THE EFFECT OF POLY GAMMA GLUTAMIC ACID ON DEMINERALISATION AND REMINERALISATION OF HUMAN DENTAL ENAMEL

ZEESHAN QAMAR

FACULTY OF DENTISTRY
UNIVERSITY OF MALAYA
KUALA LUMPUR

2017

THE EFFECT OF POLY GAMMA GLUTAMIC ACID ON DEMINERALISATION AND REMINERALISATION OF HUMAN DENTAL ENAMEL

ZEESHAN QAMAR

THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

FACULTY OF DENTISTRY
UNIVERSITY OF MALAYA
KUALA LUMPUR

2017

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: Dr. Zeeshan Qamar

Matric No: DHA 130009

Name of Degree: Doctor of Philosophy

Title of Thesis: "The Effect of Poly Gamma Glutamic Acid on Demineralisation and Remineralisation of Human Dental Enamel"

Field of Study: Oral Biology

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date:

Subscribed and solemnly declared before,

Witness's Signature Date:

Name:

Designation:

ABSTRACT

It has been suggested that the interaction of glutamic residues in statherin with the hydroxyapatite mineral of human tooth surfaces reduces the kinetics of enamel dissolution during simulated caries challenges. A Japanese traditional foodstuff 'Natto' containing poly-γ-glutamic acid (PGGA) may have a similar action. In this study, the efficacy of PGGA in inhibiting demineralisation and promoting remineralisation of human dental enamel under caries-inducing conditions were investigated. Seventy two orthodontically extracted sound premolars (54 for demineralisation study and 18 for remineralisation study, were all varnished, leaving a 2x2 mm^2 window on the mid-buccal surfaces), 10.8 g HAp-powder of uniform composition (dental enamel crystallite analogue- 0.2 g/ treatment group for demineralisation study) and 18 HAp pellets of uniform porosity (analogue of dental enamel for remineralisation study) were used in this study. The teeth and the HAp powder were respectively pretreated before being exposed to demineralising solution at various pHs (4.0, 4.5 and 5.0) to induce artificial caries lesions. They were pretreated with PBS (negative control), NaF (0.01%, 0.1% and 0.5% respectively - positive controls) and PGGA (1% and 2%w/v respectively - test groups). The Ca^{2+} release into the demineralising solution by the teeth and HAp powder (demineralisation study) and the Ca^{2+} uptake by the teeth and HAp pellets (remineralisation study) were measured using a Ca^{2+} selective electrode, later used to calculate the rate of calcium loss or gain (R_{Ca2+}). Cross sectional microhardness (CSMH) was used to determine the mineral density changes at various depths of induced caries lesions. The results from both these techniques were collated to assess the effect of PGGA in the inhibition of caries lesion formation and mineral recovery. In order to determine the possible mechanism of PGGA in inhibiting demineralisation and promoting remineralisation, the teeth and HAp pellets were treated with PGGA solutions (1% and 2% respectively) and subjected to Fourier Transform Infra-Red (FT-

IR) analysis. The density of PGGA solutions (1% and 2%) was also measured in order to determine their dynamic viscosities. In this study, it was found that 2% PGGA inhibits the demineralisation of enamel and HAp powder and is more potent compared with the positive controls (0.01% NaF, 0.1% NaF and 0.5% NaF) at the various pHs of demineralising solution (4.0, 4.5 and 5.0). Further 2% (w/v) PGGA was also shown to promote remineralisation of the enamel and HAp pellets at the pH values below the critical pH and was more effective compared with all three concentrations of the positive controls. From the FT-IR analysis, it was shown that PGGA has the capability of coating the enamel and the HAp pellets. The coating ability of PGGA may contribute to the inhibition of dental enamel demineralisation. From the FT-IR analysis, the presence of the COO^- group in PGGA may suggest its potential to bind with free Ca^{2+} present in the solution, remineralising the teeth, as evident by the increasing hardness of enamel from the CSMH study. In this study, it appears that 2% PGGA has a better effect compared with 0.5% NaF in inhibiting enamel demineralisation and promoting remineralisation under caries-inducing conditions.

ABSTRAK

Telahpun dicadangkan bahawa interaksi antara sisa-sisa glutamik pada statherin dengan mineral hidroskiapatit pada permukaan gigi manusia mengurangkan kinetiks pendemineralan semasa cabaran simulasi karies. Makanan tradisional Jepun 'Natto' mengandungi asid Poli-γ-glutamik (PGGA) mungkin mempunyai fungsi yang sama. Dalam kajian ini, keberkesanan PGGA dalam menghalang pendemineralan dan menggalakkan pemineralan semula enamel gigi manusia di bawah keadaan simulasi karies disiasat. Tujuh puluh dua gigi premolar yang dicabut dalam rawatan ortodontik (54 untuk kajian demineralisasi dan 18 untuk kajian pemineralan semula, semuanya divarnis sehingga meninggalkan tetingkap 2x2mm^2 pada permukaan pertengahan bukal), 10.8gm HAp serbuk berkomposisi seragam (analog kepada kristalit enamel gigi - 0.2gm / kumpulan rawatan untuk kajian demineralisasi) dan 18 pelet HAp berliangan seragam (analog kepada enamel gigi untuk kajian pemineralan semula) telah digunakan dalam kajian ini. Gigi dan serbuk HAp itu masing-masing dirawat sebelum terdedah kepada larutan pendemineralan (0.1M asid asetik) pada pelbagai pH (4.0,4.5 dan 5.0) untuk mengaruhkan karies tiruan. Ianya diberi rawatan awal dengan PBS (kawalan negatif), NaF (0.01%, 0.1% dan 0.5% masing-masing - Kawalan positif) dan PGGA (1% dan 2% w / v masing-masing - kumpulan ujian). The Ca^{2+} yang dibebaskan ke dalam larutan pendemineralan dari permukaan gigi dan serbuk HAp (kajian demineralisasi) dan Ca^{2+} yang dideposit pada gigi dan pelet HAp (kajian remineralisation) diukur menggunakan elektrod terpilih Ca^{2+}, yang masing-masing kemudiannya digunakan untuk mengira kadar kalsium (RCa^{2+}) dibebaskan atau kadar kalsium (RCa^{2+}) didepositkan. Mikrokekerasan (mikrohardness) keratan silang (CSMH) gigi telah digunakan untuk menentukan perubahan ketumpatan mineral di pelbagai kedalaman lesi karies tiruan yang diaruhkan. Keputusan daripada kedua-dua teknik-teknik ini telah dikumpul untuk menilai kesan PGGA dalam perencatan

pembentukan lesi karies aruhan dan pemineralan semula. Dalam usaha untuk menentukan mekanisme kemungkinan PGGA dalam menghalang pendemineralan dan menggalakkan pemineralan semula, gigi dan pelet HAp telah dirawat dengan larutan PGGA (masing-masing 1% dan 2%) dan diguna dalam analisis Fourier Transform Infra-Red (FT-IR). Ketumpatan larutan PGGA (1% dan 2% masing-masing) juga diukur untuk menentukan kelikatan dinamiknya. Dalam kajian ini, didapati bahawa 2% PGGA menghalang pendemineralan enamel dan serbuk HAp dan adalah lebih kuat berbanding dengan kawalan positif (0.01% NaF, 0.1% NaF dan 0.5% NaF) pada pelbagai pH larutan pendemineralan (4.0 ,4.5 dan 5.0). Selain itu, 2% (w / v) PGGA juga telah ditunjukkan menggalakkan pemineralan semula enamel dan pelet HAp di bawah pH kritikal dan lebih berkesan berbanding dengan kawalan positif pada kesemua kepekatan. Daripada analisis FT-IR, ia telah menunjukkan bahawa PGGA mempunyai keupayaan membentuk lapisan pada enamel dan pelet HAp. Sifat penyalutan permukaan bagi PGGA boleh dikatakan berperanan dalam perencatan pendemineralan enamel gigi. Berdasarkan analisis FT-IR, kumpulan COO^- yang hadir dalam PGGA mungkin mencadangkan potensinya untuk mengikat dengan Ca^{2+} bebas dalam larutan, memineralkan semula gigi sepertimana ditunjuk dengan peningkatan dalam kekerasan enamel melalui kajian CSMH. Dengan ini, boleh dikatakan bahawa 2% PGGA mempunyai kesan yang lebih baik daripada 0.5% NaF dalam menghalang pendemineralan dan menggalakkan pemineralan semula enamel di bawah keadaan simulasi-karies.

ACKNOWLEDGEMENTS

"I AM THANKFUL TO ALMIGHTY ALLAH FOR HELPING ME IN ALL MY ENDEAVOURS."

"This thesis is dedicated to my beloved father (late) Mr Khan Qamar"

I am extremely thankful to my supervisors Prof Dr Zubaidah @ Jubaidah Binit Haji Abdul Rahim and Dr Chew Hooi Pin for their constant guidance, time and advice which helped me throughout in every aspect of my project. I am grateful for their support in completion of my project and thesis. Their professional competence, well timed advice and continuous support have inspired me to keep doing better.

I am grateful to Prof Dr Paul Anderson (Queen Mary University of London) for his vital assistance and guidance in using Calcium Ion Selective Electrode as a part of my project.

I would like to thank the Department of Chemistry for helping me in devising and performing experiments for remineralisation studies and for helping me to study the mechanism of action.

Most importantly I would like to thank my mother, father (late) and wife; without their prayers, encouragement and support I would have not achieved anything and reached the place where I am now.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

ATR	:	Attenuated total reflectance
Ca^{2+}	:	Calcium ion
CSMH	:	Cross sectional microhardness
F^-	:	Fluoride ion
FF	:	Fluid friction
FT-IR	:	Fourier transform infra-red
HAp	:	Hydroxyapatite
ISE	:	Ion selective electrode
LMR	:	Longitudinal microradiography
NaF	:	Sodium fluoride
OCT	:	Optical Coherence Tomography
PBS	:	Phosphate buffered saline
PGGA	:	Poly-γ- glutamic acid
QLF	:	Quantitative Light-Induced Fluorescence
R_{Ca2+}	:	Rate of change in calcium ion concentration
SMR	:	Scanning microradiography
SEM	:	Scanning Electron Microscopy
SS	:	Shear stress
TEM	:	Transmission Electron Microscopy
TMR	:	Transverse microradiography
TS	:	Tensile stress

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Poly-γ-glutamic acid (PGGA) is a homo polyamide (HPA) occurring naturally. It is made up of D- and L- forms of glutamic acid units which are connected by the amide linkages between the α-amino group of an amino acid and the γ-carboxyl of an adjacent amino acid. Thus, it is resistant against the protein degrading enzymes known as proteases (enzymes cleaving typical peptide bonds) (Candela & Fouet, 2006).

It has been reported that PGGA is a component of the capsule of *Bacillus anthracis* (Ivanovics & Erdos, 1937) and accumulated in a culture broth of *Bacillus subtilis* as a product of fermentation (Bovarnick, 1942). In a mixture with fructan, PGGA is present in 'Natto'; as first reported by Sawamura (1913). Natto is a traditional Japanese food which is formed by the fermentation of Soya beans.

There are other *Bacillus* species (such as *Bacillus megaterium, Bacillus chungkaakjang*) that secrete PGGA into the growth medium, as an end product of soya bean fermentation (Troy, 1973; Kubota et al., 1992). PGGA can exist in many forms which can be broadly classified as free acid or salt forms. The salt forms can be of the type containing Na^+, Mg^{2+}, K^+, NH_4^+ or Ca^{2+}.

PGGA can exhibit 5 different types of conformational changes which are dependent on environmental changes. These conformational changes are alpha helix, beta sheet, helix to random coil transition, random coil and enveloped aggregate (Yang et al., 2008).

Statherin, an acidic polypeptide secreted by major salivary glands, particularly parotid and submandibular glands, has a high content of proline, tyrosine, and glutamic acid, the latter being an amino acid which is also present in large amount in PGGA. It is a phospho-peptide consisting of 43 amino acid residues, most commonly found in the saliva (Schlesinger & Hay, 1977).

Statherin organizes the Ca^{2+} in the mouth by inhibiting hydroxyapatite (HAp) crystal nucleation and salivary calcium phosphate salts precipitation (Hay et al., 1984; Schwartz et al., 1992; Kosoric et al., 2007; Fabian et al., 2012). It has been found that statherin is the first protein that binds to recently cleaned enamel (Hay et al., 1989; Kosoric et al., 2007). It is also involved in inhibiting unwanted precipitation of calcium phosphate from supersaturated saliva. It plays a major role in pellicle layer formation, which provides protection for the teeth (Vitkov et al., 2004; Kosoric et al., 2007).

PGGA, which is similar to statherin in possessing a large proportion of glutamic acid residues, is able to inhibit demineralisation at a pH 4.0 below the critical pH for saliva (Qamar et al., 2012). HAp pellets were used as the study sample. In that study, the rate of demineralisation was detected by Scanning Microradiography (SMR) at different points on the HAp pellets. It was found that the rate of demineralisation of PGGA-treated HAp pellet at pH 4 was significantly different from that of the control fluid (0.1M acetic acid) of pH 4.0. It was also found that PGGA has a similar effect to that of the salivary protein statherin in inhibiting demineralisation of HAp (Qamar et al., 2012).

Many diseases, like dental caries, result in the destruction of tooth structure (Paolinelis et al., 2006; Mohammed et al., 2014a). Dental caries is the most common cause of oral pain (Fejerskov & Kidd, 2008). It is an infectious disease where the destruction of tooth structure is caused by acids produced by acidogenic bacteria that ferment dietary carbohydrates (Marsh & Martin,1992; Dawes, 2003; Young & Dawes, 2011). Bacteria colonise non shedding surfaces like teeth (Marsh & Martin, 1992) and produce acids that include lactic, acetic and formic acid (Featherstone, 2008).

Dental erosion is not the same as dental caries. It is defined as an irreversible loss of dental hard tissue by acid which is not bacterial in origin (Larsen, 1990). It is the type of tooth wear that is caused by chemical means, usually acidic, and the causative factor can

either be intrinsic or extrinsic in origin (Pickles, 2006) and it is not caused by any mechanical or physical factors, caries or trauma (O'Sullivan & Milosevic, 2008). Erosion has a multifactorial aetiology, involving intrinsic factors such as vomiting and gastro-oesophageal reflux (due to stomach acids) and extrinsic factors such as acidic foods and drinks, some medicines and occupational influences like acid fumes in the air (dietary or environmental) (Fejerskov & Kidd, 2008).

Dental caries is a dynamic process which involves demineralisation and remineralisation of the tooth enamel in an ebb and flow process (Rosin-Grget & Lincir, 2001; Featherstone, 2008). Under normal conditions, remineralisation and demineralisation are in equilibrium. If this equilibrium is disturbed, it may lead to the development of dental caries (Featherstone, 2008). The enamel demineralisation occurs when the pH of the surrounding environment is below the critical pH level (5.2-5.4). The saliva is then under-saturated with the mineral content; therefore demineralisation of the dental tissue will continue until the surrounding environment becomes saturated again with mineral content (Dawes, 2003).

Demineralisation and remineralisation can occur concurrently in all kinds of carious lesions but the deeper the lesion, the harder it is to remineralize (ten Cate & Featherstone, 1991). The pathological and protective factors determine whether the lesion will progress, stay the same or reverse (Featherstone 2000). The caries process can be stopped or reversed if the protective factors that include increase in salivary flow and ionic content (fluoride, calcium and phosphate) predominate (Featherstone 2000).

A caries preventing agent like fluoride is commonly found in commercially available mouthwashes. Fluoride is an ionic form of fluorine (halogen & electronegative), the 13th most abundant element in the earth's crust. It can serve as an inhibitor of demineralisation and a promoter of remineralisation. Therefore it is suggested that

fluoride-containing products can protect enamel and serve as reservoirs of fluoride ions (Lee et al., 2015). Fluoride ions (F^-) will penetrate to the subsurface of enamel and adsorb onto the apatite crystal surface along with the acids produced by the bacteria and protect the crystals from dissolution (Buzalaf et al., 2011), promoting remineralisation. Fluoride ions (F^-) combine with hydrogen ions (reversible process) to form hydrogen fluoride (HF). Formation of HF is favored in an environment where the pH is lower than the pKa value of 3.45 (Sharma et al., 2010).

However using fluoride in dental products may have its adverse effects. The use of fluoride-containing dental products without supervision in children when teeth are developing can lead to fluorosis (Seow, 2015). It may also cause burning mouth syndrome, sore tongue, nausea, vomiting, diarrhoea, increased saliva, stomach pain or cramp, muscle weakness, seizures if swallowed in high dosage (Choi et al., 2012; Dey & Giri, 2016; Waugh et al., 2016).

An oral health care product having minimum or no adverse effects is needed to maintain the integrity of tooth enamel. It has been reported in various studies that PGGA, which has been used as a food product, increases calcium absorption from the intestine (Yang et al., 2008; Ogunleye et al., 2015). Qamar et al., (2012) have demonstrated that it inhibits demineralisation of HAp pellets at pH 4.0. Hydroxyapatite pellet is an analogue of tooth enamel and has been used in studies related to hard tissue dissolution (Kosoric 2006). Being inexpensive and with the ability to a) increase calcium absorption from the intestine and b) inhibit demineralisation, PGGA, may have a revolutionary potential in the world of dentistry.

Qamar et al., (2012) used similar methodology to that of Featherstone et al., (1983) and reported that PGGA has a potential to inhibit the dissolution of HAp pellets under caries-inducing conditions on investigation by SMR. However, the role of PGGA towards dental enamel in a caries lesion (demineralisation and remineralisation) needs to be evaluated.

Aim

The aim of this study was to determine *in vitro* the effect of PGGA on demineralisation and remineralisation of dental enamel under caries-inducing conditions.

Specific objectives

1. To determine the effect of PGGA on the integrity of dental enamel under caries-inducing conditions with respect to demineralisation at a pH equal to or below the critical pH
    a) To investigate *in vitro* the concentration of PGGA effective in controlling enamel demineralisation at the stated pHs.
    b) To investigate *in vitro* the effect of PGGA on the early developing enamel lesion in relation to the depth of demineralisation from the enamel surface.
2. To determine the effect of PGGA on dental enamel under caries-inducing conditions with respect to remineralisation at pH values below, equal to or above critical pH (pH 4.0, pH 5.0 and pH 6.0 respectively).
    a) To determine *in vitro* the concentration of PGGA effective in promoting enamel remineralisation at the stated pHs.
    b) To compare the remineralising effect between PGGA and a known remineralising agent (sodium fluoride) on the early enamel lesion.
3. To propose a possible mechanism of action of PGGA on dental enamel under simulated caries challenges.
    a) Interaction of PGGA with dental enamel and HAp pellets
    b) The plausible effect of the viscosity and density of PGGA

CHAPTER 2: LITERATURE REVIEW

2.1. Dental Enamel

2.1.1. Structure

Enamel is the outermost protective covering of the crown of a tooth which is 85% inorganic, 3% organic and 12% water with respect to volume by volume (Robinson et al., 2000; Featherstone & Lussi, 2006; Buzalaf et al., 2011). The inorganic component of enamel is mainly in the form of hydroxyapatite crystals (Berkovitz et al., 2011). Hydroxyapatite (HAp) is written as Ca_{10} $(PO_4)_6$ $(OH)_2$ (Kay et al., 1964; Cole & Eastoe, 1988; West & Joiner 2014). Enamel is non-vital and irreparable (Berkovitz et al., 2011; Ganss et al., 2014). The organic component of enamel is mainly the residual enamel matrix proteins which almost disappear upon enamel maturation (Eastoe, 1960, 1963).

Figure 2.1 shows the symmetrically arranged atomic structure of calcium hydroxyapatite. The structure contributes towards the chemical stability. It is very insoluble with excellent thermal and insulating properties (Anderson & Creanor, 2016).

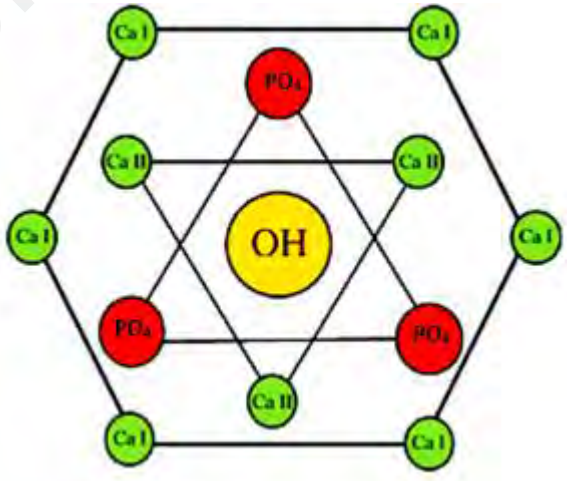


Figure 2.1: Atomic arrangement of chemical groups within the hydroxyapatite unit cell projected down long axis of the unit cell. Note that there are two different calcium positions, labeled Ca I (hexagonal calcium) and Ca II (trigonal calcium), PO_4 is the inorganic phosphate (Anderson & Creanor, 2016; Robinson 2009; Robinson et al., 1995).

Millions of enamel crystallites arise from the Tomes process of the respective ameloblasts extending perpendicularly to the cell surface. Hexagonal cross-section of a crystallite measures 25 nm which extends up to a length of 2 mm to the enamel surface from the Dentino Enamel Junction (DEJ) (Boyde, 1964). It is suggested that the crystallites are bound together by the residual enamel proteins (Anderson & Creanor, 2016).

Enamel crystallites arising in millions from the ameloblasts coalesce to form enamel prisms (Anderson & Creanor, 2016; Lynch et al., 2010), which measure 4-5 μm in width (West & Joiner, 2014). The width of enamel prisms increases along the length extending from the DEJ to the enamel surface (Al-Jawad & Anderson, 2014; Anderson & Creanor, 2016).

In HAp crystals, the calcium may be replaced by sodium or magnesium, making it calcium deficient (Anderson & Creanor, 2016). The fluoride levels are highest at the surface and decrease towards the DEJ (Berkovitz et al., 2011).

Enamel, being brittle due to high inorganic content, requires support from underlying dentine which is essential for enamel function (Berkovitz et al., 2011). Thus the occlusal enamel, if not supported by sound dentine, is more prone to fracture (Latino et al., 2001).

The thickness of enamel is different at different surfaces of the tooth. The enamel layer is thickest under the cusps and thinnest at the cervical margin (Nanci, 2014). The thickness of enamel covering the cusps is about 2.5 mm (Nanci, 2014). Enamel present at the surface is harder, radio-opaque and has decreased porosity as compared with subsurface enamel (Berkovitz et al., 2011). Due to its hardness, enamel has increased

resistance to wear and potential to bear load, making it distinctive from all the other dental tissues (He & Swain, 2008; Shahmoradi et al., 2014).

Teeth become dark with ageing. The reason for the darkening is enamel's reduced thickness which occurs because of wear, trauma and other factors, as a result of which the color of dentine beneath the enamel is reflected on the surface (Nanci, 2014; West & Joiner 2014).

2.1.2. Substitutions of ions in apatite structure

Although enamel is a dead tissue, its permeability allows ionic exchange which occurs between enamel and saliva (Nanci, 2014). Many changes occur in apatite structure and ionic substitution is responsible for these changes. Carbonate ion can substitute for hydroxyl ions. Similarly, magnesium can replace calcium and fluoride can replace hydroxyl ions in the apatite crystal structure (Weatherell et al., 1975; Robinson 2009). These substitutions cause a change in behaviour and solubility of apatite. The incorporation of fluoride in the apatite decreases its solubility product (K_{sp}), making the apatite structure more acid resistant (Robinson 2009).

Eanes *et al.,* (1979) also proposed that the displacement of hydroxyl ions by fluoride has a stabilizing outcome on the crystal lattice. This action of fluoride is significant in caries prevention. Frazier et al., (1967) found that the inclusion of fluoride in apatite structure during tooth development enhances the crystallinity of the mature enamel apatite.

During demineralisation, the areas of the lattice containing carbonate and lacking calcium are very prone to attack by hydrogen ions from acid (West & Joiner 2014; Featherstone 1999). Molybdenum makes hydroxyapatite crystals more resistant to acid attacks and may also have an anti-cariogenic effect (Nirmala & Reddy, 2011). A logical explanation for this is that incorporation of molybdenum during tooth development may

decrease the depth of fissures which in turn would lead to decreased stagnation sites resulting in decreased enamel caries (Scully, 2002).

2.1.3. Hardness and Elastic modulus of Enamel

The hardness of enamel is similar to that of mild steel (Newbrun & Pigman, 1960) which is 3.5 GPa (O'Donnell, 2011). The hardness of enamel gradually decreases with increasing depth, where it maintains stability in hardness at a distance of 100-600µm from DEJ (2-2.5 GPa). Zhang et al., (2014) found the hardness of enamel to be between 3.9 GPa to 3.8 GPa whereas the elastic modulus is approximately 80 GPa at the surface enamel which decreases up to 60 GPa near the DEJ (An et al., 2012).

It has been proposed that every 1% reduction of hydroxyapatite volume fraction would cause a 3 GPa reduction in elastic modulus of enamel (Staines et al., 1981).

2.1.4. Formation of enamel (Amelogenesis)

It commences at the late bell stage of the tooth development. During enamel formation internal enamel epithelial (IEE) cells undergo a number of morphological changes, each of which is related to different functions (Mitsiadis, 2013).

Initially in the pre-secretory stage the initial signs of differentiation of ameloblasts occur at the cuspal tip or the incisal edges, which gradually then spread down the sides of the crown. The cells of IEE become more columnar. They tend to show 'reversal of polarity' at this stage with the migration of the nucleus towards the stratum intemedium (Berkovitz et al., 2011; Shahmoradi et al., 2014).

Further it is characterized by the synthesis and secretion of enamel matrix and its initial mineralization. The morphology of the ameloblasts is highly columnar in this stage. Ameloblasts secrete an initial thin layer of aprismatic enamel. Later, ameloblasts develop Tomes processes for the prismatic structure formation (Papagerakis &

Mitsiadis, 2013). Organic matrix is secreted which is immediately mineralized. The composition of this matrix is approximately 30% inorganic, 30% organic and the remaining 40% is water. The matrix is formed to its final thickness and its surface layer is prismless.

As the final enamel thickness is already laid, the ameloblasts start to become short and many of them undergo apoptosis. Enamel matrix secretion ceases and the selectively slow withdrawal of the organic component starts followed by the matrix mineralization (Al-Jawad & Anderson, 2014). Later during enamel maturation the ameloblasts secrete kallikrein-related peptidase-4 (KLK4). The KLK4 peptidase helps in the removal of previously secreted and partially hydrolysed matrix proteins from enamel layer. The removal of the proteins provides space for the expansion of rod and inter-rods crystallites within the enamel layer.

The enamel matrix maturation continues by the removal of water and organic components which include the amelogenin proteins and this is followed by the incorporation of calcium and the phosphate ions in the matrix. This leads to the formation of highly mineralized matrix consisting of 85% inorganic, 3% organic and 12% water with respect to volume by volume.

2.2. Dental Caries

Oral diseases are the most common chronic diseases (Frencken et al., 2012) affecting civilized man. These diseases include dental caries, periodontal disease, dental erosion and developmental defects of enamel. Although the aetiology of caries and erosion are quite different from each other, both conditions are characterized by the demineralisation of dental tissue resulting from the action of acids. In caries, acid is produced by the metabolic activity of cariogenic bacteria (Hemmens et al., 1946; Heymann et al., 2013) and in erosion, the acid is mainly extrinsic or intrinsic in origin (Hemmens et al., 1946; Ren, 2011; Hara et al., 2015; Scaramucci et al., 2015). In this study the focus will be on the analysis of enamel mineral loss in caries-inducing conditions and hence further discussion includes dental caries only.

2.2.1. Aetiology of Dental Caries

Dental caries, also known as tooth decay, is a pathological process caused by plaque bacteria which results in localized destruction of the tissues of the tooth (Banerjee & Watson, 2015). "It is the demineralisation of enamel, dentine or cementum caused by organic acids produced by acidogenic bacteria in dental plaque which metabolise fermentable carbohydrates" (Wilson & Plasschaert, 2007). Studies suggest that caries is the most prevalent disease in adults which results in pain, infection and eventually tooth loss if left untreated (Urzua et al., 2012). Tooth loss results in difficulty to eat a healthy diet consisting of fiber-rich foods like fruits and vegetables and may result in malnutrition. Teeth also have an important role in speech and facial appearance and dental diseases can have an adverse effect on them (Moynihan & Petersen, 2004). Treatment of caries is costly, even more than heart disease, diabetes or osteoporosis (Moynihan & Petersen, 2004).

A caries lesion starts below the surface of enamel and may advance to dentine and pulp. Caries can be reversed in its early stages and the mineral can be regained especially in the presence of fluoride (ten Cate, 1990; Ekstrand & Oliveby, 1999; Songsiripradubboon et al., 2014). In young people caries of enamel is more common whereas in an older age group, root surface caries is more common due to the receding gums exposing cementum which is even more susceptible to the acidic challenges (Marsh & Martin, 2009).

2.2.2. Protective and Pathological Factors in Enamel Destruction

Dental caries occurs as a result of an imbalance between the continuous and dynamic processes of demineralisation and remineralisation of the tooth (Young & Featherstone 2013). An imbalance between the protective and pathological factors also contributes to the development of caries (Figure 2.2). The protective factors are saliva, modified diet with restriction of sugars, plaque control and use of fluoride (Young & Featherstone 2013). Salivary protective factors are 1) salivary flow clearing the bacteria and acids, 2) salivary phosphate and bicarbonate buffers neutralizing the acid produced by acidogenic bacteria, 3) salivary antimicrobial components, 4) pellicle formation and 5) calcium and phosphate super-saturation of saliva preventing demineralisation and enhancing remineralisation. The diet can be modified by reducing the intake of carbohydrate-rich diet. Plaque control can be carried out by mechanical (brushing, scaling) and chemical removal (mouth rinses) of the stagnant food and reducing the bacterial content.

Fluoride is the most effective anti-caries factor as it is considered to promote the process of remineralisation (ten Cate, 2013). Pathological factors are the acidogenic bacteria especially *Strep. mutans* and lactobacilli, poor oral hygiene, dietary carbohydrates and reduced salivary flow (Featherstone 2000).

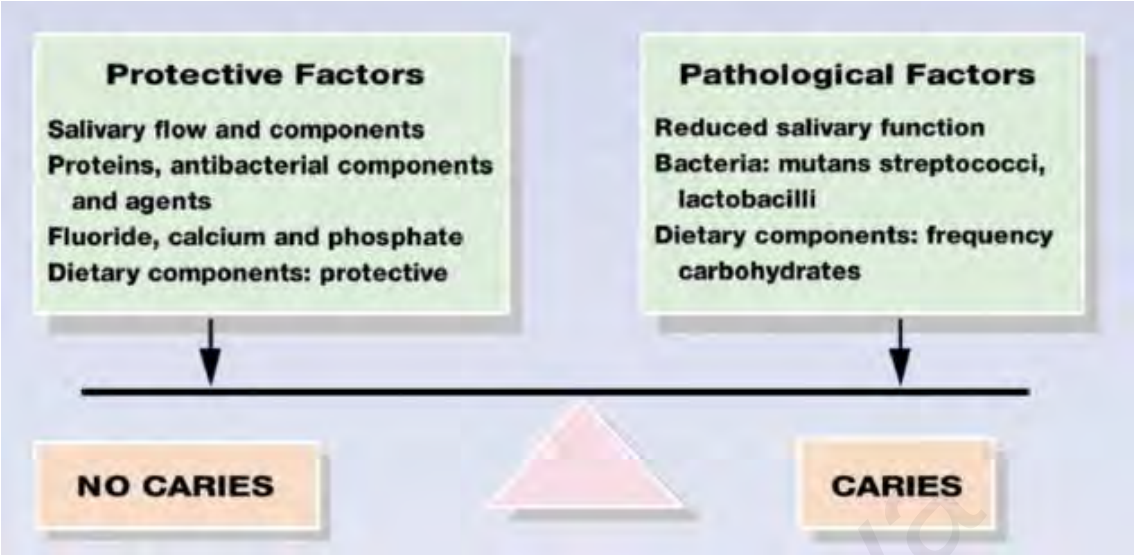


Figure 2.2: Pathological and protective factors influencing caries (Featherstone 2000)

2.2.3. Histological Changes during Caries Development

Four zones of a white spot lesion are seen on histological examination using polarized light and imbibition media, namely a surface zone, body of the lesion, dark zone and translucent zone (Heymann et al., 2013).

The surface zone is a comparatively intact area 1-2% porous, which remains intact until the commencement of cavitation (Robinson et al., 2000). The main component of the lesion is called the body of the lesion, it appears translucent and lies under the intact surface zone (Robinson et al., 2000; Heymann et al., 2013). It is 25-50% porous (Robinson et al., 2000). The next zone appears to be dark, has 5-10% porosity and is called the dark zone because it is positively birefringent (Robinson et al., 2000; Darling, 1961). The dark zone indicates the occurrence of reprecipitation of calcium at the demineralisation site where remineralisation occurs (Nurul Islam, 2009). The zone following it is the translucent zone with enamel porosity about 1-2% with the pore size big enough to allow entry of quinoline.

Since quinoline has the same refractive index as that of enamel, it makes the tissue translucent (Robinson et al., 2000). As is suggested by Silverstone (1973), the body of the lesion and the translucent zones are areas of demineralisation and the superficial and dark zones are areas of remineralisation.

2.2.4. Development of a Caries Lesion

The frequency and total amount of sugar intake are strongly linked with dental caries (Heymann et al., 2014). Caries is a bacterial disease and among the bacteria related to it are *lactobacilli* sp. and *Strep. mutans*. Dental plaque is an important risk factor and prerequisite for dental caries which develops due to the metabolic activities of the dental plaque bacteria (Marsh & Martin, 2009).

Caries prevalence can be modified by implementing preventive measures, physiological and socioeconomic factors (Vargas et al., 1998; Petersen & Phantumvanit, 2012; Lewis, 2014) and in spite of having a complex aetiology, prevention is an achievable aim.

On exposure to saliva, the cleaned enamel surface in a few seconds gets covered by an adsorbed layer of molecules mainly glycoproteins (salivary glycoproteins) forming acquired pellicle, providing a surface for microorganisms to adhere. These microorganisms multiply and synthesize extracellular matrix polymers, thus providing a surface for other bacterial species to adhere to them rather than the pellicle. The close proximity of various bacterial species leads to different interactions (synergistic and antagonistic) in dental plaque. In a healthy mouth the bacterial colonization varies according to the position of the teeth. For example the maxillary anterior teeth have less plaque deposit as compared with maxillary posterior teeth (Soames & Southam, 2005).

Cariogenic bacteria in dental plaque metabolize dietary sugars into acids (mainly lactic acid). This leads to a fall in plaque pH by about 2 units within ten minutes of ingestion of sugar. But after 30-60 minutes the pH rises back to its original level due to diffusion of sugar and acids out of the plaque, and the diffusion of saliva into the plaque (buffering to neutralize the pH). A see-sawing of ions is observed at the plaque-enamel interface due to change in the chemical environment of plaque. Some mineral ions are lost due to diffusion out of plaque into saliva during the acid phase. Repeated mineral loss leads to demineralisation and initiation of caries (Soames & Southam, 2005).

