The University Of Sheffield.

The Use of Nanoparticles as a Strategy for the Remineralisation of Dentine Affected by Caries or Acid Erosion

Eleanor Ashworth BSc (Hons)

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

The University of Sheffield

Faculty of Medicine, Dentistry and Health

School of Clinical Dentistry

September 2016

Abstract

There is a clinical need to remineralise dentine beneath the surface, relatively deep within the collagen matrix. Nanoparticles have the potential to infiltrate between the collagen fibres, and previous studies have demonstrated that silica and hydroxyapatite nanoparticles may enhance remineralisation. Dentine remineralisation research uses *in vitro* models based on fully or partially demineralised dentine prepared by exposure to non-specific acids, and to date this approach has been based on the assumption that these acids affect dentine in a similar manner. Moreover, for methodological reasons, the demineralised dentine requires fixation, but it is unknown if this additional step affects the soundness of the model. The aim of this study was therefore to undertake a detailed and systematic study of dentine remineralisation by silica nanoparticles in the presence of low molecular weight organic acids, with additional experiments directed at understanding the effect of chemical fixation on the validity of the model. Each demineralising agent that was investigated had a unique effect on the dentine composition and structure and it was concluded that acids reported to date have substantial limitations when used to simulate the effects of caries or erosion acids. A novel infiltration method was developed which suggested that silica nanoparticles infiltrated through collagen networks and adhered to collagen fibres, creating a nanoparticle-collagen complex or scaffold. The use of chemical fixatives and different demineralising agents did not appear to inhibit the adherence of nanoparticles. Furthermore, by monitoring the zeta potential of the nanoparticles under various conditions, it was discovered that the negatively charged silica nanoparticles had a high affinity for calcium ions that were present in a remineralising solution. This appeared to form part of the mechanism responsible for dentine remineralisation by infiltrated silica nanoparticles. This interpretation was further supported by studies using energy dispersive spectroscopy in the electron microscope. This research has added considerably to current knowledge of remineralisation of dentine using inorganic nanoparticles, with signs of remineralisation detected beneath the tissue surface and paving the way towards a therapeutic intervention.

Acknowledgements

First and foremost, I would like to thank GSK and BBSRC for funding this PhD project.

I would also like to thank my supervisors Professor Nicolas Martin, Dr Cheryl Miller and Professor Christopher Deery from The University of Sheffield and Dr Christabel Fowler from GSK.

There are numerous staff members from the Department who I would like to thank for helping me from my early ventures in the lab to my final thesis-writing days: Dr Lynne Bingle for her immunohistochemistry expertise, Mr Dave Thompson for his assistance with electron microscopy preparation, Dr Robert Moorehead for all of his help in and out of the lab (statistsics, in particular), and Dr Kathryn Hurrell-Gillingham and Miss Katy D'Apice for helping me with my ethics application. From outside of the Department I would like to thank Mr Chris Hill, Dr Peter Korgul and Dr Le Ma for electron microscopy training, Dr Andrew Morse for DLS training, Dr Nik Reeves for XRD training and Professor David Mowbray for his help with complicated physics calcuations.

My sincere thanks also goes to Dr Naeima Betamar for the many hours she spent in the lab with me, contributing substantially to my project.

I am so lucky to have made good friends with my office colleagues (the D22 crew); Caroline, Harriet, Sarah, Saima, Katie and Rich. Thank you for all the fun times in the lab, the office, at conferences and Friday 'chevs' in the pub.

Last but not the least, I would like to thank my friends and family for all of their support and encouragement; my Mum and Dad, Geraldine and Dave, my brother and sister, Shaun and Lorraine, and last but not least my nieces and nephews; Declan, Ronan, Connor and Molly for being wonderful and hilarious. I owe my deepest gratitude to Amy for being a truly supportive friend and her daughter, my Goddaughter, Amelia, whose smile and laughter makes everything worthwhile.

Finally, I am forever indebted to Kai for making me happy even on the most difficult days.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ABC	Avid and Biotinylated macromolecular Complex
ACP	Amorphous Calcium Phosphate
AFM	Atomic Force Microscopy
AmF	Amine Fluoride
ART	Atraumatic Restorative Treatment
AU	Arbitrary Units
BSP	Bone Sialoprotein
Ca^{2+}	Calcium Ions
CaO	Calcium Oxide
CaP	Calcium Phosphate
CDJ	Cementum Dentine Junction
Cl^-	Chloride Ions
CPP	Casein Phosphopeptide
DAB	Diaminobenzidine tetrahyrochloride
DGP	Dentine Glycoprotein
DLS	Dynamic Light Scattering
DMFT	Decayed, Missing and Filled Teeth surfaces
DMP1	Dentine Matrix Protein 1
DPP	Dentine Phosphoprotein
DSP	Dentine Sialoprotein
DSPP	Dentine Sialophosphoprotein
EDS	Energy Dispersive Spectroscopy
EDTA	Ethylenediaminetetraacetic Acid
FTIR	Fourier Transform Infrared Spectroscopy
GAG	Glycosaminoglycan
Gly	Glycine
GORD	Gastro Oesophageal Reflux Disease
H+	Hydrogen Ions
HA	Associated Ion
H	Dissociated Ion

HCA	Hydrocarbonate Apatite
HCl	Hydrochloric Acid
HEPES	N-2Hydroxyethylpiperazine-N'-Ethanethesulphonic Acid
HMDS	Hexamethyldisilazane
Hyp	Hydroxyproline
ICDD	International Centre for Diffraction Data
K	Stability Constant
Ka	Dissociation Constant
KHN	Knoop Hardness Number
M	Moles
CT	Computed Tomography
ml	Millilitre
mm	Millimeter
mM	Millimole
MMP	Matrix Metalloproteinases
mV	Millivolts
Na+	Sodium Ions
n-CAP	Nano-sized Carbonate Apatite
n-CHA	Nano-Carbonate Hydroxyapatite
NCP	Non Collagenous Proteins
NHS	National Health Service
nm	Nanometers
nmol	Nanomole
P	Phosphorous
Pa	Pascal
PAA	Polyacrylic Acid
PBS	Phosphate Buffered Saline
PDF	Powder Diffraction Files
PG	Proteoglycan
PILP	Polymer Induced Liquid Precursors
PO_4^-	Phosphate
POs-Ca	Phosphoryl Oligosaccharides of Calcium

ppm	Parts Per Million
Pro	Proline
PVPA	Polyvinylphosphonic Acid
Rpm	Revolutions per minute
S.mutans	Streptococcus mutans
SAA	Surface Area Analysis
SAPs	Self Assembling Peptides
SEM	Scanning Electron Microscopy
SHMP	Sodium Hexametaphosphate
SMFP	Sodium Monofluorophosphate
STMP	Sodium Trimetaphosphate
TCP	Tricalcium Phosphate
TEM	Transmission Electron Microscopy
TPCK	L-1-Tosylamide-2-Phenylethyl Chloromethyl Letone
µg	Microgram
µm	Micrometer
VHN	Vickers Hardness Number
wt	Weight
X5P	Xylitol-5-Phosphate
XRD	X-ray Diffraction
XRF	X-ray Fluorescence

1. Introduction

Bacterial-induced dental caries, along with erosion caused by diet and gastric acids, are two processes that cause demineralisation at the tooth surface. Enamel is situated on the outer surface of the tooth and therefore it is demineralised first, but once it is worn away the underlying dentine is exposed and becomes vulnerable to demineralisation too. Dentine is a complex tissue consisting of hydroxyapatite mineral (70 % by weight), type I/III collagen (20 % by weight) and many non-collagenous proteins (10 % by weight). When dentine is affected by severe caries, bacteria may denature the collagen fibre network. Likewise, during severe acid erosion, collagen fibres may become damaged due to chemical interaction combined with direct mechanical trauma to the surfaces exposed to the oral environment. The effect on the non-collagenous proteins is currently unknown.

Although demineralisation may be prevented by maintaining good oral hygiene and a low-sugar low-acid diet, most individuals will suffer some demineralisation of the dentition in their lifetime. Usually, a dentist will remove areas of demineralisation and restore the missing structure with an appropriate material. However, sometimes healthy dentine and/or partially demineralised dentine that has the potential to be remineralised is also removed. Thus, the main problem with this traditional approach is the unnecessary removal of a considerable amount of dentine, compromising the future survival of the tooth. In recent years, a more conservative approach has been favoured whereby less of the tooth is removed during cavity preparations and smaller restoratives are placed. However, this requires a remineralisation strategy for the partially demineralised areas that remain.

To an extent, remineralisation will occur naturally from the mineral ions in saliva and the addition of ions from toothpastes/mouth rinses. There is extensive ongoing research exploring additional ways to enhance the remineralisation process in enamel that mainly relies on surface mineral deposition, often involving the delivery of fluoride, calcium, or bioactive glasses to the demineralised area. Such strategies are noted to be effective in certain presentations of caries affecting root-dentine that has become

exposed to oral biofilms following gingival recession (Fejerskov and Kidd, 2008a). Dentine is an entirely different substrate to enamel that mostly requires an alternative strategy to achieve remineralisation deep beneath its surface and throughout the collagen fibre network.

Nanotechnology is increasingly considered as a potential way of enhancing remineralisation beneath the dentine surface. Of particular interest is the research undertaken by Besinis *et al* in the School of Clinical Dentistry, The University of Sheffield. Besinis *et al* performed proof of concept studies demonstrating that nanoparticles of silica and hydroxyapatite are small enough to move between dentine collagen fibres, and once in a colloidal suspension, can infiltrate deep within the dentine organic matrix. Subsequently, when the dentine specimens are then exposed to artificial saliva, the nanoparticles can facilitate the nucleation of mineral within the dentine organic structure.

This research was in early proof of concept stage and the exact mechanism of remineralisation has not yet been elucidated. Specifically, (i) whether the nanoparticles adhere to the collagen fibres or simply become entangled in the fibre matrix; (ii) concentration of the nanoparticle solutions, the exposure times, and the effects of chemical fixatives (required for *in vitro* specimen preparation); (iii) the effect of different acids on the dentine substrate.

Dentine remineralisation research studies require a demineralised dentine model. Clinically, dentine is demineralised by a range of acids of different origins (bacterial, gastric and dietary) but many demineralised dentine models are produced using laboratory acids, with the assumption that all acids affect dentine in the same manner. The effects of these different acids, especially on the organic integrity of dentine are rarely considered even though it is fundamental for remineralisation to occur.

2. Literature Review

2.1 Dental Anatomy

Humans are diphyodont, which means that they develop two sets of teeth; primary and permanent. Primary teeth start to erupt at around 6 months of age and there are usually 20 in total; 10 in the maxilla (upper jaw) and 10 in the mandible (lower jaw). From the age of about 5 to 14 years old, these are gradually replaced by permanent teeth, with the final 'wisdom' teeth (third molars) erupting in the early twenties. There are a total of 32 permanent teeth, 16 in the mandible and 16 in the maxilla, their arrangement in the oral cavity is illustrated below in Figure 2.1.

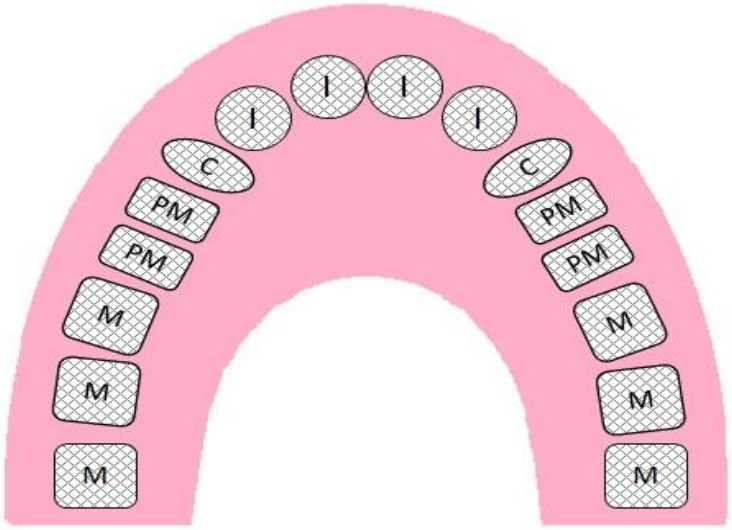

Figure 2.1: Illustration to show the human permanent dentition; I = incisor, C = canine, PM = premolar, M = molar

2.2 Tooth Structure

Although different types of teeth have slightly different morphologies, they all have the same essential structure and consist of four fundamental components: enamel, dentine, cementum and pulp; as demonstrated in the ground section (Figure 2.2).

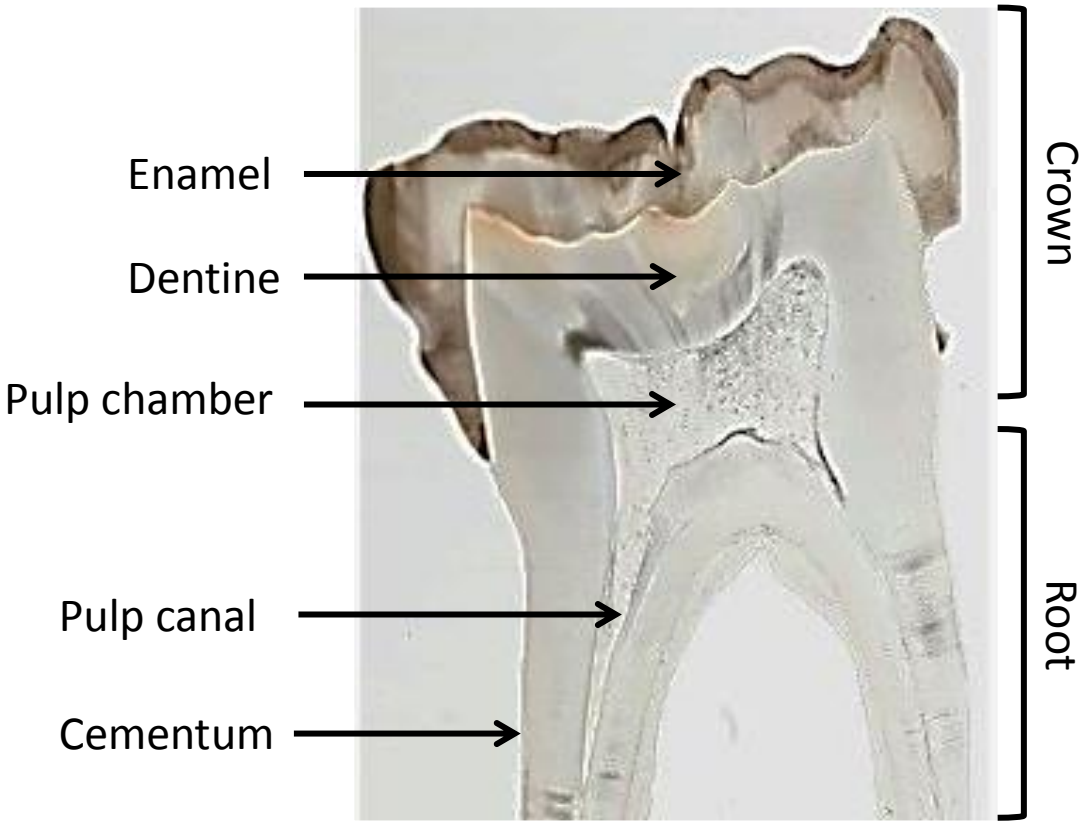

Figure 2.2: A ground section of a human permanent first molar, demonstrating the basic structure of a tooth including the enamel, dentine, pulp and cementum.

2.2.1 Enamel

Enamel forms the surface layer of the anatomical crown of the tooth and although it is actually colourless, it appears brown on Figure 2.2 due to the light from the microscope, and appears a slight yellowish colour in the oral cavity due to the underlying yellowish dentine. Dental enamel is an acellular component and is the most mineralised tissue of the body (96 % by weight) with the remaining 4 % consisting of water and organic material (Donoghue *et al*., 2006, Kawasaki and Weiss, 2008). The primary mineral in enamel is hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], a calcium phosphate that is also the main

component in bone. Enamel hydroxyapatite crystals are extremely long and thin, 50 nm in diameter and more than 100 μm long, and are tightly packed in a repeated arrangement that form enamel prisms. Due to the high mineral content, the properties of enamel and hydroxyapatite are very similar; for example, hydroxyapatite has a density of 3.16 g/cm^3 and a hardness of 430 KHN whilst enamel has a density of 2.95 g/cm^3 and a hardness of 370 KHN, which reflects the strong adherence between the crystals (Fejerskov and Kidd, 2008b).

