonia, and consequently favoured the colonisation and metabolic activity of microorganisms such as *S. sanguinis* [Reynolds and Riley, 1989]. As subjects with a history of dental caries have previously been shown to have lower free salivary arginine than caries-free adults [Van Wuyckhuyse et al., 1995], chewing CPP-ACP gum may provide these individuals with a supplementary form of arginine and other ammonia-generating amino acids, thereby promoting colonisation/emergence of ammonia-producing bacteria in plaque.

The sugar-free chewing gum significantly increased the abundance of *S. gordonii/mitis* compared to the non-chewing treatment period (these two species that were unable to be differentiated with the current analysis). There was also a trend for an increase in these species after chewing the CPP-ACP gum. Both *S. mitis* and *S. gordonii* are AD positive, H$_2$O$_2$ producing bacteria, though they are believed to play a less significant role in caries inhibition than *S. sanguinis* [Carlsson et al., 1983; Kreth et al., 2008; Nobbs et al., 2007].

Chewing the CPP-ACP gum or sugar-free gum significantly increased the proportion of *C. durum* and *R. dentocariosa* when compared to the non-chewing period. In a recent study assessing the plaque microbiome in caries-free and caries-active children by Agnello et al. [2017], the *Rothia* and *Corynebacterium* genera were both significantly higher in caries-free children in addition to *S. sanguinis*. Both *C. durum* and *R. dentocariosa* are nitrate reducing bacteria and are now considered commensals [Doel et al., 2005]. Evidence of a caries preventative effect by nitrate reducing bacteria has been demonstrated by Doel et al. [2004] who determined that individuals with high salivary nitrate levels and a high nitrate reducing capacity of resident oral bacteria experienced less dental caries than individuals with low salivary nitrate and low bacterial nitrate reducing capacity. The bacterial enzyme nitrate reductase (NR) is active in certain species to enable nitrate metabolism during anaerobic conditions when their preferred oxygen dependent metabolism is impeded. Intraoral nitrate (NO$_3^-$) is mainly sourced from saliva and is reduced to nitrite (NO$_2^-$) through bacterial NR; during acidic conditions NO$_2^-$ is protonated to nitrous acid (HNO$_2$) which is inherently unstable and forms dinitrogen trioxide (N$_2$O$_3$), nitrogen dioxide (NO$_2$) and nitric oxide (NO) [Lundberg et al., 2004].
Expression of NR inhibits drops in plaque pH through the acidification of nitrite and additionally inhibits *S. mutans* and *Lactobacillus casei* levels due to production of NO [Mendez *et al.*, 1999]. As ingested nitrite is absorbed in the gut and converted to NO in the bloodstream, intraoral bacterial NR activity has also been proposed to have an important role in maintaining circulatory NO essential for vascular health [Wade, 2013]. Stimulated saliva has an increased buffering capacity and total intraoral nitrate output [Dawes, 2008; Granli *et al.*, 1989], while CPP-ACP additionally increases plaque pH and buffering capacity [Caruana *et al.*, 2009; Heshmat *et al.*, 2014; Peric *et al.*, 2015]. As NR activity is promoted when the salivary pH increases from 6 to 8 and when salivary nitrate levels increase [Xu *et al.*, 2001], it was unsurprising that in the current study chewing either gum significantly increased bacteria that possess NR.

In addition to the significant increase in the proportion of *C. durum* and *R. dentocariosa*, chewing the sugar-free or CPP-ACP gum significantly decreased the proportion of *A. massiliensis*, *L. shahii*, *L. sp./wadei* and genus *Lautropia* when compared to the non-chewing treatment period. No aetiological or protective role for these microorganisms has been established with regards to dental caries and previous plaque cultivation studies have observed these species in both caries active and caries free sites. According to a report by Richards *et al.* [2017], *C. durum* was more frequently found in plaque from caries free sites, while some *Leptotrichia* species (in particular *L. wadei*) were found more frequently in plaque from caries active sites. *A. massiliensis* has been cultivated from plaque in both high and low caries risk individuals [Tanner *et al.*, 2011]. The other species that significantly decreased following CPP-ACP chewing gum when compared to the control treatment period (*L. mirabilis/sp., L. buccalis*, and genus *Propionibacterium*) similarly have no known causative role in oral disease and are found to colonise healthy individuals; however they have been implicated as opportunistic pathogens, particularly in the immunocompromised [Couturier *et al.*, 2012; Eribe and Olsen, 2008; He *et al.*, 2017].