When the teeth covered with dental plaque are exposed to dietary carbohydrates, the cariogenic bacteria will ferment the sugars, producing organic acids. The pH of the dental plaque drops below 5.5 causing mineral loss in the enamel called demineralisation. Saliva has components neutralizing the acid causing the pH to rise and as a result, the lost mineral may be redeposited (Ellwood et al., 2008). The redeposition of mineral is known as remineralisation. This cycle of demineralisation and remineralisation happens many times in a day due to oscillating conditions of pH, which may result in a net loss of calcium and phosphate giving rise to a carious lesion which can be detected clinically as a 'white spot lesion' (Fejerskov & Kidd, 2008). On the other hand, sometimes the loss is so insignificant that there is no clinically detectable carious lesion. The early subsurface carious lesion of enamel is clinically very important because there is a chance of reversing the lesion at this stage by remineralisation while the enamel surface is still intact (Dowker et al., 1999).

The minor components of the enamel mineral are particularly important to the kinetics of demineralisation as removal of carbonate and its replacement by fluoride ions result in decreased loss of mineral (Robinson et al., 2000).

2.2.5. Demineralisation and Remineralisation of Enamel

In normal circumstances, saliva and oral fluids are supersaturated with respect to hydroxyapatite and fluorapatite but with a drop in pH, they become undersaturated with respect to hydroxyapatite which is more soluble than fluorapatite (Higham 2014). Demineralisation is the dissolution of tooth minerals, mainly calcium and phosphate by the plaque acids. The hydroxyapatite having the chemical formula as $Ca_{10}(PO_4)_6(OH)_2$ indicates that it is composed of about 40% calcium, 57% phosphate and 2% hydroxyl ions (Robinson et al., 1995). Hydroxyapatite forms the bulk of the enamel mineral and the drop in plaque pH slowly makes it unstable. The H^+ causes the protonation of trivalent PO_4^{3-} to divalent HPO_4^{2-}. These trivalent phosphate ions are holding calcium ions in place (in the lattice) and depletion will lead to the dissolution, releasing some of the calcium from hydroxyapatite (Robinson et al., 1995). With the decrease of one unit in pH, the dissolution of hydroxyapatite increases by about a factor of 10 resulting in a subsurface carious lesion with 30-50% of mineral loss and relatively intact mineralized surface layer (Fejerskov, 2004).

Remineralisation, on the other hand, is the redeposition of the same minerals to rebuild the damaged rods preferably in the presence of fluoride when the pH is increasing (Harris et al., 2009; Owens, 2013). The early subsurface demineralisation is very important clinically because it can be reversed by remineralisation. Hence we can say that caries is a dynamic process and episodes of demineralisation and remineralisation are occurring concurrently (Robinson et al., 2000).

Stephan (1940) explained the process of demineralisation and remineralisation by exposition of a curve showing pH of dental plaque before, during and after glucose rinse in an in situ study. The curve shows three main characteristics: i) the pH of dental plaque measured under resting conditions which is constant ranging between 6.9-7.2; ii) upon exposure to sugary food, the pH drops within few minutes to a level ranging 5.2-5.5 (critical pH) leading to mineral loss, where recurrent decrease in pH leads to extensive mineral loss resulting in initiation of dental caries and iii) the return of pH to its normal value within approximately 30-60 minutes and under supersaturated calcium phosphate, remineralization is initiated.

2.2.6. Caries -Inducing Conditions (Development of Artificial Caries)

In vitro techniques are the most commonly used in dental research. Over the years these *in vitro* models have been established and enhanced. Studies of mineral changes in enamel can be classified: i) demineralisation ii) remineralisation and iii) pH cycling (Featherstone et al., 1990; ten Cate, 1990).

During the process of demineralisation unsaturated solution of low pH (acidic) is used (Anderson et al., 1998; Anderson et al., 2004). The types of acids produced on the metabolism of dietary sugar leading to demineralisation are dependent on the composition of microbial florae of dental plaque. Geddes (1972, 1975) reported lactic, acetic and propionic acids are produced in major proportion, whereas those produced in minor proportion are formic, succinic, butyric, isobutyric and valeric acids.

Featherstone (1981) in an *in vivo* study compared the effect of acids produced in major content to develop an artificial demineralised lesion. He reported acetic acid buffer has potential to produce a deeper lesion as compared to lactate buffers. As the acids with higher unionized concentration have a potential to diffuse the enamel structure and develop lesion at a rapid rate.

For remineralisation experiments, supersaturated calcium phosphate solutions of constant volume and uniform composition are used (Zahradnik, 1979). Secondly, for demineralisation experiments intact enamel is used whereas for remineralisation, enamel that is artificially demineralised or has naturally occurring caries is used. A pH cycling model denotes experiments in which the substrate (i.e. enamel) is exposed to a combinational therapy of demineralisation and remineralisation, thereby mimicking the *in vivo* condition and it is known as a method of choice (White 1995). These systems suffer limitations from a biological point of view, as it is impossible to simulate all the complexities of the oral cavity.

Researchers have designed various artificial mouth models using saliva and bacterial plaque for carious lesion formation, but have not been successful (ten Cate & Marsh, 1994; Marsh, 1995). The reason given was variations due to i) composition of tooth ii) volume and composition of saliva iii) difficulty to modulate supersaturation with calcium phosphate during a demineralisation / remineralisation experiment.

Acidic solutions (for example acetic acid, lactic acid (Featherstone & Rodgers, 1981; Shellis, 1984)) can be used to develop *in vitro* artificial lesions, making the lesion formation faster and less time consuming. It may not necessarily produce a subsurface lesion as the tooth is isolated from the natural continuous dynamic process of remineralisation and demineralisation, thus etching the surface enamel. Therefore a steady gradient of demineralisation is observed involving the surface enamel towards the bulk of enamel (Robinson et al., 1995; Siddiqui et al., 2014).

2.2.7. Remineralisation of caries lesion

There is good evidence from many *in vitro* and *in vivo* studies that remineralisation is a real phenomenon. ten Cate (2001) regarded the process of remineralisation as non-restorative repair of demineralised enamel. Various studies done on animal models and humans showed that remineralisation takes place successfully when calcium and phosphate are available in appropriate concentrations near pH 7, in the presence of fluoride (Briner et al., 1974). When the pH drops below about 5.5 (critical pH) the HAp of enamel starts to demineralize, whereas in the presence of fluoride the flurohydoxyapatite (FHAp) may develop at the enamel surface (pH above 4.5). The FHAp crystals may develop on the surface enamel reducing enamel demineralisation with continuing dissolution of subsurface HAp (below pH 5.5) (Ellwood et al., 2008).

2.2.8. Role of Fluoride in Remineralisation

Fluoride has a vital role in the process of remineralisation, though it is known that during the process of remineralisation the crystal length and structure will not be restored to those in the original structure. The process usually occurs in incipient/ early carious lesions rather than deep cavities (Mohammed et al., 2014).

Currently research is being carried out in order to develop oral health care products having a potential for the management of early carious/ incipient lesions (Carvalho & Lussi, 2014). As a secondary prevention, intervention of such products decreases the factors for development of a lesion and initiates the process of remineralisation (ten Cate & Featherstone, 1991; Laurance-Young et al., 2011). Enamel which has undergone dissolution remineralizes on exposure to supersaturated solutions *in vitro* and to the oral environment *in situ* (Li et al., 2014). Various topical formulations have been used for remineralisation such as fluoride or calcium-phosphate (Mohammed et al., 2013; Mohammed et al., 2014 b).

Many *in vivo* and *in vitro* studies have focused on maintaining the fluoride concentration in oral fluids, being effective in reducing the rate of enamel dissolution. Bioavailable fluoride reservoirs persistently maintain/increase the concentration of F^- at the site of dissolution to initiate remineralisation (Marinho, 2008; Buzalaf et al., 2012). The deposits upsurge on enamel with an increasing concentration and frequency of fluoride application (ten Cate 2013). Fluoride-containing agents of pH less than 5 tend to produce more CaF_2 deposits than those of neutral pH. Primary sources of F^- are in the form of calcium fluoride (CaF_2) mineral deposits in the oral cavity. These deposits are formed by action of tooth-bound Ca^{2+} with F^- on fluoride application (ten Cate 2013). The mechanism of action of sodium fluoride in prevention of dissolution is particularly related to the formation of CaF_2 deposits on the surface (Ganss et al., 2007; Borges et al., 2014).

The role of calcium fluoride is two-fold: firstly it provides a physical barrier against interaction of acids with underlying tissue and secondly it acts as a mineral reservoir (Magalhaes et al., 2011).

2.3. Saliva

Whole saliva is a clear complex bio-fluid which coats the oral tissues and originates from a mixture of different fluids of glandular secretions and non-glandular secretions. Glandular secretions are secreted from salivary glands (Patel & Hoffman, 2014). Non-glandular secretions include crevicular fluids, which contain oral microorganisms and host cells (Cunha-Cruz et al., 2013).

Salivary glands are classified according to their size. The major glands include the paired parotid, submandibular and sublingual glands (Berkovitz, 2011; Knosp et al., 2012; Kidokoro et al., 2014; Patel & Hoffman, 2014; Varga, 2015). Normal salivary pH ranges between 6.0 and 7.0 and this indicates that it is slightly acidic to neutral. However, the pH can vary from 5.3 to 7.8 according to the flow rate, with the lowest pH at low flow rate and the higher values at peak saliva flow (Kuriakose et al., 2013). The hydrogen bicarbonate balance of saliva determines the salivary pH and buffering capacity of the saliva (Naveen et al., 2014).

Saliva is 99% watery fluid, becomes viscoelastic (thick and sticky) depending on the amount of protein that is present (Carpenter, 2013; Cunha-Cruz et al., 2013). The average flow rate of unstimulated saliva in normal healthy individuals is about 600 ml per day (Watanabe & Dawes, 1988). In the state of stimulation such as during meals, saliva secreted by parotid glands is about 60-65% of whole salivary volume, 20-30% by submandibular and sublingual glands (Carpenter, 2013; Dijkema, 2013) and approximately 10% by minor salivary glands. In the resting state the condition is different; approximately 50% of the whole saliva is secreted by submandibular glands. (C. Dawes, 1974; Dijkema, 2013).

Saliva secretion is stimulated by mechanical, gustatory, olfactory, or pharmacological stimuli and the stimulated saliva represents 80-90% of the whole daily saliva (Proctor & Carpenter, 2014).

Many factors affect the rate of salivary flow, including hydration level, body location, light effects, preceded stimulus, circadian and circannual rhythms, the gland volume and the medications that the patient may use (Proctor, 2016).

Determination of salivary flow rate varies in a wide range of different studies due to the different methods that have been used to collect saliva from individuals. Some studies depend on the individual responses to health questionnaires and clinical evaluation outcome. Other studies preferred detecting the qualitative or quantitative salivary flow changes by saliva collection from individual glands and/or the whole saliva; however, collection methods were carried out in different ways. Saliva collection methods have to be identical and precise in order to obtain an accurate comparison (Navazesh & Kumar, 2008).

2.3.1. Composition of saliva

Saliva is composed of 99% of water, and the remaining 1% is organic and inorganic compounds (Navazesh & Kumar, 2008). Saliva is hypotonic with plasma (Berkovitz et al., 2011; Varga, 2012).

Sodium, potassium, chloride and bicarbonate are the most common ions in saliva. Calcium, phosphate, fluoride, thiocyanate, magnesium, sulphate and iodide are also present in saliva (Berkovitz et al., 2011; Ali & Creanor, 2016). Saliva also contains immune substances, proteins, enzymes, mucins and other substances like urea and ammonia (Levine, 1993).

The hypotonicity of saliva has many beneficial effects, as it helps the recognition of different tastes by the taste buds without being affected by the presence of sodium in saliva. It also helps in expansion and hydration of mucin glycoproteins that cover the oral tissues as a protective cover.

The major salivary glands secrete a larger volume of whole saliva rich in inorganic content, whereas the minor salivary glands secrete a smaller proportion of whole saliva but with a higher organic content (Varga, 2012). Saliva contains various salivary proteins with various functions as described in Figure 2.3 (Levine, 1993). These include immunoglobulin (IgA) similar to serum protein (except that it is present as a dimer and also contains secretory component), enzymes (amylases, lysozymes, peroxidase, kallikrein, acid phosphatase), glycoproteins (mucins) and small molecular weight proteins (proline-rich proteins, statherin, tyrosine rich protein, histatins, histidine-rich proteins) (Figure 2.3).

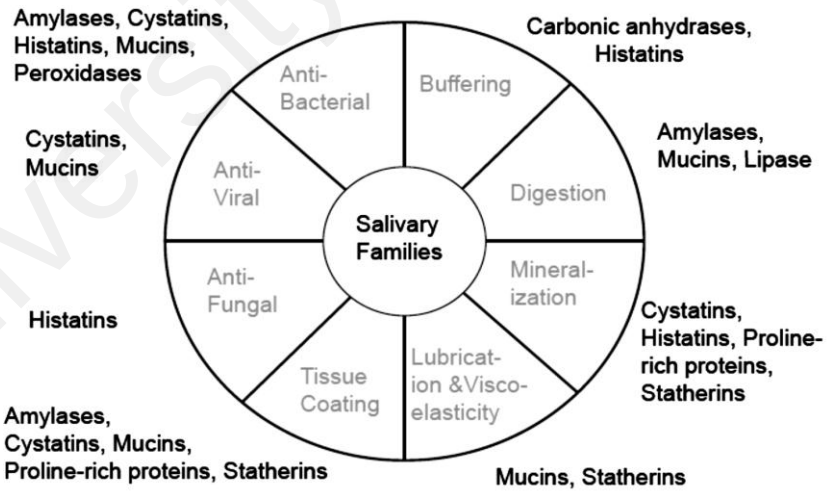


Figure 2.3: Different salivary proteins (Levine, 1993)

2.3.2. Salivary Statherin

It is a phospho – peptide with 43 amino acid residues, most commonly found in the saliva produced in the oral cavity (Schlesinger & Hay, 1977; Isola et al., 2011). It has unusual characteristics. It is an acidic peptide which is secreted by different salivary glands, typically by major salivary glands. It has high content of PRO (proline), TYR (tyrosine) and GLN (glutamine) (Schlesinger & Hay, 1977). Glutamine is a derivative of glutamic acid, a residue found in poly-γ-glutamic acid (PGGA).

Statherin is a typical salivary protein with charge and structural asymmetry (Raj et al., 1992). Statherin carries most of the negative charges at the N-terminal and will have strong affinity towards calcium ions which carry positive charge (Naganagowda et al., 1998), and hence HAp will bind to it (Gururaja & Levine, 1996). It has an efficiency to inhibit crystal growth and unwanted precipitation from a supersaturated calcium phosphate solution (Hay et al., 1984).

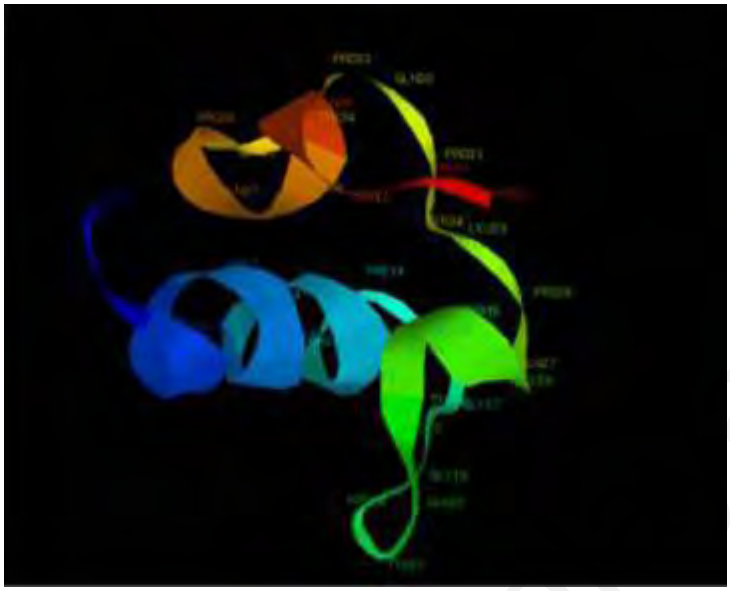
It is also thought that the statherin can take part in the transportation of the minerals particularly calcium and phosphate (Schlesinger & Hay, 1977; Raj et al., 1992) in its secretion from salivary glands, thus contributing in transporting ions at the mucosal level of the surface. In view of its strong affinity for the surfaces of HAp and enamel, statherin has major involvement in the formation of layers on the tooth surface, i.e. the so-called “acquired enamel pellicles”.

Tooth enamel integrity is maintained by this protein film which acts as a boundary on the surface (Douglas et al., 1991). Bacterial colonization specificity is easily possible due to the typical interaction between the proteins present in the pellicle and the bacterial surfaces during initial stages of plaque formation (Gibbons & Hay, 1988).

2.3.2.1. Structure of Statherin

Statherin is a peptide that has been found in the secretion of parotid and submandibular glands (Elgavish et al., 1984; Manconi et al., 2010). It has been confirmed by Long et al*.,* (2001) using solid-state NMR studies that the N terminus of statherin is the active part that is concerned in HAp binding and preventing HAp precipitation, whereas the middle and the C-terminus were transportable with no affinity to bind HAp. Naganagowda et al**.**, (1998) assumed that this may take place because of the N-terminus carrying almost all of the negative charge. It is believed that the N-terminus of statherin is responsible for 'anchoring' the molecule to the HAp surface by structural changes to an alpha-helix form leading to adsorption, even though additional lengths of the molecule may be implicated in adsorption stabilization (Raj et al., 1992; Shah et al., 2011).

(a)



DpSpSEEKFLRRIGRFGYGYGPYQPVPEQPLYPQPYQPQYQ

(b)

DpSpSEEKFLRRIGRFGYGYGPY

Figure 2.4: Structure of (a) statherin; and b) StN21 (Kosoric et al., 2007)(www.robetta.org)

2.3.2.2. Functions of Statherin

Statherin is the first protein that binds to cleaned enamel (Hay et al., 1989; Kosoric et al., 2007). Statherin organizes the Ca^{2+} in the mouth by inhibiting HAp crystal nucleation and salivary calcium phosphate salts precipitation (Hay et al., 1989; Schwartz et al., 1992; Kosoric et al., 2007; Fabian et al., 2012). Statherin in conjunction with other salivary proteins acidic proline-rich proteins, histatins and cystatins maintain the supersaturated state of saliva with respect to calcium phosphate salts. Salivary calcium phosphate supersaturation is required for the recalcification and stabilization of tooth enamel (Raj et al., 1992).

It also plays a major role in pellicle layer formation which provides protection for the teeth (Vitkov et al., 2004; Kosoric et al., 2007). It has been shown that there are elevated levels of statherin present at the air interface of the biofilm present in the mouth (Proctor et al., 2005) which leads to collecting and clearing of bacteria to the stomach rather than by surface adhesion (Fabian et al., 2012).

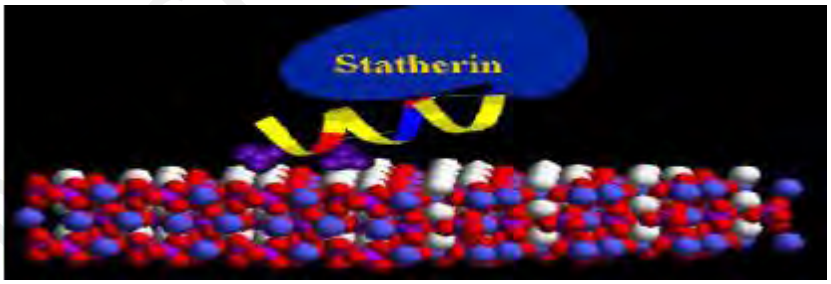


Figure 2.5: Statherin binding to HAp surfaces (Long et al., 2001)

It is also believed that statherin is involved in the inhibition of cariogenic bacteria such as *Strep. mutans* adhesion to the tooth by inhibiting the adsorption of high molecular weight glycoproteins onto the tooth surface (Fabian et al., 2012). According to Leito et al., (2009) statherin can be considered as an oral defence against fungi as it enhances *Candida albicans'* hyphae transition to yeast (Fabian et al., 2012). Furthermore, it has

been suggested by Gibbons and Hay (1988) that *A.viscosus* accumulates a high quantity of statherin onto the pellicle.

Also, the lubrication properties of statherin (together with other proteins present in the pellicle) depend on the protein structure following adsorption on the HAp that makes statherin recognized by other proteins (Goobes et al., 2006).

It has been reported by Chin et al., (1993) and Wikiel et al., (1994) that at pH 6.0 and 7.0, statherin and the N-terminus of statherin-like pentapeptides inhibit the dissolution of HAp. However, these experiments were carried out under strictly controlled environments simulating carious and/or erosive challenges, but it was questionable if these effects may take place in the more complex oral cavity (Kosoric et al., 2007).

Large conformational changes have been seen in proteins upon adsorption onto solid surfaces (Haynes & Norde, 1995) that influence their biological activity significantly (Agashe et al., 2005). This is seen mainly in proteins that act as a substrate during interaction with surfaces where they unfold upon adsorption (Andrade & Hlady, 1986; Haynes & Norde, 1995). Unfolding associated adsorption has revealed that statherin has the ability to prevent calcium phosphate crystallization by binding to the nuclei of the early crystals and inhibiting crystal growth by adsorption on the nucleated crystals (Hay et al., 1984; Douglas et al., 1991). Studies using isothermal titration calorimetry and equilibrium adsorption isotherm, indicated that most of the protein adsorbs by a process that is thermo-neutral and only a small part of the protein adsorbs with detectable heat (Goobes et al., 2006).

Additionally, a study showed that the calcium concentration in a multilayer complex salivary film is 500 times more than the calcium concentration in saliva (Proctor et al., 2005). Electrophoresis of salivary proteins of different individuals shows the presence of salivary proteins in fewer quantities in the salivary films other than statherin with variability between individuals and type of saliva. The occurrence of these minor proteins in the saliva films is considered not important for the creation and maintenance of these films as they are detachable by washing (Proctor et al., 2005) but the statherin is observed to be the major active protein moving rapidly to air interface. Therefore it is suggested that having high affinity to solid surfaces, statherin plays a major role in film development and to withstand the washing effect from saliva as compared to other salivary proteins.

2.3.2.3. StN21- and other statherin like peptides

Statherin-like peptide with 21 amino acids matched to the N –terminus of statherin (StN21) has been used to understand the effect of this part of statherin on enamel homeostasis shown in Figure 2.4. According to Long *et al*. (2001) the α-helical secondary structure is thought to play an important role in HAp binding (Moreno et al., 1979) and it is confirmed that application of this peptide to HAp surfaces result in a reduction in mineral loss by 40% compared with controls (Kosoric et al., 2007).

It has been suggested that StN21 may control the kinetics of the demineralisation process by either behaving as a semi-permeable film, or by changing the concentration of Ca^{2+} near to HAp surfaces together with the saturation level of the surrounding solution, or HAp surface stabilization. Generally it is more likely that these methods are working together to gain HAp surface stabilization (Kosoric et al., 2007).

An Scanning microradiography (SMR) study confirmed that to get cariostatic effects of statherin, a statherin-like peptide was necessary with N-terminus residues of 15 or more (StN15 or StN21) as they reduced the HAp demineralisation rates by 50-60%, unlike StN10 and StN5 peptides as previously suggested by other studies (Shah et al., 2011).

It is also suggested that at the air-saliva interface, a statherin-rich film is formed. Due to the amphipathic structure (having both hydrophilic and hydrophobic ends) of statherin it has been predicted to move quickly to the air interface acting as a very surface-active protein in such a way that the hydrophilic end points towards the air part while the hydrophobic end points towards the aqueous part. It has also been confirmed that there is a great affinity between statherin and solid hydrophobic surfaces (Lindh et al., 2002). Adding calcium increases the ability of binding with the possibility of multilayer formation (Lindh et al., 2002).

An NMR study suggested that there may be another mechanism for StN15 and HAp interaction in addition to the one that led to conformational changes close to the N-terminal. The proposed mechanism may require no major conformational changes of the protein as it may occur as a simple electrostatic interaction between StN15 and HAp (Chen et al., 2009).

2.4. Poly-γ-glutamic acid (PGGA)

PGGA a naturally occurring homo-polyamide; made of D- and L- forms of glutamic acid units by amide linkages. The amide linkages are formed between the α-amino group of one glutamic acid residue and the γ-carboxyl group of the adjacent glutamic acid residue as shown in Figure 2.6 (de Cesaro et al., 2014). The linkage has a strong resistance against enzymes known as proteases (enzyme cleaving the amide bonds) (Kedia et al., 2010). PGGA is hydrophilic and negatively charged at pH above 2.2.

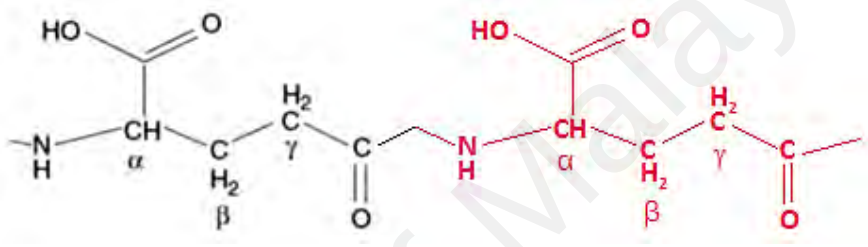


Figure 2.6: Poly-γ-glutamic acid chain(Kedia et al., 2010)

PGGA exists in various forms; broadly it can be classified as a free acid form and a salt form. The salt form of PGGA contains Na^{+}, or Mg^{2+}, or K^{+}, or NH_4^{+} or Ca^{2+}. The characteristics and chemical structure of the acidic or salt forms of PGGA can be determined by FT-IR. Depending on the environment, PGGA can exhibit 5 different types of conformational changes. These modifications are known as α-helix, β-sheet, helix to random coil transition, random coil and enveloped aggregate (Troy, 1973; Ogunleye et al., 2015).

PGGA, being a constituent of a traditional food product, is edible, non-toxic to humans and the environment. It has a high molecular weight and is not easily biodegradable (Shih & Van, 2001). Most importantly it has non immunogenic reactions, probably linked to the degradation of PGGA into glutamic acid residues (Buescher & Margaritis, 2007).

2.4.1. Production of PGGA

PGGA has a diverse history since being known for the last 75 years, but the mechanism and major substrates involved in its production are still unclear. Studies have been carried out in order to determine the conditions, nutritional requirements to improve cell growth and develop variations in molecular weight. Thus, ultimately it was suggested that for the production of PGGA, the nutritional value varies according to the type of bacterial strain.

Therefore, based on the nutritional obligation, PGGA-producing bacterial strains are divided into two groups; i) L-glutamic acid and ii) D-glutamic acid (Cheng et al., 1989; Kubota et al., 1992; Shih & Van, 2001).

The production of PGGA is enhanced by bacterial strains using carbon as a source on addition of glycerol (other source of carbon), glucose, sucrose and citric acid in the medium. It is presumed that the L-glutamic acid acts as an activator for the enzymatic system for PGGA production (Birrer et al., 1994).

Bacillus licheniformis (ATTC strain 9945a) and *Bacillus subtilis* are among the strains which are most commonly involved in the production of PGGA. Leonard et al., (1958) optimized Medium E for *Bacillus Licheniformis 9945a* in order to produce PGGA. *Bacillus subtilis*, a key strain to produce 'natto', is nutrient specific and is reliant on biotin or vitamin supplementation.

2.4.2. Chemical Structural Characteristics of PGGA

The glutamic acid residue, a major building block of PGGA, is comprised of three functional groups which can be illustrated in the sequence of their chemical activity α – NH_2, α - COOH and γ - COOH. The hydrogen dissociation constants for these are: pKα (=pK1) = 2.13-2.2. pKγ(= pK2) = 4.25-4.32, and pK_{NH} (pK3) = 9.7-9.95 (Ho et al., 2006). During the polymerisation (chemically catalysed) of glutamic acid, α-peptide bonds develop between the active groups *α*-COOH and α-NH_2, producing α-poly glutamic acid as an end product.

Lin et al ., (2007) used acid titration method to determine the pK_a value of PGGA and it has been reported as 2.9. The minimum pH for PGGA to be in an ionized form is 2.5, for presence of a free carboxyl group.

During the process of submerged fermentations, L-glutamic acids are largely racemized to D-glutamic acids. Later on, both the D- and L- glutamic acids are co-polymerized via formation of γ-peptide bonds between less reactive γ-COOH and α-NH_2 resulting in the formation of γ-(D,L)-poly glutamic acid as an end product.

2.4.3. Properties of PGGA

2.4.3.1. Molecular Weight of PGGA

PGGA produced synthetically most often has a molecular weight below 10,000 kDa; thus limiting its utility. However, the one produced by bacteria has a molecular weight greater than 10,000 kDa (Kedia et al., 2010). Both are easily degradable into lesser molecular weight fractions, as needed for its specific application (Kedia et al., 2010).

2.4.3.2. Microbial Resistance

The α-peptide bond is commonly found in protein structures and it can be hydrolysed by most proteases. The γ-peptide bond of PGGA can only be hydrolysed by a rare but naturally occurring protease, γ-glutamyl transpeptidase (Morelli et al., 2014). None of the other proteases have an ability to hydrolyze the bond of PGGA. Thus the PGGA has potent resistance against microbial attack.

2.4.3.3. Stability of PGGA

On thermal analysis of PGGA, Ho *et al* (2006) reported that it contains 10% hydrated water. Whereas the dehydration temperature is 109°C, the melting point (Tm) is 160°C and the decomposition temperature (Td) is 340°C. This suggests that the structure of PGGA can remain stable at room temperature.

2.4.3.4. Capability of PGGA to bind metals

The most important point related to the chemical structure of PGGA is that it has the capability to bind with different metals (Ho et al., 2006). The important metals required are bio-available, most vital among those include Ca^{2+} and Mg^{2+} binding via ionic complex mechanism. The complexes formed are known as calcium poly-γ-glutamate and magnesium poly-γ-glutamate. These bonds are of vital importance, being stable coordinate ionic complexes (Ho et al., 2006).

2.4.4. Applications of PGGA

PGGA is found to have various applications in different fields such as a thickener particularly for paints and cosmetic products. It is also being used as a humectant, bitterness revealing agent in edible products, biopolymer flocculants, heavy metal absorber and cryoprotectant (Bhat et al., 2013). It has a very important role in cancer therapy being a drug carrier with a capability of sustained release of chemotherapeutic drugs (Ko & Gross, 1998; Shih & Van, 2001; Tian et al.,2012; Ogunleye et al., 2015).

2.5. Methods of Detecting Demineralisation and Remineralisation of Dental Hard Tissues

2.5.1. Quantitative Assessment

It is a direct approach by assessing minerals, ionic exchange during dissolution or correlating the degree of demineralisation with i) physical properties (surface hardness) ii) changes in optical properties of softened enamel (Arends & ten Bosch, 1992).

Early dissolution can also be quantitatively evaluated by determining the demineralisation in the subsurface or thickness of enamel. There are various quantitative techniques which have been used as listed below:

i) Profilometry

ii) Surface hardness

   a) Microhardness

   b) Nanohardness

iii) Ionic changes

iv) Microradiography

v) Optical methods

   a) Optical coherence tomography (OCT)

   b) Quantitative Light-Induced fluoresence (QLF)

2.5.1.1. Profilometry

Contact diamond profilometry probably is the most commonly and conventionally applied technique for surface profile and roughness measurements (Bollen et al., 1997; Reisner et al., 1997; Whitehead et al., 1999; Joniot et al., 2000; Kakaboura et al., 2007). The surface loss of enamel and its surface roughness could be determined by scanning with a small metal or diamond contact stylus (with a diameter of 2 - 20 μm) or a non-contact laser beam. In order to determine the effect of demineralising agent using contact profilometry, some part of the tooth is covered with a nail varnish while rest exposed to the solution. It helps in comparing the treated and untreated areas of the tooth surface. The use of non-contact optical profilometer-based laser scanning techniques overcomes some of the disadvantages of contact profilometry as they do not touch the surface of the sample and the diameter of the light spot is much smaller than that of a usual stylus tip.

2.5.1.1.1. Advantages of Profilometry

The main advantage of contact profilometry is being a simple and fast assessment method over a relatively wide area of enamel.

As for the non-contact profilometry no specimen pretreatment is required. Thus a non-contact optical profilometer may be used to evaluate the same sample longitudinally (i.e. before and after treatment). The 3D topographic map of tooth or material surface can be recorded using the confocal principle (Rodriguez et al., 2009).

2.5.1.1.2. Disadvantages of Profilometry

The main disadvantage of contact profilometry is it cannot penetrate micro-irregularities, because of its stylus size, and the stylus tip may damage the enamel surface by scratching the soft, eroded enamel (Barbour & Rees, 2004) or distort flexible materials such as impression materials and abrade hard surfaces such as dental stones

(Rodriguez et al., 2009). Moreover, this technique cannot be used to depict the 3-D characteristics of a dental surface. It can only determine the 2-D surface roughness parameter (Ra) which is considered to be less realistic than the 3-D surface roughness parameter (Sa) (Kakaboura et al., 2007).

The non-contact profilometry provides a 3D topographic map of tooth or material surface but it cannot display the true colors of the tooth, which is a limitation when trying to display the surface features before and after treatment (Ren et al., 2009).

2.5.1.2. Surface Hardness

Enamel hardness is a measure of mechanical resilience done by indenter penetration (Featherstone et al., 1983; Kielbassa et al., 1999). Hardness testing can be used to determine the loss or gain of minerals as a result of demineralisation or remineralisation. Surface hardness is one of the extensively used methods to determine demineralisation and remineralisation, particularly in relation to incipient lesions.

The basic principle involved in microhardness measurement is indenting with a diamond tip of known geometrical dimensions with specific load and time. Hardness of enamel can be determined by two commonly used techniques i) Microindentation and ii) Nanoindentation.

2.5.1.2.1. ***Microindentation***

Microindentation is an established and traditional technique which produces its data in arbitrary units [knoop hardness number (KHN) or Vickers hardness number (VHN)]. The main difference between Knoop and Vickers microindentation is the penetration depth by the indenter (penetration by Vickers indenter is 4.5 times deeper in comparison with Knoop indenter) (Knoop et al., 1939). The hardness measured is affected by the surrounding material to a distance of approximately 10 times the dimensions of the

indentation (Tsui & Pharr, 1999). Therefore it could be suggested the enamel microhardness measures the mechanical properties at the indent site and a few tens to hundreds of micrometers on the surface.

Microindentation has also been used to compare developing incipient lesions by various acidic solutions and was found to produce statistically significant results. It has been shown that tooth samples having incipient lesions remineralize on exposure to saliva, milk and other remineralising products (White & Featherstone, 1987).

2.5.1.2.1.1. Advantages of microindentation

The main reason behind the popularity of microhardness is due to low start-up cost and ease of acquiring data. It is much faster and convenient to use in comparison with nanoindentation.