2.2.2 Cementum

The cementum is a calcified substance that surrounds the tooth root (Figure 2.2). Composed of hydroxyapatite (50 % by weight) and collagen (50 % by weight); cementum functions to anchor the tooth to the alveolar bone. In terms of composition, there are two types of cementum: acellular and cellular. Acellular cementum covers the entire length of the root whereas the cellular cementum is continually growing and is produced, as needed, to maintain the integrity of the tooth (Fejerskov and Kidd, 2008b).

2.2.3 Pulp

The pulp is situated at the centre of the tooth and as illustrated in Figure 2.2; the crown of the tooth contains the pulp chamber and the root of the tooth contains the pulp canal. The pulp is composed of connective tissue and nerve tissue consisting of blood vessels and lymph vessels.

2.3 Dentine

Dentine will be discussed in more detail as it is the tooth component focused on in this investigation. Dentine is yellowish in colour and is situated between the enamel and the pulp (Figure 2.2) where it can protect the pulp from microbial damage whilst also providing essential support to enamel. It is mainly composed of mineral (70 % by weight) and collagen (20 % by weight), the remaining 10 wt. % consisting of water and non-collagenous proteins (Nanci, 2008).

2.3.1 Dentine Mineral

The mineral phase of dentine is composed of hydroxyapatite. This is similar to enamel, but the crystallites in dentine are a lot smaller (3 - 30 nm in diameter and 50 nm long) and are hexagonal or plate-like in morphology, unlike the rod-shaped crystals found in enamel. This means that dentine hydroxyapatite has a much larger surface area than enamel hydroxyapatite, resulting in a more reactive mineral phase (Orvig, 1968).

Prior to mineralisation, non-mineralised dentine is referred to as 'pre-dentine'. For dentine mineralisation to succeed there must be an appropriate level of inorganic ions present, enough energy available for these to form clusters, and the level of crystal formation inhibiters must be regulated because they increase the energy barrier required for mineralisation. There are two types of mineralisation: homogeneous and heterogeneous. Homogeneous nucleation occurs when the concentration of inorganic ions is increased and ion clusters form, whereas heterogeneous nucleation involves the presence of nucleation substances which lower the energy barrier or inactivate crystal formation inhibitors. This involves matrix vesicles such as alkaline phosphatases, pyro phosphatases, calcium adenylpyrophosphatases (ATPases), metalloproteinases or proteoglycans. These macromolecules bind to calcium and inorganic phosphate to form calcium phosphate complexes. Additionally, heterogeneous nucleation can occur without vesicles and instead mineralisation occurs directly on collagen fibres, in the presence of non-collagenous proteins (NCPs), although it is not certain of the exact mechanism (Fejerskov and Kidd, 2008b).

2.3.2 Dentine Non-collagenous Proteins

Odontoblasts produce collagen and non-collagenous proteins (NCPs) (Linde and Goldberg, 1993). The NCPs are distributed between the collagen fibres, often accumulating on the tubule walls (Nanci, 2008). Crystal growth on the collagen fibres is thought to be controlled and promoted by NCPs (Qin *et al*., 2004, Qin *et al*., 2007) and mutations in NCP genes have been shown to be associated with phenotypic abnormalities in the mineralisation of dentine (Qin *et al*., 2004, Zhang *et al*., 2001, Lu *et al*., 2007, Feng *et al*., 2002,

Sreenath *et al*., 2003). There is evidence that calcium ions are transported to collagen fibres *via* a complicated signalling pathway involving calcium-activated ATPases, sodium/calcium exchangers, calcium channels and calcium binding proteins (Carafoli, 1987 and Granstrom and Linde, 1981). The space between the individual collagen fibres is called the 'gap zone' and this contains many of the NCPs such as proteoglycans (PGs). PGs enhance the interaction between calcium ions and collagen fibres whilst proteoglycanases simultaneously degrade the PGs, leaving just the calcium ions behind. This enzymatic function then allows other NCPs, such as the phosphoproteins, to bind to the collagen. Subsequently, alkaline phosphatases dephosphorylate the phosphoproteins resulting in the cleavage and release of phosphate ions; allowing calcium phosphate complexes to form. These complexes easily convert to hydroxyapatite crystals and spread between the fibres to fully mineralise the tissue (Stetlerstevenson and Veis, 1986, Goldberg and Boskey, 1996). Table 2.1 illustrates the various NCPs found in dentine.

Table 2.1: A list of non-collagenous proteins found in human dentine. Adapted from (Orsini *et al*., 2012)

Non-collagenous Protein Families	Non-collagenous Proteins
Proteoglycans	Decorin
	Biglycan
	Fibromodulin
	Lumican
	Osteoadherin
	Versican
Glycoproteins	Osteocalcin
	Osteonectin
	Osteopontin
	Dentine matrix protein 1
	Bone sialoprotein
	Dentine sialophosphoprotein
	Matrix extracellular phosphoglycoprotein
Serum proteins	Albumin
	IgG
	Transferin
	Fetuin-A
Enzymes	Matrix metalloproteinases (8, 2, 9, 20)
	Cathepsin
Growth factors	Insulin-like growth factor I
	Skeletal growth factor II
	Transforming growth factor B
	Platelet derived growth factor
	Vascular endothelial growth factor
	Placental growth factor
	Fibroblast growth factor 2
	Epidermal growth factor

2.3.2.1 Proteoglycans

Proteoglycans (PGs) are key proteins in the mineralisation of dentine and are thought to have metabolic, functional and structural roles (Iozzo, 1999, Hall *et al*., 1999, Iozzo, 1998, Waddington *et al*., 2004, Embery *et al*., 2001, Milan *et al*., 2004). PGs consist of a core protein molecule which is covalently attached to linear, negative polysaccharides called glycosaminoglycans (GAGs). Due to the different combinations of core proteins and GAGs available, there is a huge molecular variation in PGs. Some PGs play an important role in dentine matrix formation and the prevention of premature mineralisation whilst others are more involved in the regulation of the mineralisation process (Embery *et al*., 2001, Waddington *et al*., 2003). However, the exact role of dentine PGs remains controversial. As described in 2.3.2, some PGs, for example decorin and biglycan, are thought to be involved in directing hydroxyapatite crystal growth and are able to bind to collagen type I fibres (Goldberg *et al*., 2002, Milan *et al*., 2005, Orsini *et al*., 2007, Goldberg *et al*., 2005).

2.3.2.2 Glycoproteins

Osteocalcin, the most abundant NCP in bone, is found in only small quantities in dentine where it binds to hydroxyapatite, either inhibiting or controlling its crystallisation (Romberg *et al*., 1986, Bleicher *et al*., 1999). Along with hydroxyapatite, osteonectin attaches to collagen and growth factors to regulate cell proliferation, angiogenesis and matrix metalloproteinases (MMPs); which are discussed in detail in section 2.4.3.1 (Gundberg *et al*., 1984). There is also evidence that osteonectin is involved in collagen fibrilliogenesis and maturation (Martinek *et al*., 2007) as well as regulating bone structure (Delany and Hankenson, 2009). Osteopontin, a sialic acid rich phosphoprotein (Butler, 1987), is present in dentine and may function as an inhibitor for apatite growth (Boskey *et al*., 1993). Similarly, the bone sialoprotein (BSP) also acts as an inhibitor for crystal growth (Hunter *et al*., 1994) whilst the multifunctional protein, dentine matrix protein 1 (DMP1), regulates cell attachment (George *et al*., 1993) and mineralisation (Narayanan *et al*., 2003). Mineral growth and deposition can be promoted by

interactions between DMP1 and collagen type 1 (He and George, 2004, He *et al*., 2005).

2.3.2.3 Dentine Sialophosphoprotein (DSPP)

There is much evidence that the dentine sialophosphoprotein (DSPP) is involved in promoting mineralisation. DSPP gene mutations have been shown to cause dentinogenesis imperfecta in humans (Golub, 2009, Xiao *et al*., 2001) and DSPP knockout mice result in defective mineralisation (Sreenath *et al*., 2003). DSPP is cleaved into dentine sialoprotein (DSP), dentine glycoprotein (DGP) and dentine phosphoprotein (DPP) (Gu *et al*., 2000, Yamakoshi *et al*., 2006), and DSP and DPP are now identified as independent proteins in dentine (MacDougall *et al*., 1997, Gu *et al*., 2000, Ritchie and Li, 2001, Yamakoshi *et al*., 2003). Although DSP is the second most abundant NCP in dentine, its function is unknown.

DPP was discovered by Veis and Perry (1967) and is the most abundant NCP in dentine (Prasad *et al*., 2010, Veis and Perry, 1967, Dickson *et al*., 1975, Dimuzio and Veis, 1978). It contains unique amino acid extensions which can form carboxyl-phosphate interactions that may be vital for mineralisation by binding to calcium ions and exposing them to collagen fibres (Butler *et al*., 1979, Takagi *et al*., 1986, Boskey *et al*., 1990). Many studies state that DPP is key in initiating and modulating dentine hydroxyapatite formation (Linde and Lussi, 1989, Lussi and Linde, 1993, Linde, 1989, Zhang *et al*., 2001, Feng *et al*., 2002, Linde, 1973, Boskey *et al*., 1990). However, more research is necessary to understand the molecular pathways governing the expression of the gene.

2.3.3 Dentine Collagen

Collagen is the name given to a large family of structurally similar proteins that form strong, insoluble fibres. The dentine organic network is composed of 90 wt. % collagen with approximately 56 wt. % of the dentine mineral phase located within this collagen network, which is composed of 80 – 240 nm wide fibres (Kitasako *et al*., 2002). All collagen molecules consist of three left-handed helical polypeptide chains twisted around each other to form a right-handed supercoil. The arrangement and distribution varies with the

classes of collagen. For example, in tendons collagen forms rope-like fibres whilst in the skin, collagen forms loosely, woven, elastic fibres. There are at least 16 types of collagen, but 80 – 90 % of collagen consists of type I, II or III (Voet and Voet, 2010).

The main component of dentine collagen is collagen type I (Gage, 1984), which is composed of an intertwined triple helix of two alpha-1 polypeptide chains and one alpha-2 polypeptide chain, often abbreviated to [$\alpha1(I)_2$ $\alpha2(I)$] (Butler, 1987). Each alpha chain is twisted into a left-handed polyproline helix. Three alpha chains are tangled together to create a right-handed superhelix making rod-like molecules, which are 1.4 nm in diameter and 300 nm in length (Rich and Crick, 1961). These molecules then connect to form fibres as shown in Figure 2.3.

Although the majority of dentine collagen is type I, there is some type III collagen (made up of 3 alpha-1 chains) present within the dentinal tubules (Becker *et al*., 1986, Nagata *et al*., 1992, Waltimo *et al*., 1994, Shuttleworth *et al*., 1978, Vanamerongen *et al*., 1983). There is also evidence of low levels of collagen type V present in human pre-dentine, although this is absent in mineralised dentine (Becker *et al*., 1986).

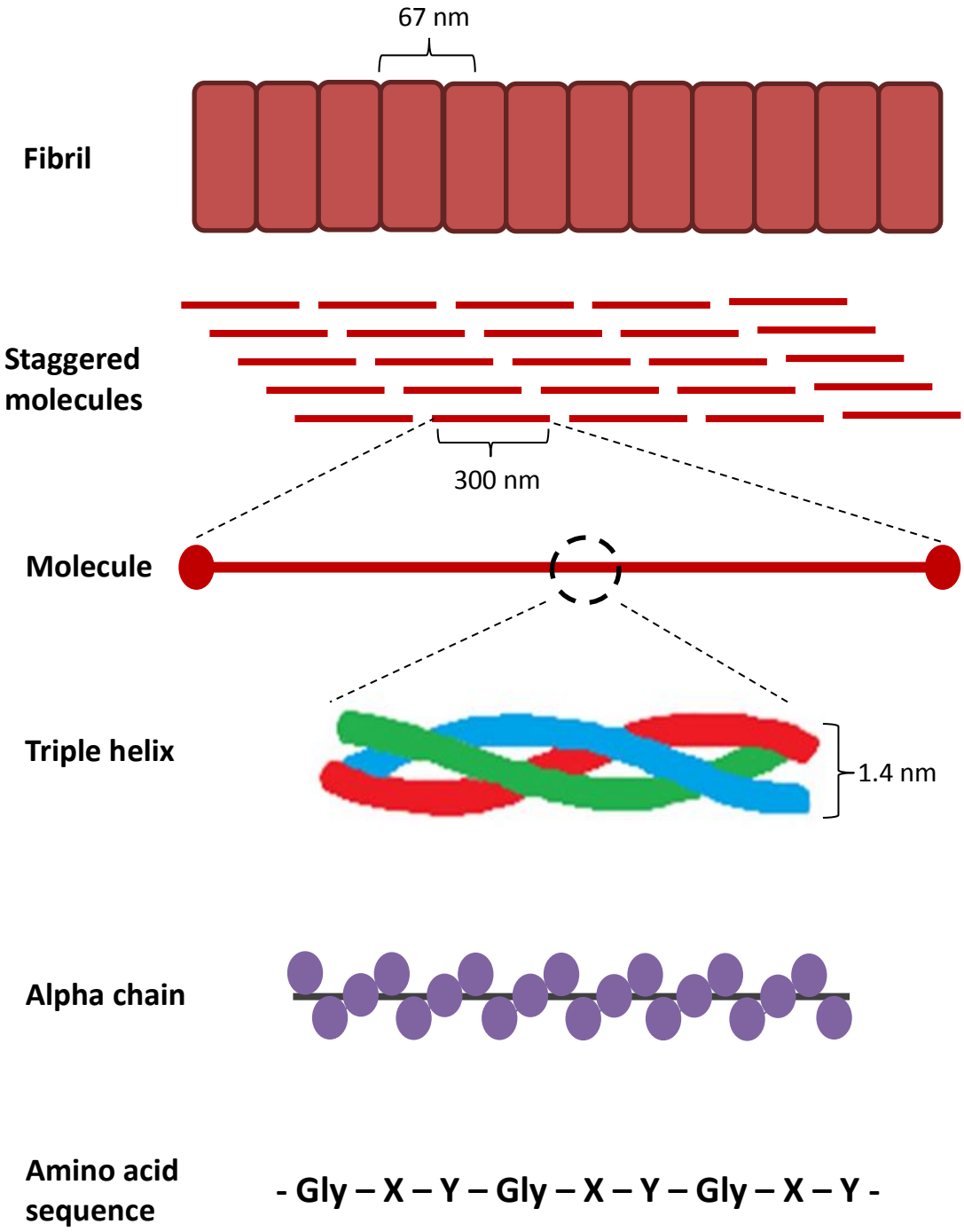

Figure 2.3: An illustration to show the stages of collagen formation from an amino acid sequence to a fibril

2.3.3.1 Collagen Type I: Amino Acid Composition

A unique characteristic of collagen is its unique amino acid composition referred to as "**Gly-X-Y**". Nearly one third of the residues are glycine (Gly) which are found at every third position of the triple helix and 15 – 30 % are

proline (Pro) residues and 4-hydroxyprolyl (4-Hyp) residues, in positions '**X**' and '**Y**', respectively. It was not until 1994 that Bella *et al* confirmed this repeating structure using x-ray crystallography (Bella *et al*., 1994). It was proposed that the Gly residue of each chain passes through the centre of the triple helix – Gly is the only residue small enough to fit. The chains are also required to be staggered so that Gly, X and Y occur at similar levels, allowing the amide of each Gly in one chain to make a hydrogen bond with the carbonyl oxygen atom of a Pro (X) on an adjacent chain. The large size and inflexibility of the Pro (X) and 4-Hyp (Y) residues confer rigidity, providing collagen with high tensile strength. The oppositely twisted directions of the collagen polypeptide chains and the triple helix prevent twists from being pulled out under tension (Beier and Engel, 1966, Kielty *et al*., 1993).