Chewing gum is known to raise saliva flow for up to 2 hours after chewing [Dawes and Kubieniec, 2004], increasing the clearance rate of nutrients and microorganisms as well as the buffering capacity, calcium phosphate level and antimicrobial activity of the saliva.
Consequently species that tolerated or thrived in biofilms with neutral pH and relatively high calcium phosphate concentrations were more favoured to dominate after chewing gum. Among a low caries risk population these species would be expected to be high abundance in supragingival plaque; therefore, only a few species were detected to significantly change in abundance.

The current study showed regular chewing of CPP-ACP gum exerted a significant increase in *S. sanguinis* abundance compared to not chewing gum; this was postulated to occur through plaque CPP-ACP incorporation promoting a biofilm with a high buffering capacity to favour neutral pH and providing a source of arginine to increase AD expression. The bacterial species detected were considered non-cariogenic which was consistent with the low caries risk individuals who participated in the study; had the study included high caries risk subjects perhaps a marked effect on acidogenic microorganisms may have resulted following the chewing gum treatment periods. The findings suggest that chewing CPP-ACP gum may have an additional anticariogenic effect apart from promoting remineralisation; prebiosis of supragingival plaque with *S. sanguinis* following CPP-ACP chewing gum may inhibit *S. mutans*, prevent drops in plaque pH and inhibit demineralisation of the dental hard tissues.

### 7.6 Conclusions

1. Compared to the non-chewing treatment period, chewing the CPP-ACP gum significantly increased the abundance of *S. sanguinis* in supragingival plaque.

2. The significant increases in bacterial abundance after chewing either gum compared to the non-chewing period included alkali-producing species positive for arginine deiminase and nitrate reductase.
8

GENERAL DISCUSSION
8.1 Enhancing remineralisation

8.1.1 Intra-lesion pH modulation

The findings of the *in vitro* remineralisation experiments conducted in Chapters 3 and 4 advocated intra-lesion pH modulation to enhance remineralisation of artificially created enamel subsurface lesions. The primary reason for this was the effect pH has on ion diffusion through the enamel surface layer as well as the DS in the lesion fluid. Remineralisation solutions with a low pH have been shown to increase remineralisation and maximise ion diffusion through the enamel surface layer by either preventing surface layer crystal growth to maintain ion diffusion channels or by increasing the activity of neutral ion pairs [Cochrane *et al.*, 2008; Flaitz and Hicks, 1996; Yamazaki and Margolis, 2008]. A high intra-lesion pH increases the DS with respect to apatite and subsequently increases the rate of apatite crystal growth [Elliott, 1994]. By modulating the pH appropriately, it was possible to combine both of these effects across a subsurface lesion to increase the rate and extent of remineralisation.

While the findings in Chapters 3 and 4 were significant, further modifications to the cyclic treatment regime may prove to be more efficacious. Previous publications have suggested incipient lesions are unlikely to ever be completely remineralised, with the most important factor being the relatively mineralised surface layer impeding ion diffusion through the lesion and restricting remineralisation to only the superficial enamel [Arends and Ten Cate, 1981; Gao *et al.*, 1993; Larsen and Fejerskov, 1989]. The low pH CPP-ACFP solutions used in Chapter 4 were intended to be supersaturated with respect to apatite to encourage crystal growth within the lesion, though this may have had the unwanted effect of reducing surface enamel pore volume. Cyclic application of a low pH solution undersaturated with respect to apatite may periodically open ion diffusion channels in the enamel surface layer, thereby allowing the potential for near complete remineralisation of incipient lesions. By combining short, periodic applications of an undersaturated low pH solution with the cyclic treatments described in 4.3.2.2, an even higher increase in the rate and extent of remineralisation may result. The potential for stannous ions to have a similar effect on surface pore volume is discussed in 8.1.2.
In perspective, intra-lesion pH modification was relevant to address the most significant challenges in remineralisation of incipient lesions. Despite the limitations of the in vitro studies, the positive remineralisation effect observed by intra-lesion pH-modulation expanded knowledge of the kinetics of remineralisation and provided a scientific basis to advocate future research and clinical treatments.