2.5.1.2.1.2. Disadvantages of microindentation

Buchalla et al., (2008) in an *in vitro* study did not recommend the calculation of mineral content from the profiles of microindentation profiles. Hardness of enamel is measured by the dimensions of the indents produced on plastic (permanent) deformation (no information regarding the elastic response or recovery). An important limitation reported is the necessity of performing tests on specimens of flat surface. Caldwell et al., (1957) accessed the natural curved specimens of enamel; a minimum of 10 indents were placed on each surface. The indents were made along a line from the cervical margin to the cuspal edge. Symmetrical indents on visual examination were measured. The hardness calculated by diagonal indent was used to calculate the hardness value corresponding to the actual indent size in mm^2 giving a Knoop hardness number (KHN).

A perfect knoop indent with a rhomboidal base has a ratio of 1:7.11 (longitudinal angle 172.5° and transverse angle 130°) (Giannakopoulos & Zisis, 2011). The ratios obtained on intact tooth surfaces were found to be different in comparison with theoretical values. The most likely cause of variation is curved surfaces.

2.5.1.2.2. ***Nanohardness***

Nanoindentation (ultra-microindentation) is an emerging technique applicable for incipient lesions. It is efficient in producing hardness values as well as reduced elastic modulus [SI unit: Pascals (Nm^2)]. The depth of nanoindents in sound enamel is around 200 nm (Finke et al., 2001).

2.5.1.2.2.1. Advantages of nanohardness

The advantages of nanohardness are listed below:

i) Displacement of the tip as a result of applied load is monitored continuously during indentation.

ii) Identifies the plastic and elastic deformation of the surface.

iii) It is beneficial for the samples having an inhomogeneous and rough surface in which flat areas can be identified for indentation. As the demineralised enamel surfaces are both inhomogeneous and irregular, it is a useful tool when used for incipient carious lesions.

iv) Data of nanoindentation load displacement are useful in calculating both the hardness and elastic modulus.

2.5.1.2.2.2. Disadvantages of nanohardness

A drawback of both microhardness and nanoindentation is that the surface is required to be flat and polished, prior to treatment with the respective acidic solution (Arends & ten Bosch, 1992). The outer layer of enamel contains a higher concentration of fluoride in comparison with other elements. Thus, when the outer layer of enamel is removed on finishing and polishing, inner enamel is exposed which is known to be more soluble than the surface enamel.

2.5.1.3. Ionic Changes

Dental enamel comprises 34-39% calcium (dry weight) and 16-18% phosphorus (dry weight) (ten Cate et al., 2003). Accordingly, demineralisation and remineralisation of enamel can be analysed using the amount of calcium or phosphate released / uptake by the hydroxyapatite of dental tissue. The examination of mineral release and uptake by teeth has been used in various situations, i.e. both *in situ* and *in vitro*. Moreover, it has also been used *in vivo* (Young et al., 2006), and is appropriate in the context of longitudinal measurements.

2.5.1.3.1. Advantages of Ionic change technique

i) Ion selective electrodes (ISEs) are comparatively economical and simply used with a wide range of areas of application, concentration and temperature.

ii) The new modern electrode models are more vigorous and long-lasting and can be used in both laboratory and field conditions.

iii) They are mainly used in applications where concentration level of a particular ion is required but can also be used to measure the pH and other ions simultaneously from the same solution.

iv) Very important for constant monitoring of concentration changes.

v) Can be used in many medical and biological applications due to the ability of direct activity measurement and not the concentration.

vi) Exactness and precision levels of ± 2 or 3% for some ions can be achieved but with careful use, proper calibration, together with the limitation awareness, this can be more favorable in comparison with other analytical methods that need more costly and complex parts.

vii) ISEs can measure both positive and negative ions which can be seen in only some techniques.

viii) Sample color and turbidity do not affect ISE accuracy.

ix) ISEs do not cause sample destruction or contamination.

2.5.1.3.2. Disadvantages of Ionic Change technique

I. Generally, precision is not better than 1%.

II. Possibility of other ion interference.

III. The electrodes are delicate with short shelf life.

IV. Electrodes record the activity of free simple ions only.

V. Physical or morphological changes are not detectable.

2.5.1.4. Microradiography

Photographic plate (Arends & ten Bosch, 1992) or a photon counter (Anderson et al., 1998) is used in microradiography to record the penetrating radiation of a beam of X-rays incident on an enamel section. The degree of blackening of the film or the photon density, together with a calibration sample, provides a map of the mineral density of the enamel. It is predominantly used in the investigation of caries, has been adopted for detecting both material loss and the extent of softening. The technique is usually divided into one of three 'generations' of microradiography. These are: transverse microradiography (TMR), used for thin sections (50–200 μm) and radiographed perpendicular to the sample; longitudinal microradiography (LMR), used for thick sections of teeth; and wavelength-independent microradiography, which is used to quantify mineral content in whole teeth (Arends et al., 1997).

2.5.1.4.1. Advantages of Microradiography

The main advantage of microradiography is it provides detailed information of the dental hard tissues.

2.5.1.4.2. Disadvantages of Microradiography

Microradiography is an expensive and time taking technique.

2.5.1.5. Optical Methods

2.5.1.5.1. Optical Coherence Tomography (OCT)

Optical coherence tomography (OCT) is a non-destructive technique which is currently extensively being used for *in vivo* studies to monitor the progression of carious lesion (Fried et al., 2002). In OCT, near-infrared light is used to capture three-dimensional images at micrometer resolution (<20μm) of the biological hard tissues.

Areas of demineralisation appear with increased reflectivity in the OCT images; this can be directly measured and is reported as integrated reflectivity ΔR (reflectivity dB units × lesion depth μm) (Kang et al., 2010).

Various studies have been conducted to correlate the ΔR measurements to the mineral loss measurements using TMR (ΔZ). A positive correlation is observed, but the strength of variable is dependent on the study undertaken with correlation coefficients *(r)* ranging between 0.5 and 0.76 (Jones et al., 2006; Louie et al., 2010). Chew et al., (2014) demonstrated a positive correlation between the demineralization measurements by OCT and QLF, though QLF was observed to be more sensitive to early changes. OCT shows promise as an evaluation technique for *in situ* studies.

2.5.1.5.1.1. Advantages of OCT

The major advantage to the use of OCT is the lacking of ionizing radiations. Adding to the advantages, no prior preparation or flattening of enamel surface is required for the measurements to be undertaken (Louie et al., 2010). In addition to this it can be used to determine the subsurface and erosive demineralization as well as *in vitro* remineralization (Louie et al., 2010).

2.5.1.5.1.2. Disadvantages of OCT

The disadvantage of OCT on surface enamel measurements is the strong specular reflection produced in the resulting images. Increase in surface reflectivity leads to difficulty in quantification of mineral loss / gain, though cross polarization can reduce the effect (Kang et al., 2010).

2.5.1.5.2. Quantitative Light-Induced Fluorescence (QLF)

The main principle for QLF measurements is the use of the enamel auto-fluorescence under certain conditions of visible light (monochromatic blue light with a wavelength of 370nm) (Pretty et al., 2003).

It has been used in conjunction for *in situ* de- and remineralization models (Higham et al., 2005). A moderate but significant linear correlation coefficient ($0.63 < r < 0.86$) has been demonstrated against TMR (Angmar-Mansson & ten Bosch, 2001; Cochrane et al., 2012). Though, the best fit relationship is two linear slopes with an initial steep line then a second line with a smaller gradient. This relationship is believed to be attributable to the greater sensitivity of QLF in detecting surface changes as well as a lower sensitivity in detecting subsurface changes of mineral content compared with TMR (Cochrane et al., 2012).

2.5.1.5.2.1. Advantages of QLF

The major advantage of QLF is that the changes in the dental hard tissue can be detected, monitored and quantified (as vol % change) without destruction of the sample, an ideal characteristic for an *in situ* caries trial (Higham et al., 2005). It can be used on intact enamel surface and also to monitor changes in the white-spot lesion intra-orally. The demineralized region of enamel appears to be less florescent on measurements (Higham et al., 2005).

2.5.1.5.2.2. Disadvantages of QLF

A potential issue that arises is the repeatability and reproducibility of QLF, though various studies validate a strong correlation coefficient with intra- and inter examiner reliability (*r* between 0.93 and 0.99) (Angmar-Mansson & ten Bosch, 2001). The QLF measurements can be affected on dehydration of lesion up to a factor of 0.15. The dehydration of lesion can occur on exposure to a still atmosphere within 15 minutes and within seconds when air dried. Thus, on an *in situ* trial it would be imperative that the sample is not left dry (even for a shorter duration of time).

2.5.2. Qualitative Assessment

2.5.2.1. Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) is a qualitative technique used to evaluate the *in situ* erosive demineralization (Featherstone, 1992; Ogaard & Rolla, 1992). SEM examines the ultra-structural characteristics at a very high resolution (~1 nm).

2.5.2.1.1. Advantages of SEM

The main advantage of SEM is visualisation of mineral deposition at high resolution provides information about the effect of remineralising agents and allows comparison with standard treatments such as fluoride. It can also be used to determine whether remineralisation is a result of primary precipitation or crystal growth (Yanagisawa & Miake, 2003).

2.5.2.1.2. Disadvantages of SEM

SEM is qualitative technique so needs to be used in conjunction with quantitative methods (Schlueter et al., 2011). Additionally, samples are placed in a vacuum and coated with non-charged substances such as gold or carbon and, therefore, only a single post-treatment observation can be made. SEM has also been used to study remineralisation *in situ* (Eisenburger et al., 2001) and *in vitro* (Reynolds, 1997; Milly et al., 2014).

The problems with conventional SEM can be overcome by environmental SEM as it can be used for wet samples as well (Donald, 2003).

2.5.2.2. Transmission Electron Microscopy (TEM)

TEM is similar to SEM, but is able to operate at a higher resolution (~0.2 nm) (Yanagisawa & Miake, 2003; Wilson & Bacic, 2012) . TEM has been used *in situ* to study pellicle formation and the effect of citric acid exposure on these layers (Hannig et al., 2003). It has also been used *in vitro* for studies of demineralisation and remineralisation at enamel crystal level (Yanagisawa & Miake, 2003) and to examine experimental nano-HA particles as a potential remineralising agent (Huang et al., 2011).

2.5.2.2.1. Advantages of TEM

Main advantage of TEM is its potential to visualize specimens at higher resolution. It can be used for various in vitro studies to determine remineralisation and demineralisation at a minute crystal level.

2.5.2.2.2. Disadvantages of TEM

The major disadvantage of this technique, along with being a qualitative method, is the need for extensive preparation of the sample, which can result in changes to the specimen (Wilson & Bacic, 2012).

2.6. Methods for Surface Coating detection

2.6.2. Fourier Transform Infra-Red (FT-IR) analysis by Attenuated total reflection (ATR) technique

ATR uses a property of total internal reflection resulting in an evanescent wave. A beam of infrared light is passed through the ATR crystal in contact with the sample. This reflection forms the evanescent wave which extends into the sample, typically to a depth of between 0.5 and 2 μm. The beam is then collected by a detector as it exits the crystal. The system then generates an infrared spectrum of the sample. FT-IR technique is widely used for the identification of the functional components, in particular organic as well as some of the inorganic compounds in the whole range of materials application such as resins, polymers, adhesives, paints, drugs and coatings (Urbaniak-Domagala, 2012). FT-IR has been used to detect dried coatings and bonding formation of materials applied on a surface (Ramesh et al., 2013). The knowledge of the functional components of materials used in coating the surface may be applied in the study of the mechanism proposed for remineralisation and demineralisation.

2.6.1.1.Advantages of FT-IR

FT-IR has some major advantages over the dispersive technique:

i) Speed: Mostly the FT-IR measurements are made in a few seconds rather than in minutes as all the frequencies are measured together (simultaneously). This phenomenon is sometimes referred to as advantage of Felgett.

ii) Sensitivity: FT-IR sensitivity is increased due to many reasons as the detectors used in it are much more sensitive. Fast scans enable the co-addition of several scans in order to reduce the random measurement noise to any desired level.

iii) Mechanical Simplicity: As the moving mirror is the only part continuously moving in the interferometer, chances of mechanical breakdown are reduced.

iv) Internally Calibrated: The instrument is self-calibrated as it employs a HeNe laser as an IWCS (internal wavelength calibration standard). Thus they do not require to be calibrated by the user.

These and several other advantages make measurements made by FT-IR highly accurate and reproducible. Therefore it is a reliable technique for identification of any sample. Small contaminants can thus be identified easily.

2.6.2. Calotest

Calotest is a simple and inexpensive technique for detection of coating thickness. In calotest, rotating sphere of a known diameter is pressed on the coating surface with a preselected load, keeping the position of the sphere relative to the sample and load constant. By adding abrasive slurry on the contact zone, a spherical shaped depression is developed both into the coating and the substrate. The optical inspection of the depression divulges the projected surfaces of the coating and substrate sections. Later X- and Y- axis parameters of the depressions are measured to calculate the coating thickness.

2.6.2.1. Advantages of Calotest

It has various advantages as it determines single and multilayered colored coating thickness in a short time by ball-cratering method. In addition to that it possesses an optical measuring system with an automatic thickness calculator, which helps in determination of coating thickness at a faster rate (Fenker et al., 2002).

2.6.2.2. Disadvantages of Calotest

As the coatings are detected via optical method, therefore it is not appropriate for use in the detection of colourless coatings.

2.7. Density and Viscosity Measurements

Density is defined as mass per unit volume. The symbol used for representing density is the Greek letter ρ. In a mathematical equation density can be measured by following equation

$$\rho = \frac{m}{V}$$

where ρ represents the density of material to be calculated, V volume of the sample and m is the mass of the sample.

The density of a material helps to determine the kinematic viscosity of the material

Viscosity can be defined as a measure of fluid resistance which is deformed either by Shear stress (SS) or tensile stress (TS). It describes the internal resistance of fluid and may be thought of as a fluid friction (FF) measurement. Thus, viscosity helps in correlating the coating thickness which may play an important role in determining possible mechanism of demineralisation inhibition.

The viscosity can be determined by two related measures dynamic viscosity (absolute viscosity) and kinematic viscosity (absolute viscosity to density). The factors affecting viscosity include temperature, composition (solutions and mixtures) and pressure.

CHAPTER 3: MATERIALS AND METHODS

3.1. Materials

3.1.1. Equipment

- Autoclave, Tomy High Pressure Steam Sterilizer (SX-500) (USA)
- Weighing Balance, Mettler Toledo (JP105DUG) (Switzerland)
- Phenom Electron Microscope, Phenom World (Netherlands)
- pH meter, Orion-pH/ISE meter (Model 710) (U.K)
- Calcium Ion Selective Electrode, NICO 2000 (U.K)
- Micracut 125 Low Speed Precision Cutter, Metkon (Turkey)
- HMV-2000 Microhardness tester, Shimadzu Corporation (Tokyo, Japan)
- Spectrum GX FT-IR System, Perkin Elmer (Germany)
- Magnetic Stirrer, Chemo Lab (Malaysia)
- Burette, Chemo Lab (Malaysia)
- Density Bottle, Chemo Lab (Malaysia)

3.1.2. Chemicals

- Poly-γ-glutamic acid (PGGA) powder, Nippon Poly-glu (Japan)
- Sodium Fluoride powder (NaF), Sodium Hydroxide pellets (NaOH), Calcium Chloride Dihydrate powder ($CaCl_2.2H_2O$), Acetic Acid (99.9%) and Ethanol (99.95%), Merck, (Germany)
- Phosphate buffered saline (PBS) tablets, Oxoid Ltd, (Hampshire, England)
- HAp powder and HAp pellets, Plasma Biotal, (United Kingdom)
- Nail Varnish, Inglot, (Poland)
- Quick Mount 2 Epoxy Resin, Quick Mount Epoxy Hardener and Castable Mounts (2 piece), Pace Technologies, (USA)
- Pipet tips (Volume 1000 µl), Eppendorf AG, (Germany)

3.1.3. Extracted teeth

Ethical approval was obtained from the Ethics Committee of the Faculty of Dentistry, University of Malaya, Kuala Lumpur, Malaysia for collection of extracted teeth [Ethics Committee Reference Number: DF OB1504/0067(P)] (Appendix A). Seventy-six extracted sound premolars were obtained after taking written consent from patients at the Ziauddin University hospital, Karachi, Pakistan. These extracted teeth used in this study were cleaned prior to extraction by scaling followed by cleaning using sterile cotton wool before storing them in a solution containing thymol. Prior to use the teeth were washed with deionised distilled water. The exclusion criteria for the selection of teeth used in this study were premolars which had i) coronal caries and / or ii) root caries and /or iii) tooth wear. The inclusion criterion was orthodontically-extracted sound premolars.

3.1.4. Preparation of the extracted teeth

The premolars were varnished, leaving an area of 2 mm x 2 mm on the middle third of the buccal surface of the crown unvarnished. The unvarnished area was then viewed under a Phenom microscope (Netherlands) at three different magnifications (1000x, 1500x and 2000x) (Please refer to Appendix B for selection of teeth). Teeth found to have tooth wear or defects on their buccal surface were excluded from the study.

3.1.5. Hydroxyapatite (HAp) pellets

These pellets are round in shape with a diameter of 12 mm (Figure 3.1) and are made from sintered powder with a particle size of approximately 4 μm and porosity of about 20%, having chemistry similar to that of dental enamel (Kosoric 2006). They have more exact mineral composition and purity compared with dental enamel (Elliot, 1994). They were used as an analogue of dental enamel in the remineralisation portion of this study.

Figure 3.1: HAp pellet

3.1.6. Hydroxyapatite (HAp) powder (Figure 3.2)

It has a surface area ranging from 12-18 m^2/g by BET method. The powder has a uniform mineral composition (Elliot et al., 2005) and was used in this study to simulate HAp crystals of human dental enamel (Higuchi et al., 1969).

Figure 3.2: HAp powder

3.2. Methods

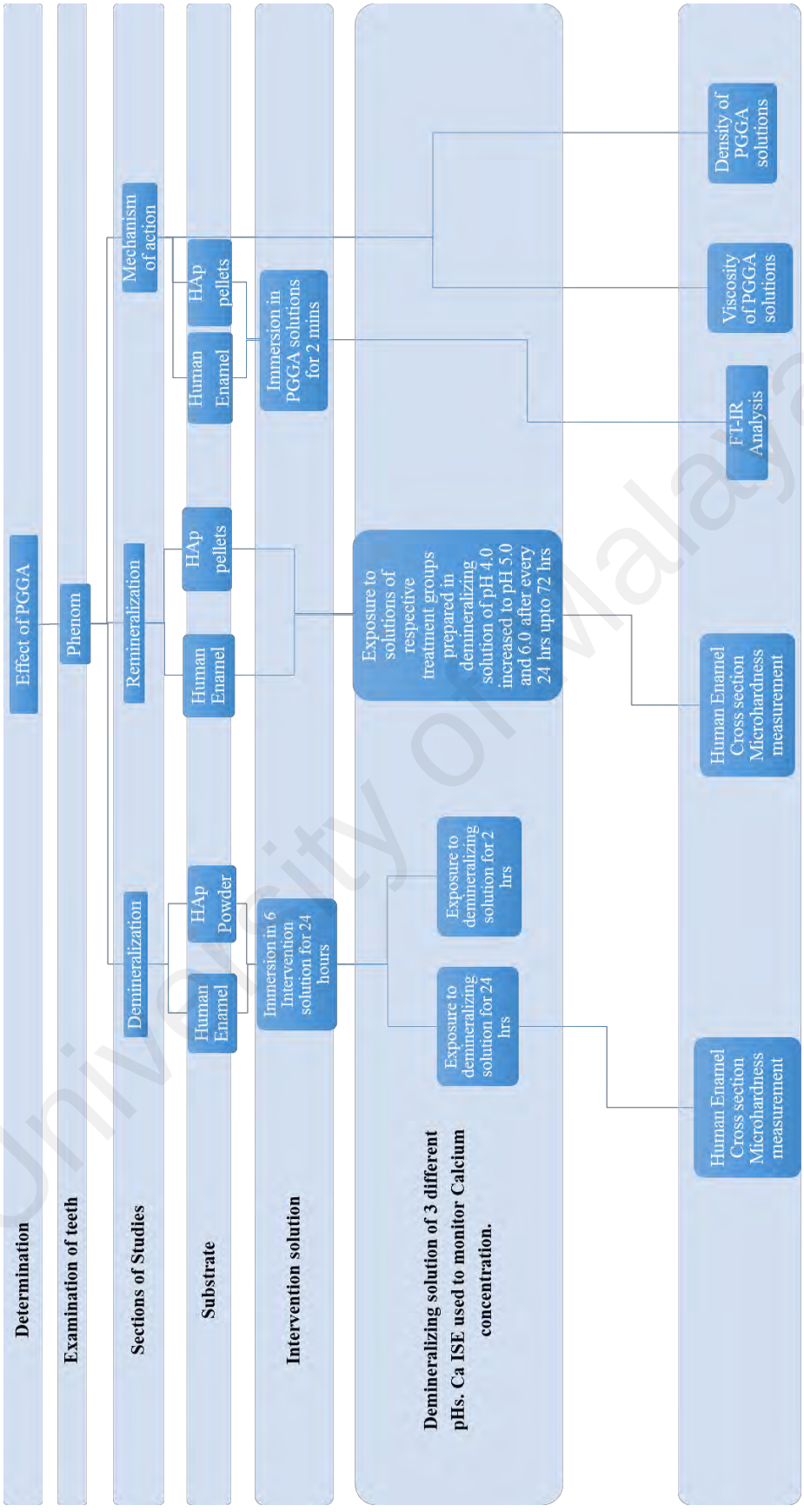


Figure 3.3: Schematic flow chart representing the design of the study

3.2.1. Preparation of the respective solutions used in the study

3.2.1.1. Poly-γ-Glutamic Acid (PGGA)

A mixture of D- and L- PGGA was used with a molecular weight > 10,000 kDa. Stock solutions of PGGA (1% and 2% respectively) were prepared in deionized-distilled water, stored at room temperature in sterilized bottles and labeled accordingly. Sterilization of the bottles was carried out by the standard method of autoclaving at a temperature of 121°C and pressure 15 p.s.i for about 15 minutes to prevent microbial growth (Elliott, 2005).

3.2.1.2. Sodium Fluoride

Three concentrations (0.01% (w/v), 0.1% (w/v) and 0.5 % (w/v)) of NaF were prepared separately in deionized-distilled water, stored in sterilized bottles at room temperature until further use. Sodium fluoride was used as the positive control. The three concentrations used were based on the concentrations normally found in oral healthcare products.

3.2.1.3. Phosphate Buffered Saline (PBS) Solution

PBS solution was prepared according to the manufacturer's instructions. One tablet of PBS was added to 100 ml of deionized water and autoclaved at a temperature of 121°C and pressure of 15 p.s.i for about 10 minutes. The solution was stored at room temperature until further use. PBS solution with pH adjusted to 7.0 was used as the negative control.

3.2.1.4. Demineralising Solution

The demineralising solution used in this study was 0.1 M acetic acid of three different pHs (4.0, 4.5 and 5.0). The demineralising solution of different pHs was prepared by adding drops of 1 M NaOH and calibrated to the respective pHs using a pH meter (Featherstone et al., 1983). The solutions were prepared in 1 L quantity, stored in bottles and labeled accordingly.

3.2.1.5. Standard Calcium Solution

Standard calcium solutions with concentrations of 0.5 mM, 1 mM and 2 mM at pH 4.0, respectively, were prepared by adding the required amount of calcium chloride to 1 liter of 0.1 M acetic acid solution. The pH of the standard solutions was calibrated to pH 4 based on the recommendation of the manufacturer. These calcium solutions were used to calibrate the calcium ion selective electrode used in this study.

3.2.2. Determination of the influence of PGGA on Demineralisation of Dental Enamel under caries-inducing conditions

Calcium ions are released into the demineralising solution during demineralisation of dental enamel and HAp powder. In this study, the calcium ions released were measured using the calcium ion selective electrode (Ca^{2+} Selective Electrode) maintaining a temperature of 37°C.

3.2.2.1. Calibration of the Ca^{2+} Selective Electrode

The electrode was first calibrated using 3 different standard calcium solutions of pH 4 (2 mM, 1 mM and 0.5 mM) in accordance with the manufacturer's instruction (Appendix C). The calibration was carried out prior to the calcium ion determination. The calibration curve was developed to check the sensitivity of the Ca^{2+}electrode (Appendix D).

3.2.2.2. Determination of the effect of PGGA on the demineralisation of enamel of the extracted teeth and HAp powder

3.2.2.2.1. ***Extracted teeth***

As the Featherstone model has been validated for artificial caries induction as discussed in Section 2.2.6 therefore we have chosen that for our demineralisation study. Acetic acid was selected as demineralising solution as it has a potential to develop lesion at a rapid rate.

Three sets of experiments using extracted teeth were carried out at different pHs (4.0, 4.5 and 5 respectively) of demineralising solutions. For each set of the experiment, 18 sound premolars were used. Sound premolars with an unvarnished 2 x 2 mm^2 window on the middle third of the buccal surface of the crown were used (Figure 3.4). They were divided into 6 different groups of immersion solution (n = 3):

i) phosphate buffered solution (PBS)

ii) 1% PGGA

iii) 2% PGGA

iv) 0.01% NaF

v) 0.1% NaF

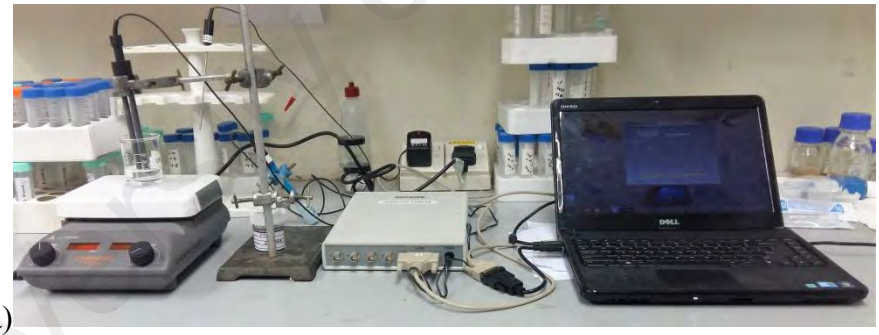
vi) 0.5% NaF

PBS was used as a negative control while the NaF solutions were used as positive controls.

Figure 3.4: Varnished tooth with an unvarnished window area of 2 mm x 2 mm

The three teeth (PBS group) were immersed separately in the PBS solutions for 24 hours, washed with deionized water to remove the excess PBS solution and then immersed in 40 ml of demineralising solution of pH 4.0 with continuous stirring (Figure 3.5). Calcium ion concentrations in the demineralising solution were monitored using the Ca^{2+} selective electrode (ISE) and recorded every one minute for 24 hours. The values obtained were later used to calculate the rate of calcium ion release (R_{Ca2+}) from the enamel. In the calculation, for all the values obtained the Ca^{2+} concentration at 0 min was first subtracted in order to give the final value of calcium concentration with time. The R_{Ca2+} release was calculated using the linear regression computation function in SPSS and expressed as mM/hr. The procedure was repeated for each of the teeth in the other remaining groups which were the NaF (0.01%, 0.1% and 0.5%) and PGGA (1% and 2%) groups, respectively.

(a)



(b)

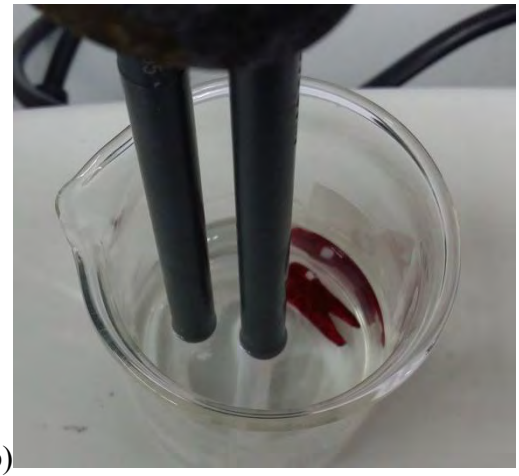


Figure 3.5: (a) Picture showing the Ca^{2+} Selective Electrode connected with Computer interface (b) tooth and Ca^{2+} selective electrode immersed in demineralising solution with continuous stirring

The same procedure was repeated with the other 2 sets of experiments where demineralising solutions were set at pH 4.5 and pH 5.0 respectively.

All of the teeth used in these experiments were kept for microhardness measurements as described in Section 3.2.2.3.

3.2.2.2.2. HAp powder

0.2 g of HAp powder was separately immersed in the respective groups of solutions (i) phosphate buffered saline solution (PBS); ii) 1% PGGA; iii) 2% PGGA; iv) 0.01% NaF, v) 0.1% NaF and vi) 0.5% NaF) of pH 4 as described in Section 3.2.2.2.1 with a little modification in the time of exposing the pretreated HAp powder to demineralising solution. In this experiment, the concentration of calcium ion in the demineralising solution at pH 4.0 was measured using the Ca^{2+} selective electrode and recorded at one minute intervals for two hours. The experiment was carried out in triplicate. A similar procedure was repeated using demineralising solutions of the other two pHs (pH 4.5 and 5.0 respectively).

3.2.2.3. Determination of the Cross-Sectional Micro Hardness (CSMH) of Enamel

The teeth that were used in the experiment described in Section 3.2.2.2.1 were first washed in deionized distilled water. Each of the teeth was then sectioned longitudinally into halves, cutting through the developed lesion, using a rotating diamond cutting saw machine.

All of the longitudinally sectioned teeth were later mounted on epoxy resin leaving the cut section area exposed for microhardness measurement (Featherstone et al., 1983), as shown in Figure 3.6.

Figure 3.6: Picture showing the longitudinal section of a tooth mounted on epoxy resin

Cross-sectional microhardness measurement was carried out using a microhardness tester with a Knoop indentor. Indents were made from the outer enamel surface to a depth of 250 μm at intervals of 50 μm (5 depth levels) on both the varnished and unvarnished areas. The indents were made with the cross-sectioned surface placed perpendicular to the direction of the load. Five indents were made at each depth with a force of 25 g and dwelling time of 5 s (Figure 3.7).

The Knoop Hardness Number (KHN) was generated automatically by the equipment by applying the formula

$$KHN = \frac{L}{d^2}$$

where L is the load applied expressed in gram force and d is the diagonal length of the indent in μm.

KHN in each depth was then averaged. Later KHN was converted to volume percentage mineral via the empirical formula below that has a proven reproducibility to micro-radiographic estimates of demineralisation and remineralisation within enamel lesions, where correlation coefficient r = 0.919 (Featherstone et al., 1983; ten Cate et al., 1985; White & Featherstone, 1987; Featherstone et al., 1988).

$$Volume\ Percentage\ Mineral\ Content = 4.3\sqrt{(KHN)} + 11.3$$

The percentage difference in the volume percent mineral content at each respective subsurface depth of unvarnished (treated with respective treatment groups) enamel was determined to know the extent of the lesion by comparing mineral content values with varnished enamel using the following formula

$$\%\ Difference\ in\ mineral\ percent\ volume = \{(T_o - T_1) \div T_O\} \times 100$$

where T_o is the mineral percent volume of the untreated (varnished) enamel at the respective depth, T_1 is the mineral percent volume of the treated (unvarnished) enamel at the respective depth.

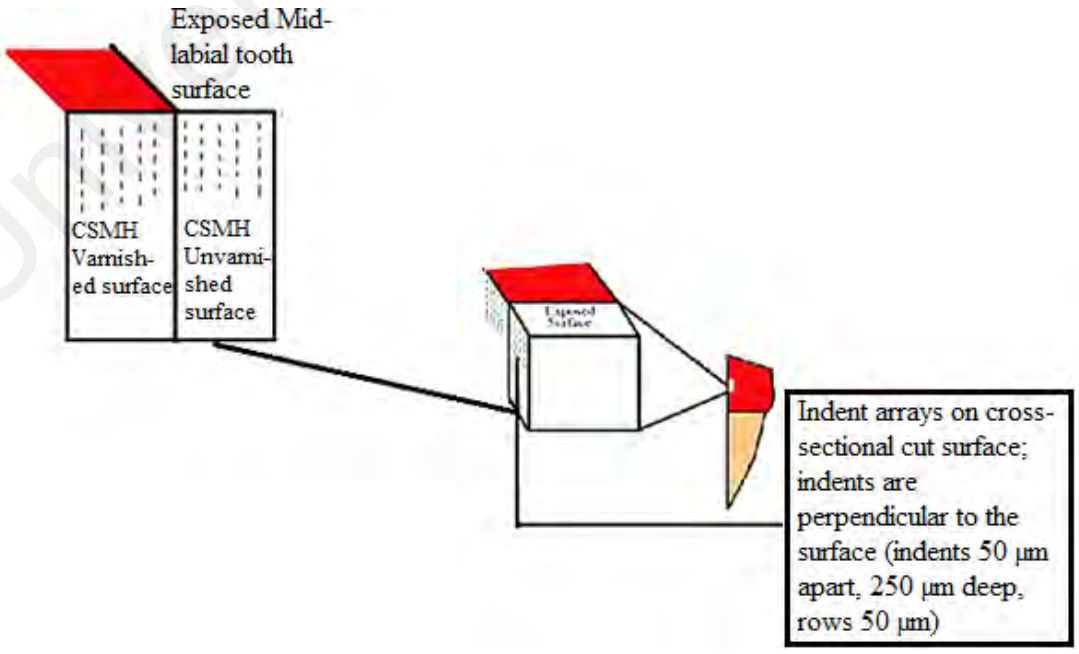


Figure 3.7: Schematic diagram showing tooth in the measurement of Cross-Sectional Microhardness (CSMH)

3.2.3. Determination of the influence of PGGA on Remineralisation of Dental Enamel under caries-inducing conditions

3.2.3.1. Calibration of the Ca^{2+}-Selective Electrode

Prior to use, the calibration of the electrode was carried out following the procedure mentioned in Section 3.2.2.1.

3.2.3.2. Determination of the effect of PGGA in demineralising solution (0.1 M Acetic acid) at different pHs (4.0, 5.0 and 6.0 respectively) on the surfaces of dental enamel and HAp pellets

3.2.3.2.1. ***Dental Enamel***

Eighteen varnished teeth, each with an unvarnished 2x2 mm^2 window on the mid-labial surfaces (Figure 3.4) were - divided into 6 groups of 3 teeth each for use in the respective solution i) 0.1 M acetic acid; ii) 1% PGGA; iii) 2% PGGA; iv) 0.01% NaF v) 0.1% NaF and vi) 0.5% NaF. The acetic acid (0.1 M) was used as the negative control; the NaF at three different concentrations (0.01%, 0.1% and 0.5% respectively) was used as the positive control. For the NaF and PGGA they were made to their respective concentrations in 0.1 M acetic acid (demineralising solution). All of the solutions used in this experiment were calibrated to pH 4.0 using a pH meter.