The enzyme prolyl hydroxylase, with cofactors ascorbic acid and α-ketoglutarate, catalyses the post-translational hydroxylation of peptide-bound Pro residues to form 4-Hyp. Collagen synthesised under conditions inactivating prolyl hydroxylase denatures at 24 °C instead of the usual 39 °C. Individuals with a lack of vitamin C (ascorbic acid) will not have sufficient use of this enzyme and are likely to form scurvy; these individuals often have bleeding gums and loose teeth because the collagen fibres are not formed correctly (Voet and Voet, 2010).

2.3.3.2 Collagen Cross links

Collagen fibres are further stabilised by covalent cross links that form between and inside the helical units. These links are partly responsible for the strength and rigidity of collagen fibres and cause collagen to be insoluble in solvents that disrupt hydrogen bonds and ionic interactions. The cross links are formed when side chains of some lysines and hydroxylysines are converted, *via* lysyl oxidase, to aldehyde groups, forming allysine and hydroxyallysine.

Lysine → lysyl oxidase → allysine

Hydroxylsine → lysyl oxidase → hydroxyallysine

These aldehydes can then interact with side chains of unoxidised lysine and hydroxylysine residues to form Schiff base cross links; usually between

collagen molecules. Allysine residues also react with other allysine residues to form cross links usually beween individual strands of the triple helix in a process called aldol condensation (Voet and Voet, 2010).

2.3.4 Dentine Tubules

Dentine is very permeable due to the presence of microscopic tubules (tunnels). Tubules run parallel to each other and perpendicular to the pulp space, as illustrated in Figure 2.4, running from the pulp to the under surface of the enamel border, often following an ‘S’ shape. The diameter of tubules ranges from 2.5 μm at the pulpal area to 900 nm near the enamel end (Ten Cate, 1994). Tubules contain tiny projections of odontoblasts which help to form and maintain the dentine and, although the projections of odontoblasts are not nerves, connect to the nerve axons in the dental pulp, causing sensitivity to an individual when dentine is exposed (Fejerskov and Kidd, 2008b).

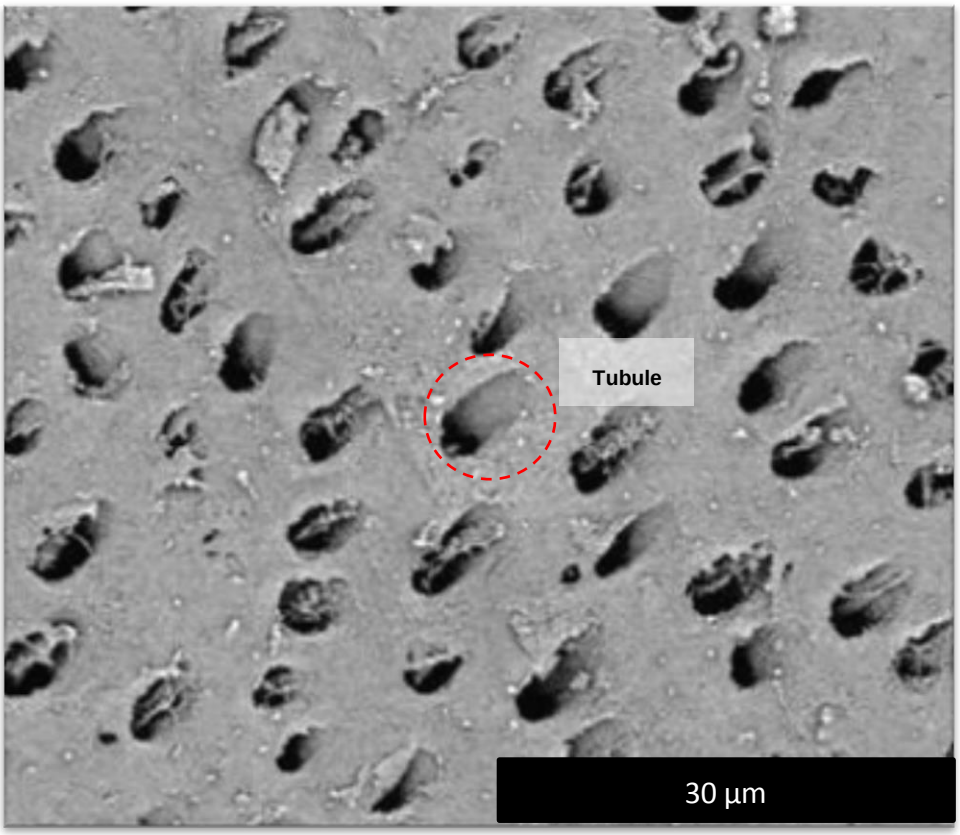

Figure 2.4: A scanning electron micrograph of human dentine showing dentinal tubules on the occlusal surface

2.3.5 Types of Dentine

In terms of structure, dentine is most commonly divided into: (a) primary dentine, (b) secondary dentine and (c) tertiary dentine and in terms of location: (d) mantle dentine, (e) root dentine and (f) peritubular dentine.

(a) Primary Dentine

Primary dentine is produced rapidly as the tooth forms, composing the majority of the tooth and providing size and morphology. After primary dentinogenesis, secondary dentine is formed (Nanci, 2008).

(b) Secondary Dentine

Secondary dentine is formed by the odontoblasts after root formation (Nanci, 2008, Bonucci, 1992) and it is similar to primary dentine but is unevenly deposited around the periphery of the pulp. Secondary dentine is said to have slightly different tubule curvature and less regular tubule structure (Nanci, 2008).

(c) Tertiary Dentine

If the tooth suffers injury then odontoblasts synthesise tertiary dentine, also known as reactive or reparative dentine (Tziafas *et al*., 2000) to increase the distance between oral microbes and the pulp. This type of dentine has a unique morphology and is often not tubular and therefore, is less permeable and prevents the diffusion of toxins or harmful substances to the pulp (Tziafas *et al*., 2000). The properties of tertiary dentine depend on the intensity of the injury; attrition is more likely to induce tertiary dentine than caries (Stanley *et al*., 1983). Reactionary dentine is produced by original odontoblasts at the direct site of the injury but if the injury is severe and odontoblasts are damaged, then reparative dentine is synthesised by odontoblast-like cells (Magloire *et al*., 1992, Yamamura, 1985, Lesot *et al*., 1993, Smith and Lesot, 2001).

(d) Mantle Dentine

Mantle dentine is the layer of dentine directly beneath the enamel. It is usually 5 – 30 µm thick (Linde and Goldberg 1993) and has an irregular organic matrix absent of phosphoproteins (Takagi *et al*., 1986, Nakamura *et al*., 1985), the collagen mainly consists of type III fibres with some small quantities of type I (Ohsaki and Nagata, 1994). It is thought that the elastic properties of teeth may be determined by the mantle dentine, allowing high loads on the occlusal surface to be endured.

(e) Root Dentine

Many studies have shown that root dentine has less tubular density than regular dentine, which further decreases towards the root tip (Mjor and Nordahl, 1996, Carrigan *et al*., 1984, Schellenberg *et al*., 1992). In addition, the cementum-dentine junction (CDJ) represents a region of interspaced 15 – 30 um wide collagen fibril bridges which differs greatly from the rest of the dentine (Linde, 1984, Ho *et al*., 2009).

(f) Peritubular Dentine

Peritubular dentine is the area around the tubule lumens. Peritubular dentine is more mineralised than intertubular dentine (Nanci, 2008) and contains

significantly less collagen (Gotliv and Veis, 2007, Weiner *et al*., 1999, Gotliv *et al*., 2006). The deposition of peritubular dentine causes the diameter of the tubule lumen to be reduced. The peritubular dentine may be formed more rapidly when there is injury or increased stimulation, and can be up to 1 µm thick (Linde and Goldberg, 1993). Furthermore, peritubular dentine is more homogenous than intertubular dentine, and has different hardness (Kinney *et al*. 1996).

2.3.6 Dentine in other Mammals

Many studies investigate dentine from a variety of species. Mouse studies have been used many times to study tooth development (Fleischmannova *et al*., 2008) and rat models are very common for measuring changes in mineral content (Frost and Jee, 1992). Furthermore, bovine teeth are often used to simulate human tooth properties, and are frequently used for erosion and abrasion studies (Rios *et al*., 2006a, Rios *et al*., 2006b, Vieira *et al*., 2006, Francisconi *et al*., 2008, Wegehaupt *et al*., 2008). In terms of hardness values and tubule number and diameter, bovine and human dentine are very alike (Schilke *et al*., 2000). However, bovine teeth have more peritubular dentine than human dentine, which means that they have a higher radio-density (Fonseca *et al*., 2008). There are many variances in structure and chemistry of dentine from different species; in addition, many of these species do not experience injuries such as caries or acid erosion (Linde and Goldberg, 1993).

2.4 Demineralisation of the Tooth

Teeth become damaged by demineralisation (the dissolution of hydroxyapatite) when they are exposed to acidic conditions. This can happen *via* two different processes; dental caries or dental erosion. Dental caries occurs when bacteria, present in the oral cavity, ferment carbohydrates on the tooth surface to produce acids, such as lactic acid, which dissolve the hydroxyapatite crystallites. Dental erosion is a more direct process by which the consumption of acidic foods and beverages, or the regurgitation of stomach acids, results in direct demineralisation of the tooth surface. Both processes result in the dissolution of hydroxyapatite, leading to a loss of

tooth structure and eventually a vulnerable tooth with a compromised future (Fejerskov and Kidd, 2008c).

Tooth erosion and tooth caries are similar in that they both result in damage to the tooth surface through a process of acid-induced demineralisation. However, the actual mechanism and clinical presentation of these two conditions is fundamentally different. Caries requires a bacterial biofilm with cariogenic bacteria in direct contact with the tooth surface over a prolonged period of time. Conversely, acid erosion is associated with the intermittent but frequent bathing of the tooth surfaces with specific acids of dietary or gastric origin; this is characterised by the absence of a visible cariogenic biofilm.

2.4.1 Dental Caries

For caries to form on a tooth surface there must be cariogenic bacteria and fermentable carbohydrates present. Dental caries can occur on any tooth surface but is most likely to occur where there are deep grooves or other areas where dental plaque can accumulate, such as between the teeth. The oral cavity contains many types of bacteria but there are some types which may be specifically linked to caries, mainly *Streptococcus mutans* (*S.mutans*) and *Lactobacilli*. These bacteria ferment sugars (glucose, fructose or sucrose) into acids, such as lactic acid. Eventually, these acids start to dissolve the hydroxyapatite component of the tooth surface, the frequency of exposure to the acidic environments affecting the outcome of the caries development (Fejerskov and Kidd, 2008b). Other bacteria linked with caries include *Streptococcus sanguinis, Streptococcus mitis, Enterococci, Actinomyces* and *Nocardia*. Figure 2.5 is a photograph of a tooth with a caries lesion.

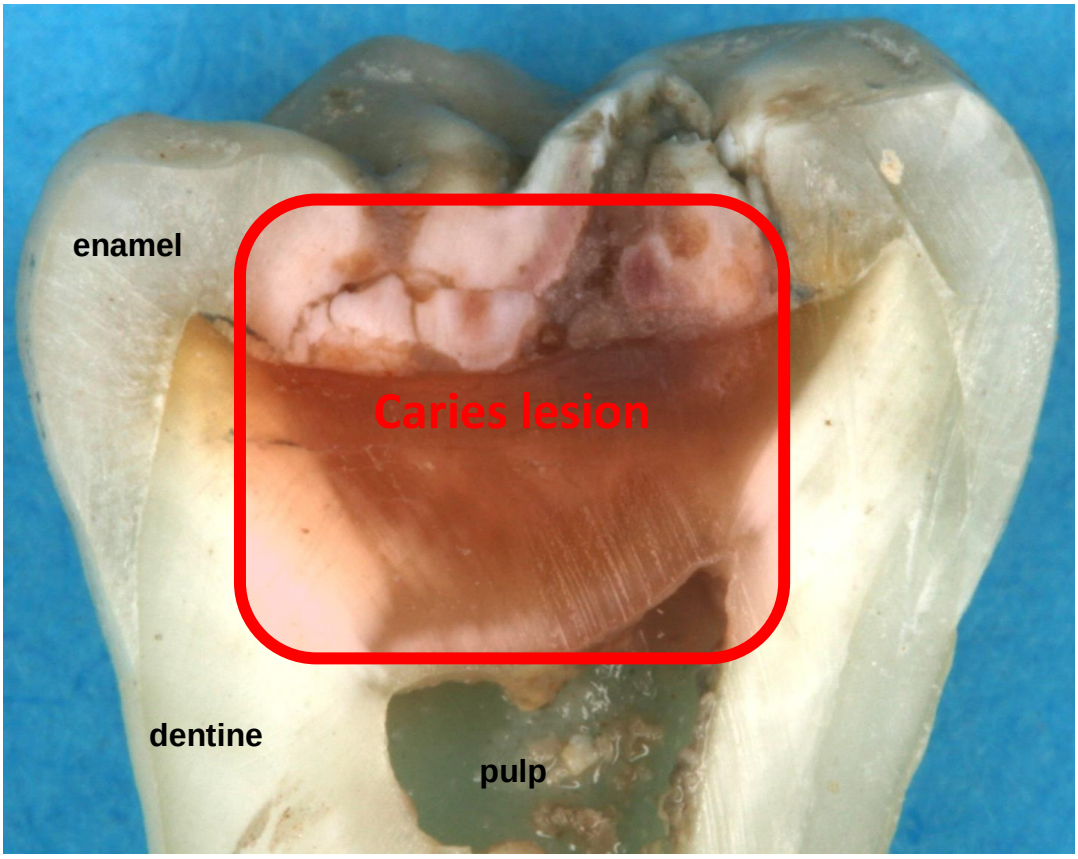

Figure 2.5: A photograph of a human molar with a deep carious lesion that has extended into the dentine.

2.4.1.1 Plaque

In order for caries to occur, a biofilm called plaque must be formed (Zaura and ten Cate, 2004); a dynamic and active community of microorganisms attached to the tooth surface in an organised manner. Plaque formation begins with the formation of a pellicle; an acellular film which contains glycoproteins, lipids and phosphoproteins. This proteinaceous film has selective permeability, restricting the transport of ions in and out. Within 0 – 4 hours, single bacterial cells, such as *Streptococci*, begin to colonise the pellicle, adhering initially *via* Van der Waal forces and electrostatic interactions (Lower, 2005). Within 4 – 24 hours, the bacterial growth results in a micro-colony. The microbes then co-aggregate and undergo succession and within 14 days there is increased species diversity on the tooth surface (Fejerskov and Kidd, 2008b).

This community of microorganisms is always metabolically active, many of the bacteria being able to ferment carbohydrates, producing acid by-products as a result. The most cariogenic bacteria, such as *S.mutans*, can not only produce organic acids quickly but are also acidophiles, meaning they thrive in extreme acidic environments. It takes less than 3 minutes for the pH in the oral environment to drop to less than pH 5, with continuous falls in pH resulting in demineralisation (Stookey, 2008).

2.4.1.2 Caries Prevalence

Over recent decades, there has been a significant decrease in the occurrence of dental caries in developed countries, primarily due to the use of fluorides (Tenuta and Cury, 2010). Figure 2.6 shows a graph from the Child Dental Health Survey (2013) displaying the prevalence of children with dentine caries in permanent teeth. The graph illustrates that between 1983 and 2003, there was a vast reduction in dentine caries but the rate of decline between 2003 and 2013 was insignificant and consequently, caries is still a major health concern (CDH Survey, 2013).

Caries is more prevalent in developing or socio-economically deprived countries and is seen in increasing levels in countries where preventive programmes have not been established (Petersen *et al*., 2005). According to the World Health Organisation fact sheet 318 (April 2012), 60 - 90 % of school children worldwide and nearly 100 % of adults had dental cavities and 30 % of people 65 - 74 years had no natural teeth whatsoever (World Health Organisation, 2012).