8.1.2 The incorporation of stannous fluoride

A successful outcome of the experiment in Chapter 6 was the incorporation of stannous ions into CPP complexes. As CPPs contain acidic negatively charged residues they inherently attract cations in solution, typically high levels of Ca$^{2+}$. As other cations are known to bind to casein such as Na$^{+}$ and Mg$^{2+}$ [O’Mahony and McSweeney, 2016], it was unsurprising that the stannous ion, with a valency of two, was complexed by interaction with negatively charged residues of the CPPs. This allowed soluble bundles of calcium, phosphate, fluoride and stannous ions to be delivered to mineral deficient tooth structure. Chapter 6 is the first experiment to report this and subsequently demonstrate an enhanced remineralisation effect by the combination of CPP-ACP and SnF$_2$.

Stability of SnF$_2$ in dental products has historically been challenging due to the risk of hydrolysis or oxidation that renders the stannous ion inactive [White, 1995]. Alkaline pH generally promotes oxidation of Sn(II) to Sn(IV) which can form unwanted insoluble Sn-containing compounds [Smith, 2012]. Meyer and Nancollas [1972] demonstrated that in a solution of pH 7.4 hydrolysed stannous ions predominated and inhibited hydroxyapatite growth by adsorbing to crystal growth sites, highlighting the importance of preventing hydrolysis of Sn(II). Sn(II) compounds such as SnF$_2$ are relatively water soluble at low pH and by association with fluoride the Sn(II) ion is more resistant to hydrolysis and oxidation in solution [Smith, 2012]. Despite this, SnF$_2$ is difficult to stabilise in dental products at higher pH and the addition of ‘sacrificial’ tin compounds and tin ‘reservoirs’ has been advocated to accommodate for the reactivity of the stannous ion and to maintain stability and bioavailability of SnF$_2$ [White, 1995]. The incorporation of SnF$_2$ by CPP-ACP was shown to allow stability of SnF$_2$ at relatively high pH levels without the addition of sacrificial tin compounds or tin reservoirs and without any insoluble compounds.
evident. This was likely because Sn(II) association with the CPP-ACP complex substantially lowered its reactivity with water and other ions in solution to provide protection from hydrolysis and oxidation. Not only did the addition of SnF$_2$ to CPP-ACP complexes permit solubility of SnF$_2$ at neutral pH, it enhanced the delivery of calcium, phosphate and fluoride ions to increase the percent remineralisation of enamel subsurface lesions in situ. The incorporation of SnF$_2$ by CPP-ACP therefore increased the overall stability of the complexes and demonstrated CPP-ACP complexes are an effective vehicle for delivery of SnF$_2$ at neutral pH.

The phases formed in or above tooth surfaces after topical SnF$_2$ application have been speculated though not confirmed. Previous evidence has proposed stannous ions adsorb to apatite crystals to inhibit both crystal dissolution and crystal growth [Lippert, 2016; Meyer and Nancollas, 1972]. The analysis of CPP-ACP + SnF$_2$ remineralised lesions in Chapter 6 revealed stannous ions were localised at the surface layer of the lesion, while the deeper zones of the lesion contained calcium, phosphate and fluoride without stannous. This suggested that the CPP-ACP + SnF$_2$ treatment promoted adsorption of stannous ions to superficial apatite crystals which prevented their remineralisation and allowed ion diffusion to deeper zones within the lesion, resulting in greater remineralisation with fluoridated apatite in the bulk of the lesion. Retention of stannous ions in the surface layer following application of a combined CPP-ACP and SnF$_2$ solution may therefore help prevent apatite dissolution from caries and erosion while simultaneously maintain diffusion channels and enhance the remineralisation effect of the CPP-ACP complexes.