Each tooth in the respective groups was initially immersed in solutions with pH 4.0 for 24-hr and the calcium ion concentrations in the respective group of solutions were determined using Ca^{2+} ISE and recorded at an interval of 15 min. After the first 24-hr, 1 M NaOH was gradually added to each of the respective solutions to raise the pH from 4.0 to 5.0 (5-min). The calcium ion concentration in the respective solutions was measured for another 24 hr at 15 min intervals. After the second 24-hr, 1 M NaOH was again gradually added to each of the respective solutions in order to increase the pH further to 6.0. The concentrations of calcium ion in the respective solutions were again

measured and recorded at 15 min interval for another 24-hr. The amount of calcium was later used to calculate the rate of calcium release and uptake using linear regression analysis. A positive rate value indicates calcium ion release and a negative one, uptake of calcium ions.

All of the teeth from the experiment were kept for use in the determination of cross-sectional microhardness of enamel as described in Section 3.2.3.3.

3.2.3.2.2. ***HAp pellets***

The procedure described in Section 3.2.3.2.1 was repeated using HAp pellets which were also varnished, leaving a window of unvarnished area of 2 mm x 2 mm.

3.2.3.3. Determination of Cross-Sectional Micro Hardness (CSMH) of Enamel

A similar procedure to that described in Section 3.2.2.3 was employed with a slight modification. In this experiment, intervals of 25 µm from the outer enamel surface to a depth of 250 µm subsurface was used instead of 50 µm intervals (Featherstone et al., 1988).

After calculating the volume percent mineral for each depth, relative mineral recovery value was then calculated. The calculation was based on the data of mineral content profile using the curve with Simpson's rule (Figure 3.8) to provide integrated mineral recovery values for each group in units Vol % x µm (depth of enamel).

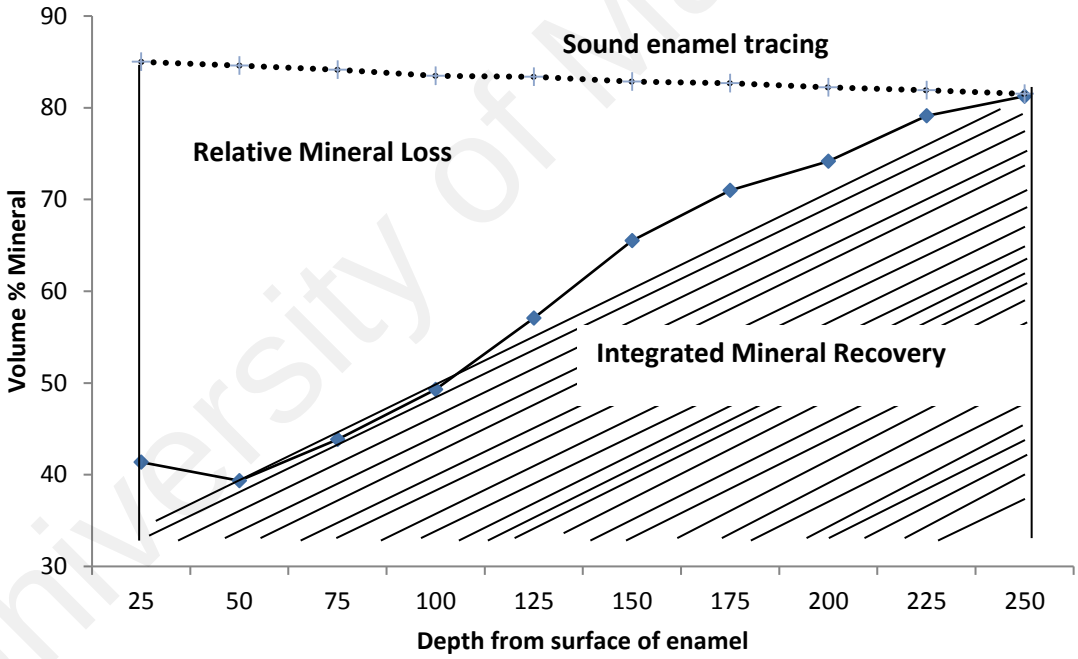


Figure 3.8: Schematic diagram (Curve with Simpsons's rule) showing integrated mineral recovery and relative mineral loss. Dotted line is the microhardness profile of sound enamel (average volume percent mineral); lower continuous line with closed circle is the microhardness profile of demineralised enamel upon remineralisation. Area with oblique lines represents the integrated mineral recovery and blank area represents the relative mineral loss.

3.2.4. Determination of the interaction between PGGA and the surfaces of i) teeth and ii) HAp pellets, respectively

In this study, the Fourier Transform Infra-Red (FT-IR) by ATR technique was employed.

3.2.4.1. FT-IR baseline absorption spectra for PGGA, tooth and HAp pellets

FT-IR analysis was carried out with the Perkin Elmer FT-IR System Spectrum GX using ATR technique. The stage of the ATR was first scrubbed with ethanol in order to remove any contaminant and then allowed to dry. PGGA powder was used to obtain the baseline absorption spectra of PGGA. To do this, the powder was placed on the stage of the analyser and the beam of light was allowed to pass through. The beam of light exiting the interferometer interacted with PGGA and a spectrum detecting various components in the PGGA was obtained. The experiment was repeated 3 times. A similar procedure was repeated with hydroxyapatite pellets (n=4) and extracted teeth (n=4) for their respective baseline spectra.

3.2.4.2. FT-IR absorption spectra for the surfaces of i) teeth- ii) HAp pellets- treated with PGGA

Prior to the FT-IR analysis, 2 teeth were immersed in the 1% PGGA (1% PGGA-treated teeth) and another 2 in 2% PGGA (2% PGGA-treated teeth) for 2 min. They were later placed in a dryer at 55°C overnight to allow the PGGA to dry, which would facilitate its placement on the stage for the FT-IR analysis. The analysis was carried out three times for each tooth.

A similar procedure was carried out for each of the 4 hydroxyapatite pellets. The FT-IR spectra were obtained using the procedure as described in Section 3.2.4.1.

3.2.5. Measurement of the Density of PGGA

Density is defined as mass per unit volume. The symbol representing density is a Greek letter "ρ". In a mathematical equation, density can be measured by

$$\rho = \frac{m}{V}$$

where "ρ" denotes the density of material to be calculated, "V" is volume of the sample and "m" is the mass of the sample.

Density-bottles were adjusted individually. The nominal capacity specified to 0.001 cm^3 is indelibly engraved on each bottle. Each bottle is calibrated with its own stopper or thermometer; hence, stoppers and thermometers are not interchangeable. Each bottle and its stopper or thermometer is marked with a unique matching identification number.

A clean, dry density bottle of 25 ml capacity was weighed first and the weight recorded before filling it with 1% w/v PGGA, the bottle was then reweighed after filling it with the PGGA solution. The experiment was carried out at room temperature (25°C) and repeated 6 times. The same procedure was carried out using 2% w/v PGGA.

The density of PGGA solutions was calculated by

i) The weight of PGGA solution = (Weight of density bottle + PGGA solution) - (Weight of density bottle)

ii) Density = mass/volume

Specific gravity of PGGA solution = Weight of PGGA solution /Volume of solution.

3.2.6. Measurement of the Viscosity of PGGA

Viscosity can be defined as a measure of fluid resistance when it is deformed either by shear stress (SS) or by Tensile Stress (TS). It describes the internal resistance of a fluid and may be thought as a FF (fluid friction) measurement.

It can be defined by the coefficients dynamic viscosity (absolute viscosity), and kinematic viscosity. The factors affecting viscosity for Newtonian fluids are temperature, composition (solutions and mixtures) and pressure. Viscosity was determined by using a simple 25 ml burette. The exit time of the liquid passing two fixed points (0.0 and 25.0) on the burette was measured (Figure 3.9). The viscosity of 1%w/v PGGA solution was measured by first filling the burette with it. Then the tap on the burette was opened and the time needed for the burette to empty the PGGA solution was recorded. The burette was then emptied and cleaned thoroughly with ethanol to remove any residual liquid and reused. The above procedure was repeated 6 times. The room temperature at which this experiment was carried out was 25 °C. A similar procedure was repeated for 2% w/v PGGA solution.

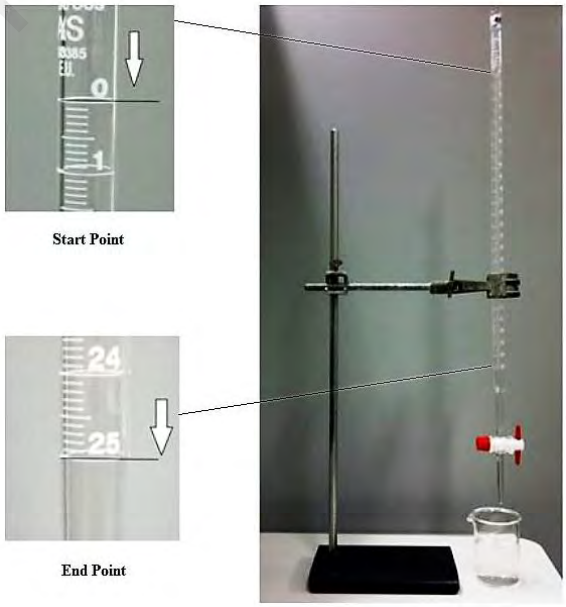


Figure 3.9: Representing burette setup to test viscosity showing the start and end points.

3.2.6.1. Determination of Kinematic Viscosity of PGGA

This applies to liquids flowing under gravitational forces like the PGGA flow in the oral cavity for its interaction with the tooth structure.

To calculate kinematic viscosity (ηk2) of the PGGA solution, the following formula was used:

$$Kinematic\ viscosity\ (\eta k2) = \frac{Viscosity\ of\ water\ (\eta k1)\ \text{x Exit time of sample}}{Exit\ time\ of\ water}$$

3.2.6.2. Determination of Dynamic Viscosity of PGGA

This applies to the flow of liquid through a tube with a constant pressure at one end.

To calculate the dynamic viscosity (η), the kinematic viscosity is multiplied by the density (in the correct units) as follows

$$Dynamic\ viscosity\ of\ sample\ (\eta) = \eta k2 \text{ x } \rho$$

where ηk2 is the kinematic viscosity and ρ is the density.

3.2.7. Statistical Analysis

The data obtained were analyzed using IBM SPSS software version 23 (Chicago, U.S.A). The relationships of Ca^{2+} release over time during demineralisation in the demineralising solution for the extracted teeth (Section 3.2.2.2.1) and in acidified PGGA and NaF for the extracted teeth and HAp pellets (Section 3.2.3.2.1 & 3.2.3.2.2) were computed with linear regression and Z test. Linear regression analysis is a technique used for predicting the unknown value of a variable from the known value of another variable, if the two variables have a linear relationship. By linear regression, we mean models with one independent and one dependent variable. It estimates the numerical relationship between two variables. In other words, linear regression gives the equation of the straight line that best describes the association and enables the prediction of one variable from the other. Therefore in this analysis, time was the independent variable whose values were used to predict the dependent variable, that is, Ca^{2+} concentration in solution with time. Linear regression was performed in order to generate a coefficient which is the rate. The rate was then used in the Z-test analysis. Z test is a parametric statistical test where normal distribution is applied and basically used for dealing with problems related to large samples where $n \geq 30$.The rate of calcium ions calculated in Sections 3.2.2.2.1, 3.2.3.2.1 and 3.2.3.2.2 was analyzed by Z-test to determine the statistical difference between the rate on treatment with different treatment groups.

As the Ca^{2+} release for HAp powder was not linear with time, therefore, the data of Ca^{2+} released in the demineralising solution from the HAp powder (Section 3.2.2.2.2) was analyzed at specific time points using a two-way Analysis of Variance (ANOVA) where time and the treatment groups were the fixed variables and Ca^{2+} concentration in the solution, the dependent variable.

The data obtained for the CSMH of enamel demineralisation and remineralisation experiments (Section 3.2.2.3 & 3.2.3.3) were analysed using a one-way Analysis of Variance (ANOVA). One-way Anova is a parametric test used to determine whether there is an interaction effect between two independent variables on a continuous dependent variable. The hardness value was the dependent variable whereas the different treatment groups were the fixed factors.

Data for density and viscosity of PGGA (Section 3.2.5 & 3.2.6) were analysed using a One-way ANOVA. The concentration was the fixed variable, with density/ viscosity of PGGA solutions as the dependent variables.

For all the analyses, the statistical significance value was set at $p<0.05$.

CHAPTER 4: RESULT

4.1. Effect of demineralising solution (0.1 M acetic acid) at pH 4.0, 4.5 and 5.0, respectively, on PGGA pre-treated enamel and HAp powder

4.1.1. Dental Enamel

The teeth were pretreated for 24-hr with PBS (negative control), NaF at three different concentrations (positive control) and PGGA at two different concentrations before being placed in the demineralising solution (0.1 M acetic acid) at pH 4.0. Figure 4.1 shows the amount of calcium ions released into the demineralising solution at pH 4.0 from dental enamel of the respective pretreated teeth (mean value of the three teeth from each group), measured at 1 min intervals for 24 hours. The rate of enamel demineralisation (measured on linear regression analysis of the calcium ions released as shown in Appendix E) from pretreated dental enamel with PBS on immersion in demineralising solution was 6.08 x 10^{-5} mM/h. It was found that the rate of calcium (R_{Ca2+}) released from the enamel pretreated with NaF was lower when compared with that of PBS-pretreated. The reduction was approximately 34% (3.98x 10^{-5} mM/h) for teeth pretreated with 0.01% NaF in comparision to R_{Ca2+} release for PBS pretreated teeth. Further reduction was observed with higher concentration of NaF; 55% (2.74x 10^{-5} mM/h) reduction for teeth pretreated with 0.1% NaF and 71% (1.77 x 10^{-5} mM/h) for teeth pretreated with 0.5% NaF in comparision to R_{Ca2+} release for PBS pretreated teeth. The reduction in the rate of demineralisation was also seen for the teeth pretreated with PGGA. It was approximately 66% (2.02x 10^{-5} mM/h) for teeth pretreated with 1% PGGA and 81% (1.18 x 10^{-5} mM/h) for teeth pretreated with 2% PGGA.

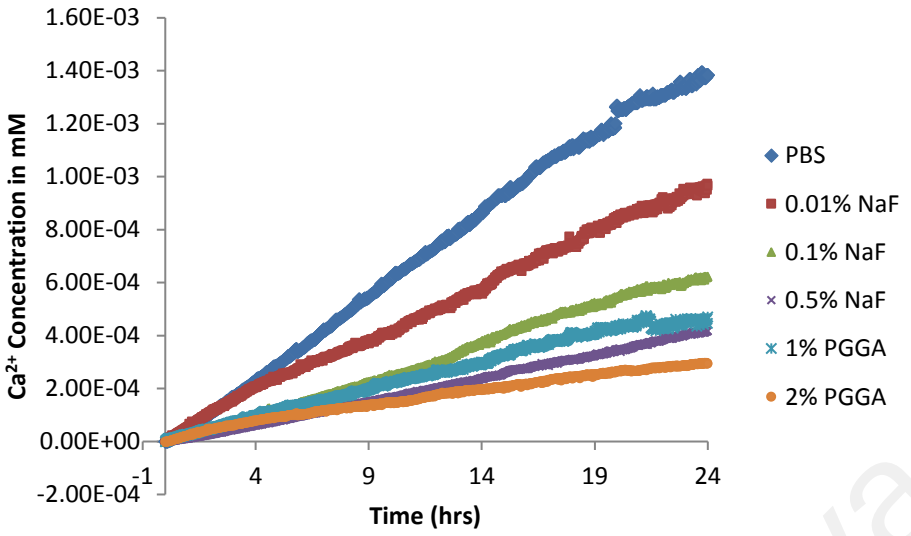


Figure 4.1: The concentration of calcium ions in mM released from enamel *pretreated* with PBS (Negative control), 0.01% NaF, 0.1% NaF, 0.5% NaF (positive controls) and PGGA at concentrations of 1% and 2%, respectively, upon exposure to 0.1 M acetic acid at pH 4.0 measured at intervals of 1 minute and monitored for 24 hours. The experiment was carried out in triplicate and the graph above represents the average of 3 values of Ca^{2+} for the respective solutions.

Using linear regression analysis, the R_{Ca2+} release was calculated and the values obtained were tabulated in Table 4.1. Upon performing the Z-test a statistically significant difference was observed between each of the six treatment groups including the negative control group (PBS). ($\rho<0.05$). (Appendix F).

Table 4.1: R_{Ca2+} release in the demineralising solution (0.1 M acetic acid at pH 4.0) from the teeth which were pretreated with the different treatments (PBS (negative control), 0.01% NaF, 0.1% NaF, 0.5% NaF (positive control). 1% PGGA and 2% PGGA).

Treatment Groups	**PBS**	**0.01% NaF**	**0.1% NaF**	**0.5% NaF**	**1% PGGA**	**2% PGGA**
R_{Ca2+} release (mM/h) Mean± SE	$6.08 \times 10^{-5} \pm 0.009 \times 10^{-5}$	$3.98 \times 10^{-5} \pm 0.005 \times 10^{-5}$	$2.74 \times 10^{-5} \pm 0.005 \times 10^{-5}$	$1.77 \times 10^{-5} \pm 0.001 \times 10^{-5}$	$2.02 \times 10^{-5} \pm 0.005 \times 10^{-5}$	$1.18 \times 10^{-5} \pm 0.003 \times 10^{-5}$

The R_{Ca2+} release in mM/h was expressed as Mean ± SE where the number of determinants (n) is 3. The R_{Ca2+} release between the different treatment groups of solutions in 24-hr was statistically significantly different ($\rho<0.05$). PBS was the negative control and NaF at three different concentrations was used as the positive control.

When the pH of the demineralising solution used was increased to 4.5 R_{Ca2+} release trends were observed to be similar (Figure 4.2) to that for pH 4.0 (Figure 4.1). The R_{Ca2+} release from dental enamel pretreated with PBS (calculated using the linear regression analysis as shown in Appendix G), was 8.66 x 10^{-6} mM/h. It was observed that the R_{Ca2+} released from the dental enamel of the teeth pretreated with different treatment groups are in the following sequence: 0.01% NaF > 0.1% NaF > 1% PGGA > 0.5% NaF > 2% PGGA (Table 4.2).

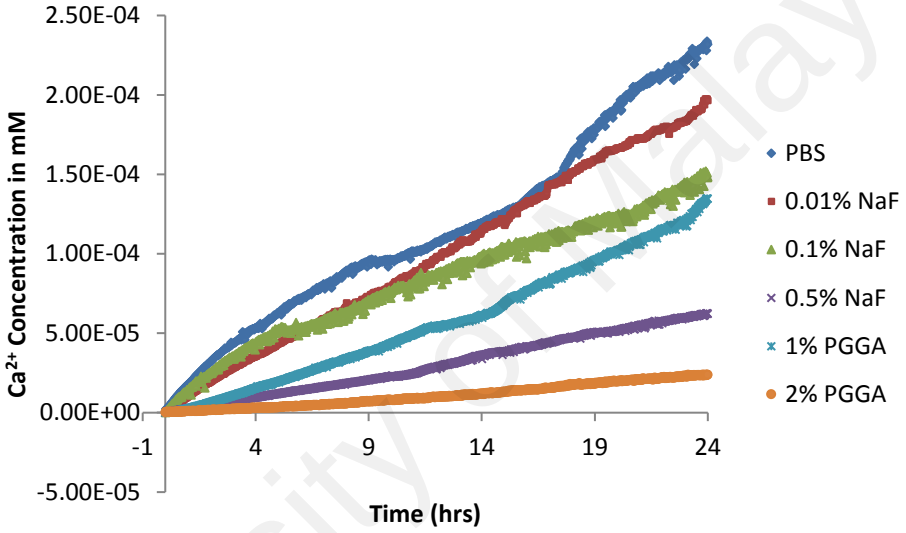


Figure 4.2: The concentration of calcium ions in mM released from enamel *pretreated* with PBS (Negative control), 0.01% NaF, 0.1% NaF, 0.5% NaF (positive controls) and PGGA at concentrations of 1% and 2% respectively upon exposure to 0.1 M acetic acid at pH 4.5 measured at intervals of 1 minute and monitored for 24 hours. The experiment was carried out in triplicate and the graph above represents the average of 3 values of Ca^{2+} for the respective solutions.

Upon performing the Z-test analysis for the six treatment groups, including the negative control group (PBS), a statistically significant difference was found between all the groups. ($\rho < 0.05$) (Table 4.2) (Appendix H).

Table 4.2: R_{Ca2+} release in the demineralising solution (0.1 M acetic acid at pH 4.5) from the teeth that were pretreated with the different treatments (PBS control (negative control), 0.01% NaF, 0.1% NaF, 0.5% NaF (positive control). 1% PGGA and 2% PGGA).

Treatment Groups	**PBS**	**0.01% NaF**	**0.1% NaF**	**0.5% NaF**	**1% PGGA**	**2% PGGA**
R_{Ca2+} release (mM/h) Mean ± SE	$8.66 \times 10^{-6} \pm 0.038 \times 10^{-6}$	$8.07 \times 10^{-6} \pm 0.009 \times 10^{-6}$	$5.46 \times 10^{-6} \pm 0.081 \times 10^{-6}$	$2.66 \times 10^{-6} \pm 0.005 \times 10^{-6}$	$5.41 \times 10^{-6} \pm 0.015 \times 10^{-6}$	$1.03 \times 10^{-6} \pm 0.003 \times 10^{-6}$

The R_{Ca2+} release in mM/h was expressed as Mean ± SE where the number of determinants (n) is 3. Comparison of the R_{Ca2+} release from pretreated teeth with different treatment groups of solutions in 24-hr were statistically significantly different ($\rho<0.05$). PBS was the negative control and NaF at three different concentrations was used as the positive control.

The calcium ion concentrations that were released from dental enamel of pretreated teeth in demineralising solution at pH 5.0, (Figure 4.3) with the exception of teeth pretreated with 0.5% NaF and 2% PGGA, were found to exhibit similar patterns as those for pH 4.0 (Figure 4.1) and pH 4.5 (Figure 4.2).

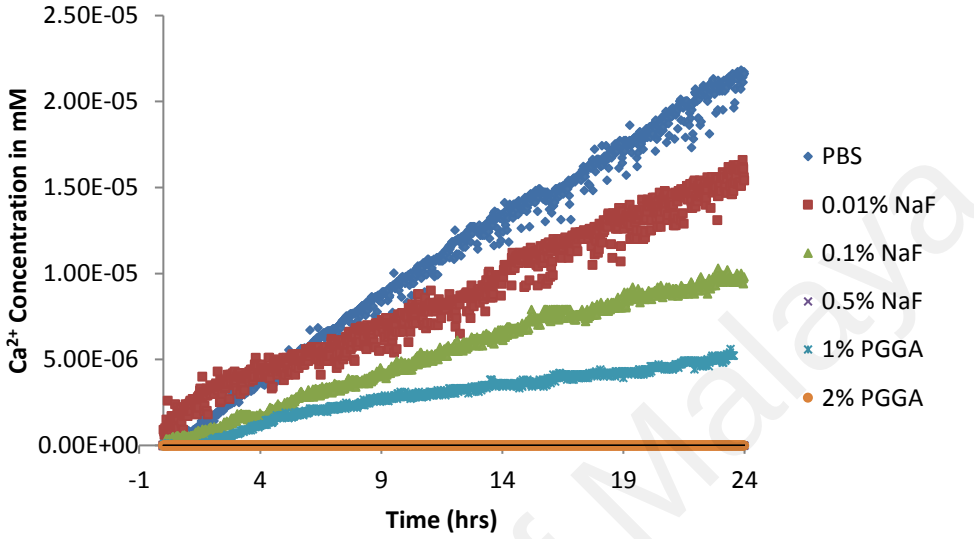


Figure 4.3: The concentration of calcium ions in mM released from enamel *pretreated* with PBS (Negative control), 0.01% NaF, 0.1% NaF, 0.5% NaF (positive controls) and PGGA at concentrations 1% and 2% respectively upon exposure to 0.1 M acetic acid at pH 5.0 measured at intervals of 1 minute and monitored for 24 hours. The experiment was carried out in triplicate and the graph above represents the average of 3 values of Ca^{2+} for the respective solutions. The color coding for 0.5% NaF and 2% PGGA were superimposed as neither exhibited any calcium release.

The R_{Ca2+} release was calculated using linear regression analysis (Appendix I) and tabulated as in Table 4.3. The analysis showed the following trend in the R_{Ca2+} release: PBS > 0.01% NaF > 0.1% NaF > 1% PGGA (Table 4.3). The teeth pretreated with 0.5% NaF and 2% PGGA respectively did not exhibit any calcium ion release in the demineralising solution at pH 5.0. Upon performing the Z-test between the four treatment groups including the control group (PBS) it was found that they are statistically significantly different (R_{Ca2+} release for 0.5% NaF and 2% PGGA pretreated teeth was found to be zero and they were therefore excluded from statistical analysis). ($\rho \leq 0.05$) (Appendix J).

Table 4.3: R_{Ca2+} release in the demineralising solution (0.1 M acetic acid at pH 5.0) from the teeth that were pretreated with the different treatments (PBS (negative control), 0.01% NaF, 0.1% NaF, 0.5% NaF (positive control). 1% PGGA and 2% PGGA).

Treatment Groups	**PBS**	**0.01% NaF**	**0.1% NaF**	**0.5% NaF**	**1% PGGA**	**2% PGGA**
R_{Ca2+} release (mM/h) Mean ± SE	$9.03 \times 10^{-7} \pm 0.019 \times 10^{-7}$	$5.96 \times 10^{-7} \pm 0.022 \times 10^{-7}$	$4.22 \times 10^{-7} \pm 0.013 \times 10^{-7}$	-*	$2.81 \times 10^{-7} \pm 0.029 \times 10^{-7}$	-*

The R_{Ca2+} release in mM/h was expressed as Mean ± SE where the number of determinants (n) is 3. Comparison of the R_{Ca2+} release from pretreated teeth with different treatment groups of solutions in 24-hr were statistically significantly different ($\rho<0.05$). PBS was the negative control and NaF at three different concentrations was used as the positive control. *Excluded from analysis because there was no calcium ion release for the calculation.

4.1.2. HAp powder

Figure 4.4 shows the concentration of calcium ions released into the demineralising solution at pH 4.0 from HAp powder pre-treated with PBS, three concentrations of NaF (0.01% w/v, 0.1% w/v and 0.5% w/v) and two concentrations of PGGA (1% w/v and 2% w/v) respectively. The calcium ion concentration (mM) released was measured at 1 min interval for 2 hr (120 min). PBS was used as the negative control and NaF as the positive control. The pretreated HAp powder upon immersion in demineralising solution of pH 4.0 showed an initial rapid increase in Ca^{2+} release which after some time attained equilibrium (Figure 4.4). The PBS pre-treated HAp powder showed the highest Ca^{2+} release. This was followed by the other treatment groups in the following sequence: 0.01% NaF > 0.1% NaF > 1% PGGA >0.5% NaF > 2% PGGA.

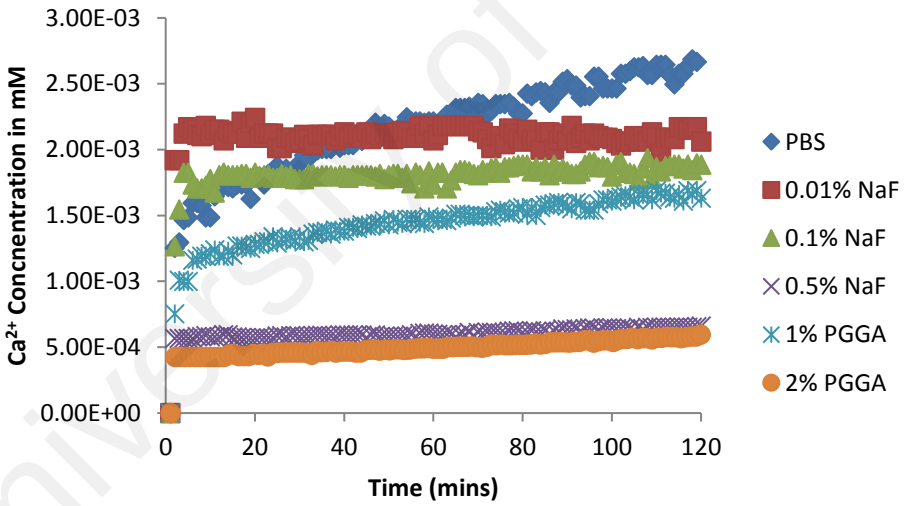


Figure 4.4: Calcium ion release expressed in mM, monitored for 120 min (2 hr) at intervals of 1 min from the HAp powder *pretreated* with PBS (negative control) and NaF (positive control)/ PGGA at various concentrations upon exposure to 0.1 M acetic acid at pH 4.0. At 0 minute, the Ca^{2+} concentrations in the demineralising solution for all the treatment groups were actually zero. The Ca^{2+} concentration was compared at 4 different time points (30 min, 60 min, 90 min and 120 min) in order to determine the difference within and between the groups with time. The individual experiment was repeated 3 times.

The Ca^{2+} concentration was compared at 4 different time points (30 min, 60 min, 90 min and 120 min) in order to determine the statistical difference within and between the groups with time as shown in Table 4.4. The power for all the two-way ANOVA analyses was found to be more than 80%.

Table 4.4: Ca^{2+} concentration at different time intervals (30 min, 60 min, 90 min and 120 min) released from pretreated HAp powder in demineralising solution at pH 4.0.

TIME INTERVAL (mins)	**Ca^{2+} Concentration (mM) Expressed as Mean ± SD x 10^{-3}**					
	PBS	**0.01%NaF**	**0.1% NaF**	**0.5% NaF**	**1% PGGA**	**2% PGGA**
30	2.23 ± 0.31^{Aa}	2.12 ± 0.07^{Ab}	1.66 ± 0.10^{Ac}	0.596 ± 0.10^{Ad}	1.28 ± 0.16^{Ae}	0.49 ± 0.10^{Af}
60	2.41 ± 0.19^{Ba}	2.23 ± 0.06^{Bb}	1.71 ± 0.02^{Ac}	0.63 ± 0.06^{ABd}	1.42 ± 0.10^{Be}	0.53 ± 0.06^{Af}
90	2.61 ± 0.08^{Ca}	2.23 ± 0.06^{Bb}	1.85 ± 0.05^{Bc}	0.68 ± 0.04^{ABd}	1.52 ± 0.10^{Ce}	0.62 ± 0.08^{Bd}
120	2.72 ± 0.07^{Da}	2.26 ± 0.08^{Cb}	1.90 ± 0.06^{BDc}	0.72 ± 0.07^{Bd}	1.63 ± 0.11^{De}	0.63 ± 0.06^{Bd}

Ca^{2+} concentration in mM was expressed as Mean ± SD where the number of determinants (n) is 3.

$^{A-D}$ different capital letters indicate a statistically significant difference in Ca^{2+} concentration at different time intervals in same group ($\rho<0.05$);

$^{a-f}$ Different lower case letters indicate a statistically significant difference for Ca^{2+} concentration at same time in different groups ($\rho<0.05$). The individual experiment was carried out in triplicate. PBS was the negative control and NaF at three different concentrations was used as the positive control.

Similar profiles were observed for the calcium ion concentration released from the HAp powder pretreated with the respective different treatment groups in the demineralising solution of pH 4.5 (Figure 4.5). The PBS pre-treated HAp powder showed the highest Ca^{2+} release in 0.1 M acetic acid pH 4.5, followed by the other pretreatment groups in the following order: 0.01% NaF > 0.1% NaF > 1% PGGA >0.5% NaF > 2% PGGA.

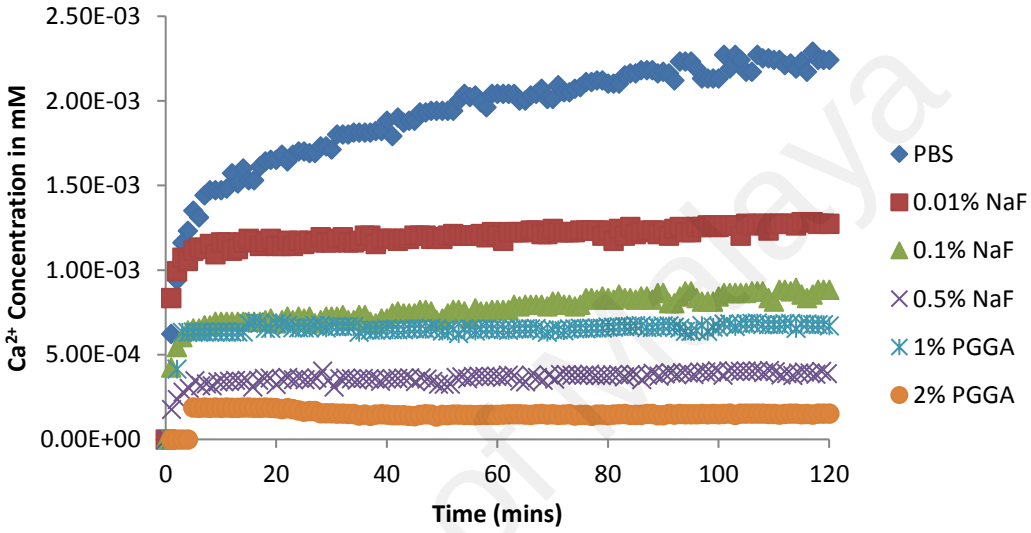


Figure 4.5: Calcium ion release expressed in mM, monitored for 120 min (2 hr) at intervals of 1 min from the HAp powder *pretreated* with PBS (negative control) and NaF (positive control) / PGGA various concentrations upon exposure to 0.1 M acetic acid at pH 4.5. At 0 minute, the Ca^{2+} concentrations in the demineralising solution for all the treatment groups were actually zero. The Ca^{2+} concentration was compared at 4 different time points (30 min, 60 min, 90 min and 120 min) in order to determine the difference within and between the groups with time. The individual experiment was repeated 3 times.

Table 4.5 shows the mean ± SD of Ca^{2+} concentration (mM) released from the HAp powder at different time intervals. All of the statistical analyses performed by the two-way ANOVA were found to have power of more than 80%.

Table 4.5: Ca^{2+} concentration at different time intervals (30 min, 60 min, 90 min and 120 min) released from pretreated HAp powder in demineralising solution at pH 4.5.

TIME INTERVAL (mins)	Ca^{2+} Concentration (mM) Expressed as Mean ± SD x 10^{-3}					
	PBS	**0.01%NaF**	**0.1% NaF**	**0.5% NaF**	**1% PGGA**	**2% PGGA**
30	1.73 ± 0.07^{Aa}	1.16 ± 0.04^{Ab}	0.73 ± 0.004^{Ac}	0.36 ± 0.002^{Ad}	0.67 ± 0.002^{Ace}	0.13 ± 0.02^{Af}
60	2.05 ± 0.05^{Ba}	1.23 ± 0.03^{Bb}	0.77 ± 0.003^{Bc}	0.38 ± 0.002^{Ad}	0.68 ± 0.003^{Ae}	0.22 ± 0.02^{Bf}
90	2.18 ± 0.07^{Ca}	1.26 ± 0.03^{BCb}	0.87 ± 0.003^{Cc}	0.39 ± 0.002^{Ad}	0.68 ± 0.002^{Ae}	0.25 ± 0.01^{Bd}
120	2.24 ± 0.05^{Da}	1.30 ± 0.03^{Cb}	0.87 ± 0.004^{CDc}	0.40 ± 0.003^{Ad}	0.68 ± 0.002^{Ae}	0.25 ± 0.01^{Bd}

Ca^{2+} concentration in mM was expressed as Mean ± SD where the number of determinants (n) is 3.