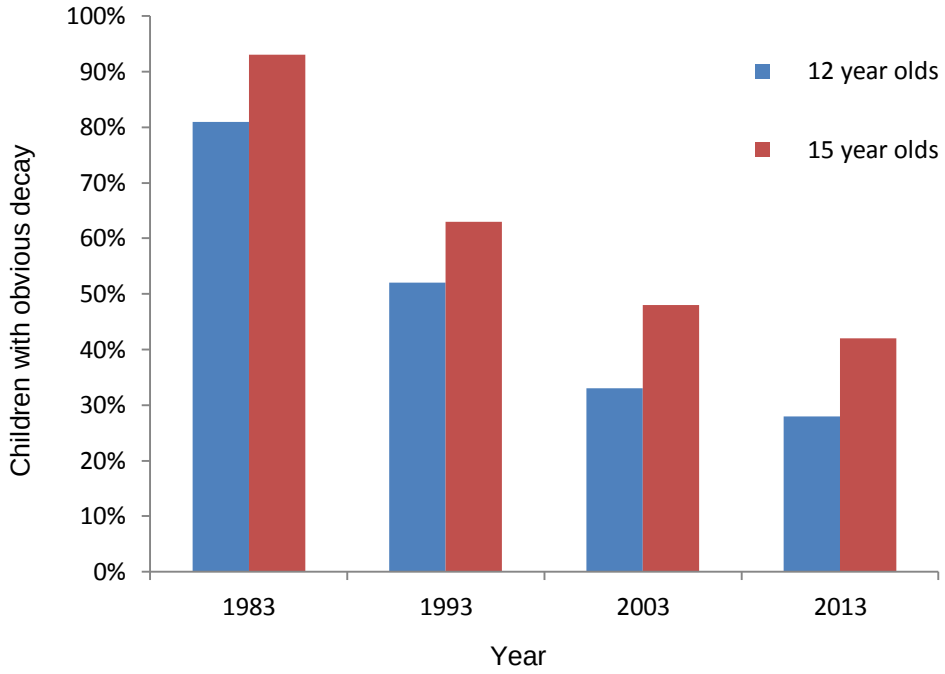

Figure 2.6: Percentage of 12 and 15 year olds with "obvious decay experience" from 1983 to 2013* (*Data for 1983 and 1993 relates to UK but data for 2003 and 2013 excludes Scotland). With permission from (CDH Survey, 2013)

2.4.2 Acid Erosion

Interest in acid erosion initiated in 1993 when it was initially mentioned in the UK Child Dental Health Survey (Downer, 1995) and later in 2006, acid erosion was described as "a challenge for the 21st century" (Ganss, 2006). Unlike caries, erosion is defined as "the irreversible loss of tooth surface by an acid not produced by bacteria" (Litonjua *et al*., 2003).

The initial phase of acid erosion involves demineralisation and softening of the tooth surface, caused by gastric acids from the stomach or external acids from diet (Bartlett, 2006). As the outer surface of the tooth is softened it becomes more vulnerable to physical wear; abrasion from tooth brushing or attrition from the grinding of teeth. As demonstrated in Figure 2.7, if there is no intervention and these factors are left unchanged then tooth surface loss can lead to an irreversible and severe change in appearance (Amaechi and Higham, 2005, Barbour and Rees, 2006).

Each time that the tooth is exposed to an acid, an ultra-thin layer of the surface is demineralised and removed. Whilst plaque is an essential

prerequisite for caries, for erosion, plaque theoretically prevents an acid attack by acting as a barrier and a buffer on the tooth surface.

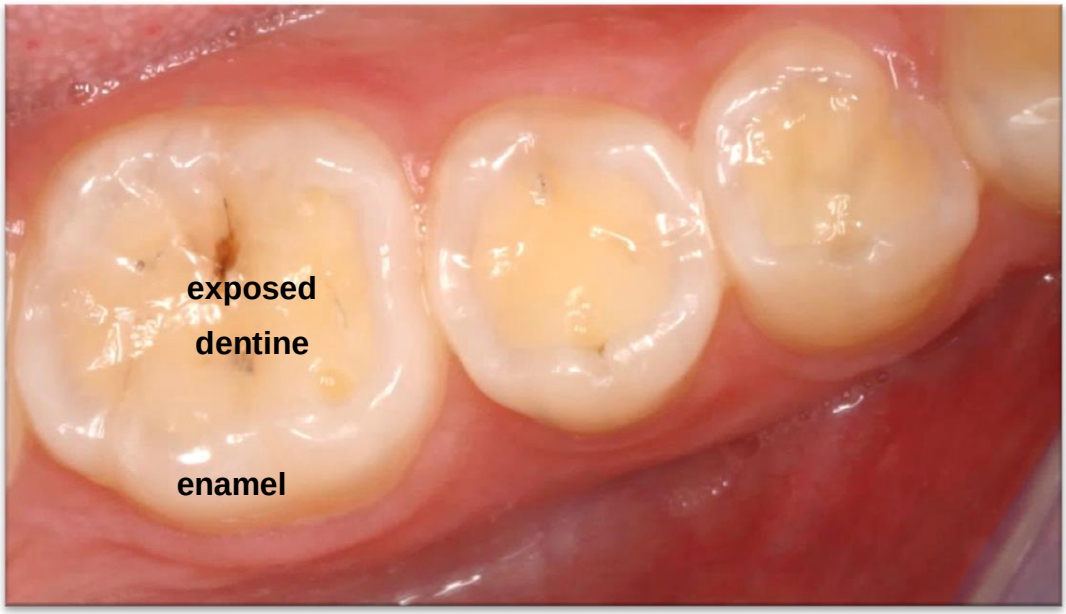

Figure 2.7: A photograph of human pre-molar and molar teeth showing tooth surface loss caused by acid-induced erosion. The enamel on the occlusal surface has been dissolved exposing the softer dentine beneath.

The main cause of erosion is from extrinsic acids in the diet (Lussi and Carvalho, 2014) and the prevalence depends on food habits, selection of food/beverage and prehistory. When an individual is drinking, the liquid flows over the tongue before flowing towards the throat and during swallowing; the tongue is pressed up against the palate and maxillary teeth. As acidic liquids flow over the lingual surfaces of maxillary teeth, erosion has the chance to occur. In a lot of circumstances, the lips and cheeks of the oral cavity can protect the labial and buccal teeth surfaces. However, some individuals have a habit of swishing the drink around, exposing the labial, buccal and occlusal surfaces to the acid (Fejerskov and Kidd, 2008a). Hydrochloric acid from the stomach can also cause dental erosion when regurgitated voluntarily (as part of eating disorders, bulimia) or involuntary (digestive functional illness).

Acid erosion is generally restricted to the few seconds that the acidic solution is present on the tooth surface. When a soft drink is consumed, the pH initially falls to a value not much lower than the drink itself and then it only

takes around a minute for the pH to reach 5.5 again, in a process known as 'cycling'. When pH is below 5.5 then demineralisation of enamel will occur, when pH is higher 5.5 then demineralisation will not occur; pH 5.5 is referred to as the "critical pH value". The increase in pH is due to saliva which has a high buffering capacity due to its high content of bicarbonate, discussed in detail in section 2.5.1.1. However, orange juice has such a high buffering capacity that the low pH is maintained for a few minutes (Fejerskov and Kidd, 2008a).

An acidic drink would have to be rinsed away within the first 30 seconds of it entering the mouth to prevent demineralisation. This emphasises the consequences that drinking habits have on acid erosion because if an individual sips the beverage over a long period of time then they expose their teeth to erosion much more than if they were to drink it in one go. This is also relevant for dental caries as each sip of a sugary drink coats the tooth surfaces with sugar.

2.4.2.1 Erosion Prevalence

Considering that every individual that has consumed fresh fruit or fruit juice in their lifetime has almost certainly been the victim of some acid erosion, it is not surprising that acid erosion is now a widespread health concern. The principal difficulty for epidemiology studies of acid erosion is in the diagnosis. Prevalence data for caries is typically given by average number of decayed, missing and filled surfaces (DMFS), but no such concept is available for acid erosion. As a whole, erosion has been much less investigated and is less understood than caries (Smith and Robb, 1996). The main solution in tackling acid erosion is probably to control the intake of dietary acids but there is a rising demand for scientists to characterise the effects of acid erosion and to discover an effective treatment for patients with regular exposure to gastric acids (e.g. individuals with eating disorders or gastro-oesophageal reflux disease (GORD)) (Uhlen *et al*., 2014). Currently, scientists are using techniques previously performed on caries, such as the use of fluoride, and reapplying this to acid erosion models (Ganss, 2008).

2.4.3 Demineralisation of Dentine

When the enamel is worn away by caries or erosion, or when the tooth root is exposed because of recession, dentine becomes exposed and can also be affected by demineralisation. Once caries has reached the dentine, it progresses more rapidly because: (i) there is less mineral content in dentine compared to enamel, (ii) there is more carbonate in the dentine mineral and (iii) the crystallites are smaller than in enamel (Hoppenbrouwers *et al*., 1987). Whereas enamel becomes demineralised at pH 5.5, dentine starts to become demineralised at around pH 6.7. Once the dentine is demineralised it can also involve bacterial invasion and damage to the pulp. This results in chronic inflammation and with no intervention, demineralisation will progress until the tooth is destroyed (Fejerskov and Kidd, 2008b). When dentine is exposed, the surfaces become sensitive to hot and cold food, dentine demineralisation not becoming obvious until individuals incur sensitivity symptoms (Addy, 2005).

Figure 2.8 is a ground section of a human molar that illustrates the effect that caries has on the dentine structure. The area of dentine that is first exposed to caries is often referred to as the ‘zone of destruction’, which becomes necrotic and sometimes liquefies causing cracks or clefts. With rapid caries, the zone of destruction is often soft and yellow but with slowly progressing caries, it is harder and browner.

As caries progresses, the tubules often become invaded by bacteria which cause demineralisation by their acid production, as well as proteolysis of key collagenous proteins. The tubules react to caries by stimulating odontoblasts to over produce mineral until the tubules become plugged with mineral deposits. This helps to slow down the acid attack and also provides protection to the pulp; the over production of mineral clinically referred to as the ‘sclerotic area’ and is illustrated on Figure 2.8. Tubules also produce tertiary dentine as a defence mechanism to caries, as discussed in section 2.3.5 (Fejerskov and Kidd, 2008b). Furthermore, sometimes odontoblasts within the tubules become denatured and these are observed as ‘dead tracts’, as illustrated on Figure 2.8.

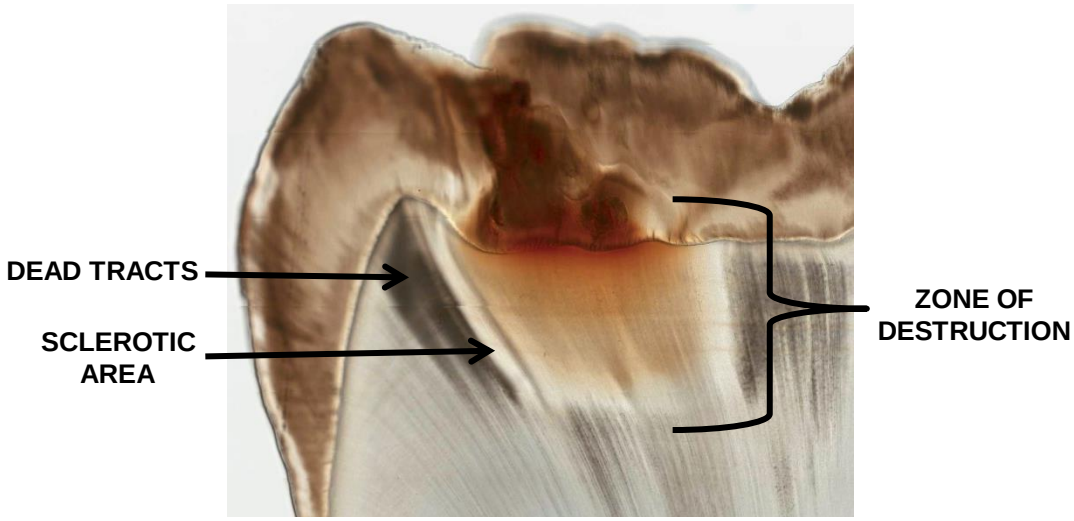

Figure 2.8: Ground section of a human molar with a caries lesion, illustrating the zone of destruction, sclerotic areas and dead tracts

2.4.3.1 Collagen Degradation

It is well accepted that caries and erosion effect dentine mineral levels, but the exact mechanism behind the denaturation of dentine collagen remains unclear. Perhaps one reason for this is the lack of techniques available to distinguish between denatured collagen and native collagen. Type I collagen lacks any enzymatic activity, which is unusual for a protein (Nimni *et al*., 1991) and eliminates the common methods used to characterise denaturation, such as loss of enzymatic activity. Although many techniques exist that allow the observation of collagen, they often require specimen dehydration, a high vacuum and/or high energy incident probes that may lead to artefacts or sample destruction. The collagen triple helix is surrounded by polar hydrogen bonding of water, which preserves intermolecular spaces and offers porosity. On exposure to dehydration, the surface quickly becomes smooth and homogenous; the collagen fibres becoming densely packed with no gaps between them (El Feninat *et al*., 2001). Despite the difficulties in characterising collagen, there are three suggested mechanisms for its denaturation in dentine: (a) bacterial proteases, (b) acidic conditions, (c) matrix metalloproteinases and cathepsins.

(a) Bacterial proteases

In 1961, pioneering work on dentine caries discovered that the majority of carious dentine maintains the same structure even after demineralisation, but following a 'second wave' of destruction the collagenous matrix becomes destroyed by bacteria (Johansen and Parks, 1961). Subsequent research then demonstrated that a dentine caries lesion can be divided into two zones: the inner zone and the outer zone (Kuboki *et al*., 1977, Dung and Liu, 1999, Fusayama, 1979, Fusayama and Kurosaki, 1972). The outer zone is fully demineralised with lots of denatured collagen and a high number of bacteria. The inner zone is only partially demineralised and has the ability to remineralise and establish new collagen cross links (Shimizu *et al*., 1981, Beeley *et al*., 2000, Ogawa *et al*., 1983).

Also, in the 1960s it was revealed that microorganisms in the oral cavity have the ability to produce proteinases, such as collagenases. These break down the collagen network (Gibbons and Macdonald, 1961, Mergenhagen and Scherp, 1960) causing the denatured collagen to lose its ability to become mineralised again (Fusayama and Kurosaki, 1972). Over the years, many studies have focused on the following genera: *Streptococcus*, *Actinomyces*, *Lactobacillus*, *Bifidobacterium*, *Rothia*, *Arachnia*, *Eubacterium*, *Propionibacterium*, *Veillonella*, *Proprionibacterium*, *Selenomonas*, *Atopobium* and *Prevotella* (Hahn *et al*., 1991, Vanhoute, 1994, Becker *et al*., 2002, Chhour *et al*., 2005, Munson *et al*., 2004, Aas *et al*., 2008); whilst later studies further demonstrated a substantial dominance of *Lactobacilli* in deep dentine caries (Gross *et al*., 2010, Gross *et al*., 2012). Furthermore, Harrington and Russell (1994) described two proteases produced by *S.mutans* that degrade human type I collagen and it has been suggested that they may be involved in dentine caries (Harrington and Russell, 1994).

Bacterial proteases cleave the collagen molecule in the telopeptide regions, as illustrated in Figure 2.9. The telopeptide regions are involved in inter- and intra- molecular cross links, and therefore, degradation at these areas may detach the collagen molecules from each other, affecting overall fibril integrity.