An additional anticariogenic effect of the stannous ion is its ability to inhibit bacterial enzymes involved in the metabolism of carbohydrates, and consequently reduce bacterial acid production [Oppermann et al., 1980]. This mechanism has been shown to inhibit acid producing species such as S. mutans while favouring growth of commensal species such as S. sanguinis that rely less on carbohydrate metabolism [Cheng et al., 2017]. The synergistic effect of a combined CPP-ACP and SnF$_2$ treatment in the context of dental caries is therefore possible to not only increase the remineralisation effect of CPP-ACP but also favourably alter the bacterial composition of plaque. Multiple clinical studies
have demonstrated the use of CPP-containing products significantly reduces mutans streptococci levels [Chandak et al., 2016; Ebrahimi et al., 2017; Fadl et al., 2016; Karabekiroğlu et al., 2017; Pukallus et al., 2013] and it was shown in Chapter 7 that a CPP-ACP-containing sugar-free gum significantly increased the abundance of *S. sanguinis*. In an active carious lesion, demineralisation opposes remineralisation; by reducing the abundance of acid-producing species in dental plaque a combined CPP-ACP and SnF₂ treatment may indirectly increase its remineralisation action by inhibiting bacterial mediated demineralisation. The addition of SnF₂ to CPP-ACP therefore appears to have clinical potential to enhance remineralisation by CPP-ACP complexes and expand their anticariogenic properties.

8.1.3 CPP-ACP-mediated prebiosis

The clinical study conducted in Chapter 7 revealed among a population of low caries-risk individuals the use of CPP-ACP-containing sugar-free chewing gum significantly increased *S. sanguinis* levels. This demonstrated that apart from the well documented remineralisation effect of CPP-ACP complexes, the use of CPP-ACP-containing products may also prevent caries by favourably altering the biofilm composition to increase the abundance of *S. sanguinis*, a species which competitively inhibits *S. mutans* and prevents pH drops in the plaque fluid [Burne and Marquis, 2000; Ferro et al., 1983; Giacaman et al., 2015]. As stated in 8.1.2, reducing the abundance of acid-producing species in dental plaque may indirectly enhance the remineralisation action of CPP-ACP-containing products.

The significance of pH in relation to the lesion fluid and crystal growth was outlined in 8.1.1, though pH also has a marked influence on plaque bacterial composition [Bowden and Hamilton, 1998]. The findings of Chapter 7 supported the contention that CPP-ACP incorporation in dental plaque has a buffering effect that resists changes in pH and maintains a higher baseline pH, as this was likely to create a more favourable environment for the proliferation of *S. sanguinis* [Caruana et al., 2009; Heshmat et al., 2014; Ozdas et al., 2015; Peric et al., 2015]. It is possible, apart from the direct remineralisation effect of CPP-ACP, that promotion of a health-associated biofilm by CPP-ACP-containing products may help prevent the onset of caries and foster the arrest of active caries through
prebiosis. Therefore, use of CPP-ACP-containing products may be an important aid in terms of caries management as they may have the potential to promote the prevention, arrest and repair of the disease.

8.2 Future directions
The research presented in this thesis has advanced the knowledge of CPP-ACP complexes.

The substantial increase in the rate of remineralisation from intra-lesion pH modulation was an exciting finding. As remineralisation is usually a slow process, any modification that results in a significant increase in the remineralisation rate should be explored with a view to translating it to clinical application. With the high incidence of white spot lesions in high caries-risk individuals (especially those undertaking orthodontic treatment), the swiftness at which these lesions can be remineralised may determine whether cavitation occurs and a restoration is required. By further investigation of the effect intra-lesion pH modulation, DS and CPP-ACP/ACFP concentration have on remineralisation, it is possible that a clinical protocol for cyclic treatment may be developed to achieve rapid remineralisation of subsurface lesions. The addition of SnF2 to the remineralisation treatment or cyclic use of an undersaturated low pH treatment may have the potential to augment this process by preserving ion diffusion channels to facilitate remineralisation of deeper zones (as discussed in 8.1).