$^{A-D}$ different capital letters mean statistically significant difference in Ca^{2+} concentration at different time intervals in same group ($\rho<0.05$);

$^{a-f}$ Different lower case letters means statistically significant difference for Ca^{2+} concentration at same time in different groups ($\rho<0.05$). The individual experiment was carried out in triplicate. PBS was the negative control and NaF at three different concentrations was used as the positive control.

At pH 5.0 of demineralising solution, a similar profile of calcium release to those of pH 4.0 and 4.5 was also observed with the exception of teeth pretreated with 0.5% NaF and 2% PGGA (Figure 4.6). The HAp powder pretreated with PBS solution appeared to have the highest Ca^{2+} concentration in the demineralising solution followed by the respective pretreatment groups in the following order: 0.01% NaF > 0.1% NaF > 1% PGGA> 0.5% NaF > 2% PGGA.

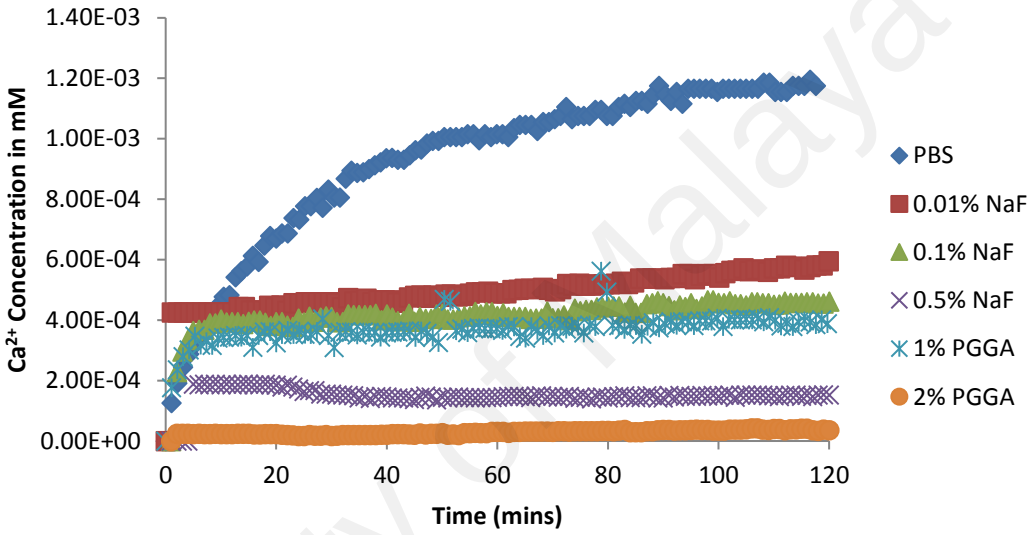


Figure 4.6: Calcium ion release expressed in mM, monitored for 120 min (2 hr) at intervals of 1 min from the HAp powder *pretreated* with PBS (negative control) and NaF (positive control) / PGGA various concentrations upon exposure to 0.1 M acetic acid at pH 5.0. At 0 minute, the Ca^{2+} concentrations in the demineralising solution for all the treatment groups were actually zero. The Ca^{2+} concentration was compared at 4 different time points (30 min, 60 min, 90 min and 120 min) in order to determine the difference within and between the groups with time. The individual experiment was repeated 3 times.

Using two-way ANOVA with time and treatment groups as independent variables, it was found that the difference in the calcium concentration was statistically significant between different groups at each time point (Table 4.6). The power for all the statistical analyses was found to be more than 80%.

Table 4.6: Ca^{2+} concentration at different time intervals (30 min, 60 min, 90 min and 120 min) released from HAp powder in demineralising solution at pH 5.0.

TIME INTERVAL (mins)	Ca^{2+} Concentration (mM) Expressed as Mean ± SD x 10^{-3}					
	PBS	**0.01% NaF**	**0.1% NaF**	**0.5% NaF**	**1% PGGA**	**2% PGGA**
30	0.81 ± 0.005^{Aa}	0.44 ± 0.002^{Ab}	0.41 ± 0.002^{Ac}	0.15 ± 0.001^{Ad}	0.31 ± 0.002^{Ae}	0.02 ± 0.0007^{Af}
60	1.05 ± 0.04^{Ba}	0.50 ± 0.002^{Bb}	0.41 ± 0.002^{Ac}	0.15 ± 0.001^{Ad}	0.38 ± 0.004^{Be}	0.03 ± 0.0002^{ABf}
90	1.13 ± 0.01^{Ca}	0.55 ± 0.002^{Cb}	0.46 ± 0.003^{Bc}	0.14 ± 0.002^{Ad}	0.40 ± 0.002^{Ce}	0.04 ± 0.0003^{Bf}
120	1.19 ± 0.02^{Da}	0.59 ± 0.002^{Db}	0.46 ± 0.002^{Bc}	0.15 ± 0.002^{Ad}	0.39 ± 0.003^{BCe}	0.11 ± 0.002^{Cf}

Ca^{2+} concentration in mM was expressed as Mean ± SD where the number of determinants (n) is 3.

$^{A-D}$ different capital letters mean statistically significant difference in Ca^{2+} concentration at different time intervals in same group ($\rho<0.05$);

$^{a-f}$ Different lower case letters means statistically significant difference for Ca^{2+} concentration at same time in different groups ($\rho<0.05$). The individual experiment was carried out in triplicate. PBS was the negative control and NaF at three different concentrations was used as the positive control

4.1.3. Cross Sectional Microhardness (CSMH) of pretreated Dental Enamel in demineralising solution (0.1 M acetic acid) at pH 4.0, 4.5 and 5.0, respectively

The result in this section will be discussed according to the respective pHs of the demineralising solution.

Demineralising solution at pH 4.0

Figure 4.7 shows the volume percent mineral of tooth specimens from the enamel surface up to 250 µm subsurface, of the various groups i.e. pretreated respectively with the following treatments: a) PBS (negative control), b) 0.01% NaF, c) 0.1% NaF, d) 0.5% NaF, e) 1% PGGA and f) 2% PGGA which were then exposed to the demineralising solution (0.1 M acetic acid) at pH 4.0. The dental enamel microhardness was measured from the tooth surface up to the 250 µm subsurface at 50 µm distance intervals. It was observed that teeth pretreated with PBS exhibited the maximum reduction in volume percent mineral forming a lesion up to 200 µm subsurface on comparing with the microhardness of the untreated enamel (subsurface microhardness of varnished enamel, for calculation please refer materials and methods). It was followed by the teeth pretreated with 0.1% NaF and 1% PGGA, showing volume percent mineral reduction up to 150 µm subsurface. The teeth pretreated with the other two groups 0.5% NaF and 2% PGGA respectively both showed the lesion up to 100 µm subsurface. (The volume percent mineral content of untreated enamel measured is shown in Appendix K)

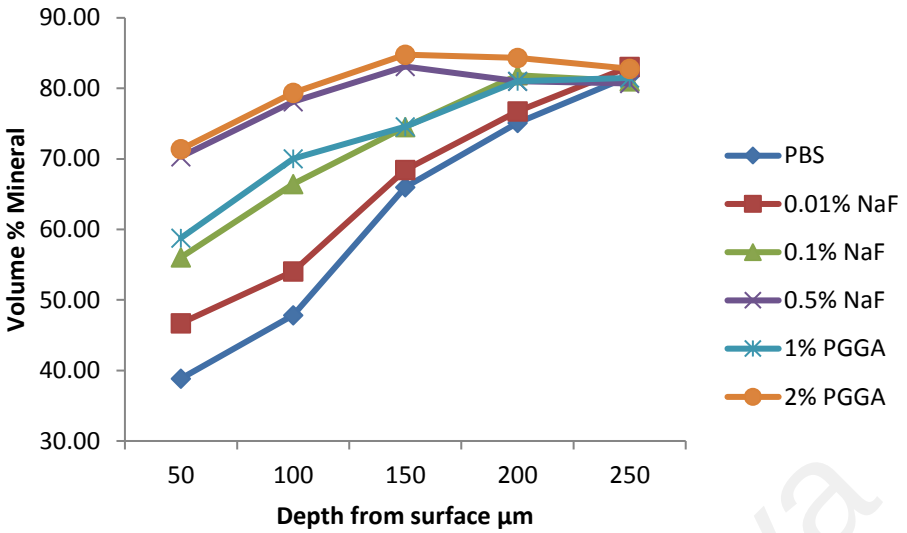


Figure 4.7: Volume percent mineral of enamel for the different groups of *pretreated* teeth placed in demineralising solution at pH 4.0. The microhardness was measured at 50 µm intervals from the tooth surface up to the 250 µm subsurface. PBS was the negative control and NaF at three different concentrations was used as the positive control. The individual experiment was carried out in triplicate and the values above represent the average of them.

Table 4.7 represents the statistical analysis of the enamel mineral volume with respect to the distance from tooth surface at 50 µm intervals up to 250 µm between the different treatment groups. Statistically significant difference was observed between the negative control (PBS) and the other treatment groups along all the depths of lesion ($\rho<0.05$). Upon comparing with the positive controls (0.01% NaF, 0.1% NaF and 0.5% NaF) it was shown that 2% PGGA had the least reduction in mineral content at depth of 100 µm and it was statistically significant ($\rho<0.05$) except with 0.5% NaF it was not significantly different. (Appendix J represents the volume percent mineral content of the varnished/ untreated enamel at various depths for the respective treatment groups)

Table 4.7: Mineral content of dental enamel after effect of demineralising solution of pH 4.0 on pretreated teeth with different groups of solutions

Distance from tooth Surface (µm)	Mineral Content Values (Mineral % Volume= 4.3(√ KHN) + 11.3)					
	PBS	**0.01% NaF**	**0.1% NaF**	**0.5% NaF**	**1% PGGA**	**2% PGGA**
50	38.85 ±1.44 [a]	46.71 ± 1.94 [b]	56.05 ± 1.75 [c]	70.32 ± 1.42 [d]	58.79 ± 1.01 [e]	71.37 ± 0.89 [d]
100	47.83± 1.47 [a]	54.06 ± 0.81 [b]	66.43 ± 1.21 [c]	78.12 ± 0.32 [d]	70.03 ± 1.33 [e]	79.37 ± 1.34 [d]
150	66.01 ± 1.21 [a]	68.43 ± 1.10 [a]	74.52 ± 2.48 [b]	83.10 ± 0.91 [c]	74.55 ± 3.47 [b]	84.78 ± 0.28 [c]
200	75.10 ± 1.14 [a]	76.76 ± 1.55 [a]	81.86 ± 2.76 [b]	80.99 ± 2.26 [b]	81.01 ± 4.17 [b]	84.33 ± 0.29 [b]
250	81.74 ± 2.99 [a]	83.08 ± 2.13 [a]	81.00± 3.67 [a]	80.71 ± 2.63 [a]	81.48 ± 3.41 [a]	83.79 ± 0.97 [a]

Mineral Content expressed as Mean ± SD, where the number of determinants (n) is 3.
[a-f] Different lower case letters mean statistically significant difference at same depth between different treatment groups ($\rho<0.05$). The individual experiment was carried out in triplicate. PBS was the negative control and NaF at three different concentrations was used as the positive control.

Demineralising solution at pH 4.5

The volume percent mineral at all depths from the tooth surface to 250 µm subsurface for the pretreated teeth immersed in demineralising solution at pH 4.5 followed the same pattern as that of the pretreated teeth immersed in demineralising solution of pH 4.0 (Figure 4.8). At this pH it was observed that the volume percent mineral reduction on calculating difference in the mineral content of treated enamel from untreated enamel for all the groups was different (at each subsurface depth) as follows: PBS (negative control) and 0.01% NaF respectively involved enamel forming lesion depth up to 150 µm subsurface: 0.1% NaF and 1% PGGA respectively up to the depth of 100 µm, teeth pretreated with 0.5% NaF and 2% PGGA respectively up to a depth of 50 µm subsurface. (The volume percent mineral content of untreated enamel measured is shown in Appendix L).

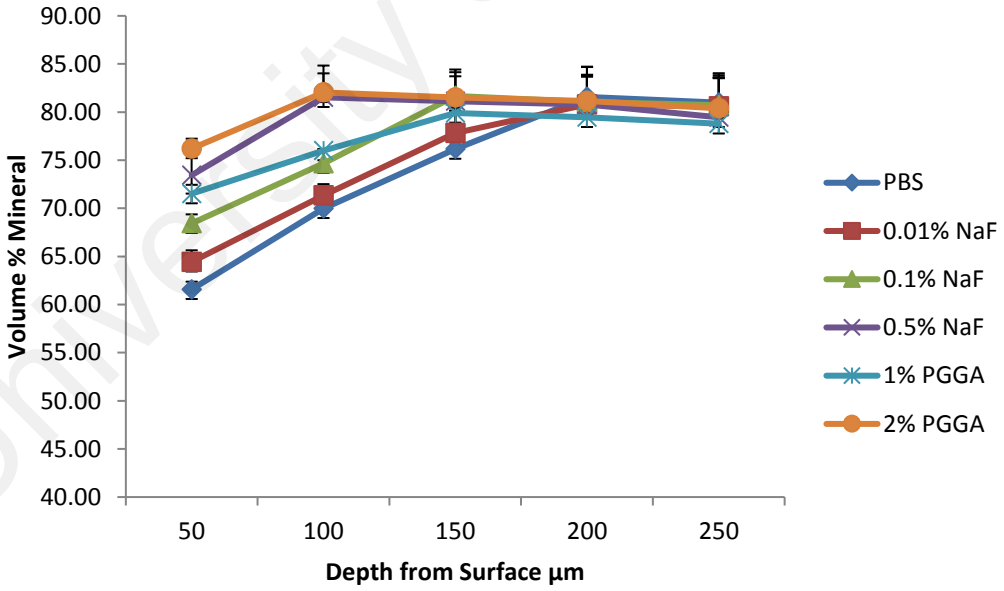


Figure 4.8: Volume percent mineral of enamel for the different groups of *pretreated* teeth placed in demineralising solution at pH 4.5. The microhardness was measured at 50 µm intervals from the tooth surface up to the 250 µm subsurface. PBS was the negative control and NaF at three different concentrations was used as the positive control. The individual experiment was carried out in triplicate and the values above represent the average of them.

Table 4.8 compares the volume percent mineral with respect to the distance from tooth surface between the different treatment groups. On comparison among the different treatment groups it was found that mineral content values for all the treatment groups including the negative control are significantly different up to the depth of 50 µm subsurface ($\rho<0.05$). The teeth pretreated with 2% PGGA showed the least reduction in the mineral content at 50 µm ($\rho<0.05$). (Appendix K represents the volume percent mineral content of the varnished/ untreated enamel at various depths for the respective treatment groups)

Table 4.8: Mineral content of dental enamel after effect of demineralising solution of pH 4.5 on pretreated teeth with different groups of solutions

Distance from tooth Surface (µm)	**Mineral Content Values (Mineral % Volume= 4.3(√ KHN) + 11.3)**					
	PBS	**0.01% NaF**	**0.1% NaF**	**0.5% NaF**	**1% PGGA**	**2% PGGA**
50	61.58 ± 0.80 [a]	64.44 ±1.20 [b]	68.44 ± 0.94 [c]	73.45 ± 2.37 [d]	71.52 ± 0.01 [e]	76.21 ± 1.03 [f]
100	69.99 ± 1.46 [a]	71.35 ± 1.17 [a]	74.68 ± 1.46 [b]	81.53 ± 3.30 [c]	76.00 ± 0.14 [b]	82.04 ± 1.98 [c]
150	76.15 ± 2.20 [a]	77.84 ± 2.31 [a]	81.68 ± 2.47 [b]	81.13 ± 3.30 [b]	79.91 ± 0.55 [b]	81.52 ± 2.21 [b]
200	81.57 ± 2.10 [a]	80.79 ± 3.00 [a]	81.06 ± 2.82 [a]	80.78 ± 3.90 [a]	79.46 ± 0.23 [a]	81.15 ± 3.58 [a]
250	80.99 ±2.53 [a]	80.60 ± 2.97 [a]	80.74 ± 3.10 [a]	79.46 ± 4.58 [a]	78.78 ± 1.16 [a]	80.39 ± 3.24 [a]

Mineral Content expressed as Mean ± SD, where the number of determinants (n) is 3.
[a-f] Different lower case letter means statistically significant difference at same depth between different treatment groups ($\rho<0.05$). The individual experiment was carried out in triplicate. PBS was the negative control and NaF at three different concentrations was used as the positive control.

Demineralising solution at pH 5.0

Figure 4.9 describes the percentage of mineral content in enamel of pretreated teeth with the various treatment groups on immersion in demineralising solution at pH 5.0 evaluated at 50 µm intervals up to 250 µm subsurface (on comparison with mineral content of untreated/varnished enamel at respective depth). The pretreated teeth with PBS (negative control) showed the maximum change in mineral content up to the depth of 100 µm subsurface (on comparing with varnished enamel at respective depths). The depth of lesion decreased from the surface for the PBS pretreated teeth on immersion in demineralising solution of pH 5.0 compared with those at pHs 4.0 and 4.5. (The volume percent mineral content of untreated enamel measured is shown in Appendix M).

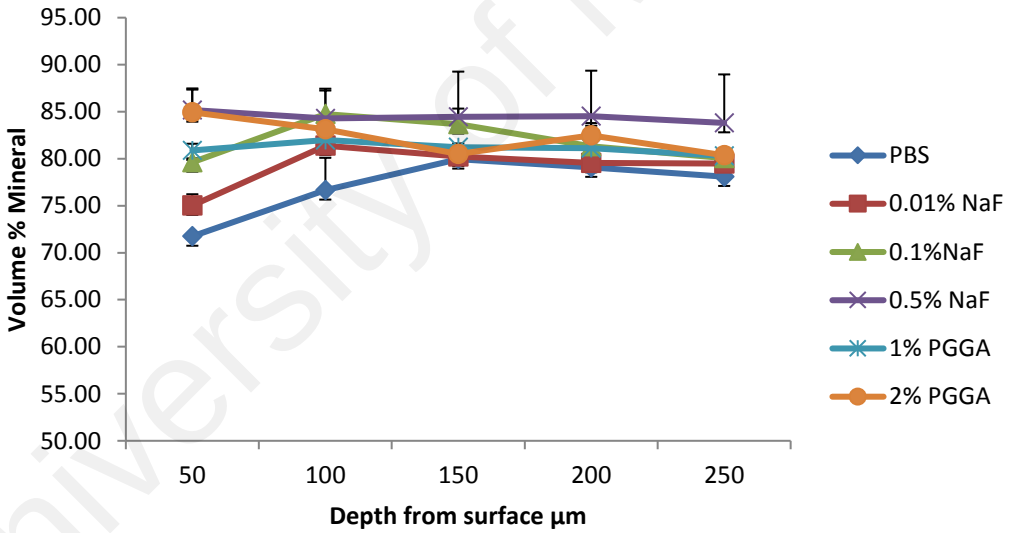


Figure 4.9: Volume percent mineral of enamel for the different groups of pretreated teeth placed in demineralising solution at pH 5.0. The microhardness was measured at 50 µm intervals from the tooth surface up to the 250 µm subsurface. PBS was the negative control and NaF at three different concentrations was used as the positive control. The individual experiment was carried out in triplicate and the values above represent the average of them.

Table **4.9** represents the statistical analysis of the mineral content values with respect to the distance from tooth surface between the different treatment groups. It was found that between the different treatment groups except 0.5% NaF and 2% PGGA the mineral content values were significantly different at depth of 50 μm ($\rho<0.05$).

Table 4.9: Mineral content of dental enamel after effect of demineralising solution of pH 5.0 on pretreated teeth with different groups of solutions

Distance from tooth Surface (μm)	**Mineral Content Values (Mineral % Volume= 4.3(√ KHN) + 11.3)**					
	PBS	**0.01% NaF**	**0.1% NaF**	**0.5% NaF**	**1% PGGA**	**2% PGGA**
50	71.74 ± 0.25 [a]	75.02 ± 1.20 [b]	78.12 ± 0.42 [c]	85.17 ± 2.30 [d]	79.86 ± 0.73 [e]	84.93 ± 2.42 [d]
100	76.65 ± 3.44 [a]	81.38 ± 2.35 [b]	84.74 ± 2.51 [b]	84.28 ± 3.17 [b]	81.98 ± 1.29 [b]	83.12 ± 0.27 [b]
150	79.95 ± 0.51 [a]	80.22 ± 0.57 [a]	83.67 ± 1.66 [b]	84.46 ± 4.80 [b]	81.21 ± 0.41 [a]	80.50 ± 0.35 [a]
200	79.07 ± 0.42 [a]	79.55 ± 0.89 [a]	81.32 ± 0.43 [a]	84.51 ± 4.86 [b]	81.13 ± 0.67 [a]	81.89 ± 1.31 [ab]
250	78.10 ± 1.34 [a]	79.49 ± 1.54 [a]	80.06 ± 0.30 [a]	83.80 ± 5.16 [b]	80.30 ± 0.47 [a]	80.36 ± 0.28 [a]

Mineral Content expressed as Mean ± SD, where the number of determinants (n) is 3.
[a-f] Different lower case letter means statistically significant difference at same depth between different treatment groups ($\rho<0.05$). The individual experiment was carried out in triplicate. PBS was the negative control and NaF at three different concentrations was used as the positive control.

4.2. Effect of PGGA on Remineralisation of Dental Enamel and HAp pellets under caries-inducing conditions

4.2.1. The effect of PGGA in demineralising solutions of different pH values on the dental enamel surface

Figure 4.10 shows the concentration of calcium ions present in acidified PGGA, acidified NaF (positive control) and acetic acid (negative control) at different pHs (4.0, 5.0 and 6.0 respectively). The effect of PGGA at different pHs will be discussed separately in the following subheadings.

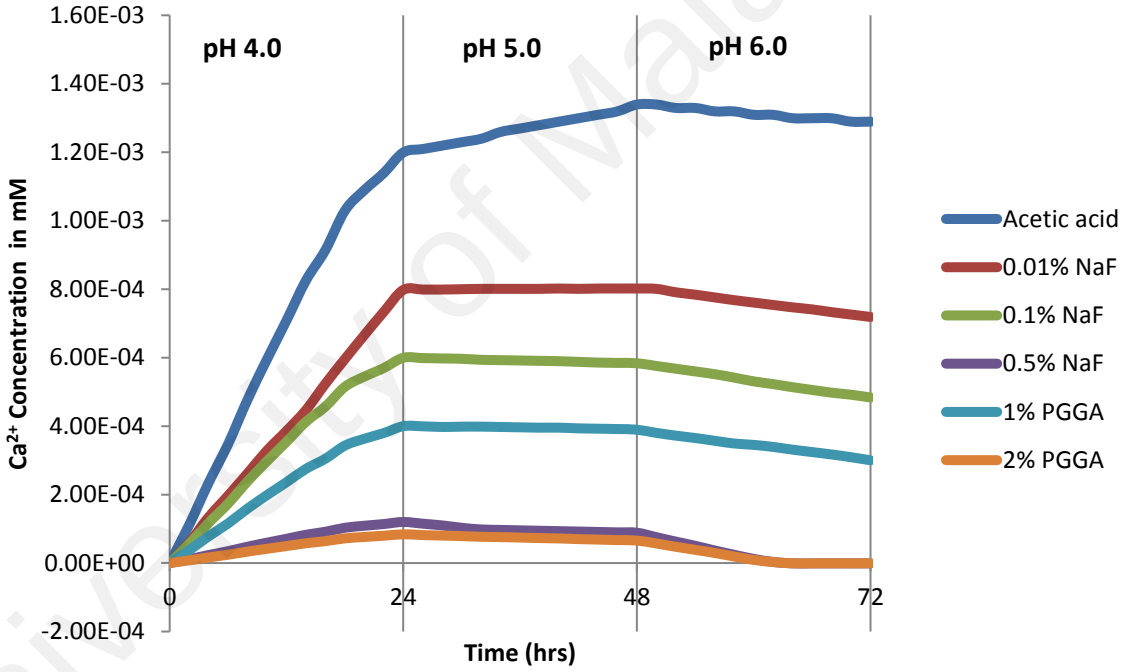


Figure 4.10: The concentration of calcium ion (mM) measured in the respective treatment solutions (Acetic acid (Negative control), acidified NaF (0.01% NaF, 0.1% NaF, 0.5% NaF respectively) (positive control) and acidified PGGA (1% and 2% respectively) containing teeth. Initially the pH of the solutions was calibrated to pH 4.0 with a pH meter. The concentration of calcium ion was measured at intervals of 15 min at pH 4.0 in the first 24-hr. The concentration was again measured in the second 24-hr after the pH had been raised to 5.0 with 1 M NaOH. The measurement was continued in the next 24-hr after the pH had been raised to 6.0. All of the measurements were carried out at intervals of 15 min for the 24-hr duration at the respective pHs. The individual experiment was carried out in triplicate and the values above represent the average of them.

4.2.1.1. Effect of PGGA in demineralising solution of pH 4.0 (1st 24 hr)

The calcium ion concentration measured at every 15 min interval was used in the linear regression analysis in which the rates were calculated and tabulated in Table 4.10. It was shown that the R_{Ca2+} release from teeth that were treated with NaF (positive control) and PGGA respectively when compared with the negative control (5.20 x 10-5 ± 0.17 x 10^{-5} mM/hr) was decreasing. When teeth treated with acidified 0.01% NaF the decrease was 36% and further decrease (93%) was observed when treated with acidified 2% PGGA.

Statistical analysis using Z-test shows that there were significant differences between the R_{Ca2+} for teeth treated with respective concentrations of acidified NaF groups (0.01% NaF, 0.1% NaF and 0.5% NaF) and the control negative group. Significant difference was also observed between R_{Ca2+} for the teeth immersed in the respective concentration of acidified PGGA treatment groups and the negative control group. Similarly, when comparing the rate between the different concentrations of NaF and between the different concentrations of PGGA the difference was statistically significant ($\rho<0.05$) (Appendix N).

Table 4.10: R_{Ca2+} release from the teeth immersed in the respective acidified treatment group solutions (Acetic acid (negative control), 0.01% NaF, 0.1% NaF, 0.5% NaF (positive control). 1% PGGA and 2% PGGA) of pH 4.0.

Treatment Groups	**0.1 M Acetic Acid**	**0.01% NaF**	**0.1% NaF**	**0.5% NaF**	**1% PGGA**	**2% PGGA**
R_{Ca2+} release (mM/h) Mean ± SE	5.20 x 10^{-5} ± 0.17x10^{-5}	3.33 x 10^{-5} ±0.023 x 10^{-5}	2.60 x 10^{-5} ±0.087 x 10^{-5}	0.519 x 10^{-5} ±0.017 x 10^{-5}	1.73 x 10^{-5} ±0.058 x 10^{-5}	0.364 x 10^{-5} ±0.012 x 10^{-5}

The R_{Ca2+} in mM/h was expressed as Mean ± SE where the number of determinants (n) is 3. The R_{Ca2+} release between the different solutions of acidified treatment groups in 24-hr were statistically significant ($\rho<0.05$). Acetic acid was the negative control and acidified NaF at three different concentrations was used as the positive control.

4.2.1.2. Effect of PGGA in demineralising solution of pH 5.0 (2nd 24 hr)

It was shown that increasing the pH of the respective treatment solutions from 4.0 to 5.0 has an influence on the calcium concentration depending on the treatment solution. Some treatment solutions showed a decreasing trend (0.1% NaF < 1% PGGA < 0.5% NaF < 2% PGGA) and some increasing trend (0.01% NaF < Acetic acid) (Figure 4.10). From the linear regression analysis R_{Ca2+} calculated for each of the respective treatment group is tabulated in Table 4.11.

Statistical analysis was performed using Z-test between the R_{Ca2+} release /uptake in the respective acidified six treatment groups with a significance value set at $\rho < 0.05$. The comparisons made between the R_{Ca2+} release/ uptake of the treatment groups were found to be statistically significant (Table 4.11) (Appendix O).

Table 4.11: R_{Ca2+} release/ uptake from the teeth immersed in the respective acidified treatment group solutions (Acetic acid (negative control), 0.01% NaF, 0.1% NaF, 0.5% NaF (positive control). 1% PGGA and 2% PGGA) of pH 5.0.

Treatment Groups	**0.1 M Acetic Acid**	**0.01% NaF**	**0.1% NaF**	**0.5% NaF**	**1% PGGA**	**2% PGGA**
R_{Ca2+} release/ uptake (mM/h) Mean ± (SE)	5.75×10^{-6} $\pm 0.13 \times 10^{-6}$	0.13×10^{-6} $\pm 0.020 \times 10^{-6}$	-0.42×10^{-6} $\pm 0.041 \times 10^{-6}$	-0.77×10^{-6} $\pm 0.0098 \times 10^{-6}$	-0.70×10^{-6} $\pm 0.021 \times 10^{-6}$	-1.05×10^{-6} $\pm 0.14 \times 10^{-6}$

The R_{Ca2+} in mM/h was expressed as Mean ± SE where the number of determinants (n) is 3. The R_{Ca2+} release/ uptake between the different solutions of acidified treatment groups in the second 24-hr were statistically significant ($\rho < 0.05$). Acetic acid was the negative control and acidified NaF at three different concentrations was used as the positive control.

4.2.1.3. Effect of PGGA in demineralising solution of pH 6.0 (3rd 24 hr)

On further increasing the pH from 5.0 to 6.0 of the six acidified respective treatments groups negative values indicated uptake of calcium ions with teeth (Figure 4.10) for the treatment groups in the following trend: Acetic acid < 2% PGGA < 0.5% NaF < 0.01% NaF < 0.1% NaF < 1% PGGA. The R_{Ca2+} uptake determined on linear regression analysis by teeth immersed in acidified treatment groups are tabulated in Table 4.12.

R_{Ca2+} uptake was analyzed using Z-test comparing the treatment groups with a significance value set at $\rho < 0.05$. The readings were found to have statistically significant difference between the negative control and the five treatment groups (Table 4.12) (Appendix P).

Table 4.12: R_{Ca2+} uptake from the teeth immersed in the respective acidified treatment group solutions (Acetic acid (negative control), 0.01% NaF, 0.1% NaF, 0.5% NaF (positive control). 1% PGGA and 2% PGGA) of pH 6.0.

Treatment Groups	**0.1 M Acetic Acid**	**0.01% NaF**	**0.1% NaF**	**0.5% NaF**	**1% PGGA**	**2% PGGA**
R_{Ca2+} Uptake (mM/h) Mean ± (SE)	$-2.24 \times 10^{-6} \pm 0.134 \times 10^{-6}$	$-3.65 \times 10^{-6} \pm 0.045 \times 10^{-6}$	$-4.29 \times 10^{-6} \pm 0.068 \times 10^{-6}$	$-3.58 \times 10^{-6} \pm 0.479 \times 10^{-6}$	$-4.50 \times 10^{-6} \pm 0.056 \times 10^{-6}$	$-2.73 \times 10^{-6} \pm 0.357 \times 10^{-6}$

The R_{Ca2+} in mM/h was expressed as Mean ± SE where the number of determinants (n) is 3. The R_{Ca2+} uptake between the different solutions of acidified treatment groups in the third 24-hr were statistically significant ($\rho<0.05$) except difference between 0.1% NaF and 1% PGGA was not statistically significant. Acetic acid was the negative control and acidified NaF at three different concentrations was used as the positive control.

4.2.2. The effect of PGGA in demineralising solutions of different pH values on the HAp pellet's surface

Figure 4.11 illustrates the release and uptake of calcium ions by the HAp pellets in the respective acidified solution of the various pHs (4.0, 5.0 and 6.0). Acetic acid was used as the negative control where the three different concentrations of acidified NaF (0.01%, 0.1% and 0.5% NaF respectively) as the positive control. The effect of PGGA at different pHs will be discussed below under separate sub-headings.

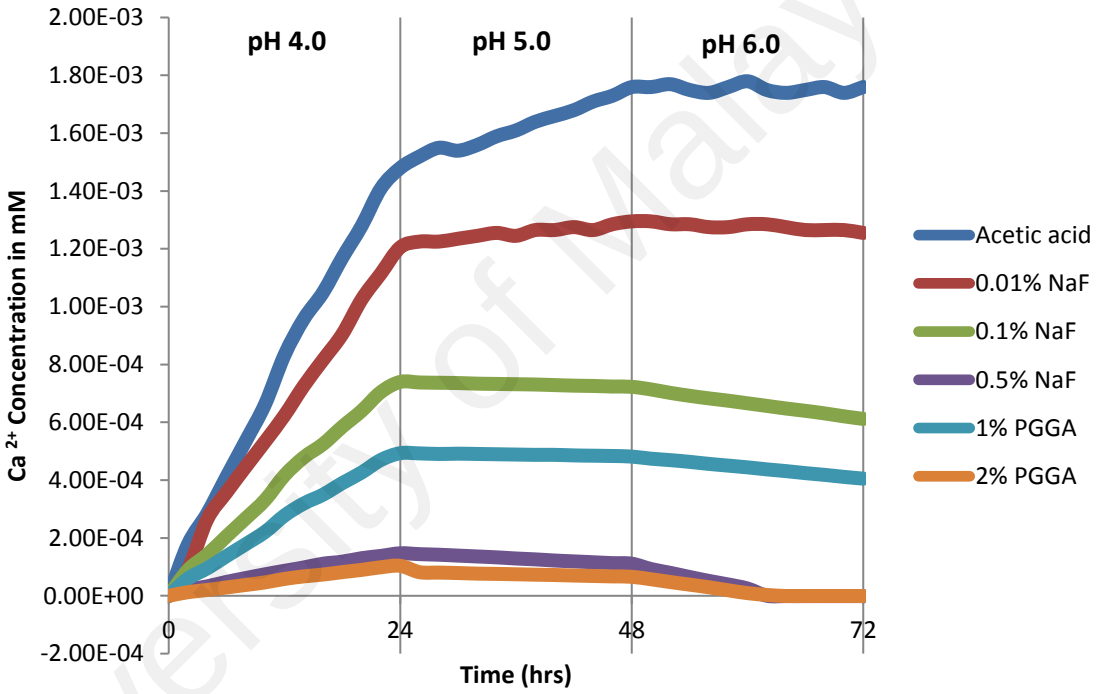


Figure 4.11: The concentration of calcium ions (mM) measured in the respective treatment solutions (Acetic acid (Negative control), acidified NaF (0.01% NaF, 0.1% NaF, 0.5% NaF respectively) (positive control) and acidified PGGA (1% and 2% respectively) containing HAp pellets. Initially the pH of the solutions was calibrated to pH 4.0 with a pH meter. The concentration of calcium ion was measured at intervals of 15 min at pH 4.0 in the first 24 hr. The concentration was again measured in the second 24-hr after the pH had been raised to 5.0 with 1 M NaOH. The measurement was continued in the next 24-hr after the pH had been raised to 6.0. All of the measurements were carried out at intervals of 15 min for the 24-hr duration at the respective pHs. The individual experiment was carried out in triplicate and the values above represent the average of them.