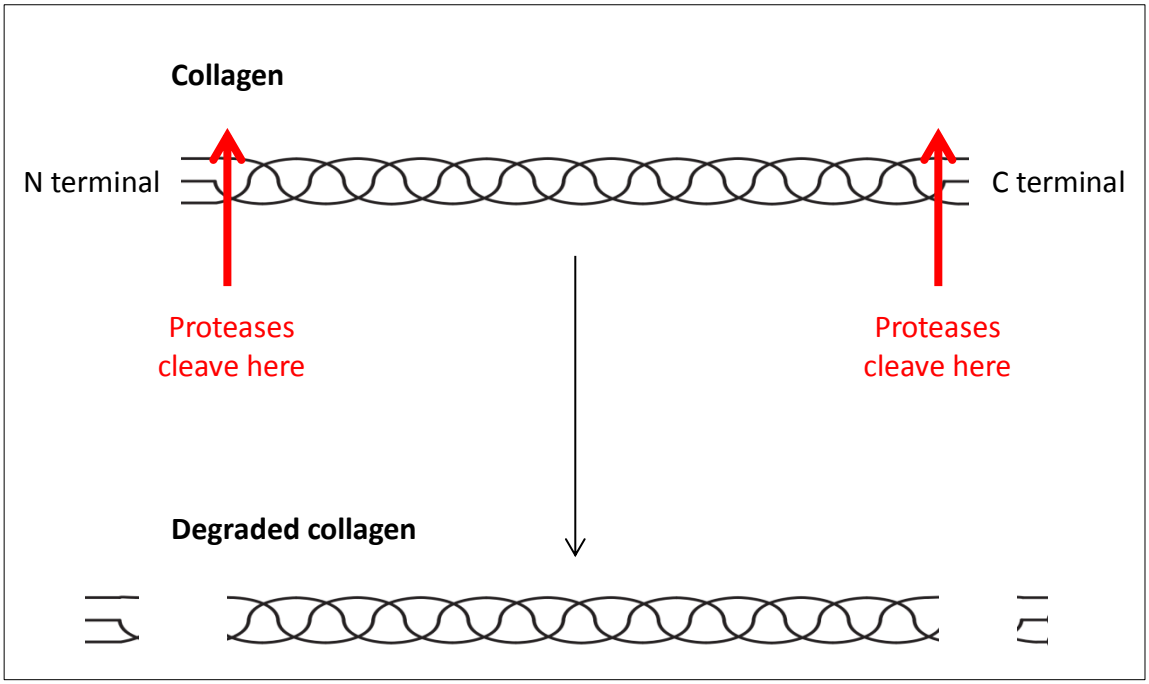

Figure 2.9: An illustration to demonstrate how bacterial proteases break down collagen by cleaving the collagen at the telopeptide regions (N and C terminals)

However, some *in vitro* studies have shown that oral bacteria lack the enzymatic competence to degrade intact collagen to the extent that has been reported (Vanstrijp *et al*., 1994, vanStrijp *et al*., 1997). This suggests that there are other mechanisms, aside from bacterial proteases, that cause collagen degradation. Furthermore, this suggests that the acid itself could be directly involved in collagen degradation and if so, collagen denaturation is not exclusive to dentine caries but may also occur during acid erosion too.

(b) Acidic conditions

Zhang *et al* (1998) state that "solutions with high hydrogen ion concentrations can alter the tertiary structure of proteins by causing protonation of amino groups and suppression of the ionisation of carboxyl groups". This addition of positive charge and decrease of negative charge may result in changes of the configuration of non-helical parts of collagen. However, it is thought that the helical portions of collagen may provide enough stability to resist degradation during short exposures to acid at room temperature (Zhang *et al*., 1998).

There are many studies investigating the direct effects of acid conditioners on dentine smear layers. A smear layer is a surface layer of mineral, 0.5 – 2 µm thick, that develops after tooth surfaces have been prepared with cutting instruments (Eick *et al*., 1970). The permeability of mineralised dentine covered with a smear layer is reduced significantly, preventing sufficient infiltration (Nakashima *et al*., 2009) and therefore, acidic conditioners are used to remove the smear layer by solubilising the mineral crystallites (Vanmeerbeek *et al*., 1993). Whilst removing the smear layer is essential for many dental treatments, it is important that acid conditioners do not affect the collagen network.

Eick and colleagues (1993) used electron microscopy to compare the dentine surface before and after treatment with an acidic conditioner and found that treatments worked well to prepare surfaces for infiltration without affecting the collagen cross-banding or structural integrity (Eick *et al*., 1993). Studies using atomic force microscopy (AFM) to observe dentine samples in hydrated and dehydrated states also showed that the collapse and denaturation of collagen was due to dehydration and not acid etching using an acidic conditioner (El Feninat *et al*., 2001).

In contrast, Marshall *et al* (1993) used AFM to observe the effects of acid on dentine collagen and showed that the intertubular matrix appeared to collapse when exposed to dilute nitric acid (Marshall *et al*., 1993). Perhaps the most detailed study in the literature involves fourier transform infrared spectroscopy (FTIR). This technique can define the molecular composition of a sample surface and verify the conformational mobility of peptide chains. The study by Eliades *et al* (1997) used multiple internal reflection (MIR) – FTIR to demonstrate that acidic conditioners result in some collagen denaturation. The changes in collagen type I were studied by deconvoluting the amide I bands of the spectra and assigning the band components to carbonyl hydrogen bonding states of the alpha helix. After exposure to an acid conditioner, the intermolecular set of hydrogen bonds was reduced and shifted to lower frequencies, and the presence of proline hydrogen bonded amine carbonyls increased (Eliades *et al*., 1997). These changes represent a decrease in bond energy on the dentine surface, which complies with

previous findings by Erickson (1992) that have shown that dentine has a low surface energy after exposure to an acid (Erickson, 1992).

Furthermore, a study by Dung *et al* (1994) indicated that dentine, once demineralised by lactic acid, was significantly more soluble to trypsin, suggesting that some denaturation had occurred. They exposed root dentine powder specimens and pure collagen type I specimens to lactic acid (pH 4 or 5.5) and then treated the specimens with trypsin before measuring the remaining soluble collagen using a hydroxyproline assay. The results indicated that dentine and collagen specimens required a pre-treatment with lactic acid, before subsequent trypsin exposure, to produce denatured collagen (Dung *et al*., 1994). These results comply with previous findings that only denatured collagen is susceptible to an enzymatic attack from trypsin (Scott and Leaver, 1974).

Immunohistochemistry has also been used to evaluate the morphological aspects of collagen fibres after phosphoric acid treatment; one particular study using an anti-collagen type I monoclonal antibody tagged with gold particles. Phosphoric acid exposure for longer than 15 seconds caused a statistically significant decrease in the labelling index, indicating that structural modifications may have occurred to the exposed collagen (Breschi *et al*., 2002). Therefore, there is some evidence to show that collagen denaturation can occur in the presence of an acid, in the absence of bacteria.

(c) Matrix metalloproteinases and cathepsins

Recent research has focused on the role of matrix metalloproteinases (MMPs) and cysteine cathepsins as an additional mechanism of collagen denaturation (Nascimento *et al*., 2011, Chaussain *et al*., 2013). MMPs were first introduced in section 2.3.2.2; they are endo-peptidase enzymes produced by odontoblasts (Hannas *et al*., 2007), expressed during normal dentine-pulp formation, and are involved in the maintenance of the organic structure of dentine (Murphy and Knauper, 1997). There have been many MMPs discovered localised in dentine, namely MMP -2, -3, -8, -9, -14 and -20 (Martin-de las Heras *et al*., 2000, Mazzoni *et al*., 2011, Boushell *et al*.,

2011) with MMP -8 being the most abundant (Sorsa *et al*., 2006, Sorsa *et al*., 2004). It is believed that once MMPs are produced by odontoblasts in the pulp, they are secreted into pre-dentine in an inactive form and then become trapped within the dentine collagen network whilst mineralisation occurs (Martin-de las Heras *et al*., 2000, Sulkala *et al*., 2007). Some studies have shown that there are more MMPs in the pulp end of dentine compared to the enamel end, suggesting that MMPs are secreted through the tubules (Niu *et al*., 2011). It is believed that MMPs cause the denaturation of collagen in four stages:

(1) Hydroxyapatite is dissolved by an acid, leaving the organic network to become exposed, including inactive MMPs.
(2) A decrease in pH causes the activation of MMPs (in particular, MMP-8) (Tjaderhane *et al*., 1998, Tezvergil-Mutluay *et al*., 2013).
(3) Due to cycling, the pH increases and the active MMPs apply proteolytic properties to degrade the collagen network (Figure 2.10).
(4) The degraded collagen can be further degraded by other MMPs or bacterial proteases, in the case of caries.

Additional studies have supported this mechanism. For example, there appears to be a significantly increased abundance of MMP-8 in saliva during a caries attack (Nordbo *et al*., 2003) and additionally, the inhibition of MMPs has been shown to prevent collagen degradation (Tjaderhane *et al*., 1998).

Cathepsins, proteases activated by low pH, have also been of key interest to researchers. They are thought to play a role in the activation of MMPs as well as being able to cleave the collagen molecule directly, leading to the denaturation of the collagen matrix (Garnero *et al*., 2003). A recent study has supported these theories and discovered a significantly higher abundance of cathepsin -B and -K in caries-affected dentine compared to healthy dentine (Vidal *et al*., 2014).

Figures 2.10 and 2.11 illustrate where MMPs and cathepsins cleave the collagen molecule; MMPs cleave the helix at the region between amino acids 775 – 776 whereas cathepsin cleaves at multiple sites within the helical portion as well as at the terminal ends.

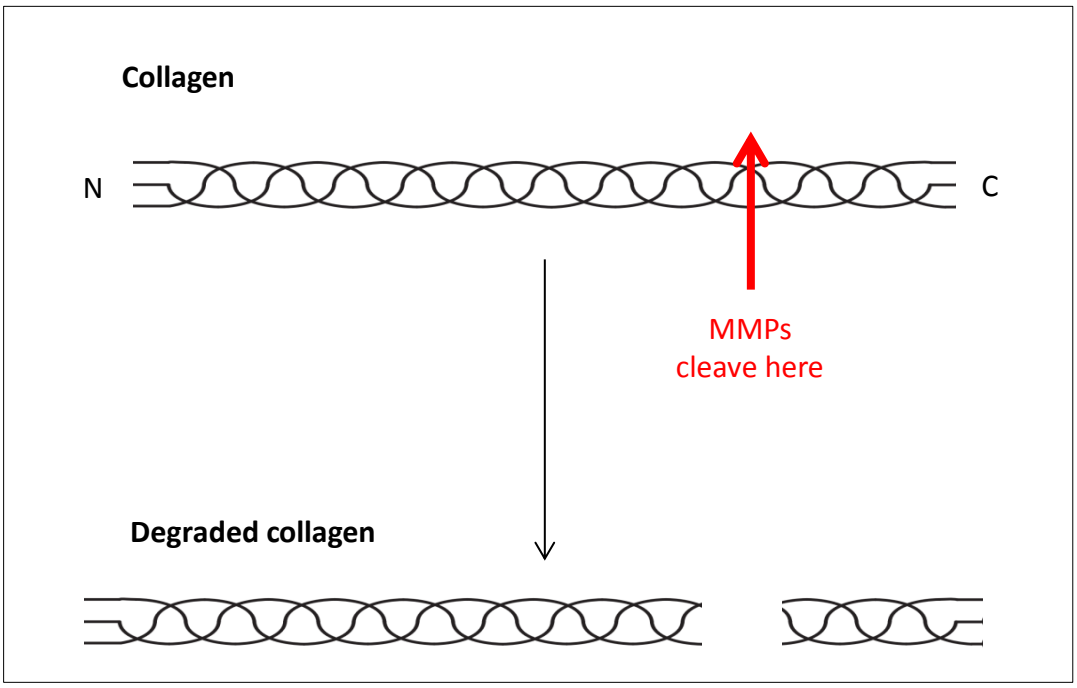

Figure 2.10: An illustration to demonstrate how MMPs break down collagen by cleaving the collagen within the helical region (Adapted from Garnero *et al.,* 2003).

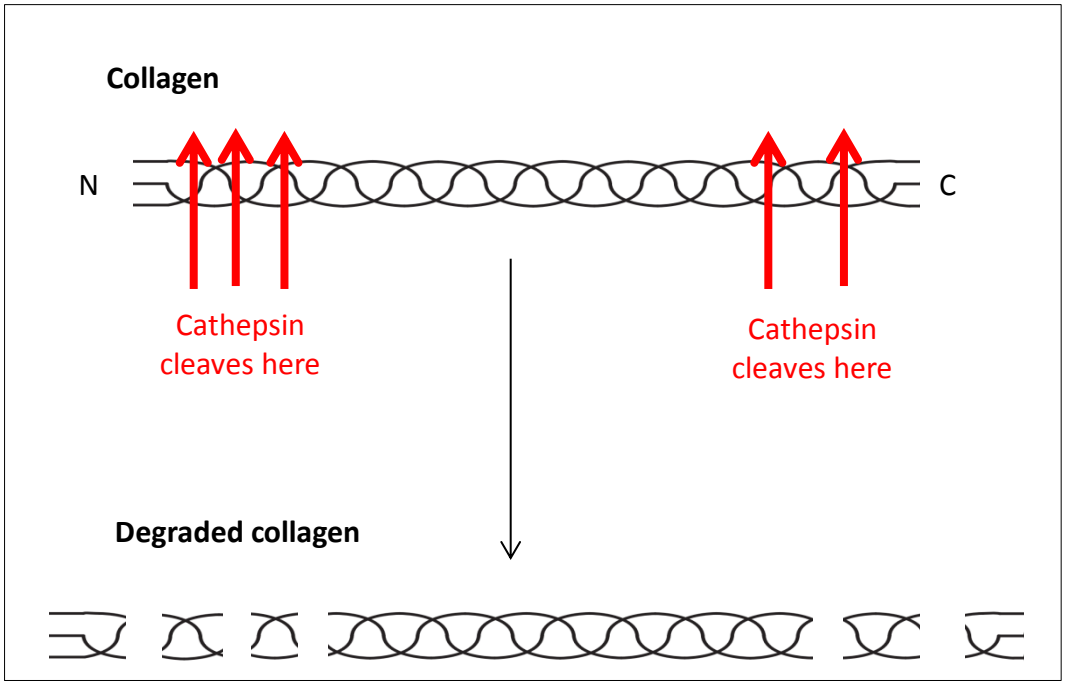

Figure 2.11: An illustration to demonstrate how cathepsins break down collagen by cleaving the collagen within the helical and telopeptide regions (Adapted from Garnero *et al.,* 2003).

Previously, it was assumed that dentine collagen is only affected during caries, but recent findings suggest that dentine collagen is also vulnerable during acid erosion; either directly from the acids or from MMPs and cathepsins. This raises concerns for the use of acid etching techniques during clinical treatments. Furthermore, recent findings show that acid conditioners may activate MMPs, which can then degrade collagen and weaken the stability of the bond between the dentine and the adhesive (Chaussain *et al*., 2013, Tjaderhane *et al*., 2013).

More research is also essential to understand if different acids, i.e. strong and weak acids, cause different degrees of degradation. In addition, it is also unclear whether the low pH environment affects the non-collagenous proteins. As discussed in section 2.3.2, non-collagenous proteins are a crucial component of dentine, and if they are affected by the acids in caries or erosion, then the integrity of dentine will be compromised.

2.5 Management of Demineralisation

The management of demineralised dentine can be divided into three categories: prevention, removal (caries only) and remineralisation.

2.5.1 Preventing Demineralisation

There are various components that can help to prevent demineralisation such as saliva, diet and oral hygiene (including fluoride and chlorhexidine). As people are living for longer, prevention is key to retaining teeth for longer. As reviewed by Walls and Meurman (2012), there are a range of approaches to prevent demineralisation (Walls and Meurman, 2012) and some are listed below.

2.5.1.1 Saliva

Saliva has a variety of roles within the oral cavity. It keeps the oral tissue lubricated which in turn allows the functions of speaking, eating and swallowing. Both the inorganic and organic components of saliva also play a critical role in protecting teeth and preventing dental caries and acid erosion (Lenander-Lumikari and Loimaranta, 2000). There are four main ways in which saliva can help to prevent caries:

(1) Saliva rinses food debris, and sometimes bacteria, from the tooth surfaces.
(2) Saliva contains specific antibodies e.g. secretory immunoglobulin A, lysozyme, lactoferrin and lactoperoxidase; that possess specific antibacterial properties against cariogenic bacteria.
(3) Saliva has a high buffering capability primarily due to its bicarbonate content. The bicarbonate buffer removes the increased amounts of hydrogen ions, from the acids, to produce more carbonic acid. This increase in carbonic acid is then removed by carbonic anhydrase which catalyses the reaction of carbonic acid to carbon dioxide and water (Lagerlof and Oliveby, 1994, Stookey, 2008, Edgar *et al*., 1994). This allows the acidic environment in the oral cavity to be quickly neutralised.
(4) Calcium, phosphate and fluoride ions found in saliva can diffuse into plaque and enhance remineralisation.