The incorporation of SnF2 into CPP-ACP complexes has resulted in evidence to help support the development of a commercial product that combines and synergises the anticariogenic properties of both agents. Further research of this combination is therefore warranted. This research should assess the effect of this combination on enamel and dentine remineralisation, erosion inhibition and plaque bacterial composition in vivo. Separately, SnF2 and CPP-ACP have been demonstrated to reduce dentine hypersensitivity [Alexandrino et al., 2017; Miller et al., 1969]. It would be sagacious to assess the effect a combined CPP and SnF2 treatment has on dentine hypersensitivity, as well as its potential to affect tooth staining.
Prebiosis is generally associated with gut microflora and it has been shown to reduce the risk of obesity, type 2 diabetes and colon cancer [Roberfroid et al., 2010]. As increasing evidence is being developed to advocate prebiosis of the oral microflora to promote alkaline-producing bacteria to help reduce caries risk, prevention of plaque related oral diseases is destined to focus on the prebiotic effect of current and future treatments [Burne and Marquis, 2000; Doel et al., 2005; Doel et al., 2004; Kreth et al., 2008; Liu et al., 2012; Nascimento et al., 2009; Nascimento et al., 2013]. The prebiotic effect of CPP-ACP observed in Chapter 7 demonstrated a significant increase in \( S. sanguinis \) in caries-free individuals and it would be rational to assess the changes in plaque microflora in caries-active individuals. As mentioned in 8.1.2, the incorporation of SnF\(_2\) into CPP-ACP complexes may augment this effect by reducing \( S. mutans \) levels and promoting \( S. sanguinis \) levels in dental plaque, resulting in a more pronounced prebiosis than SnF\(_2\) treatment alone.

Apart from advancing the knowledge of CPP-ACP complexes, this thesis explored a relatively new approach to assess remineralisation using XMT. Chapter 5 demonstrated some of the limitations the Australian synchrotron and a C\(\mu\)-CT scanner (Bruker Skyscan 1172, Kontich, Belgium) possessed for analysis of remineralisation in vitro when compared to TMR. Despite the observed inferiority to TMR when calculating the lesion mineral density, it was apparent that XMT would still be useful for visualisation of the morphology of natural carious lesions and other areas of interest within natural teeth. In addition to this, use of a radiopaque dye may have the potential to assess changes in tooth porosity using XMT [Kawabata et al., 2008], giving insight into treatments for dentine hypersensitivity as well as surface layer porosity in relation to demineralisation and remineralisation. As the technology is constantly improving, there is scope in the future for a computer-aided approach to be employed for these purposes as well as tooth mineralisation studies, especially for longitudinal studies which require intact samples for repeated analysis.
8.3 Conclusions
Enhancement of remineralisation using CPP-ACP complexes was investigated with in vitro, in situ and clinical studies. The primary findings were that modifications to the intra-lesion pH and the incorporation of SnF$_2$ into CPP-ACP complexes increased remineralisation and the anticariogenic properties of CPP-ACP complexes. Furthermore, CPP-ACP-induced prebiosis of *S. sanguinis* in supragingival plaque was also demonstrated in a clinical trial. It was observed that intra-lesion BSA did not inhibit remineralisation by CPP-ACFP, NaOCl interaction with CPP-ACFP destabilised and precipitated calcium, phosphate, and fluoride ions, and that TMR was superior to the Australian Synchrotron or a Cµ-CT (Bruker Skyscan 1172, Kontich, Belgium) for analysis of remineralisation. The findings suggested modifications to the formulation and application of CPP-ACP-containing products may be useful to enhance their remineralisation efficacy. Further research is warranted to expand on these results and provide a pathway for changes in clinical treatment.
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Enhancing remineralisation using casein phosphopeptide complexes

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