4.2.2.1. Effect of PGGA in demineralising solution of pH 4.0 (1st 24 hr)

The readings of calcium ion concentration recorded at 15 min interval were used to calculate the R_{Ca2+} with linear regression analysis tabulated in Table 4.13. The acetic acid (0.1 M) treated HAp pellet showed highest R_{Ca2+} release ($6.21 \times 10^{-5} \pm 0.11 \times 10^{-5}$ mM/hr). The R_{Ca2+} release decreased approximately by 23% for 0.01% NaF-treated ($4.91 \times 10^{-5} \pm 0.09 \times 10^{-5}$ mM/hr). The R_{Ca2+} release for the remaining respective treatment groups was observed to be in the following sequence: 0.1% NaF> 1% PGGA> 0.5% NaF > 2% PGGA.

Statistical analysis using Z-test shows that there were significant differences between R_{Ca2+} release from HAp pellets on immersion in the respective acidified concentration of NaF treated groups (0.01% NaF, 0.1% NaF and 0.5% NaF) and the control negative group. Significant difference was also observed between R_{Ca2+} release from the HAp pellet treated with the respective concentration of acidified PGGA treatment groups and the negative control group. Similarly, when comparing the rate between the different concentrations of NaF and between the different concentrations of PGGA the difference was statistically significant ($\rho<0.05$).

Table 4.13: R_{Ca2+} release from the surface of HAp pellets immersed in the respective acidified treatment group solutions (Acetic acid (negative control), 0.01% NaF, 0.1% NaF, 0.5% NaF (positive control). 1% PGGA and 2% PGGA) of pH 4.0.

Treatment Groups	**0.1 M Acetic Acid**	**0.01% NaF**	**0.1% NaF**	**0.5% NaF**	**1% PGGA**	**2% PGGA**
R_{Ca2+} release (mM/h) Mean ± SE	$6.21 \times 10^{-5} \pm 0.11 \times 10^{-5}$	$4.91 \times 10^{-5} \pm 0.09 \times 10^{-5}$	$3.11 \times 10^{-5} \pm 0.054 \times 10^{-5}$	$0.601 \times 10^{-5} \pm 0.018 \times 10^{-5}$	$2.07 \times 10^{-5} \pm 0.036 \times 10^{-5}$	$0.44 \times 10^{-5} \pm 0.007 \times 10^{-5}$

The R_{Ca2+} in mM/h was expressed as Mean ± SE where the number of determinants (n) is 3. The R_{Ca2+} release between the different solutions of acidified treatment groups in 24-hr were statistically significant ($\rho<0.05$). Acetic acid was the negative control and acidified NaF at three different concentrations was used as the positive control.

4.2.2.2. Effect of PGGA in demineralising solution of pH 5.0 (2nd 24 hr)

On increasing the pH to 5.0, it was observed that PGGA affects the concentration of calcium ions differently compared with pH 4.0 according to the respective treatment groups. Some of the treatment groups continued to show an increasing trend of calcium ion concentration in the solution where the increase was higher for acetic acid-treated HAp pellet as compared with 0.01% NaF-treated. As for the other treatment groups it was observed that the calcium ion concentration decreases with negative values observed for 0.1% NaF, 0.5% NaF, 1% PGGA and 2% PGGA. The negative values indicated uptake of calcium ions by HAp pellets which was less for 0.1% NaF-treated compared with 0.5% NaF-treated. Maximum uptake of calcium ions was observed for 2% PGGA-treated HAp pellet. The R_{Ca2+} calculated from linear regression analysis is tabulated in Table 4.14.

Statistical analysis using Z-test was performed between the R_{Ca2+} release/uptake by HAp pellets treated with the six different treatment groups with a significance level set at $\rho < 0.05$. Comparison of the R_{Ca2+} (release or uptake) between the different treatment groups was found to be statistically significant ($\rho < 0.05$), except between 1% PGGA and 0.5% NaF.

Table 4.14: R_{Ca2+} release/ uptake from the surface of HAp pellets immersed in the respective acidified treatment group solutions (Acetic acid (negative control), 0.01% NaF, 0.1% NaF, 0.5% NaF (positive control). 1% PGGA and 2% PGGA) of pH 5.0.

Treatment Groups	**0.1 M Acetic Acid**	**0.01% NaF**	**0.1% NaF**	**0.5% NaF**	**1% PGGA**	**2% PGGA**
R_{Ca2+} release/ Uptake (mM/h) Mean ± SE	$1.10 \times 10^{-5} \pm 0.044 \times 10^{-5}$	$0.30 \times 10^{-5} \pm 0.025 \times 10^{-5}$	$-0.046 \times 10^{-5} \pm 0.0031 \times 10^{-5}$	$-0.076 \times 10^{-5} \pm 0.0010 \times 10^{-5}$	$-0.073 \times 10^{-5} \pm 0.0018 \times 10^{-5}$	$-0.16 \times 10^{-5} \pm 0.0026 \times 10^{-5}$

The R_{Ca2+} in mM/h was expressed as Mean ± SE where the number of determinants (n) is 3. The R_{Ca2+} release/ uptake between the different solutions of acidified treatment groups in the second 24-hr were statistically significant ($\rho<0.05$) except difference between 1% PGGA and 0.5% NaF was not significant. Acetic acid was the negative control and acidified NaF at three different concentrations was used as the positive control.

4.2.2.3. Effect of PGGA in demineralising solution of pH 6.0 (3rd 24 hr)

By increasing the pH of the demineralising solution to 6.0, it was found that the concentration of the calcium ions decreased with respect to the treatment groups (Figure 4.11). All the values were negative suggesting calcium uptake. The uptake by the HAp pellet was the least with 0.01% NaF-treated group and highest with 1% PGGA-treated group compared with the negative control. The R_{Ca2+} uptake was calculated by linear regression analysis and tabulated in Table 4.15.

The R_{Ca2+} uptake by HAp pellets treated with respective treatment groups was analyzed using the Z-test. The difference between all the treatment groups was found to be statistically significant, except between 0.1% NaF and 1% PGGA.

Table 4.15: R_{Ca2+} uptake from the surface of HAp pellets immersed in the respective acidified treatment group solutions (Acetic acid (negative control), 0.01% NaF, 0.1% NaF, 0.5% NaF (positive control). 1% PGGA and 2% PGGA) of pH 6.0.

Treatment Groups	**0.1 M Acetic Acid**	**0.01% NaF**	**0.1% NaF**	**0.5% NaF**	**1% PGGA**	**2% PGGA**
R_{Ca2+} Uptake (mM/h) Mean ± SE	-0.042×10^{-5} $\pm 0.053 \times 10^{-5}$	-0.142×10^{-5} $\pm 0.025 \times 10^{-5}$	-0.452×10^{-5} $\pm 0.005 \times 10^{-5}$	-0.367×10^{-6} $\pm 0.0023 \times 10^{-5}$	-0.458×10^{-5} $\pm 0.0561 \times 10^{-5}$	-0.276×10^{-5} $\pm 0.036 \times 10^{-5}$

The R_{Ca2+} in mM/h was expressed as Mean ± SE where the number of determinants (n) is 3. The R_{Ca2+} uptake between the different solutions of acidified treatment groups in the third 24-hr were statistically significant ($\rho<0.05$) except difference between 0.1% NaF and 1% PGGA was not statistically significant. Acetic acid was the negative control and acidified NaF at three different concentrations was used as the positive control.

4.2.3. The effect of PGGA in demineralising solutions of different pH on the Cross-Sectional Micro Hardness (CSMH) of Enamel

The profiles for volume percent mineral vs depth from the surface of enamel are illustrated in Figure 4.12. It can be observed that the volume percent mineral increased between the depth from 25 µm to 50 µm. Acetic acid-treated tooth tended to show the least increase of the subsurface volume percent mineral which was followed by 0.01% NaF < 0.1% NaF< 1% PGGA < 0.5% NaF < 2% PGGA.

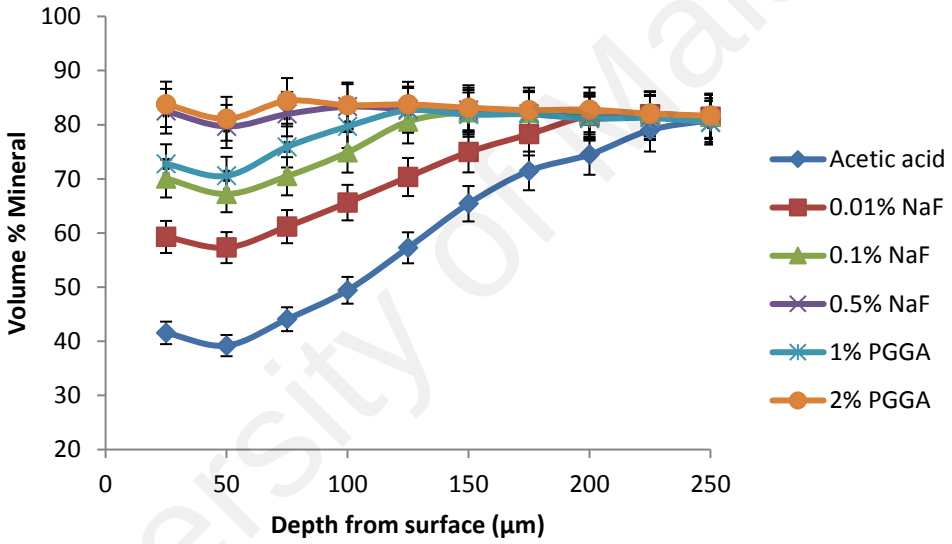


Figure 4.12: Comparative cross-sectional mineral profiles for teeth treated with respective treatment groups. The microhardness was measured at 25 µm intervals from the tooth surface up to 250 µm subsurface. PBS was the negative control and NaF at three different concentrations was used as the positive control. The individual experiment was carried out in triplicate and the values above represent the average of them.

Table 4.16 illustrates the statistical comparison in the volume percent mineral across different depths of the same group from 25 μm to 250 μm and the comparison of the mineral content within the same depth among the different treatment groups. The volume percent of minerals was statistical analyzed by two-way analyses among the groups.

On analysis of the volume percent mineral content measured at 25 μm intervals across the subsurface enamel, statistically significant difference was observed for teeth treated with acetic acid (negative control) between the depths up to the subsurface 250 μm ($\rho<0.05$), whereas the volume percent mineral for teeth treated with 0.01% NaF in demineralising solution a statistically significant difference was observed between the depths up to 200 μm subsurface ($\rho<0.05$). The teeth treated with 0.1% NaF- and 1% PGGA-in demineralising solution showed significant difference of volume % mineral between the depths up to 125 μm subsurface ($\rho<0.05$). For the teeth treated with 0.5% NaF and 2% PGGA in demineralising solution, statistically significant difference was observed between the depths up to 50 μm subsurface ($\rho<0.05$).

On further analysis between the volume % mineral between the treatment groups within the respective depth statistically significant difference was observed at the subsurface of 25 μm in the mineral percent volume of the enamel ($\rho<0.05$), where 0.01% NaF-treated teeth showed lowest volume % mineral in comparison with acetic acid (negative control) and highest with 2% PGGA.

Table 4.16: The volume percent mineral values of dental enamel on exposure to the respective acidified treatment group.

Distance from tooth Surface (µm)	Mineral Content Value (Mineral % Volume= 4.3(√ KHN) + 11.3)					
	Acetic Acid	**0.01% NaF**	**0.1% NaF**	**0.5% NaF**	**1% PGGA**	**2% PGGA**
25	41.55 ±0.2 ^{Aa}	59.27±0.2 ^{Ab}	70.07±0.3 ^{Ac}	82.49±0.5 ^{Ad}	72.75±0.3 ^{Ae}	83.76±0.7 ^{Af}
50	39.20±0.2 ^{Ba}	57.30±0.1 ^{Bb}	67.20±0.6 ^{Bc}	79.68±1.0 ^{BCd}	70.55±0.3 ^{Be}	81.09±0.2 ^{Bd}
75	44.08±0.2 ^{Ca}	61.18±0.3 ^{Cb}	70.48±0.5 ^{Ac}	81.96±0.3 ^{Ad}	75.89±0.5 ^{Ce}	84.40±0.4 ^{Af}
100	49.42±0.5 ^{Da}	65.61±0.9 ^{Db}	74.90±1.1 ^{Cc}	83.33±1.4 ^{Ad}	79.68±1.0 ^{DFe}	83.61±0.7 ^{ACd}
125	57.26±0.2 ^{Ea}	70.34±1.0 ^{Eb}	80.57±0.5 ^{Dc}	82.83±1.8 ^{ABd}	82.64±1.2 ^{Ed}	83.74±0.5 ^{Ad}
150	65.14±0.2 ^{Fa}	74.94±1.6 ^{Fb}	82.30±2.1 ^{Ec}	82.68±1.4 ^{ABc}	81.86±2.3 ^{EFc}	83.13±1.2 ^{ADCc}
175	71.46±0.6 ^{Ga}	78.23±2.0 ^{Gb}	82.18±2.1 ^{DEc}	81.97±1.8 ^{ABCc}	81.92±1.9 ^{EFc}	82.71±1.6 ^{ACc}
200	74.48±0.4 ^{Ha}	81.62±1.6 ^{Hb}	81.83±2.3 ^{DEb}	81.32±2.7 ^{ABCb}	81.12±1.8 ^{EFb}	82.76±0.9 ^{ACb}
225	78.99±1.2 ^{Ia}	81.94±1.8 ^{Hb}	82.03±1.7 ^{Deb}	81.45±1.8 ^{BCb}	81.24±2.3 ^{EFb}	82.04±1.7 ^{BCb}
250	80.86±1.3 ^{Ja}	81.44±2.0 ^{Ha}	81.49±1.9 ^{DEa}	80.36±3.6 ^{Ca}	80.43±2.3 ^{Fa}	81.67±1.6 ^{BDa}

Mineral content expressed in Mean± SD where the number of determinants (n) is 3.
$^{A-J}$ different capital letters mean statistically significant difference for mineral content value of same group at different depth ($\rho<0.05$);
$^{a-f}$ Different lower case letter means statistically significant difference at same depth in different groups ($\rho<0.05$). The individual experiments were carried out in triplicate. PBS was the negative control and NaF at three different concentrations was used as the positive control.

Table 4.17 describes the recovery of enamel integrated mineral after the effect of various acidified solutions. The 0.01% NaF treated teeth showed the least integrated mineral recovery content as compared with the integrated mineral content (untreated/ varnished enamel) of the respective teeth, where 2% PGGA showed to be most effective for integrated mineral recovery of the enamel. Statistical analysis was performed using one-way Anova and the significance level was set at $\rho< 0.05$. The difference was found to be statistically significant between the mineral recovery of the teeth treated with respective acidified treatment groups ($\rho<0.05$). (The volume percent mineral content of untreated enamel measured is shown in Appendix Q)

Table 4.17: Integrated Mineral Recovery Value of Enamel by the effect of various acidified solutions

Treatment Groups	Integrated Mineral Recovery
Acetic Acid	12833.75 ± 55.04
0.01% NaF	15349.83 ± 242.24
0.1% NaF	16724.80 ± 291.64
0.5% NaF	17728.60 ± 346.63
1% PGGA	17078.63 ± 290.09
2% PGGA	17946.51 ± 168.08

Integrated mineral recovery expressed as Mean ± SD, the number of determinants (n) is 3. Statistically significant difference was found for integrated mineral content by effect of various treatment groups ($\rho<0.05$). The individual experiment was carried out in triplicate. PBS was the negative control and NaF at three different concentrations was used as the positive control.

4.3. Interaction of PGGA with Dental Enamel and HAp pellet

4.3.1. FT-IR Analysis

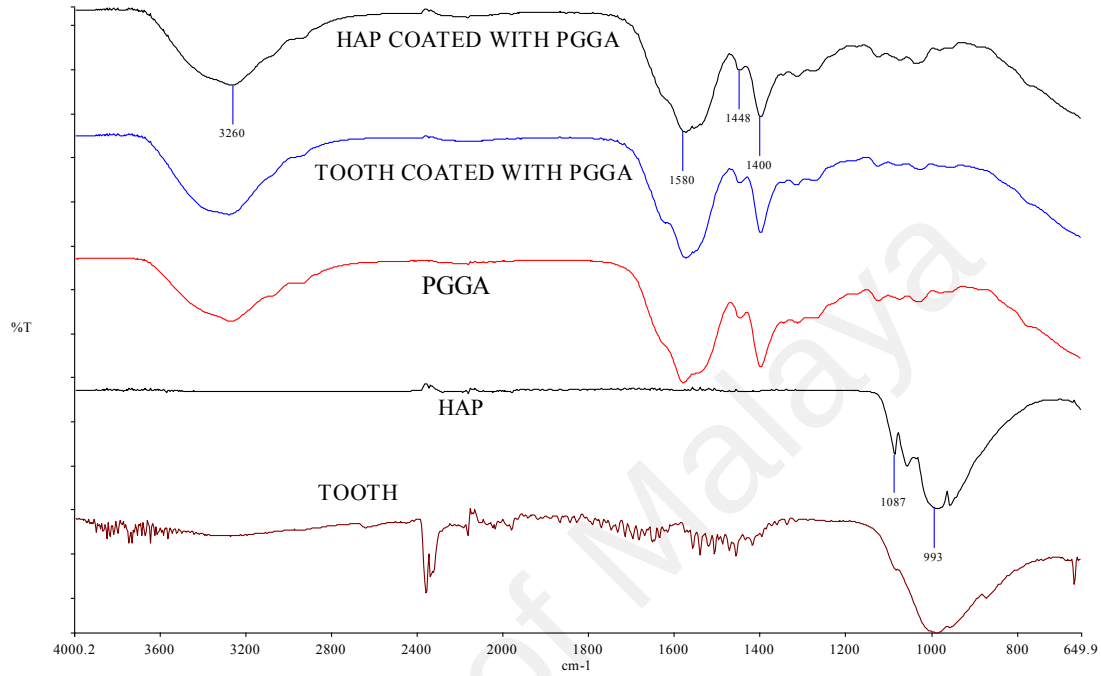


Figure 4.13: Infrared (FT-IR) transmission spectra of tooth, HAp pellet (HAP), PGGA powder; and tooth and HAp coated with 1% PGGA solution. The Y-axis is the percentage of transmission (%T) and the X-axis is the wave number in cm^{-1}. The experiment was repeated in triplicate and the values above represent one of them.

The FT-IR spectra of enamel and HAp obtained prior to and after immersing (coating) with 1% PGGA are shown in Figure 4.13. Both the uncoated HAp pellet and tooth showed a broad band with multiple peaks in the region of 650-1100 cm^{-1}. The band was not observed in the samples that had been coated; presumably the surfaces have been covered by a layer of coating thicker than 2 µm.

The spectra of the coatings on the HAp pellet and tooth surface showed similar transmission peaks as those of the PGGA powder. There was broad peak at 3260 cm^{-1} due to the –OH and –N-H groups which could overlap in this region. A strong peak at 1580 cm^{-1} and a weaker shoulder at 1620 corresponded to the amide linkages contributed by –C=O stretching and –N-H bending modes. The $–CH_2-$ bending modes were seen at 1448 and 1400 cm^{-1}.

4.3.2. Density

It was shown that the density of PGGA increases with concentration and the difference in the density for the two concentrations was statistically significant ($p < 0.05$) (Table 4.18)

Table 4.18: Density of PGGA solution in de-ionized water

Concentration of Poly-γ-glutamic acid **w/v**	**Density** **g/cm^3**
1%	1.0048 ± 0.0001
2%	1.0099 ± 0.0003

The density was expressed in g/cm^3 as the mean±SD of 6 determinations

4.3.3. Viscosity

i) Kinematic Viscosity of PGGA

Table 4.19 describes that there is an increase in the kinematic viscosity with an increase in the concentration of PGGA.

Table 4.19: Kinematic viscosity of PGGA in de-ionized water

Concentration of Poly-γ-glutamic acid w/v	**Kinematic Viscosity (m^2sec^{-1})**
1%	1.504 ± 0.010
2%	2.054 ± 0.13

The kinematic viscosity was expressed in m^2sec^{-1} as the Mean ± SD of 6 determinations

ii) Dynamic Viscosity of PGGA

Table 4.20 describes the dynamic viscosity of the PGGA in deionized water, by flowing different concentrations of solutions through the burette at one end under a constant pressure. It was observed that the dynamic viscosity also increases with the PGGA concentration

Table 4.20: Dynamic viscosity of PGGA in de-ionized water

Concentration of Poly-γ-glutamic acid w/v	**Dynamic Viscosity (Pa. sec)**
1%	1.51 ± 0.02
2%	2.07 ± 0.34

The dynamic viscosity was expressed in Pa. sec as the mean±SD of 6 determinations

CHAPTER 5: DISCUSSION

The type of teeth, demineralising solution and its pH, positive control and the techniques used to assess the parameters for demineralisation and remineralisation were given due consideration. The orthodontically extracted pre-molars were selected to conduct our study for various reasons (Featherstone & Rodgers, 1981). Being extracted in the early stage of life, usually between the ages of 14 to 22 years, the premolars are considered to be devoid of defects such as erosion, caries, attrition etc. The average enamel thickness on the mid-labial surface of premolars is 1-1.5 mm (Feeney et al., 2010).

Featherstone and Rodgers (1981) reported that subsurface lesions produced by acetic acid are approximately one and a half times deeper than those produced by lactic acid at the same pH. In this study, 0.1 M acetic acid was used as the demineralising solution and its effect at three different pHs (4, 4.5 and 5) on the demineralisation and depth of subsurface lesion was determined and compared. Fluoride is commonly used as a gold standard for the treatment of incipient lesions (Weyant, 2013; Weyant et al., 2013). Based on the fluoride content in commercially-available oral healthcare products (see footnote [1,2]) three different concentrations of fluoride were used as the positive controls.

The changes in the mineral content leading to demineralisation /remineralisation can be determined using various *in-vitro* techniques. In this study, a Ca^{2+} selective electrode (Ca^{2+}SE) and cross sectional microhardness (CSMH) were respectively used to determine the rate of mineral loss/gain and changes in the lesion formation. The analysis of demineralisation and remineralisation with ISE is a well-established,

[1] Colgate Palmolive, USA.

[2] Glaxo Smithkline, Australia.

sensitive and accurate technique (Arends & ten Bosch, 1992). The CSMH was employed as it has been used in the determination of subsurface mineral content loss and deposition (Arends & ten Bosch, 1992; White et al., 1992; Huang et al., 2010; da Silva et al., 2015). Featherstone *et al* (1983) reported a strong correlation between the microradiography and the microhardness of enamel mineral profiles (r=0.919). Therefore CSMH is regarded as an accurate and inexpensive technique in order to determine the subsurface mineral volume loss leading to subsurface lesion formation in enamel.

5.1. Effect of PGGA on Enamel demineralisation

5.1.1. The Effect of PGGA on the Inhibition of Demineralisation of enamel at different pHs (pH 4.0, 4.5 and 5.0) of demineralising solutions.

In this investigation the enamel was pretreated with the PGGA at different concentration respectively (1% and 2%) before immersing in the demineralising solution at the respective pHs (4.0, 4.5 and 5.0). The rate of demineralisation was recorded at one minute intervals for 24-hr at 37°C. It was observed that the respective pretreated teeth on immersion in demineralising solution followed a linear pattern of calcium release at each pH (4.0, 4.5 and 5.0). From the graph it was observed that there was an increase in the calcium ion concentration even after 1 min which was more obvious after 24 hrs. For this reason, the rate of demineralisation was measured using the calcium ion concentration released after 24 hrs and expressed as milli molar calcium ion concentration per hr.

Comparison of the rate of demineralisation of the teeth pretreated with PBS (negative control) in demineralising solution between the three different pHs (4.0, 4.5 and 5.0) showed a higher rate at pH 4.0 followed by pH 4.5 and lowest at pH 5.0. This could be attributed to the different H^+ concentration in the demineralising solution. Featherstone and Rodgers (1981) have reported that 0.1 M acetic acid of pH 4.0 (0.085 M unionized

acid) contains higher H^+ concentration compared to pH 4.5 (0.063 M unionized acid). The H^+ plays a role in determining the acidity of a solution. This can be explained further below. The H^+ and OH^- when in equal amount form water thus maintaining a neutral pH (Dawes, 2003). The ionic product of water, $K_w = [H^+] [OH^-] = 10^{-14}$ $(mol/L)^2$ to stabilize pH (Larsen & Bruun, 1986).

$$H^+ + OH^- \leftrightarrow H_2O$$

Therefore increasing H^+ and decreasing OH^- in a solution makes it acidic leading to enamel demineralisation. The lower the pH, the higher will be the concentration of H^+. For the teeth pretreated with 1% and 2% PGGA respectively, their rates of demineralisation in demineralising solution of pH 4.0 were also higher than that of pH 4.5. We also determined the rate of demineralisation of teeth pretreated with respective concentrations of NaF (0.01%, 0.1% and 0.5%) in the demineralising solution of pH 4.0 and 4.5. The rate of demineralisation was also higher at pH 4.0 compared to at pH 4.5. Similar explanation on the role of H^+ concentration can also be used for teeth pretreated with respective different concentrations of PGGA and NaF at the pH 4.0 and pH 4.5.

In this study, the PGGA pretreated teeth were exposed to a pH below the critical pH of saliva (5.2-5.4) (Dawes, 2003). This suggests that PGGA inhibits demineralisation. The pattern of decreasing demineralisation with increasing PGGA concentration is in agreement with what was reported by Qamar et al., (2012) who used HAp pellets instead of teeth.

When the pH of demineralising solution was increased further to 5.0 which is more or less the critical pH, there was no calcium ions release observed from the teeth pretreated with 2% PGGA and 0.5% NaF respectively. This implies there was no demineralisation at this pH compared to pHs 4.0 and 4.5 which can be due to the lower H^+ concentration. Comparison of the effect produced by 2% PGGA and that of 0.5% NaF (the maximum concentration incorporated in oral health care product) showed to be more or less

similar. This may imply that 2% PGGA was as effective as the 0.5% NaF. However, Lussi et al., (2012) have suggested that short-term application of 0.3% fluoride preparation leads to formation of CaF_2 deposits inhibiting demineralisation. Thus, it appears that 2% PGGA is a better alternative to be incorporated in oral health care product compared to 0.5% NaF. Despite saying that, this needs further investigation for its relevance in clinical practise.

The mechanism by which PGGA inhibits enamel demineralisation could be attributed to the coating potential being a viscous material, as the viscosity in our current study increased with increasing PGGA concentration. We attempted to measure the coating thickness using calotest technique in Anton Paar laboratory (Switzerland) which could not provide us with the result (Appendix R).

5.1.2. The effect of PGGA on lesion depth in enamel upon immersion in demineralising solution at different pHs (pH 4.0, 4.5 and 5.0)

All of the teeth that have been used in the ISE study were reused for the CSMH assessment which determined the depth of the lesion. On CSMH of the respective pretreated teeth with different treatment groups and immersed in demineralising solution of pH 4.0, maximum mineral volume loss and depth of lesion formed was observed for the negative control pretreated teeth that was up to 200 μm subsurface enamel. This is in agreement with the results reported by Featherstone and Rodgers (1981) for the depth of lesion developed upon treatment with the 0.1 M acetic acid. Featherstone and Rodgers (1981) compared the depth of subsurface lesion developed by different organic acids at different pHs. The teeth pretreated with 0.01% NaF immersed in demineralising solution of pH 4.0 were observed to develop a similar depth of lesion as that in the negative control pretreated teeth, but the volume % mineral loss was less at each respective depth. Reduction in the depth of lesion was observed for the teeth pretreated with increasing NaF concentrations. The teeth pretreated with 0.5% NaF

showed lesions developed up to 100 μm enamel subsurface. The 1% PGGA pretreated teeth showed lesion development up to the depth of 150 μm subsurface enamel, the lesion depth decreased for the teeth pretreated with 2% PGGA by 50 μm up to a depth of 100 μm subsurface enamel. The depth of lesion developed for the teeth pretreated with 0.5% NaF and 2% PGGA was same, but the loss of volume % mineral was higher for the teeth pretreated with 0.5% NaF. This suggests that teeth on pretreatment with 2% PGGA have the potential to inhibit development of subsurface lesion formation.

On CSMH of the respective pretreated teeth immersed in demineralising solution of pH 4.5, similar profiles of lesion formation were observed to those of teeth treated with demineralising solution of pH 4.0 except that the depth of subsurface lesion developed was reduced for all of the teeth. The depth of lesion formed for the negative control treated teeth immersed in demineralising solution of pH 4.5 reduced to 150 μm subsurface as compared with the negative control pretreated teeth immersed in demineralising solution of pH 4.0. This is in disagreement with Featherstone and Rodgers (1981) who reported 0.1 M acetic acid solution of pH 4.5 developed lesions up to a depth of 150 μm subsurface in 2 days, whereas in our study the lesion developed for PBS pretreated teeth is similar but it formed within 1 day. The possible reason could be variation of the mineral content present in the surface of dental enamel, such as more carbonate, magnesium ions might have been present in the surface of teeth we used for our study which would have led to faster demineralisation and deeper subsurface lesion formation. The teeth pretreated with 0.01% NaF on immersion in demineralising solution of pH 4.5 developed similar lesions with depth of 150 μm subsurface as that for negative control pretreated teeth except the changes in mineral volume were different at each lesion depth. The depth of subsurface lesion reduced with increasing concentration of NaF for pretreatment of teeth. The teeth pretreated with 0.1% NaF showed a lesion developed to a depth of 100 μm subsurface whereas for 0.5% NaF pretreated teeth up to

50 µm. The depth of lesion developed for 1% PGGA pretreated teeth was similar to that of 0.1% NaF pretreated teeth on immersion in demineralising solution of pH 4.5 except the reduction in mineral volume was less for 1% PGGA pretreated teeth at each subsurface depth. Teeth pretreated with 2% PGGA developed a lesion up to a depth of 50 µm subsurface similar to 0.5% NaF, except less reduction was observed in the mineral volume for teeth pretreated with 2% PGGA.

The pretreated teeth in demineralising solution of pH 5.0 followed a similar pattern to those at pH 4.0 and 4.5. The maximum lesion depth was observed for the negative control pretreated enamel up to 100 µm subsurface on immersion in demineralising solution pH 5.0. Teeth pretreated with 0.01% NaF, 0.1% NaF and 1% PGGA on immersion in demineralising solution of pH 5.0 developed a lesion up to 50 µm subsurface, but a difference in the mineral volume reduction was observed. Teeth pretreated with 0.01% NaF showed higher mineral reduction followed by 0.1% NaF and 1% PGGA pretreated teeth at depth of 50 µm subsurface. Teeth pretreated with 0.5% NaF and 2% PGGA did not show development of subsurface lesion formation, correlating with the data of ISE where no loss of Ca^{2+} was observed for the respective groups.

5.1.3. The effect of PGGA on inhibition of Ca^{2+} release from pretreated HAp powder on immersion in respective demineralising solution (pH 4.0, 4.5 and 5.0)

The HAp powder (analogue to enamel crystallites) was used for demineralisation experiments as Qamar et al., (2012) used HAp pellets (analogue to dental enamel) for demineralisation study. The HAp powder pretreated with respective treatment groups showed release of Ca^{2+} at a faster rate and came to equilibrium after a few minutes on immersion in demineralising solution for all three pHs (4.0, 4.5 and 5.0). Therefore the experiments were stopped after 2-hr. The possible reason for quick Ca^{2+} release in the

initial few minutes is due to increased surface area of HAp powder in contact with demineralising solution. Higuchi et al., (1969) reported a similar pattern of calcium release from HAp powder on immersion in demineralising solution and he stopped the experiments after a few minutes. Similarly Wong et al., (1987) compared the rate of demineralisation between HAp powder and HAp pellets treated with different fluoride concentrations. She reported a pattern of demineralisation of HAp powder similar to that of our study whereas the pattern for HAp pellets was linear with time. Wong et al., (1987) suggested that the dissolution of HAp pellets is diffusion controlled and therefore is linear with time, unlike HAp powder.

The negative control pretreated HAp powder on immersion in the demineralising solution of pH 4.0 showed faster release of Ca^{2+} as compared with HAp powder pretreated with other treatment groups. The Ca^{2+} release reduced for the HAp powder pretreated with 0.01% NaF. The release of Ca^{2+} was observed to decrease further with increasing concentration of NaF for pretreatment of HAp powder. For HAp powder pretreated with 0.1% NaF, decrease in Ca^{2+} release was observed as compared with that for 0.01% NaF pretreated HAp powder on immersion in demineralising solution of pH 4.0. Further decrease in release of Ca^{2+} was observed for 0.5% NaF pretreated HAp powder. Wong et al., (1987) also reported the decrease in Ca^{2+} release with increasing concentration of fluoride. She used fluorohyroxyapatite (FHAp) powder comprising different concentrations of fluoride content, immersed in 0.1 M acetic acid solutions of pH 3.0 and 5.0. She determined the demineralisation rate by comparing the data of FHAp pellets and FHAp powder (Wong et al., 1987). She concluded that with increasing concentration of fluoride in the powder, decrease in rate of demineralisation was observed, which is in agreement with our results. Similar to NaF, on increasing the concentration of PGGA treatment from 1% to 2% for HAp powder, a decrease in Ca^{2+}

release was observed. Thus, 2% PGGA pretreated HAp powder was more effective in inhibiting release of Ca^{2+} than was 0.5% NaF pretreated HAp powder.

Similarly the release of Ca^{2+} from pretreated HAp powder in demineralising solution of pH 4.5 was not linear with time as was that for pH 4.0. The negative control pretreated HAp powder on immersion in demineralising solution of pH 4.5 showed maximum release of Ca^{2+} in comparison with HAp powder pretreated with various NaF and PGGA concentrations. The amount of Ca^{2+} released was lower for the negative control pretreated HAp powder on immersion in demineralising solution of pH 4.5 as compared with the negative control pretreated HAp powder immersed in demineralising solution of pH 4.0. The probable reason for the decrease in the Ca^{2+} release is due to increase in pH of demineralising solution, thus decreasing the unionized acid from 0.085 M (0.1 M acetic acid of pH 4.0) to 0.063 M (Featherstone & Rodgers, 1981). Decrease in Ca^{2+} release was also observed for the pretreated HAp powder with respective treatment groups (NaF and PGGA) immersed in demineralising solution of pH 4.5 as compared with Ca^{2+} release from pretreated HAp powder on immersion in demineralising solution of pH 4.0. The release of Ca^{2+} was observed to decrease for 0.01% NaF pretreated HAp powder in comparison with that negative control pretreated, on immersion in demineralising solution of pH 4.5. The decrease in Ca^{2+} was further observed for the HAp powder pretreated with 0.5% NaF. The HAp powder pretreated with 1% PGGA showed more decrease in Ca^{2+} release than that of 0.1% NaF pretreated. The 2% PGGA pretreated HAp powder was the most effective concentration in inhibiting the release of Ca^{2+} than the other treatment groups on immersion in demineralising solution of pH 4.5.