The flow rate of saliva is very important; a healthy person secretes between 0.5 L – 1.0 L of saliva every day and teeth are usually bathed in 0.5 ml of unstimulated saliva at all times; forming a layer approximately 10 µm thick (Nissim *et al*., 2006). Saliva has a better buffer capacity when the flow rate is high, allowing the pH to be neutralised quicker and bacteria to be cleared from the surface (Miura *et al*., 1991). A reduced saliva flow rate can lead to severe caries or acid erosion because the acidic environment remains for longer when saliva quantity is diminished (Spak *et al*., 1994).

2.5.1.2 Dietary Habits

Diet plays a significant role in caries and erosion prevention. All acidic food and drinks such as fruit and fruit juices cause acid erosion, and a diet that involves a high frequency of sugar intake can cause caries (Kidd, 2004). Demineralisation occurs in enamel when the pH drops to 5.5 and occurs in dentine when the pH reaches 6.2 (Dawes, 2003). Consumption of fermentable carbohydrates or acidic drinks can leave the oral cavity with a pH as low as 4 and if carbohydrate or acid intake is regular, then the pH can remain low for a while, resulting in demineralisation. Therefore, it is the frequency of the food and drink intake, rather than the quantity, that is the

key cause (Kandelman, 1997). Demineralisation could be reduced in a diet low in fermentable carbohydrates and acids.

2.5.1.3 Oral Hygiene

Oral hygiene (including toothpastes, mouth rinses and dental flosses) is imperative to maintain healthy teeth and control the formation and progression of caries and erosion (Fejerskov and Kidd, 2008b). However, as described below, it is believed to be the fluoride present in the products that makes the actual difference in caries prevention.

2.5.1.4 Fluoride

There are four ways in which fluoride can help to hinder the demineralisation process:

(1) When fluoride is present in low concentrations it can help to eliminate demineralisation and even promote remineralisation. When pH is above 4.5, low levels of fluoride cause fluorhydroxyapatite to form on the surface layers of the teeth whilst the hydroxyapatite dissolves. When the pH rises to above 5.5 again, fluoride ions react with the free calcium ions and hydrogen phosphate ions to form fluorapatite crystals. Fluorapatite is less soluble than hydroxyapatite and is more resistant to acid dissolution in environments with pH as low as 4.5 (Smales and Yip, 2006).

(2) When fluoride is present in high concentrations (>100 ppm), it reacts with calcium to form calcium fluoride. This acts as a fluoride reservoir which can later dissolve to release fluoride and interfere with caries or erosion by either forming fluorapatite (as described above, (1)) or inhibiting bacterial acid production (as described below, (3)). The rate of fluoride dissolving from calcium fluoride increases when the pH reduces. The layer of calcium fluoride also protects the surface due to its low solubility (Fejerskov and Kidd, 2008b).

(3) Fluoride can inhibit ATPase activity in oral *Streptococci* resulting in less acid production. This affects the ability of bacteria into the cells and consequently reduces lactic acid formation. It further prevents the production

of glycogen which subsequently prevents the bacteria from producing acid without the presence of sugars.

(4) Fluoride may affect the morphology of the teeth making them less likely to develop caries. When fluoride is present during tooth formation it may cause teeth to have shallower fissures, resulting in less food and plaque accumulation on the tooth surfaces (Fejerskov and Kidd, 2008b).

One of the main reasons for the success of fluoride has been the ease and variety of ways that it can be administrated. Fluoride is most commonly delivered by: (a) drinking water, (b) dentifrices (toothpastes), (c) mouth rinses and (d) gels or varnishes. Other sources of fluoride include (e) highly concentrated pastes, (f) tablets for children and (g) milk.

(a) Fluoridated Water

The fluoridation of drinking water is a cost effective way of delivering fluoride to a large population of people and consequently, there are currently over 210 million people worldwide that consume fluoridated water at 1 ppm (Pollard *et al*., 2000). Fluoridated water has a topical effect and for it to be effective, it must be ingested continuously where it can then exist in the oral cavity from the secretion of saliva. The fluoride concentration of people living in fluoridated areas is approximately 0.02 ppm compared to 0.01 ppm in non-fluoridated areas. This increase in fluoride has demonstrated a tremendous effect in reducing dental caries (Proskin and Volpe, 1995).

(b) Dentifrices (toothpastes)

Fluoride in toothpaste is one of the major factors influencing the decrease of dental caries. It is estimated that over 500 million people worldwide use fluoridated toothpaste and in some countries, fluoride toothpastes denote over 95 % of the total dental products (World Health Organisation, 1994). Tooth brushing with fluoride toothpaste is beneficial because it controls caries by removing the biofilm and also by enriching un-removed biofilm with fluoride. The additional fluoride can remain in saliva for 1 – 2 hours and in the biofilm for up to 10 hours after brushing (Zamataro *et al*., 2008).

The effectiveness of fluoride dentifrices is affected by pH and other ingredients that are present. There are four main fluoride formulations used in dentifrices:

- Sodium fluoride (NaF)
- Sodium monofluorophosphate (SMFP)
- Stannous fluoride (SnF_2)
- Amine fluoride (AmF)

NaF and SMFP, found in the majority of toothpastes, release fluoride ions at different rates; NaF fluoride ions are available more readily due to the requirement of SMFP to be broken down. Preferably however, SMFP is combined with calcium carbonate abrasives, comparably cheaper than inert abrasives, such as silica, required for NaF toothpastes. Calcium carbonates react with fluoride ions from NaF leading to dentifrice inactivation (Cury *et al*., 2004).

(c) Mouth Rinses

The levels of fluoride in mouth rinses tend to be 225 ppm for daily usage or 1000 ppm for weekly usage. They have been very successful in helping to control demineralisation, especially when combined with fluoridated water, or when patients have an extremely high risk of caries. The main disadvantage with mouth rinses is that they are unsuitable for children below the age of 6 as the accidental swallowing of mouth rinses may lead to fluorosis (Fejerskov and Kidd, 2008b).

(d) Gels and Varnishes

Gels and varnishes usually have a very high level of fluoride and are applied by a dental care professional to an individual with a high risk of dental caries. Products with a high concentration of fluoride, such as a varnish (22500 ppm) or gel (from 9000 – 123000 ppm) can be applied to a tooth surface, allowing fluorapatite to be precipitated onto the tooth surface (Tenuta *et al*., 2008). A recent review of 13 clinical trials has shown that, on average, fluoride varnishes reduced the number of decayed, missing or filled teeth by 43 % in young people (Marinho *et al*., 2013).

(e) High Fluoride Pastes

High fluoride toothpastes have a concentration of fluoride of up to 5000 ppm. These high fluoride toothpastes are used to help prevent and control caries in high-risk patients, they are not suggested for children. They are also effective in controlling root caries.

Mannaa *et al* reported significant effects of using high fluoride toothpastes. There was recently a 6 week clinical trial testing a 5000 ppm fluoride toothpaste. Clinical examination and analysis of the saliva has shown that the highly concentrated fluoride paste is able to reduce the risk of caries (Mannaa *et al*., 2014). Furthermore, Ekstarand *et al* performed a randomised clinical trial to examine the anti-caries effect of a 5000 ppm fluoridated toothpaste compared to a regular 1450 ppm fluoridated toothpaste. Results showed that the highly concentrated paste was significantly better for controlling root caries and promoting remineralisation than the regular toothpaste (Ekstrand *et al*., 2013).

(f) Tablets for Children

During the 1950s, fluoride supplements (tablets and lozenges) were made available to those living in areas with non-fluoridated water. Since 1994, the use of fluoride tablets has been reconsidered in many countries.

In 2011, Tubert-Jeannin *et al* reviewed the literature to evaluate the efficacy of fluoride tablets (Tubert-Jeannin *et al*., 2011). The results showed that, regarding permanent teeth, fluoride supplements compared to no supplements were associated with a 24 % reduction in DMFS. However, the effect on primary teeth was inconclusive.

(g) Fluoridated Milk

Since 1932 there have been reports outlining the general benefits of milk consumption for dental health (Sprawson *et al.* 1932). In the 1950s, along with the introduction of fluoridated water, the benefits of fluoridated milk were also explored (Inamura *et al.*1959).

In 2015, Yeung *et al* updated their review on benefits of drinking fluoridated milk. They concluded that there is little evidence to suggest that this fluoride

source would be substantially beneficial to children and they suggested that better quality trials are required to make definite conclusions (Yeung *et al*., 2015).

2.5.1.5 Chlorhexidine

Many mouth rinses and varnishes available contain chlorhexidine to help prevent the progression of caries (Lorenz *et al*., 2006). Chlorhexidine is an antimicrobial agent that is used in dentistry because it binds to bacterial cell walls and may inhibit plaque formation and bacterial colonisation. Current chlorhexidine-containing products include Cervitec® varnish with 1 % chlorhexidine, Cervitec® gel containing 0.2 % chlorhexidine and Cervitec® mouth rinse with 0.1 % chlorhexidine (Ozdemir *et al*., 2014, Jentsch *et al*., 2014).

A 10 % chlorhexidine varnish (Chlorzoin™) was investigated for its ability to reduce the spread of *S.mutans* from mother to child. The results showed that *S.mutans* was reduced in the children, but the incidence of caries was not altered (Dasanayake *et al*., 2002). In fact, there is little additional evidence that chlorhexidine is actually effective in caries prevention (James *et al*., 2010).

The prevention of adult caries study (PACS) was a placebo-controlled, randomised clinical trial that aimed to assess the effectiveness of a 10 % chlorhexidine solution. There was no significant difference observed between the two study arms (Papas *et al*., 2012).

In 2015, Walsh *et al* reviewed chlorhexidine varnishes and gels for their abilities to reduce caries and they suggested that current evidence is inconclusive (Walsh *et al*., 2015).

Furthermore, in 2009, chlorhexidine was investigated for its ability to inhibit MMPs and cathepsins (discussed in section 2.4.3.1) (Breschi *et al*., 2010, Breschi *et al*., 2009, Brackett *et al*., 2009, Loguercio *et al*., 2009). Currently, more research is required to verify these results but chlorhexidine could prove to be useful for maintaining the integrity of the collagen structure.

2.5.2 Removal of Dentine

When prevention is unsuccessful, the demineralised area must be removed, leaving behind only sound, healthy tooth. The cavity is then restored with a restorative material; as illustrated in Figure 2.12. There are many techniques available to a dentist for the removal of the demineralised area, for example; (a) rotary cutting instruments, (b) air abrasion, (c) air polishing, (d) ultrasonic systems, (e) lasers and (f) chemo-mechanical systems; all of which are explained in more detail as follows:

(a) Rotary cutting instruments

Rotary cutting instruments can be used to remove the demineralised part of the tooth by chipping or grinding the area away. Steel burs were the first type of rotary cutting instrument to be invented but there are other materials in use too, such as tungsten carbide and diamond (Mount *et al*., 2005). Rotary cutting instruments can be used to cut enamel and dentine quickly and easily but they can be too non-selective for dentine resulting in the unnecessary removal of non-demineralised areas.

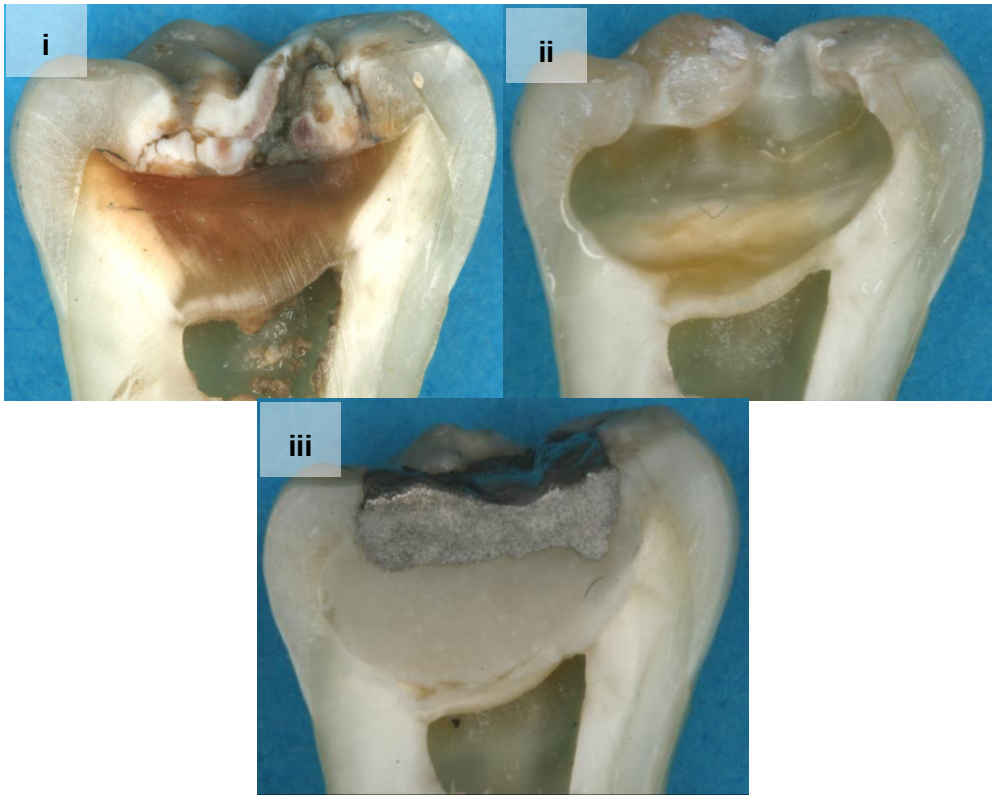

Figure 2.12: Photographs showing (i) a tooth with a deep caries lesion; (ii) the tooth after the carious lesion is removed; (iii) a restorative material in place

(b) Air abrasion

Air abrasion involves blowing aluminium oxide particles at the tooth surface using compressed air, the particles hitting the tooth and gently chipping away at the surface. The size of the particles and the speed of the compressed air can be altered to suit the size of the problem. This technique can be used to remove very early decay before a cavity is even visible. However, air abrasion can often cause difficulty for the dentist when selecting specific areas to remove, and can lead to the removal of some non-demineralised surfaces (Fejerskov and Kidd, 2008b).

(c) Air polishing

Air polishing is similar to air abrasion in that it involves the use of compressed air, but air polishing consists of water-soluble sodium bicarbonate and tri-calcium phosphate particles (Banerjee *et al*., 2000b). The fact that air polishing contains water-soluble abrasives means that the

abrasives do not escape far from the intended tooth surface and therefore, is more accurate and usually used to remove carious dentine towards the end of a cavity preparation (Fejerskov and Kidd, 2008b).

(d) Ultrasonic systems

Ultrasonic vibrations in combination with abrasive slurries can be used to prepare teeth for restorative treatment. This technique is often more pleasant for the patient because there is no heat or pressure. However, there is a limited choice of tips and the system can be quite time-consuming (Yip and Samaranayake, 1998).

(e) Laser techniques

High intensity, coherent beams of light, can be used to remove demineralised surfaces. The energy from the laser beams is absorbed by water molecules that promptly evaporate and chip away at the surface. As with the previous techniques mentioned, laser beams also have a risk of removing healthy areas of the tooth (Keller and Hibst, 1997, Moritz *et al*., 1996).

(f) Chemo-mechanical

The current problems of the techniques listed above are usually discomfort to the patients or the unnecessary removal of healthy surfaces. Following various microbiology investigations, there appears to be no difference between the number of bacteria on hard dentine and soft dentine; this suggests that some partially-demineralised dentine can be left behind during cavity preparations, allowing the tooth a better chance of survival. An alternative to conventional caries removal techniques is a chemo-mechanical system called Carisolv™ (successor to Caridex™). It is considered to remove infected dentine, leaving only affected and sound dentine behind.

Carisolv™ contains a red gel which consists of amino acids (glutamic acid, leucine, and lysine), sodium chloride, erythrocin, carboxymethylcellulose, water and sodium hydroxide. It also includes a clear liquid containing 0.5 % sodium hypochlorite. The red gel and the clear liquid are mixed together, at a 1:1 ratio, and applied to the carious dentine for 60 seconds. Carisolv™

claims to soften and dissolve carious dentine, which is then scraped away with a specially designed hand instrument, leaving only affected dentine behind. Results from clinical studies have been very promising, and it is especially useful for children or anxious patients. However, this technique can be time-consuming compared to the other removal processes (Banerjee *et al*., 2000a, Ericson *et al*., 1999).

Of the above methods, only the hand instruments (a) involve direct contact with the tooth surface and use tactile perception to distinguish between soft, demineralised dentine and hard, mineralised dentine.

2.5.3 Remineralisation

Remineralisation is the process of re-establishing minerals to the hydroxyapatite latticework structure. Natural remineralisation is continuously occuring but the level of activity varies according to the conditions in the mouth.

2.5.3.1 Natural Remineralisation

Saliva has already been mentioned as a key component in preventing demineralisation because of its ability to neutralise acidic conditions (section 2.5.1.1) but saliva has also been extensively studied for its remineralisation potential as well (Silverstone, 1973, Tencate and Featherstone, 1991, Tencate and Duijsters, 1982). As mentioned in 2.5.1.1, saliva contains high levels of bicarbonate which can remove hydrogen ions from acidic solutions to produce carbonic acid. Saliva also contains a high content of mineral ions that are dissolved by carbonic acid, which is unstable and quickly transforms to carbon dioxide and water. This permits the mineral ions to precipitate out and incorporate onto the tooth surface. In order for remineralisation to be successful, carbonic acid must be produced in proximity to mineral ions in the saliva and the mineral ions must be precipitated onto a tooth surface (Fejerskov and Kidd, 2008b).

2.5.3.2 Remineralisation Techniques

There is increased interest into ways of improving the natural remineralisation process. An enhanced remineralisation system would be

beneficial because it would allow more conservative dentistry to occur, as demonstrated in the photographs in Figure 2.13. As mentioned in section 2.4.3.1, caries lesions can be defined as having 'inner' and 'outer' zones. The outer zones are severely demineralised and the damage in this area is irreversible and needs to be removed, as shown in Figure 2.13. However, the demineralisation in the inner zone is much less severe, and the damage here is reversible. As demonstrated in Figure 2.13 (ii), if this inner zone can be remineralised and re-hardened, then it does not need to be removed during cavity preparation and instead, a smaller restorative material can be placed on top of the remineralised inner zone. There is no evidence that partial caries removal is damaging, in fact, there is evidence that it is actually more beneficial to the pulp (Ricketts *et al*., 2013). Furthermore, in order to achieve this conservative approach to restorative dentistry, a remineralisation technique that can infiltrate deep into the inner carious zone is needed.

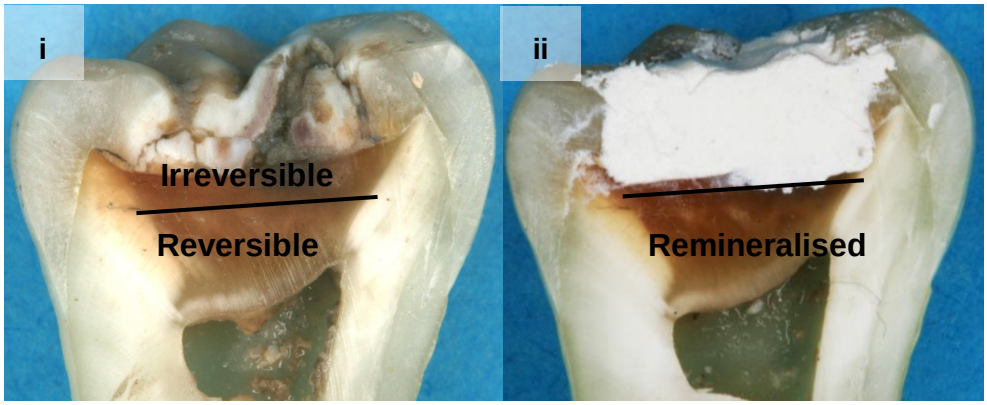

Figure 2.13: Photographs to illustrate the idea behind more conservative dentistry; (i) a caries lesion with irreversible and reversible damage, (ii) a small restorative placed above remineralised dentine

There are various remineralisation strategies that are currently commercially available, or in the process of being investigated, which are explained below. These include indirect strategies such as (a) sugar substitutes and (b) saliva substitutes, and direct strategies such as (c) fluoride delivery, (d) calcium delivery, (e) fluoride and calcium delivery, (f) bioactive materials, (g) arginine, (h) polymer-induced liquid-precursors, (i) NCP analogues and (j) self-assembling peptide scaffolds.

(a) Sugar Substitutes

Sugar substitutes are non-fermentable alternatives to sugar which can be applied to a variety of items, such as chewing gums, to decrease the amount of acid produced by bacteria from the fermentation of sugar. The most common sugar substitutes are xylitol, found in Trident™ and sorbitol, found in Wrigley's Orbit™ (Scheinin and Banoczy, 1985).

Xylitol is the most widely-used substitute and there are several claims regarding its remineralisation potential (Essig *et al*., 1985). In particular, there are three proposed mechanisms:

(1) Xylitol is non-fermentable and therefore prevents the production of acid and the subsequent dramatic fall in pH of the oral environment (Edwardsson *et al*., 1977).

(2) *Streptococci* ingest xylitol, converting it to xylitol-5-phosphate (X5P). X5P then degrades the cell membranes of the *Streptococci*, resulting in the decrease of acid production (Assev and Rolla, 1986).

(3) Xylitol chewing gum increases the quantity of saliva and therefore the quantity of calcium and phosphate ions in the oral environment, which can facilitate remineralisation.

In 2009, a study by Milgrom *et al* showed that xylitol can decrease the likelihood of caries developing in children when they intake a xylitol syrup, every day for a year, as a baby. They also showed that the effects of xylitol are much more significant than sorbitol in reducing caries (Milgrom *et al*., 2009). However, previous to this study, there were many publications indicating that xylitol is no more anti-cariogenic than other sugar substitutes. A publication from 2004 states that there is "no evidence for caries-therapeutic effect of xylitol" (van Loveren, 2004). Furthermore, many studies show that xylitol is only successful when chewed more than three times a day at 5 – 6 g per time (Ly *et al*., 2006) and this encourages the concept that its success is merely due to the increased saliva quantity that regular chewing gum can provide.

The efficacy of xylitol lozenges in adult caries preventation was tested in a placebo-controlled randomised trial (X-ACT: The Xylitol for Adult Caries Trial). Daily use of xylitol lozenges did not result in statistically significant reduction in caries (Bader *et al*., 2013).

More recently, Sharif *et al* further suggested that the effects of xylitol are still unproven. They state that there is very limited evidence that xylitol effects caries (Sharif *et al*., 2015) and to increase confidence in xylitol, there needs to be more *in vivo* studies and better designed clinical trials with more participants of all ages.

(b) Saliva Substitutes

Section 2.5.1.1 describes the benefits of having a good quality and quantity of saliva. Artificial salivas aim to replenish the quality and quantity of saliva, relieving the symptoms of dry mouth (known clinically as xerostomia) in order to prevent demineralisation and encourage remineralisation. Oralube® is an artificial saliva containing sodium, calcium, potassium, magnesium, chloride, phosphate, fluoride and also the sweetener sorbitol. It allows individuals with xerostomia to have an increased rinsing effect from saliva and increased precipitation of calcium and phosphate (Fejerskov, 2008a).

(c) Fluoride Delivery

Fluoride is found in a variety of formulations and can be administrated in many different ways to promote the deposition of fluorapatite, a less soluble form of hydroxyapatite. More information can be found in section 2.5.1.4.

(d) Calcium Delivery

There is lots of evidence proposing that high concentrations of calcium ions can encourage tooth remineralisation (Duckworth and Huntington, 2006, Shaw *et al*., 1983, Pearce *et al*., 2002, Masamura *et al*., 1995). Moreover, it is suggested that small increases in calcium concentration have a much greater effect on remineralisation than the same increase in phosphate concentration (Lynch, 2004). In fact, it has been proposed that calcium may be twenty times more powerful than phosphate at inducing remineralisation (Lynch and ten Cate, 2005, Tanaka and Kadoma, 2000). When plaque fluid

was investigated, the calcium:phosphate ratio was found to be approximately 0.3 (Margolis, 1990) but studies have demonstrated that remineralisation occurs best when the plaque fluid has a calcium:phosphate ratio of 1.6 (Lynch, 2004), which furthermore suggests that calcium concentration is the main rate-limiting component in remineralisation. Moreover, some *in vitro* experiments have shown that more remineralisation occurs when the calcium concentration is increased and the fluoride concentration is maintained. Therefore, current remineralisation research focuses on the delivery of calcium, rather than the addition of phosphate (ten Cate, 1994).

For example, calcium chloride has been tested as a delivery source of calcium in mouth rinses (Pearce, 1982, Pearce and Nelson, 1988) whilst phosphoryl oligosaccharides of calcium (POs-Ca) and tricalcium phosphate (TCP) have been added to chewing gums (Kitasako *et al*., 2011, Karlinsey *et al*., 2010a, Karlinsey *et al*., 2009)

Another example is Amorphous Calcium Phosphate (ACP) which has the trademark name Enamelon™. Enamelon™ contains calcium sulphate and ammonium phosphate which are delivered separately to provide a reservoir on the tooth surface; dissolving as they mix with saliva to release calcium and phosphate ions and precipitate to ACP (or amorphous calcium fluoride phosphate (ACFP) if fluoride ions are present). However, ACP/ACFP is very unstable and quickly converts to hydroxyapatite or fluorapatite; a more stable and insoluble phase (Takagi *et al*., 1998). Most studies with Enamelon™ have been animal models, *in vitro* and *in situ* caries models, which all show promise for this to encourage remineralisation, with evidence from clinical trials demonstrating that Enamelon™ can decrease root caries and increase caries regression compared to fluoride alone (Papas *et al*., 1999, Papas *et al*., 2008, Reynolds, 2008).

Furthermore, Recaldent™, the trade mark name for Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP), has also been shown to reduce demineralisation and enhance remineralisation (Aimutis, 2004). CPP, from the main protein of milk, can stabilise calcium, phosphate and fluoride ions as soluble amorphous complexes and therefore they help to increase remineralisation by regulating the availability of these ions to the tooth (Cross

et al., 2007, Schupbach *et al*., 1996). The stabilising abilities are important because it ensures delivery onto the tooth surface before they precipitate or crystallise. CPP-ACP binds to the tooth surface and acts as a reservoir of bioavailable calcium and phosphate; releasing calcium and phosphate under acidic conditions, increasing remineralisation. The presence of CPP-ACP has been shown to significantly reduce caries in a dose-dependent manner (Meyer-Lueckel *et al*., 2015, Vyavhare *et al*., 2015, Rezvani *et al*., 2015, Agrawal *et al*., 2015).

Sometimes fluoride can be introduced to create CPP-ACFP. The addition of fluoride makes the complex even more successful due to the co-localisation of the many ions: 1 molecule of CPP binds 25 calcium ions, 15 phosphate ions and 5 fluoride ions (Aimutis, 2004, Reynolds *et al*., 1995, Mehta *et al*., 2013, Beerens *et al*., 2010).

As with ACP, most investigations are animal models, *in vitro* or *in situ* caries models. However, there has been a two year clinical trial study which demonstrated a significant difference in caries reduction between CPP-ACP chewing gum against a control chewing gum (Morgan *et al*., 2008). CPP-ACP can be found in many dentifrices such as GC tooth mousse® (Poggio *et al*., 2014), Trident™ chewing gum (Prestes *et al*., 2013) and Recaldent Gum™ (Zero, 2009).

(e) Fluoride and Calcium Delivery

There is evidence that fluoride and calcium can work synergistically because an increase in calcium concentrations allows fluoride to be retained for longer (Magalhaes *et al*., 2007). For example, some *in vivo* studies have demonstrated that the use of a calcium pre-rinse before a fluoride rinse can increase the fluoride concentration in saliva better than a usual sodium fluoride mouth rinse alone (Vogel *et al*., 2006, Vogel *et al*., 2008). Furthermore, clinical trials have shown that more remineralisation occurs when calcium glycerophosphate (CaGP) or calcium carbonate is added to fluoride toothpaste (Joiner *et al*., 2009, Duke *et al*., 1979, Sidi and Wilson, 1991, Lynch and ten Cate, 2006, Lynch, 2004). In addition, more remineralisation has also been demonstrated when hydroxyapatite is added

to fluoride toothpastes (Hornby *et al*., 2009, Schaefer *et al*., 2009) or tricalcium phosphate added to fluoride (Karlinsey and Mackey, 2009, Karlinsey *et al*., 2010b, Mathews *et al*., 2012).

(f) Bioactive Materials

Traditionally, a bioactive material is one which stimulates a beneficial response from the body by bonding to host bone and forming a calcium phosphate layer (Hench and Wilson, 1984). Bioactive materials include bioactive glasses, glass-ceramics and bioceramics (Zhao *et al*., 2008, Hench and Wilson, 1984, Zhao *et al*., 2005)). Due to their ability to form a calcium phosphate layer, they are of increased interest in restorative dentistry.

Bioglasses are composed of calcium, sodium, phosphate and silicate and were discovered by Hench and Anderson in 1969 (Hench, 2006). They are reactive when exposed to body fluids and can deposit calcium phosphates during a two-step mechanism (Andersson and Kangasniemi, 1991). First, the bioglass undergoes dissolution and in turn, the dissolution products accumulate and cause a change in pH and chemical composition. This provides surface sites and a pH advantageous for calcium phosphate nucleation.

So far, the use of bioglass in dentistry has been focused on its ability to occlude dentinal tubules and treat dentine hypersensitivity (Mitchell *et al*., 2011, Curtis *et al*., 2010). However, their ability to form hydroxyapatite-like material on tubules surfaces suggests that they have potential as a remineralisation agent (Earl *et al*., 2011, Gillam *et al*., 2002, Pradeep and Sharma, 2010) and it has already been suggested that bioactive glasses can encourage remineralisation more so than fluoride (Burwell *et al*., 2009).

Novamin® is the trademark of a successful bioactive glass acquired by GlaxoSmithKline in 2009, initially used to treat sensitivity by depositing particles to occlude dentine tubules (Litkowski *et al*., 1997). When Novamin® particles are exposed to aqueous solutions such as saliva, the sodium ions immediately (less than 60 seconds) exchange with hydrogen cations (Andersson and Kangasniemi, 1991). This allows calcium and phosphate to be released, resulting in a layer of silanol (Si-OH).

$$Si\text{-}O\text{-}Na^{+} + H^{+} + OH^{-} \rightarrow Si\text{-}OH\text{-} + \mathbf{Na}^{+} + \mathbf{OH}^{-}$$

This exchange of hydrogen for sodium increases the pH in the oral environment, encouraging calcium and phosphate, due to supersaturation in saliva, to precipitate out of the particles and from saliva to form a calcium-phosphate coating on the tooth. As this continues, the layer crystallises into hydrocarbonateapatite (HCA) which is similar (chemically and structurally) to hydroxyapatite (Andersson and Kangasniemi, 1991).

Bioactive glass has also been shown to be beneficial when combined with air-abrasion. Air abrasion using bioactive glass powder has been proven to cause minimal damage to the enamel surface compared to more traditional methods, such as alumina air-abrasion (Banerjee *et al*., 2008).

(g) Arginine

In 2009, Colgate® introduced a new toothpaste for dentine hypersensitivity called Sensitive Pro-Relief™ containing Pro-Argin™ technology. This novel formula consists of 8 % arginine, calcium carbonate and 1450 ppm fluoride. It has been validated in two clinical trials plus many *in vitro* studies (Ayad *et al*., 2009, Docimo *et al*., 2009).