On immersion of pretreated HAp powder in demineralising solution of pH 5.0, a similar pattern of Ca^{2+} release was observed to that for pretreated HAp powder on immersion in demineralising solution of pH 4.0 and 4.5. Negative control pretreated HAp powder showed maximum release of Ca^{2+} on immersion in demineralising solution of pH 5.0 as

compared with Ca^{2+} release from HAp powder pretreated with other treatment groups immersed in demineralising solution of pH 5.0. The release of Ca^{2+} was observed to decrease for HAp powder pretreated with respective treatment groups on immersion in demineralising solution of pH 5.0 as compared with the negative control pretreated HAp powder immersed in demineralising solution of pH 4.0 and 4.5. This is attributed to decrease in the concentration of H^+ present in demineralising solution due to increase in pH to 5.0. A similar decrease in Ca^{2+} release was observed for the pretreated HAp powder with NaF and PGGA on immersion in demineralising solution of pH 5.0 as compared with that of pH 4.5. The HAp powder pretreated with 0.01% NaF showed decrease in Ca^{2+} release as compared with negative control pretreated HAp powder on immersion in demineralising solution of pH 5.0. Further decrease in Ca^{2+} release was observed for HAp powder pretreated with increasing NaF concentrations. HAp powder pretreated with 1% PGGA showed the potential of decreasing Ca^{2+} release more potently than 0.1% NaF pretreated HAp powder. On increasing concentration of PGGA to 2% for pretreatment of HAp powder, further reduction in Ca^{2+} release was seen, suggesting that 2% PGGA is more potent in inhibiting demineralisation than 0.5% NaF pretreated HAp powder.

5.2. The effect of PGGA on enamel remineralisation

The methodology for remineralisation was devised without exogenous calcium in the treatment solutions, using a standard paradigm to maintain enamel minerals as suggested by Featherstone (2000). According to Featherstone (2000) the paradigm shifts from demineralisation to remineralisation due to increase in pH and with the super-saturation with respect to hydroxyapatite in the environment. Therefore to determine the potential of PGGA for remineralisation, the teeth and HAp pellets were immersed in acidified treatment groups of pH 4.0 in order to release natural mineral content from teeth in the initial 24-hr without any exogenous minerals. Further the pH of the respective acidified solutions was raised to pH 5.0 and remained at that pH for another 24-hr. This was to determine the potential of PGGA to promote remineralisation below the critical pH values of saliva in contact with the enamel. Later the pH was raised further to 6.0 and remained at that pH for another 24-hr (second 24-hr) to determine remineralisation potential above the critical pH value of enamel in saliva.

5.2.1. The effect of acidified PGGA on Ca^{2+} for enamel remineralisation

The teeth were immersed in the solutions of respective treatment groups acidified to pH 4.0 to allow mineral content release from human enamel. The Ca^{2+} release was linear with time measured at 15-min intervals maintaining a temperature of 37°C. The Ca^{2+} release was highest for the teeth immersed in 0.1 M acetic acid of pH 4.0, the Ca^{2+} release decreased for the teeth immersed in acidified 0.01% NaF at pH 4.0. The release of Ca^{2+} was observed to decrease from teeth with increasing concentration of NaF, thus teeth treated with acidified 0.5% NaF was more effective than acidified 0.01% and 0.1% NaF solutions in inhibiting release of Ca^{2+}. The possible reason for the Ca^{2+} release from teeth immersed in acidified 0.1% NaF and 0.5% NaF is due to the undersaturated condition (absence of Ca^{2+}) to develop CaF_2 deposits. This is in agreement with Saxegaard and Rolla (1988) who reported the decreasing pH of the acidified fluoride

solution leads to etching of the surface enamel and makes the Ca^{2+} available to develop CaF_2 deposits. Acidified 1% PGGA showed decrease in rate of Ca^{2+} release more potently than acidified 0.1% NaF. Further reduction in the rate of demineralisation was observed on increasing concentration of acidified PGGA solution to 2%.

After the first 24-hr the pH of the respective acidified solutions was increased to 5.0. For the blank control (the teeth immersed in 0.1 M acetic acid), the teeth continued to show release of Ca^{2+}. The release of Ca^{2+} from the teeth immersed in demineralising solution of pH 5.0 is in agreement with the critical pH of saliva or plaque fluids in contact with the enamel as reported by other researchers that is, above 5.0 (5.2-5.4) (Dawes, 2003). Similarly the teeth immersed in acidified 0.01% NaF continued to show release of Ca^{2+} but at a slower rate. However it is not in agreement with a study reported by Larsen and Jensen (1994) who, on x-ray diffraction, found that the CaF_2 deposits start to develop in acidified 0.01% NaF solution of pH 5.0 in the presence of calcium ions in the solution. The possible reason for disagreement is the amount of calcium present in the acidified 0.01% NaF solution. In our study the amount of Ca^{2+} concentration was extremely low as compared with the Ca^{2+} present in their solution that was 4.8 mmol/l (Larsen & Jensen 1994). The teeth immersed in 0.1% NaF solution acidified to pH 5.0 started to show decrease in Ca^{2+} from the solution suggesting an uptake to remineralize the teeth. The rate of Ca^{2+} uptake was observed to increase for the teeth immersed in 0.5% NaF solution acidified to pH 5.0. On the other hand the rate of Ca^{2+} uptake by the teeth immersed in acidified 1% PGGA was lower than that for acidified 0.5% NaF solution but higher than that for the teeth immersed in acidified 0.1% NaF solution. The teeth immersed in 2% PGGA solution acidified to pH 5.0 showed more potent uptake of Ca^{2+} in comparison with teeth immersed in acidified- 1% PGGA and -NaF respectively. The potential of PGGA to cause decrease of Ca^{2+} concentration in the respective solutions acidified to pH 5.0 is attributed to the presence

of free COO^- groups as confirmed by FT-IR analysis. The free COO^- groups help bind free Ca^{2+} in the solution. Ho et al., (2006) reported the free COO^- in PGGA has a strong potential to bind free Ca^{2+} present in the solution.

On increasing the pH to 6.0 after the second 24-hr (after 48-hr) in acidified solutions, uptake of calcium ions occurred. The rate of calcium uptake was slowest by the teeth immersed in 0.1 M acetic acid solution. The rate of Ca^{2+} uptake increased for the teeth immersed in acidified 2% PGGA followed by acidified 0.5% NaF solution. The decrease of Ca^{2+} was more potent for the teeth immersed in acidified 0.01% NaF followed by acidified 0.1% NaF as compared with the teeth in 0.5% NaF solution acidified to pH 6.0. This is in disagreement with Lussi et al (2012) who suggested that the rate of Ca^{2+} reduction from solution increases (due to CaF_2 precipitation) in the *in-vitro* conditions with 0.03% or more fluoride at neutral pH. Where in our study the rate for Ca^{2+} uptake decreased with increase in pH, the reason could be the absence of Ca^{2+} ions in the solution as obvious from the ISE data. The amount of Ca^{2+} at the 52^{nd} hour was approximately zero, thus decreasing the rate of Ca^{2+} uptake for the teeth immersed in acidified 0.5% NaF. A similar pattern of decreasing rate of Ca^{2+} uptake was observed for the teeth immersed in 2% PGGA solution acidified to pH 6.0 this could also be attributed to absence of Ca^{2+} at nearly the 52^{nd}-hr.

5.2.2. The effect of acidified PGGA on enamel mineral recovery

Teeth from the ISE study were reused to determine the CSMH. From the CSMH the mineral percent volume was calculated. There was an increase in the mineral content was observed up to a depth of 25 μm which is the superficial enamel layer. This was observed for all the teeth treated with the acidified PGGA solutions at various pHs. This implies that there was mineral uptake by the teeth. On comparing the effect between acidified 1% - and 2% - PGGA, the teeth immersed in acidified 2% PGGA were

observed to have higher mineral content. The teeth immersed in acidified 2% PGGA also showed increase in mineral volume at a subsurface depth of 75 μm which is the body of the demineralised lesion. The teeth immersed in acidified 0.5% NaF solution only show mineral recovery at the 25 μm subsurface enamel. This suggests that 2% PGGA is better than 0.5% NaF for having the potential to remineralize the body of the demineralised lesion. In this study it can be explained that the Ca^{2+} release into the solution are taken up by the tooth enamel in a similar way where polyphenols in Galla Chinensis showing to have potential to remineralize the body of the carious lesion in bovine enamel (Chu et al., 2007). The normal variation in CSMH in untreated controlled teeth was in the range 78- 85% (Appendix Q). Despite what has been said the diffusion coefficient for PGGA molecules and calcium ions may need to be taken into consideration in future studies.

5.2.3. The effect of acidified PGGA on Ca^{2+} uptake for HAp pellet remineralisation

HAp pellets were used in contrast to human teeth for decreasing the variability, as the HAp pellets have similar chemistry to that of human enamel with uniform mineral composition and porosity (Elliot, 1994; Kosoric 2006). A similar methodology for remineralisation study of HAp pellets was followed as that for human teeth.

The rate of Ca^{2+} release was highest for the HAp pellet immersed in 0.1 M acetic acid of pH 4.0, the Ca^{2+} release decreased for the HAp pellets in 0.01% NaF solution acidified to pH 4.0. The rate of demineralisation was observed to decrease for HAp pellets immersed in the respective acidified solutions with higher concentration of NaF, thus pellets treated with acidified 0.5% NaF was more effective in reducing rate of demineralisation than 0.01 %- and 0.1 % -NaF solutions acidified to pH 4.0. HAp pellets immersed in acidified 1% PGGA showed a more potent decrease in rate of Ca^{2+}

release than in acidified 0.1% NaF. Acidified 2% PGGA solution showed further reduction in rate of demineralisation of the HAp pellets. The rate of demineralisation observed for all HAp pellets immersed in the respective acidified treatment groups at pH 4.0 during first 24-hr was found to be faster in comparison with that of human teeth. The possible reason could be differences in crystal size between the teeth and the pellets affecting the rate of demineralisation.

On increasing the pH of the respective acidified treatment groups from 4.0 to 5.0 after the first 24-hr, HAp pellets in 0.1 M acetic acid continued to show demineralisation but at a slower rate than at pH 4.0. Similarly the HAp pellets immersed in acidified 0.01% NaF solution (pH 5.0) continued to show demineralisation at a slower rate than the pellets immersed in 0.1 M acetic acid solution of pH 5.0. Uptake of Ca^{2+} was observed by the HAp pellets immersed in other concentrations of acidified-NaF (0.1% NaF and 0.5% NaF) and -PGGA (1% and 2%). The HAp pellets immersed in 0.1% NaF showed the slowest rate of Ca^{2+} uptake in comparison with the HAp pellets immersed in acidified 0.5% NaF and the respective acidified PGGA solutions (1% and 2%). The increase in uptake of Ca^{2+} was observed by HAp pellets immersed in acidified 1% PGGA which was followed by acidified 0.5% NaF at pH 5.0. The HAp pellets immersed in acidified 2% PGGA showed the highest rate of decrease in the Ca^{2+} in comparison with HAp pellets treated with various acidified- NaF and -1% PGGA solution. On comparing the rate of Ca^{2+} uptake by the HAp pellets immersed in acidified treatment groups (0.1% NaF, 0.5% NaF, 1% PGGA and 2% PGGA) to that of the teeth at pH 5.0, a faster rate of Ca^{2+} reduction was observed for the HAp pellets immersed in the respective acidified treatment groups.

On increasing the pH from 5.0 to 6.0 of the respective acidified treatment groups after the second 24-hr (after 48-hr), calcium uptake was observed by all of the HAp pellets immersed in their respective acidified solutions of pH 6.0. The rate of Ca^{2+} uptake was observed to be slowest for the HAp pellets immersed in 0.1 M acetic acid. The rate of Ca^{2+} uptake was observed to increase for the HAp pellets immersed in acidified 0.01% NaF, it was followed in increasing pattern by the pellets immersed in acidified- 2% PGGA and -0.5% NaF. The rate of Ca^{2+} uptake further increased for HAp pellets immersed in acidified 0.1% NaF followed by acidified 1% PGGA. This is in disagreement with studies reporting that the CaF_2 deposit formation is directly proportional to fluoride concentration in the acidified solutions (ten Cate, 1999) which can relate to the increase in the rate of Ca^{2+} reduction from the solution. The possible reason for decrease in the rate of Ca^{2+} uptake by HAp pellets immersed in acidified 0.5% NaF could be the available amount of Ca^{2+} in the solution, as at the 56^{th} hour no free calcium was observed in the ISE graph. A similar pattern of decreasing rate of Ca^{2+} uptake was observed for the HAp pellets immersed in acidified 2% PGGA which could also have been attributed to absence of Ca^{2+} at nearly 56^{th}-hr. Apart from decrease in rate of Ca^{2+} uptake by HAp pellets in 2% PGGA acidified to pH 6.0, the results for 2% PGGA acidified to pH 5.0 showed a strong potential of remineralisation in comparison with NaF.

5.3. Proposed Mechanism of Action of PGGA

5.3.1. Demineralisation Inhibition

The FT-IR spectra of the PGGA coatings on the HAp pellet and tooth surface showed similar transmission peaks as those of the PGGA powder. The broad peak at 3260 cm^{-1} was due to the –OH and –N-H groups which could overlap in this region. A strong peak at 1580 cm^{-1} and a weaker shoulder at 1620 correspond to the amide linkages contributed by –C=O stretching and –N-H bending modes. The $–CH_2-$ bending modes were seen at 1448 and 1400 cm^{-1}. Thus both teeth and HAp pellets after immersion in 1% and 2% PGGA showed similar spectra to that of powdered PGGA, indicating that their surfaces are coated by the PGGA. This is in agreement with what has been reported by Ho et al., (2006) for PGGA FT-IR spectra. The PGGA solution is viscous and its viscosity increases with concentration. The viscosity may assist in the coating. The PGGA coating the tooth may give protection to the surface and hence plays a role in maintaining the integrity of the tooth.

5.3.2. Remineralisation Promotion

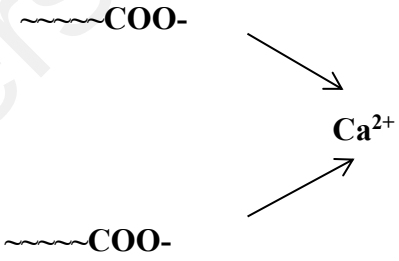


Figure 5.1: Binding of Ca^{2+} to poly-γ-glutamic acid

In this study PGGA was observed to have a potential in remineralising enamel and HAp pellets. It was demonstrated by the uptake of Ca^{2+} from the solution, using a Ca^{2+} Selective electrode and increased mineral volume confirmed by cross sectional Knoop microhardness testing. This could be attributed to the presence of free $-COO^-$ groups in PGGA which have an efficiency to bind with Ca^{2+} present in the environment (Ho et al.,

2006). The ionic interaction between the negative charge of $-COO^-$ group of two γ-glutamic acid residues in PGGA and the positive charges of the free Ca^{2+} ions is shown in Figure 5.1.

In the human saliva, the Ca^{2+} is among the trace elements which are observed in higher concentration in comparison with other elements (Chicharro et al., 1999). Therefore it can be suggested intra-orally Ca^{2+} ions can bind PGGA more potently than the other trace elements.

Studies have proven that PGGA when used in food products, increases absorption of Ca^{2+} from intestine (Tanimoto et al., 1995; Tanimoto et al., 2007). With this understanding, it is proposed that calcium bound to PGGA diffuses onto the enamel surface and is made available for the remineralisation. PGGA may promote incorporation of Ca^{2+} at a faster rate than the normal equilibrium shift mechanism even at low pH.

CHAPTER 6: CONCLUSION

The study showed that PGGA may attach to enamel and efficiently **inhibit demineralisation** and promote **remineralisation** of human dental enamel under caries-inducing conditions. This could be attributed to its high viscosity and presence of free -COO- groups. Being edible and biodegradable, it is an environmentally friendly ingredient if incorporated as an ingredient in mouthrinses as compared with other commercially-available oral healthcare products. Hence, it is suggested that the use of PGGA- based mouthrinses should be advocated for patients who are at high caries risk, suffering from xerostomia or any other salivary gland disorder.

Future Works

As PGGA has shown potential for inhibiting enamel demineralisation and promoting remineralisation of dental enamel and HAp, further work is required to quantify the effectiveness of PGGA under different conditions. Therefore there are other novel ideas which require investigation with the active ingredient PGGA, as listed below

i) The potential of PGGA should be investigated with a specific amount of calcium and treating the enamel in a pH cycling model.
ii) Phosphorus content of the solution alongside of calcium to provide a better idea with respect to the mineral phase.
iii) Diffusion coefficient for PGGA molecules and calcium ions.
iv) The binding affinities for calcium (PGGA/ HAp), the effect of pH on PGGA binding calcium and HAp surfaces.

v) The effectiveness of the PGGA should be investigated in an intra-oral environment.

vi) The effectiveness of PGGA to inhibit demineralisation in normal oral health conditions particularly for patients with or prone to develop incipient lesions.

vii) The efficacy of PGGA to remineralise incipient lesions in patients with normal oral health.

viii) Other than being incorporated into a mouthrinse, the effect of PGGA as a main ingredient in artificial saliva for patients with xerostomia or other salivary gland disorders should be determined.

Limitation of the Study

The limitation of the study was due to difficulty in availability of certain equipment and financial constrain with respect to research grant. In order to determine the effect of PGGA in a pH-cycling model and the combinational effect with different ions such as phosphates would have been better investigated while using Micro-CT. But due to unavailability of quality imaging equipment and programmer required to reconstruct the images it was not possible. Though the material is edible, but it requires further testing as listed above before its trial in a complex intra-oral environment.

REFERENCES

Agashe, M., Raut, V., Stuart, S. J., & Latour, R. A. (2005). Molecular simulation to characterize the adsorption behavior of a fibrinogen gamma-chain fragment. *Langmuir, 21*(3), 1103-1117.

Al-Jawad, M., & Anderson, P. (2014). Biomineralization and Biodemineralization of Enamel. *Handbook of Oral Biomaterials*, (1st ed.,pp 57-80):CRC Press.

Ali, K., & Creanor, S. (2016). Saliva. *Essential Clinical Oral Biology*, (1st ed.,pp 99-104): John Wiley & Sons Ltd,U.K.

An, B. B., Wang, R. R., & Zhang, D. S. (2012). Region-dependent micro damage of enamel under indentation. *Acta Mechanica Sinica, 28*(6), 1651-1658.

Anderson, P., Bollet-Quivogne, F. R. G., Dowker, S. E. P., & Elliott, J. C. (2004). Demineralization in enamel and hydroxyapatite aggregates at increasing ionic strengths. *Archives of Oral Biology, 49*(3), 199-207.

Anderson, P., & Creanor, S. (2016). Enamel, *Essential Clinical Oral Biology* (1st ed.,pp 23-33): John Wiley & Sons Ltd, U.K.

Anderson, P., Levinkind, M., & Elliott, J. C. (1998). Scanning microradiographic studies of rates of in vitro demineralization in human and bovine dental enamel. *Archives of Oral Biology, 43*(8), 649-656.

Andrade, J. D., & Hlady, V. (1986). Protein Adsorption and Materials Biocompatibility - a Tutorial Review and Suggested Hypotheses. *Advances in Polymer Science, 79*, 1-63.

Angmar-Mansson, B., & ten Bosch, J. J. (2001). Quantitative light-induced fluorescence (QLF): a method for assessment of incipient caries lesions. *Dentomaxillofacial Radiology, 30*(6), 298-307.

Arends, J., Ruben, J. L., & Inaba, D. (1997). Major topics in quantitative microradiography of enamel and dentin: R parameter, mineral distribution visualization, and hyper-remineralization. *Adv Dent Res, 11*(4), 403-414.

Arends, J., & Tenbosch, J. J. (1992). Demineralization and Remineralization Evaluation Techniques. *Journal of Dental Research, 71*, 924-928.

Banerjee, A., & Watson, T. F. (2015). Dental Hard Tissue Pathologies, aetiology, and their clinical manifestation. *Pickard's Guide to Minimally Invasive Operative Dentistry.* (10 ed., pp. 1-15): OUP Oxford.

Barbour, M. E., & Rees, J. S. (2004). The laboratory assessment of enamel erosion: a review. *Journal of Dentistry, 32*(8), 591-602.

Berkovitz , B. K. B., Moxham, B. J., Linden, R. W. A., & Sloan, A. J. (2011). Dental tissue I, Enamel: structure, composition and development *Master Dentistry, Oral Biology* (1st ed., Vol. 3, pp. 142-160): Churchill Livingstone Elsvier.

Bhat, A. R., Irorere, V. U., Bartlett, T., Hill, D., Kedia, G., Morris, M. R., . . . Radecka, I. (2013). Bacillus subtilis natto: a non-toxic source of poly-gamma-glutamic acid that could be used as a cryoprotectant for probiotic bacteria. *Amb Express,* 3(1),36.

Birrer, G. A., Cromwick, A. M., & Gross, R. A. (1994). Gamma-Poly(Glutamic Acid) Formation by Bacillus-Licheniformis 9945a - Physiological and Biochemical-Studies. *International Journal of Biological Macromolecules, 16*(5), 265-275.

Bollen, C. M. L., Lambrechts, P., & Quirynen, M. (1997). Comparison of surface roughness of oral hard materials to the threshold surface roughness for bacterial plaque retention: A review of the literature. *Dental Materials, 13*(4), 258-269.

Borges, A. B., Scaramucci, T., Lippert, F., Zero, D. T., & Hara, A. T. (2014). Erosion Protection by Calcium Lactate/Sodium Fluoride Rinses under Different Salivary Flows in vitro. *Caries Research, 48*(3), 193-199.

Bovarnick, M. (1942). The formation of extracellular D(-)glutamic acid polypeptide by Bacillus subtilis. *Journal of Biological Chemistry*(145), 415–424.

Boyde, A. (1964). Tooth Enamel. In M. V. Stack & R. W. Fearnhead (Eds.), *The structure of developing mammalian dental enamel.* (pp. 63-167 & 192-194). Bristol.: Wright.

Briner, W. W., Gray, J. A., & Francis, M. D. (1974). Significance of enamel remineralization. *J Dent Res, 53*(2), 239-243.

Buchalla, W., Imfeld, T., Attin, T., Swain, M. V., & Schmidlin, P. R. (2008). Relationship between nanohardness and mineral content of artificial carious enamel lesions. *Caries Research, 42*(3), 157-163.

Buescher, J. M., & Margaritis, A. (2007). Microbial biosynthesis of polyglutamic acid biopolymer and applications in the biopharmaceutical, biomedical and food industries. *Critical Reviews in Biotechnology, 27*(1), 1-19.

Buzalaf , M. A., Pessan , J. P., Honorio , H. M., & ten Cate , J. M. (2011). Mechanisms of action of fluoride for caries control. [Review]. *Monogr Oral Sci, 22*, 97-114.

Buzalaf, M. A. R., Hannas, A. R., & Kato, M. T. (2012). Saliva and dental erosion. *Journal of Applied Oral Science, 20*(5), 493-502.

Caldwell, R. C., Muntz, M. L., Gilmore, R. W., & Pigman, W. (1957). Microhardness studies of intact surface enamel. *J Dent Res, 36*(5), 732-738.

Candela, T., & Fouet, A. (2006). Poly-gamma-glutamate in bacteria. *Molecular Microbiology, 60*(5), 1091-1098.

Carpenter, G. H. (2013). The Secretion, Components, and Properties of Saliva. *Annual Review of Food Science and Technology, Vol 4, 4*, 267-276.

Carvalho, T. S., & Lussi, A. (2014). Combined effect of a fluoride-, stannous- and chitosan-containing toothpaste and stannous-containing rinse on the prevention of initial enamel erosion-abrasion. *Journal of Dentistry, 42*(4), 450-459.

Chen, M., Briscoe, W. H., Armes, S. P., & Klein, J. (2009). Lubrication at Physiological Pressures by Polyzwitterionic Brushes. *Science, 323*(5922), 1698-1701.

Cheng, C., Asada, Y., & Aida, T. (1989). Production of Gamma-Polyglutamic Acid by Bacillus-Licheniformis A35 under Denitrifying Conditions. *Agricultural and Biological Chemistry, 53*(9), 2369-2375.

Chew, H. P., Zakian, C. M., Pretty, I. A., & Ellwood, R. P. (2014). Measuring Initial Enamel Erosion with Quantitative Light-Induced Fluorescence and Optical Coherence Tomography: An in vitro Validation Study. *Caries Research, 48*(3), 254-262.

Chicharro, J. L., Serrano, V., Urena, R., Gutierrez, A. M., Carvajal, A., Fernandez-Hernando, P., & Lucia, A. (1999). Trace elements and electrolytes in human resting mixed saliva after exercise. [Clinical TrialComparative StudyResearch Support, Non-U.S. Gov't]. *Br J Sports Med, 33*(3), 204-207.

Chin, K. O. A., Johnsson, M., Bergey, E. J., Levine, M. J., & Nancollas, G. H. (1993). A Constant Composition Kinetics Study of the Influence of Salivary Cystatins, Statherin, Amylase and Human Serum-Albumin on Hydroxyapatite Dissolution. *Colloids and Surfaces a-Physicochemical and Engineering Aspects, 78*, 229-234.

Choi, A. L., Sun, G. F., Zhang, Y., & Grandjean, P. (2012). Developmental Fluoride Neurotoxicity: A Systematic Review and Meta-Analysis. *Environmental Health Perspectives, 120*(10), 1362-1368.

Chu, J. P., Li, J. Y., Hao, Y. Q., & Zhou, X. D. (2007). Effect of compounds of Galla chinensis on remineralisation of initial enamel carious lesions in vitro. *Journal of Dentistry, 35*(5), 383-387.

Cochrane, N. J., Walker, G. D., Manton, D. J., & Reynolds, E. C. (2012). Comparison of quantitative light-induced fluorescence, digital photography and transverse microradiography for quantification of enamel remineralization. *Australian dental journal, 57*(3), 271-276.

Cole, A. S., & Eastoe, J. E. (1988). Chapter 33 - The oral environment *Biochemistry and Oral Biology (Second Edition)* (pp. 475-489): Butterworth-Heinemann.

Cunha-Cruz, J., Scott, J., Rothen, M., Mancl, L., Lawhorn, T., Brossel, K., . . . Northwest Practice-based, R. C. i. E.-b. D. (2013). Salivary characteristics and dental caries: evidence from general dental practices. [Research Support, N.I.H., Extramural]. *J Am Dent Assoc, 144*(5), e31-40.

da Silva, A. P. P., Goncalves, R. S., Borges, A. F. S., Bedran-Russo, A. K., & Shinohara, M. S. (2015). Effectiveness of plant-derived proanthocyanidins on demineralization on enamel and dentin under artificial cariogenic challenge. *Journal of Applied Oral Science, 23*(3), 302-309.

Darling, A. I. (1961). The selective attack of caries on the dental enamel. *Ann R Coll Surg Engl, 29*, 354-369.

Dawes, C. (1974). Rhythms in salivary flow rate and composition. [Review]. *Int J Chronobiol, 2*(3), 253-279.

Dawes, C. (2003). What is the critical pH and why does a tooth dissolve in acid? *Journal-Canadian Dental Association, 69*(11), 722-725.

de Cesaro, A., da Silva, S. B., da Silva, V. Z., & Ayub, M. A. Z. (2014). Physico-chemical and rheological characterization of poly-gamma-glutamic acid produced by a new strain of Bacillus subtilis. *European Polymer Journal, 57*, 91-98.

Dey, S., & Giri, B. (2016). Fluoride Fact on Human Health and Health Problems: A Review. *Medical & Clinical Reviews*, 2(1:2), 1-6.

Dijkema, T. (2013). Salivary gland sparing radiotherapy. PhD thesis, Utrecht University, The Netherlands, (pp. 9-22).

Donald, A. M. (2003). The use of environmental scanning electron microscopy for imaging wet and insulating materials. *Nature Materials, 2*(8), 511-516.

Douglas, W. H., Reeh, E. S., Ramasubbu, N., Raj, P. A., Bhandary, K. K., & Levine, M. J. (1991). Statherin - a Major Boundary Lubricant of Human Saliva. *Biochemical and Biophysical Research Communications, 180*(1), 91-97.

Dowker, S. E. P., Anderson, P., & Elliott, J. C. (1999). Real-time measurement of in vitro enamel demineralization in the vicinity of the restoration-tooth interface. *Journal of Materials Science-Materials in Medicine, 10*(6), 379-382.

Eanes, E. D. (1979). Enamel apatite: chemistry, structure and properties. [Comparative Study]. *J Dent Res, 58*(Spec Issue B), 829-836.

Eastoe, J. E. (1960). Organic matrix of tooth enamel. *Nature, 187*, 411-412.

Eastoe, J. E. (1963). The Amino Acid Composition of Proteins from the Oral Tissues. Ii. The Matrix Proteins in Dentine and Enamel from Developing Human Deciduous Teeth. *Arch Oral Biol, 8*, 633-652.

Eisenburger, M., Hughes, J., West, N. X., Shellis, R. P., & Addy, M. (2001). The use of ultrasonication to study remineralisation of eroded enamel. *Caries Research, 35*(1), 61-66.

Ekstrand, J., & Oliveby, A. (1999). Fluoride in the oral environment. *Acta Odontologica Scandinavica, 57*(6), 330-333.

Elgavish, G. A., Hay, D. I., & Schlesinger, D. H. (1984). H-1 and P-31 Nuclear Magnetic-Resonance Studies of Human Salivary Statherin. *International Journal of Peptide and Protein Research, 23*(3), 230-234.

Elliot, J. (1994). Hydroxyapatite and nonstoichiometric apatites. *Structure and Chemistry of the Apatites and Other Calcium Orthophosphates. Studies in Inorganic Chemistry, 18*, 111-190.

Elliot, J., Bollet-Quivogne, F., Anderson, P., Dowker, S., Wilson, R., & Davis, G. (2005). Acidic demineralization of apatites studied by scanning X-ray microradiography and microtomography. *Mineralogical Magazine, 69*(5), 643-652.

Elliott, J. (2005). *Using narrative in social research: Qualitative and quantitative approaches,* (1st ed.,pp 1-170): Sage Publications,U.K.

Ellwood, R., Fejerskov, O., Cury, J. A., & Clarkson, B. (2008). Fluorides in caries control *Dental Caries:The Disease and its Clinical Management* (2 ed., pp. 287-328): Blackwell Munksgaard, U.K.

Fabian, T. K., Hermann, P., Beck, A., Fejerdy, P., & Fabian, G. (2012). Salivary Defense Proteins: Their Network and Role in Innate and Acquired Oral Immunity. *International Journal of Molecular Sciences, 13*(4), 4295-4320.

Featherstone, J. D. B., (1992). Consensus conference on intra-oral models: evaluation techniques. [Consensus Development Conference Review]. *J Dent Res, Spec No 71*, 955-956.

Featherstone, J. D. B., (2008). Dental caries: a dynamic disease process. *Australian dental journal, 53*(3), 286-291.

Featherstone , J. D. B., Glena, R., Shariati, M., & Shields, C. P. (1990). Dependence of in vitro demineralization of apatite and remineralization of dental enamel on fluoride concentration. [Research Support, U.S. Gov't, P.H.S.]. *J Dent Res, 69 Spec No*, 620-625; discussion 634-626.

Featherstone, J. D. B., & Lussi, A. (2006). Understanding the chemistry of dental erosion. *Monogr Oral Sci, 20*, 66-76.

Featherstone , J. D. B. (1999). Prevention and reversal of dental caries: role of low level fluoride. *Community Dentistry and Oral Epidemiology, 27*(1), 31-40.

Featherstone , J. D. B. (2000). The science and practice of caries prevention. *Journal of the American Dental Association, 131*(7), 887-899.

Featherstone , J. D. B., & Rodgers, B. E. (1981). Effect of Acetic, Lactic and Other Organic-Acids on the Formation of Artificial Carious Lesions. *Caries Research, 15*(5), 377-385.

Featherstone, J. D. B., Shariati, M., Brugler, S., Fu, J., & White, D. J. (1988). Effect of an Anticalculus Dentifrice on Lesion Progression under Ph Cycling Conditions Invitro. *Caries Research, 22*(6), 337-341.

Featherstone , J. D. B., ten Cate, J. M., Shariati, M., & Arends, J. (1983). Comparison of Artificial Caries-Like Lesions by Quantitative Microradiography and Microhardness Profiles. *Caries Research, 17*(5), 385-391.

Feeney, R. N., Zermeno, J. P., Reid, D. J., Nakashima, S., Sano, H., Bahar, A., . . . Smith, T. M. (2010). Enamel thickness in Asian human canines and premolars. *Anthropological Science, 118*(3), 191-198.

Fejerskov, O. (2004). Changing paradigms in concepts on dental caries: Consequences for oral health care. *Caries Research, 38*(3), 182-191.

Fejerskov , O., & Kidd, E. A. M. (2008). Defining the disease: an introduction *Dental Caries: The Disease and Its Clinical Management* (2nd ed., pp. 3-6): Wiley-Blackwell.

Fenker, M., Balzer, M., Jehn, H. A., Kappl, H., Lee, J. J., Lee, K. H., & Park, H. S. (2002). Improvement of the corrosion resistance of hard wear resistant coatings by intermediate plasma etching or multilayered structure. *Surface & Coatings Technology, 150*(1), 101-106.

Finke, M., Hughes, J. A., Parker, D. M., & Jandt, K. D. (2001). Mechanical properties of in situ demineralised human enamel measured by AFM nanoindentation. *Surface Science, 491*(3), 456-467.

Frazier, P. D., Little, M. F., & Casciani, F. S. (1967). X-ray diffraction analysis of human enamel containing different amounts of fluoride. *Arch Oral Biol, 12*(1), 35-42.

Frencken, J. E., Peters, M. C., Manton, D. J., Leal, S. C., Gordan, V. V., & Eden, E. (2012). Minimal intervention dentistry for managing dental caries - a review. *International Dental Journal, 62*(5), 223-243.

Fried, D., Xie, J., Shafi, S., Featherstone, J. D. B., Breunig, T. M., & Le, C. (2002). Imaging caries lesions and lesion progression with polarization sensitive optical coherence tomography. *Journal of Biomedical Optics, 7*(4), 618-627.

Ganss, C., Lussi, A., & Schlueter, N. (2014). The histological features and physical properties of eroded dental hard tissues. [Review]. *Monogr Oral Sci, 25*, 99-107.

Ganss, C., Schlueter, N., & Klimek, J. (2007). Retention of KOH-soluble fluoride on enamel and dentine under erosive conditions - A comparison of in vitro and in situ results. *Archives of Oral Biology, 52*(1), 9-14.

Giannakopoulos, A. E., & Zisis, T. (2011). Analysis of Knoop indentation. *International Journal of Solids and Structures, 48*(1), 175-190.

Gibbons, R. J., & Hay, D. I. (1988). Human Salivary Acidic Proline-Rich Proteins and Statherin Promote the Attachment of Actinomyces-Viscosus Ly7 to Apatitic Surfaces. *Infection and Immunity, 56*(2), 439-445.

Goobes, G., Raghunathan, V., Louie, E. A., Gibson, J. M., Olsen, G. L., & Drobny, G. P. (2006). A REDOR study of diammonium hydrogen phosphate: A model for distance measurements from adsorbed molecules to surfaces. *Solid state nuclear magnetic resonance, 29*(1), 242-250.

Gururaja, T. L., & Levine, M. J. (1996). Solid-phase synthesis and characterization of human salivary statherin: A tyrosine-rich phosphoprotein inhibitor of calcium phosphate precipitation. *Peptide Research, 9*(6), 283-289.

Hannig, M., Hess, N. J., Hoth-Hannig, W., & De Vrese, M. (2003). Influence of salivary pellicle formation time on enamel demineralization--an in situ pilot study. [Comparative Study Research Support, Non-U.S. Gov't]. *Clin Oral Investig, 7*(3), 158-161.

Hara, A. T., Carvalho, J. C., & Zero, D. T. (2015). Causes of dental erosion: extrinsic factors *Dental Erosion and Its Clinical Management* (pp. 69-96): Springer.

Harris, J. C., Elcock, C., Sidebotham, P. D., & Welbury, R. R. (2009). Safeguarding children in dentistry: 2. Do paediatric dentists neglect child dental neglect? *British Dental Journal, 206*(9), 465-470.

Hay DI, Moreno EC. Statherin and the acidic proline-rich proteins. In: Tenovuo J, editor. Human Saliva: Clinical Chemistry and Microbiology. I. Boca Raton, FL, USA: CRC Press; 1989. pp. 131–150.

Hay, D. I., Smith, D. J., Schluckebier, S. K., & Moreno, E. C. (1984). Relationship between Concentration of Human Salivary Statherin and Inhibition of Calcium-Phosphate Precipitation in Stimulated Human-Parotid Saliva. *Journal of Dental Research, 63*(6), 857-863.

Haynes, C. A., & Norde, W. (1995). Structures and Stabilities of Adsorbed Proteins. *Journal of Colloid and Interface Science, 169*(2), 313-328.

He, L. H., & Swain, M. V. (2008). Understanding the mechanical behaviour of human enamel from its structural and compositional characteristics. *Journal of the Mechanical Behavior of Biomedical Materials, 1*(1), 18-29.

Hemmens, E. S., Blayney, J. R., & et al. (1946). The microbic flora of the dental plaque in relation to the beginning of caries. *J Dent Res, 25*, 195-205.

Heymann , H. O., Swift, E. J., Ritter, A. V., & Sturdevant, C. M. (2013). In Dental caries: etiology, clinical characteristics,risk assessment, and management. *Sturdevant's art and science of Operative Dentistry.* (6 ed., pp. 41-88): Elsevier/Mosby, St. Louis, Mo. .

Heymann, H. O., Swift Jr, E. J., & Ritter, A. V. (2014). *Sturdevant's art & science of operative dentistry*: Elsevier Health Sciences.

Higham , S. (2014). Caries Process and Prevention Strategies: Demineralization/Remineralization. *dentalcare.com Continuing Education Course, 501*, 1-21.

Higham, S. M., Pretty, I. A., Edgar, W. M., & Smith, P. W. (2005). The use of in situ models and QLF for the study of coronal caries. *Journal of Dentistry, 33*(3), 235-241.

Higuchi, W. I., Mir, N. A., Patel, P. R., Becker, J. W., & Hefferen, J. J. (1969). Quantitation of enamel demineralization mechanisms. 3. A critical examination of the hydroxyapatite model. *J Dent Res, 48*(3), 396-409.

Ho, G. H., Ho, T. I., Hsieh, K. H., Su, Y. C., Lin, P. Y., Yang, J., . . . Yang, S. C. (2006). gamma-polyglutamic acid produced by Bacillus subtilis (natto): Structural characteristics, chemical properties and biological functionalities. *Journal of the Chinese Chemical Society, 53*(6), 1363-1384.

Ho, G. H., Ho, T. I., Hsieh, K. H., Su, Y. C., Lin, P. Y., Yang, J., . . . Yang, S. C. (2006). γ- Polyglutamic Acid Produced by Bacillus Subtilis (Natto): Structural Characteristics, Chemical Properties and Biological Functionalities. *Journal of the Chinese Chemical Society, 53*(6), 1363-1384.

Huang, S., Gao, S., Cheng, L., & Yu, H. (2011). Remineralization Potential of Nano-Hydroxyapatite on Initial Enamel Lesions: An in vitro Study. *Caries Research, 45*(5), 460-468.

Huang, S. B., Gao, S. S., Cheng, L., & Yu, H. Y. (2010). Combined effects of nano-hydroxyapatite and Galla chinensis on remineralisation of initial enamel lesion in vitro. *Journal of Dentistry, 38*(10), 811-819.

Isola, M., Solinas, P., Proto, E., Cossu, M., & Lantini, M. S. (2011). Reduced statherin reactivity of human submandibular gland in diabetes. *Oral Diseases, 17*(2), 217-220.

Ivanovics, G., & Erdos, L. (1937). The capsule hapten of anthrax bacilli. *Zeitschriftfur Immunitatsforschung Experimentelle Therapie*(90), 5-19.

Jones, R. S., Darling, C. L., Featherstone, J. D., & Fried, D. (2006). Remineralization of in vitro dental caries assessed with polarization-sensitive optical coherence tomography. [Research Support, N.I.H., Extramural Research Support, U.S. Gov't, Non-P.H.S.]. *J Biomed Opt, 11*(1), 014016. doi: 10.1117/1.2161192

Joniot, S. B., Gregoire, G. L., Auther, A. M., & Roques, Y. M. (2000). Three-dimensional optical profilometry analysis of surface states obtained after finishing sequences for three composite resins. *Operative Dentistry, 25*(4), 311-315.

Kakaboura, A., Fragouli, M., Rahiotis, C., & Silikas, N. (2007). Evaluation of surface characteristics of dental composites using profilometry, scanning electron, atomic force microscopy and gloss-meter. *Journal of Materials Science-Materials in Medicine, 18*(1), 155-163.

Kang, H., Jiao, J. J., Lee, C., Darling, C. L., & Fried, D. (2010). Imaging early demineralization with PS-OCT. *Proc SPIE Int Soc Opt Eng, 7549*. doi: 10.1117/12.849343.

Kay, M. I., Young, R. A., & Posner, A. S. (1964). Crystal Structure of Hydroxyapatite. *Nature, 204*, 1050-1052.

Kedia, G., Hill, D., Hill, R., & Radecka, I. (2010). Production of Poly-gamma-Glutamic Acid by Bacillus subtilis and Bacillus licheniformis with Different Growth Media. *Journal of Nanoscience and Nanotechnology, 10*(9), 5926-5934.

Kidokoro, M., Nakamoto, T., Mukaibo, T., Kondo, Y., Munemasa, T., Imamura, A., . . . Hosokawa, R. (2014). Na+-K+-2Cl(-) cotransporter-mediated fluid secretion increases under hypotonic osmolarity in the mouse submandibular salivary gland. *American Journal of Physiology-Renal Physiology, 306*(10), F1155-F1160.

Kielbassa, A. M., Wrbas, K. T., Schulte-Monting, J., & Hellwig, E. (1999). Correlation of transversal microradiography and microhardness on in situ-induced demineralization in irradiated and nonirradiated human dental enamel. *Archives of Oral Biology, 44*(3), 243-251.

Knoop, F., Peters, C. G., & Emerson, W. B. (1939). A sensitive pyramidal-diamond tool for indentation measurements. *Journal of Research of the National Bureau of standards, 23*(1), 39-61.

Knosp, W. M., Knox, S. M., & Hoffman, M. P. (2012). Salivary gland organogenesis. *Wiley Interdisciplinary Reviews-Developmental Biology, 1*(1), 69-82.

Ko, Y. H., & Gross, R. A. (1998). Effects of glucose and glycerol on gamma-poly(glutamic acid) formation by Bacillus licheniformis ATCC 9945a. *Biotechnology and Bioengineering, 57*(4), 430-437.

Kosoric , J. (2006). *Influence of salivary proteins as biomineralisation of hydroxyapatite and enamel using scanning microradiography: the effect on the N-terminus of statherin on demineralisation of hydroxyapatite.* Queen Mary, University of London.

Kosoric, J., Williams, R. A. D., Hector, M. P., & Anderson, P. (2007). A synthetic peptide based on a natural salivary protein reduces demineralisation in model systems for dental caries and erosion. *International Journal of Peptide Research and Therapeutics, 13*(4), 497-503.

H. Kubota, H. Fukuda, H. Takebe, T. Endo (1992), US Patent 5,118,784 (Meiji Seika Kabushiki Kaisha).

Kuriakose, S., Sundaresan, C., Mathai, V., Khosla, E., & Gaffoor, F. M. (2013). A comparative study of salivary buffering capacity, flow rate, resting pH, and salivary Immunoglobulin A in children with rampant caries and caries-resistant children. [Comparative Study]. *J Indian Soc Pedod Prev Dent, 31*(2), 69-73.

Larsen, M. (1990). Chemical events during tooth dissolution. *Journal of Dental Research, 69*(2 suppl), 575-580.

Larsen, M. J., & Bruun, C. (1986). Enamel/saliva – inorganic chemical reactions. In A. Thylstrup & O. Fejerskov (Eds.), *Textbook of cariology* (pp. 181–203). Copenhagen: Munksgaard.

Larsen , M. J., & Jensen , S. J. (1994). Experiments on the Initiation of Calcium-Fluoride Formation with Reference to the Solubility of Dental Enamel and Brushite. *Archives of Oral Biology, 39*(1), 23-27.

Latino, C., Troendle, K., & Summitt, J. B. (2001). Support of undermined occlusal enamel provided by restorative materials. *Quintessence International, 32*(4), 287-291.

Laurance-Young, P., Bozec, L., Gracia, L., Rees, G., Lippert, F., Lynch, R. J. M., & Knowles, J. C. (2011). A review of the structure of human and bovine dental hard tissues and their physicochemical behaviour in relation to erosive challenge and remineralisation. *Journal of Dentistry, 39*(4), 266-272.

Lee, B. S., Chou, P. H., Chen, S. Y., Liao, H. Y., & Chang, C. C. (2015). Prevention of enamel demineralization with a novel fluoride strip: enamel surface composition and depth profile. [Research Support, Non-U.S. Gov't]. *Sci Rep, 5*, 13352. doi: 10.1038/srep13352

Leito, J. T., Ligtenberg, A. J. M., Nazmi, K., & Veerman, E. C. I. (2009). Identification of salivary components that induce transition of hyphae to yeast in Candida albicans. *Fems Yeast Research, 9*(7), 1102-1110.

Leonard, C. G., Housewright, R. D., & Thorne, C. B. (1958). Effects of some metallic ions on glutamyl polypep-tide synthesis by Bacillus subtilis. *Journal of Bacteriology, 76*, 499-503.

Levine, M. J. (1993). Salivary macromolecules. *Annals of the New York Academy of Sciences, 694*(1), 11-16.

Lewis, C. W. (2014). Fluoride and dental caries prevention in children. *Pediatr Rev, 35*(1), 3-15.

Li, X., Wang, J., Joiner, A., & Chang, J. (2014). The remineralisation of enamel: a review of the literature. *J Dent, 42 Suppl 1*, S12-20.

Lin, Y.-H., Chen, C.-T., Liang, H.-F., Kulkarni, A. R., Lee, P.-W., Chen, C.-H., & Sung, H.-W. (2007). Novel nanoparticles for oral insulin delivery via the paracellular pathway. *Nanotechnology, 18*(10), 105102.

Lindh, L., Glantz, P. O., Stromberg, N., & Arnebrant, T. (2002). On the adsorption of human acidic proline-rich proteins (PRP-1 and PRP-3) and statherin at solid/liquid interfaces. *Biofouling, 18*(2), 87-94.

Long, J. R., Shaw, W. J., Stayton, P. S., & Drobny, G. P. (2001). Structure and dynamics of hydrated statherin on hydroxyapatite as determined by solid-state NMR. *Biochemistry, 40*(51), 15451-15455.

Louie, T., Lee, C., Hsu, D., Hirasuna, K., Manesh, S., Staninec, M., . . . Fried, D. (2010). Clinical Assessment of Early Tooth Demineralization Using Polarization Sensitive Optical Coherence Tomography. *Lasers in Surgery and Medicine, 42*(10), 738-745.

Lussi, A., Hellwig, E., & Klimek, J. (2012). Fluorides—mode of action and recommendations for use. *Schweizer Monatsschrift fur Zahnmedizin, 122*(11), 1030.

Lynch, C. D., O'Sullivan, V. R., Dockery, P., McGillycuddy, C. T., & Sloan, A. J. (2010). Hunter-Schreger Band patterns in human tooth enamel. *Journal of Anatomy, 217*(2), 106-115.

Magalhaes, A. C., Wiegand, A., Rios, D., Buzalaf, M. A., & Lussi, A. (2011). Fluoride in dental erosion. [Review]. *Monogr Oral Sci, 22*, 158-170.

Manconi, B., Fanali, C., Cabras, T., Inzitari, R., Patamia, M., Scarano, E., . . . Sanna, M. T. (2010). Structural characterization of a new statherin from pig parotid granules. *Journal of Peptide Science, 16*(6), 269-275.

Marinho, V. C. (2008). Evidence-based effectiveness of topical fluorides. [Research Support, Non-U.S. Gov'tReview]. *Adv Dent Res, 20*(1), 3-7.

Marsh, P., & Martin, M. (1992). Dental caries. *Oral microbiology* (pp. 133-166): Springer.

Marsh, P. D. (1995). The role of microbiology in models of dental caries. [Review]. *Adv Dent Res, 9*(3), 244-254; discussion 255-269.

Marsh, P. D., & Martin, M. V. (2009). Dental plaque is an important risk factor and prerequisite for dental caries which develops due to the metabolic activities of the dental plaque bacteria. *Oral Microbiology.* (5 ed., pp. 103-145). U.K.: Churchill Livingstone, Elsevier.

Milly, H., Festy, F., Watson, T. F., Thompson, I., & Banerjee, A. (2014). Enamel white spot lesions can remineralise using bio-active glass and polyacrylic acid-modified bio-active glass powders. *Journal of Dentistry, 42*(2), 158-166.

Mitsiadis, T. (2013). Development, Pathology and Regeneration of Dental and Orofacial Tissues: A Molecular Approach. *Mol Biol, 2*, e107.

Mohammed, N. R., Kent, N. W., Lynch, R. J. M., Karpukhina, N., Hill, R., & Anderson, P. (2013). Effects of Fluoride on in vitro Enamel Demineralization Analyzed by F-19 MAS-NMR. *Caries Research, 47*(5), 421-428.

Mohammed, N. R., Lynch, R. J. M., & Anderson, P. (2014). Effects of fluoride concentration on enamel demineralization kinetics in vitro. *Journal of Dentistry, 42*(5), 613-618.

Mohammed, N. R., Mneimne, M., Hill, R. G., Al-Jawad, M., Lynch, R. J. M., & Anderson, P. (2014). Physical chemical effects of zinc on in vitro enamel demineralization. *Journal of Dentistry, 42*(9), 1096-1104.

Morelli, C. F., Calvio, C., Biagiotti, M., & Speranza, G. (2014). pH-Dependent hydrolase, glutaminase, transpeptidase and autotranspeptidase activities of Bacillus subtilis gamma-glutamyltransferase. *Febs Journal, 281*(1), 232-245.

Moreno, E. C., Varughese, K., & Hay, D. I. (1979). Effect of human salivary proteins on the precipitation kinetics of calcium phosphate. [Research Support, U.S. Gov't, P.H.S.]. *Calcif Tissue Int, 28*(1), 7-16.

Moynihan, P., & Petersen, P. E. (2004). Diet, nutrition and the prevention of dental diseases. *Public Health Nutrition, 7*(1A), 201-226.

Naganagowda, G. A., Gururaja, T. L., & Levine, M. J. (1998). Delineation of conformational preferences in human salivary statherin by H-1, P-31 NMR and CD studies: Sequential assignment and structure-function correlations. *Journal of Biomolecular Structure & Dynamics, 16*(1), 91-107.

Nanci, A. (2014). Enamel:Composition, formation and structure, *Ten Cate's Oral Histology:Development, Structure and Function* (8th ed. pp 122-164): Elsevier, USA.

Navazesh, M., & Kumar, S. K. S. (2008). Measuring salivary flow - Challenges and opportunities. *Journal of the American Dental Association, 139*, 35s-40s.

Naveen, S., Asha, M., Shubha, M., Bajoria, A., & Jose, A. (2014). Salivary Flow Rate, pH and Buffering Capacity in Pregnant and Non Pregnant Women–A Comparative Study. *JMED Res, 2014. doi: 10.5171/2014.506946.*

Newbrun, E., & Pigman, W. (1960). The hardness of enamel and dentine*. *Australian dental journal, 5*(4), 210-217.

Nirmala, S., & Reddy, V. S. (2011). A comparative study of pH modulation and trace elements of various fruit juices on enamel erosion: an in vitro study. *Journal of Indian Society of Pedodontics and Preventive Dentistry, 29*(3), 205.

Nurul Islam (2009). Histopathology of Dental Caries. Retrieved Oct 29, 2009 from http://www.kck.usm.my/ppsg/histology/Histopathology_of_dental_Dental_caries_1.ppt

O'Donnell, M. D. (2011). Predicting bioactive glass properties from the molecular chemical composition: Glass transition temperature. *Acta Biomaterialia, 7*(5), 2264-2269.

O'Sullivan, E., & Milosevic, A. (2008). UK National Clinical Guidelines in Paediatric Dentistry: diagnosis, prevention and management of dental erosion. *International Journal of Paediatric Dentistry, 18*, 29-38.

Ogaard, B., & Rolla, G. (1992). The in vivo orthodontic banding model for vital teeth and the in situ orthodontic banding model for hard-tissue slabs. [Review]. *J Dent Res, 71 Spec No*, 832-835.

Ogunleye, A., Bhat, A., Irorere, V. U., Hill, D., Williams, C., & Radecka, I. (2015). Poly-γ-glutamic acid: production, properties and applications. *Microbiology, 161*(Pt 1), 1-17.

Owens, G. (2013). *In vitro caries: dental plaque formation and acidogenicity* (Doctoral dissertation, University of Liverpool).

Paolinelis, G., Watson, T. F., & Banerjee, A. (2006). Microhardness as a predictor of sound and carious dentine removal using alumina air abrasion. *Caries Research, 40*(4), 292-295.

Papagerakis, P., & Mitsiadis, T. (2013). Development and structure of teeth and periodontal tissues. *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism,* (8th ed.,pp 491-495): *New York, John Wiley & Sons, USA*.

Patel, V. N., & Hoffman, M. P. (2014). Salivary gland development: A template for regeneration. *Seminars in Cell & Developmental Biology, 25*, 52-60.

Petersen, P. E., & Phantumvanit, P. (2012). Perspectives in the Effective Use of Fluoride in Asia. *Journal of Dental Research, 91*(2), 119-121.

Pickles, M. J. (2006). Tooth wear. [Review]. *Monogr Oral Sci, 19*, 86-104.

Pretty, I. A., Smith, P. W., Edgar, W. M., & Higham, S. M. (2003). Detection of in vitro demineralization adjacent to restorations using quantitative light induced fluorescence (QLF). *Dental Materials, 19*(5), 368-374.

Proctor, G. B. (2016). The physiology of salivary secretion. *Periodontology 2000, 70*(1), 11-25.

Proctor, G. B., & Carpenter, G. H. (2014). Salivary secretion: mechanism and neural regulation. [Review]. *Monogr Oral Sci, 24*, 14-29.

Proctor, G. B., Hamdan, S., Carpenter, G. H., & Wilde, P. (2005). A statherin and calcium enriched layer at the air interface of human parotid saliva. *Biochemical Journal, 389*, 111-116.

Qamar, Z., Anderson, P., & Hill, R. (2012). *Poly Gamma Glutamic Acid: Effect On Inhibiting Dissolution Of Hydroxyapatite And Enamel*: LAP Lambert Academic Publishing.

Raj , P. A., Johnsson, M., Levine, M. J., & Nancollas, G. H. (1992). Salivary Statherin - Dependence on Sequence, Charge, Hydrogen-Bonding Potency, and Helical Conformation for Adsorption to Hydroxyapatite and Inhibition of Mineralization. *Journal of Biological Chemistry, 267*(9), 5968-5976.

Ramesh, K., Nor, N. M., Ramesh, S., Vengadaesvaran, B., & Arof, A. (2013). Studies on Electrochemical Properties and FTIR analysis of Epoxy Polyester Hybrid Coating System. *Int. J. Electrochem. Sci, 8*, 8422-8432.

Reisner, K. R., Levitt, H. L., & Mante, F. (1997). Enamel preparation for orthodontic bonding: A comparison between, the use of a sandblaster and current techniques. *American Journal of Orthodontics and Dentofacial Orthopedics, 111*(4), 366-373.

Ren, Y. F., Zhao, Q., Malmstrom, H., Barnes, V., & Xu, T. (2009). Assessing fluoride treatment and resistance of dental enamel to soft drink erosion in vitro: Applications of focus variation 3D scanning microscopy and stylus profilometry. *Journal of Dentistry, 37*(3), 167-176.

Reynolds, E. C. (1997). Remineralization of enamel subsurface lesions by casein phosphopeptide-stabilized calcium phosphate solutions. *Journal of Dental Research, 76*(9), 1587-1595.

Robinson , C. (2009). Fluoride and the caries lesion: interactions and mechanism of action. [Review]. *Eur Arch Paediatr Dent, 10*(3), 136-140.

Robinson, C., Kirkham, J., & Shore, R. (1995). *Dental enamel: formation to destruction*: CRC.

Robinson , C., Shore, R. C., Brookes, S. J., Strafford, S., Wood, S. R., & Kirkham, J. (2000). The chemistry of enamel caries. *Critical Reviews in Oral Biology & Medicine, 11*(4), 481-495.

Rodriguez, J. M., Curtis, R. V., & Bartlett, D. W. (2009). Surface roughness of impression materials and dental stones scanned by non-contacting laser profilometry. *Dental Materials, 25*(4), 500-505.

Rosin-Grget, K., & Lincir, I. (2001). Current concept on the anticaries fluoride mechanism of the action. *Collegium Antropologicum, 25*(2), 703-712.

Sawamura, S. (1913). On Bacillus natto. *J. Coll Agric* 5(2), 189-191.

Saxegaard, E., & Rolla, G. (1988). Fluoride acquisition on and in human enamel during topical application in vitro. *Scand J Dent Res, 96*(6), 523-535.

Scaramucci, T., Carvalho, J. C., Hara, A. T., & Zero, D. T. (2015). Causes of dental erosion: intrinsic factors *Dental Erosion and Its Clinical Management* (pp. 35-67): Springer.

Schlesinger, D. H., & Hay, D. (1977). Complete covalent structure of statherin, a tyrosine-rich acidic peptide which inhibits calcium phosphate precipitation from human parotid saliva. *Journal of Biological Chemistry, 252*(5), 1689-1695.

Schlueter, N., Hara, A., Shellis, R. P., & Ganss, C. (2011). Methods for the Measurement and Characterization of Erosion in Enamel and Dentine. *Caries Research, 45*, 13-23.

Schwartz, S. S., Hay, D. I., & Schluckebier, S. K. (1992). Inhibition of Calcium-Phosphate Precipitation by Human Salivary Statherin - Structure-Activity-Relationships. *Calcified Tissue International, 50*(6), 511-517.

Scully, C. (2002). Odontogenesis *Oxford handbook of applied dental sciences*(1st ed., pp 109-116): Oxford Press, U.K..

Seow, W. K. (2015). Dental Enamel Defects in the Primary Dentition: Prevalence and Etiology. In K. B. Drummond & N. Kilpatrick (Eds.), *Planning and Care for Children and Adolescents with Dental Enamel Defects: Etiology, Research and Contemporary Management* (pp. 1-14). Berlin, Heidelberg: Springer Berlin Heidelberg.

Shah, S., Kosoric, J., Hector, M. P., & Anderson, P. (2011). An in vitro scanning microradiography study of the reduction in hydroxyapatite demineralization rate by statherin-like peptides as a function of increasing N-terminal length. *European Journal of Oral Sciences, 119*, 13-18.

Shahmoradi, M., Bertassoni, L. E., Elfallah, H. M., & Swain, M. (2014). Fundamental structure and properties of enamel, dentin and cementum *Advances in calcium phosphate biomaterials* (pp. 511-547): Springer.

Sharma, R., Tsuchiya, M., Skobe, Z., Tannous, B. A., & Bartlett, J. D. (2010). The acid test of fluoride: how pH modulates toxicity. [Research Support, N.I.H., Extramural]. *PLoS One, 5*(5), e10895.

Shellis, R. P. (1984). Relationship between Human-Enamel Structure and the Formation of Caries-Like Lesions Invitro. *Archives of Oral Biology, 29*(12), 975-981.

Shih, I. L., & Van, Y. T. (2001). The production of poly-(gamma-glutamic acid) from microorganisms and its various applications. *Bioresource Technology, 79*(3), 207-225.

Siddiqui, S., Anderson, P., & Al-Jawad, M. (2014). Recovery of crystallographic texture in remineralized dental enamel. [Research Support, Non-U.S. Gov't]. *PLoS One, 9*(10), e108879.

Soames, J. V., & Southam, J. C. (2005). Dental Caries *Oral Pathology* (4 ed., pp. 19-32): Oxford, U.K.

Songsiripradubboon, S., Hamba, H., Trairatvorakul, C., & Tagami, J. (2014). Sodium fluoride mouthrinse used twice daily increased incipient caries lesion remineralization in an in situ model. *Journal of Dentistry, 42*(3), 271-278.

Staines, M., Robinson, W. H., & Hood, J. A. A. (1981). Spherical Indentation of Tooth Enamel. *Journal of Materials Science, 16*(9), 2551-2556.

Tanimoto, H., Fox, T., Eagles, J., Satoh, H., Nozawa, H., Okiyama, A., . . . Fairweather-Tait, S. J. (2007). Acute effect of poly-γ-glutamic acid on calcium absorption in post-menopausal women. *Journal of the American College of Nutrition, 26*(6), 645-649.

Tanimoto, H., Sato, H., Kuraishi, C., Kido, K., & Seguro, K. (1995). *U.S. Patent No. 5,447,732*. Washington, DC: U.S. Patent and Trademark Office.

ten Cate, J. M. (1990). In vitro studies on the effects of fluoride on de- and remineralization. [Review]. *J Dent Res, 69 Spec No*, 614-619; discussion 634-616.

ten Cate, J. M. (1999). Current concepts on the theories of the mechanism of action of fluoride. *Acta Odontologica Scandinavica, 57*(6), 325-329.

ten Cate , J. M. (2001). Remineralization of caries lesions extending into dentin. *Journal of Dental Research, 80*(5), 1407-1411.

ten Cate, J. M. (2013). Contemporary perspective on the use of fluoride products in caries prevention. *British Dental Journal, 214*(4), 161-167.

ten Cate , J. M. (2013). Contemporary perspective on the use of fluoride products in caries prevention. *British Dental Journal, 214*(4), 161-167.

ten Cate, J. M., & Featherstone, J. D. B. (1991). Mechanistic Aspects of the Interactions between Fluoride and Dental Enamel. *Critical Reviews in Oral Biology and Medicine, 2*(3), 283-296.

ten Cate, J. M., & Marsh, P. D. (1994). Procedures for Establishing Efficacy of Antimicrobial Agents for Chemotherapeutic Caries Prevention. *Journal of Dental Research, 73*(3), 695-703.

ten Cate., J. M., Larsen, M. J., Pearce, E. I., & Fejerskov, O. (2003). Chemical interactions between the tooth and oral fluids. In O. Fejerskov & E. A. M. Kidd (Eds.), Dental Caries. The Disease and Its Clinical Management (pp. 49–70): Copenhagen, Blackwell Munksgaard.

ten Cate, J. M., Shariati, M., & Featherstone, J. D. B. (1985). Enhancement of (Salivary) Remineralization by Dipping Solutions. *Caries Research, 19*(4), 335-341.

Tian, H. Y., Tang, Z. H., Zhuang, X. L., Chen, X. S., & Jing, X. B. (2012). Biodegradable synthetic polymers: Preparation, functionalization and biomedical application. *Progress in Polymer Science, 37*(2), 237-280.

Troy, F. (1973). Chemistry and biosynthesis of the poly(-D-glutamyl) capsule in Bacillus licheniformis. Properties of memberane mediated biosynthetic reaction. *Journal of Biological Chemistry*(248), 305-315.

Tsui, T. Y., & Pharr, G. M. (1999). Substrate effects on nanoindentation mechanical property measurement of soft films on hard substrates. *Journal of Materials Research, 14*(1), 292-301.

Urbaniak-Domagala, W. (2012). *The use of the spectrometric technique FTIR-ATR to examine the polymers surface*: INTECH Open Access Publisher.

Urzua, I., Mendoza, C., Arteaga, O., Rodriguez, G., Cabello, R., Faleiros, S., . . . Gamonal, J. (2012). Dental caries prevalence and tooth loss in chilean adult population: first national dental examination survey. *Int J Dent, 2012*, 810170. doi: 10.1155/2012/810170

Varga, G. (2012). Physiology of the salivary glands. *Surgery (Oxford), 30*(11), 578-583.

Varga, G. (2015). Physiology of the salivary glands. *Surgery (Oxford), 33*(12), 581-586.

Vargas, C. M., Crall, J. J., & Schneider, D. A. (1998). Sociodemographic distribution of pediatric dental caries: NHANES III, 1988-1994. *Journal of the American Dental Association, 129*(9), 1229-1238.

Vitkov, L., Hannig, M., Nekrashevych, Y., & Krautgartner, W. D. (2004). Supramolecular pellicle precursors. *Eur J Oral Sci, 112*(4), 320-325.

Watanabe, S., & Dawes, C. (1988). The effects of different foods and concentrations of citric acid on the flow rate of whole saliva in man. [Clinical Trial Comparative Study Controlled Clinical Trial Research Support, Non-U.S. Gov't]. *Arch Oral Biol, 33*(1), 1-5.

Waugh, D. T., Potter, W., Limeback, H., & Godfrey, M. (2016). Risk Assessment of Fluoride Intake from Tea in the Republic of Ireland and its Implications for Public Health and Water Fluoridation. *International Journal of Environmental Research and Public Health, 13*(3). doi: 10.3390/ijerph13030259

Weatherell, J. A., Deutsch, D., Robinson, C., & Hallsworth, A. S. (1975). Fluoride concentrations in developing enamel. *Nature, 256*(5514), 230-232.

West , N. X., & Joiner , A. (2014). Enamel mineral loss. *J Dent, 42 Suppl 1*, 2-11.

Weyant, R. J. (2013). Topical Fluoride for Caries Prevention: Executive Summary of the Updated Clinical Recommendations and Supporting Systematic Review (vol 144, pg 1279, 2013). *Journal of the American Dental Association, 144*(12), 1335-1335.

Weyant, R. J., Tracy, S. L., Anselmo, T., Beltran-Aguilar, E. D., Donly, K. J., Frese, W. A., . . . Sci, A. D. A. C. (2013). Topical fluoride for caries prevention Executive summary of the updated clinical recommendations and supporting systematic review. *Journal of the American Dental Association, 144*(11), 1279-1291.

White, D. J., Faller, R. V., & Bowman, W. D. (1992). Demineralization and Remineralization Evaluation Techniques - Added Considerations. *Journal of Dental Research, 71*, 929-933.

White, D. J., & Featherstone, J. D. B. (1987). A Longitudinal Microhardness Analysis of Fluoride Dentifrice Effects on Lesion Progression Invitro. *Caries Research, 21*(6), 502-512.

Whitehead, S. A., Shearer, A. C., Watts, D. C., & Wilson, N. H. F. (1999). Comparison of two stylus methods for measuring surface texture. *Dental Materials, 15*(2), 79-86.

Wikiel, K., Burke, E. M., Perich, J. W., Reynolds, E. C., & Nancollas, G. H. (1994). Hydroxyapatite Mineralization and Demineralization in the Presence of Synthetic Phosphorylated Pentapeptides. *Archives of Oral Biology, 39*(8), 715-721.

Wilson , N., & Plasschaert, A. (2007). Dental Caries, Minimally Invasive Dentistry and Evidence-based Clinical Practice. *Minimally Invasive Dentistry: The Management of Caries.* (pp. 1-6): Quintessenz,Germany.

Wilson, S. M., & Bacic, A. (2012). Preparation of plant cells for transmission electron microscopy to optimize immunogold labeling of carbohydrate and protein epitopes. *Nature Protocols, 7*(9), 1716-1727.

Wong, L., Cutress, T. W., & Duncan, J. F. (1987). The influence of incorporated and adsorbed fluoride on the dissolution of powdered and pelletized hydroxyapatite in fluoridated and non-fluoridated acid buffers. *J Dent Res, 66*(12), 1735-1741.

Yan-Fang Ren, D. (2011). Dental Erosion: Etiology, Diagnosis and Prevention.

Yanagisawa, T., & Miake, Y. (2003). High-resolution electron microscopy of enamel-crystal demineralization and remineralization in carious lesions. *Journal of Electron Microscopy, 52*(6), 605-613.

Yang, L.-C., Wu, J.-B., Ho, G.-H., Yang, S.-C., Huang, Y.-P., & Lin, W.-C. (2008). Effects of poly-γ-glutamic acid on calcium absorption in rats. *Bioscience, biotechnology, and biochemistry, 72*(12), 3084-3090.

Young, A., Thrane, P. S., Saxegaard, E., Jonski, G., & Rolla, G. (2006). Effect of stannous fluoride toothpaste on erosion-like lesions: an in vivo study. *European Journal of Oral Sciences, 114*(3), 180-183.

Young , D. A., & Featherstone , J. D. B. (2013). Caries management by risk assessment. *Community Dentistry and Oral Epidemiology, 41*(1), e53-e63.

Young, W. G., & Dawes, C. (2011). Dental Diagnosis and the Oral Medicine of Toothwear. *Toothwear: The ABC of the Worn Dentition*, 89-110.

Zahradnik, R. T. (1979). Modification by salivary pellicles of in vitro enamel remineralization. [Research Support, U.S. Gov't, P.H.S.]. *J Dent Res, 58*(11), 2066-2073.

Zhang, Y. R., Du, W., Zhou, X. D., & Yu, H. Y. (2014). Review of research on the mechanical properties of the human tooth. *International Journal of Oral Science, 6*(2), 61-69.