Arginine is believed to adsorb onto the surface of calcium carbonate, forming agglomerates which then bind to the oppositely charged (negative) dentine surfaces and occlude the tubules. Consequently, pH rises due to the deposition of calcium and phosphate from saliva (Kleinberg, 2002). Overall, when the arginine and calcium carbonate interact, this results in phosphate, with arginine, calcium and carbonate, depositing onto the dentine and within the tubules (Petrou *et al*., 2009). This strengthens dentine, making it less susceptible to demineralisation whilst encouraging remineralisation.

In 2013, a double-blind, randomised study was undertaken in the Dominican Republic to compare the effects of an arginine toothpaste and mouthwash to a potassium toothpaste and mouthwash and a fluoride toothpaste and mouthwash (control). The arginine products were shown to be the most

effective treatment as they provided the greatest reduction of hypersensitivity (Boneta *et al*., 2013).

(h) Polymer-induced liquid-precursors

As described so far, there are many remineralisation strategies that begin with inorganic ion precursors to encourage the nucleation of hydroxyapatite. However, these systems tend to produce randomly orientated hydroxyapatite crystals onto a surface. Some components, such as dentine, are composed of a collagen network and require intra- and inter-fibrillar mineralisation; surface mineralisation is inadequate and suggested to actually prevent further mineralisation beneath the surface (Cui *et al*., 2007, Doi *et al*., 1996).

An alternative remineralisation approach that has become the focus of recent research is the use of polymer-induced liquid-precursors (PILPs) (Olszta *et al*., 2007, Olszta *et al*., 2003a). Gower *et al* suggested that the PILP system may encourage remineralisation within collagen networks (Gower, 2008, Jee *et al*., 2010a). The PILP system requires adding acidic peptides (e.g. polyaspartic acid) into a remineralising solution. This anionic polymer sequesters calcium ions, until a positive charge builds up which can then attract counter ions, such as phosphate ions. The amorphous precursor is extremely hydrated and has fluidic properties allowing it to flow into gaps and grooves of the collagen fibres; and once the collagen fibres have become infiltrated, the amorphous calcium phosphate crystallises into hydroxyapatite, parallel to the fibril axis (Olszta *et al*., 2007, Gower, 2008, Dai *et al*., 2008). So far, the PILP system has proven to be very successful in mineralising collagen type I, even including collagen that does not contain remnant mineral (Olszta *et al*., 2003a, Jee *et al*., 2010a, Thula *et al*., 2011, Olszta *et al*., 2003b).

Furthermore, there are currently studies exploring enzyme-aided PILP, which is more likely to mimic the biochemistry of natural mineralisation. As described in section 2.3.3, alkaline phosphatase catalyses the release of inorganic phosphate groups from phosphoesters through dephosphorylation (Doi *et al*., 1996, Doi *et al*., 1992). Gower *et al* have investigated the use of alkaline phosphatase to deliver a controlled release of inorganic phosphate

groups from a commercial phosphoester (rac-glycerol 1-phosphate). Initial studies have shown that the enzyme-aided PILP provides less mineralisation that original PILP, but with further optimisation it could provide a closer mimic to the actual biochemical process (Jee *et al*., 2010b).

(i) Non-collagenous Protein Analogues

As discussed in section 2.3.2, although non-collagenous proteins (NCPs) only make up 10 wt. % of the dentine, they are believed to play a crucial role in mineralisation (Cao *et al*., 2013, Boukpessi *et al*., 2008). NCPs such as dentine matrix protein-1 (DMP-1) and dentine phosphoprotein (DPP) are suggested to have a high affinity for calcium ions due to their highly phosphorylated serine and threonine residues (He and George, 2004). Therefore, it seems appropriate to study NCPs as part of a remineralisation strategy, with research focusing on creating an NCP analogue due to the difficulty to extract and purify NCPs.

Polyacrylic acid (PAA) and polyvinylphosphonic acid (PVPA) have so far been studied as NCP analogues (Tay and Pashley, 2008). PAA is believed to mimic the calcium phosphate binding sites of DMP-1 whilst PVPA is believed to mimic the collagen-binding function of DMP-1 (Tay and Pashley, 2008). PAA and PVPA have been added to remineralising solutions to stabilise nano ACP precursors which can infiltrate through dentine, Tay and Pashley have used this method to remineralise intra- and inter-fibrillar dentine (Gu *et al*., 2011, Liu *et al*., 2011, Tay and Pashley, 2008). This idea of a dual system of a calcium phosphate binding site (e.g. PAA) and a collagen-binding ability (e.g. PVPA) have been investigated further using other analogues.

Sodium trimetaphosphate (STMP) is a substance already in use in the food industry as a phosphorylating reagent and is known to absorb type I collagen *via* phosphorylation (Zhang *et al*., 2012b, Li and Chang, 2008, Gu *et al*., 2010); the phosphorylated collagen attracting calcium ions and leading to the nucleation of inter- and intra-fibrillar remineralisation (Liu *et al*., 2011). Moreover, studies have shown that non-phosphorylated collagen cannot form intra-fibrillar remineralisation as efficiently as phosphorylated collagen

(Cao *et al*., 2013)). STMP has also recently been investigated for its anti-caries action in enabling fluoride to be preserved for longer in the oral cavity (Forsback *et al*., 2004, Jee *et al*., 2010a, Vollenweider *et al*., 2007, Cao *et al*., 2014). The reduction in demineralisation and increase in remineralisation by STMP is believed to much more significant than the use of fluoride alone (Cao *et al*., 2014).

(j) Self-assembling Peptide Scaffolds

In 1997, Aggeli *et al* discovered peptides, which under specific environmental conditions can self-assemble into fibrillar scaffolds (Aggeli *et al*., 1997b, Aggeli *et al*., 1997a). The fibrillar scaffolds begin as micro-meter long beta sheets but can further assemble into ribbons, fibrils and fibres (Nyrkova *et al*., 2000, Aggeli *et al*., 2001), these fibres forming scaffolds which are capable of inducing mineral deposition (Kirkham *et al*., 2007). Although the mechanism underpinning this mineralisation is unknown, it has been suggested that it may occur due to the precipitation of mineral within an assembled scaffold or the peptides enable the mineral to become more stabilised in a low pH environment reducing the amount of demineralisation (Kirkham *et al*., 2007).

2.5.3.4 Remineralisation using Nanotechnology

Further to the strategies above, nanotechnology is becoming increasingly prevalent in dental remineralisation studies. Defined as the development of materials, devices or systems using particles less than 100 nm in size, nanotechnology was first proposed as a major advance in medicine in 1960 by Richard Feynman when he stated that nanomedicine “cannot be avoided” (Feynman, 1960). Currently, nanotechnology is experiencing fast growth in dentistry and its potential is not fully uncovered. Nanoparticles are thought to possess great antibacterial properties because they have a large surface area and are small enough to interact directly with bacterial cell walls, particularly if they are less than 10 nm (Morones *et al*., 2005, Verran *et al*., 2007). In particular, silver, zinc and copper nanoparticles have been investigated for their antibacterial mechanisms, as reviewed by Besinis *et al* (Besinis *et al*., 2015).

Silver nanoparticles are believed to possess stronger antibacterial properties than zinc (Besinis *et al*., 2014a, Besinis *et al*., 2014b) but often zinc or copper nanoparticles are favoured because they are cheaper and more stable, allowing them to be incorporated with polymers (Ren *et al*., 2009). Zinc nanoparticles have demonstrated the ability to inhibit biofilm formation by *S.mutans* and consequently, in 2010, zinc oxide nanoparticles were combined into dental composites and displayed an 80 % reduction of biofilm formation (Sevinc and Hanley, 2010). Furthermore, in 2012, AFM and SEM studies showed that nanoparticles of zinc oxide can damage bacterial cell membranes and cause leakage of the cytoplasm (Chao *et al*., 2012). In addition, silica nanoparticles have been reported to inhibit bacterial adherence and reduce biofilm formation. The mechanism of this is unknown but it is speculated that it is an indirect effect from the silica nanoparticles which effects adherence (Cousins *et al*., 2007, Gaikwad and Sokolov, 2008)

Nanoparticles have been used in the following ways to encourage remineralisation; (a) nano-fillers, (b) nano calcium fluoride, (c) nano-hydroxyapatite, (d) nano carbonated apatite, (e) nano carbonate hydroxyapatite, (f) nano bioactive glass and (g) silica nanoparticles.

(a) Nano-fillers

Caries at the restoration borders is a common cause for replacement of existing restorations, which accounts for 50 – 70 % of all restorations. Nano-fillers have been developed that release calcium and phosphate at the composite-tooth interface, the advantage of using nanoparticles is that they can be incorporated into a resin and still match the strength of other commercial composites. Nano-composites with amorphous calcium phosphate (ACP) have been shown to neutralise lactic acid solution of pH 4 by quickly increasing the pH to 5.7 in fewer than 10 minutes (Xu *et al*., 2007, Moreau *et al*., 2011).

Furthermore, the release of fluoride from nano-fillers would be a substantial benefit for dental restoration, because fluoride has the potential to enrich dentine and reduce secondary caries (as described in section 2.5.1.4). Studies have shown that the addition of calcium fluoride nanoparticles (50 -

60 nm) to resin composites can result in fluoride release (Xu *et al*., 2007). Recently, nano composites with calcium fluoride and dicalcium phosphate anhydride have been tested; they release fluoride, calcium and phosphate and lead to the precipitation of fluorapatite, potentially aiding inhibition of caries at these susceptible sites (Cheng *et al*., 2012).

Moreover, nano-filers have been developed that contain antibacterial agents such as chlorhexidine and/or silver nanoparticles in addition to calcium and phosphate. The incorporation of the antibacterial components has been shown to reduce biofilm colonies, metabolic activity and lactic acid production by *S.mutans* (Cheng *et al*., 2012). In 2013, Zhang *et al* revealed the dual antibacterial effects of silver nanoparticles with 12-methacryloyloxydodecylpyridinium bromide (MDPB) (Zhang *et al*., 2013). MDPB has been extensively investigated for its antibacterial properties against various oral bacteria such as *Streptococci, Actinomyces, Lactobacilli* (Izutani *et al*., 2011, Imazato *et al*., 1994, Kuramoto *et al*., 2005, Rolland *et al*., 2011). Biofilm inhibition by MDPB combined with silver nanoparticles could be applied to a range of dental materials such as adhesives, sealants or composites. However, current research on antibacterial resins has not included any randomised clinical trials (Pereira-Cenci *et al.,* 2013).

(b) Nano Calcium Fluoride

Nano calcium fluoride acts as an anti-caries agent by increasing the concentration of fluoride ions in the oral cavity (Sun and Chow, 2008), the synergy of calcium and fluoride was discussed previously, in section 2.5.3.2. Nano calcium fluoride has high reactivity and therefore, may function as a source of fluoride when delivered in a toothpaste or mouthwash. In 2008, Sun and Chow showed that a nano calcium fluoride rinse produced seven times more fluoride on the tooth surface than a sodium fluoride rinse with the same concentration of fluoride. This suggests that the nano calcium fluoride may have a higher affinity to oral substrates and could be more effectively retained in the mouth, therefore providing a longer lasting source of fluoride (Sun and Chow, 2008).

Additionally, nano calcium fluoride could also be used to reduce dentine permeability, particles needing to be small enough to penetrate the tubules, which possess openings usually less than 5 µm. Moreover, calcium fluoride nanoparticles may be more useful when combined with calcium phosphate, which could produce more fluoride-containing apatite (Cherng *et al*., 2004).

(c) Nano-hydroxyapatite

Nano-hydroxyapatite (n-HA) is considered a very biocompatible and bioactive material (Hannig and Hannig, 2010), and in regards to morphology and structure, these nano-sized particles have similarity to the enamel mineral (Vandiver *et al*., 2005). There are some reports that have shown that n-HA has the potential to repair enamel, but little information is available for the remineralisation effect on established dentine lesions. For remineralisation of enamel lesions by n-HA, different formulations have been developed (Hannig and Hannig, 2010); n-HA toothpastes producing better remineralisation for enamel than an amine fluoride toothpaste *in vitro* (Tschoppe *et al*., 2011). They have also shown increased hardness in the enamel surfaces as well as reduced bacterial colonisation in dentine (Jeong *et al*., 2006, Lv *et al*., 2007, Tschoppe *et al*., 2011).

Besinis *et al* also produced synthetic nano-hydroxyapatite using a sol-gel method (Besinis *et al*., 2012). Hydroxyapatite was considered because it is a bioactive material, similar to the mineral component of human teeth (Phillips *et al*., 2003). Initially, the hydroxyapatite nanoparticles (shown in Figure 2.14) did not infiltrate well and agglomerated onto the surface, but this was improved when a deflocculant agent (sodium hexametaphosphate) was added (Besinis *et al*., 2012)**.**

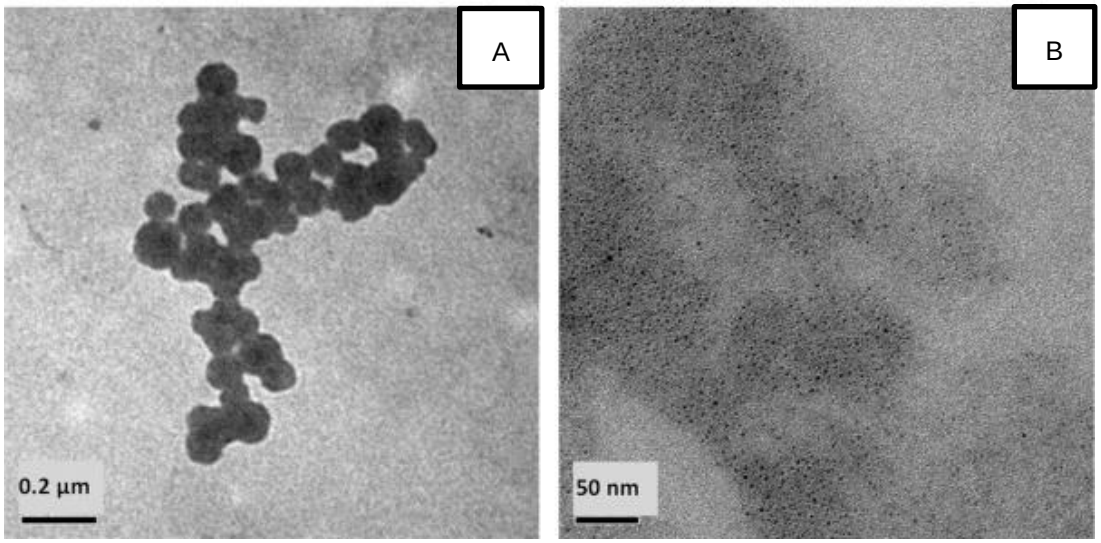

Figure 2.14: TEM images of sol-gel hydroxyapatite nanoparticles. (A) hydroxyapatite agglomerate; (B) hydroxyapatite with sodium hexametaphosphate (SHMP). With permission from Besinis *et al*., 2012.

(d) Nano Carbonated Apatite

Although hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] is widely used, carbonate apatite [$Ca_5(PO_4)_3CO_3$] has recently been used as a coating material on dental implants because of its superior biocompatibility. It is known that nano-sized carbonate apatite (n-CAP) has a much higher solubility and a more neutral pH than previous micro-sized versions and therefore, might have potential use in desensitising dentifrices and remineralisation. A recent study demonstrated, using hardness tests and electron microscopy, that a fluoride dentifrice containing 5 % n-CAP was effective in remineralising artificial caries lesions. Studies by Jeong *et al* (2007) utilising electron microscopy analysis, also confirmed the success of using n-CAP to penetrate the dentine tubules (Jeong *et al*., 2007).

(e) Nano Carbonate Hydroxyapatite

Nano carbonate hydroxyapatite [$Ca_5(PO_4,CO_3)_3OH$] has been used to replenish mineral and to create scaffolds that mimic the enamel structure (Roveri *et al*., 2008). The application of nano carbonate hydroxyapatite crystals (n-CHA) has been described as a “novel remineralising technique for enamel” (Nakashima *et al*., 2009) and dentine (Rimondini *et al*., 2007); the remineralisation effect induced by n-CHA is due to deposition of n-CHA into

the demineralised surface forming a mineral coating. These n-CHA crystals have similar size, morphology, chemical composition and crystallinity to that of dentine. The remineralising effect has been studied using electron microscopy analysis and has shown occlusion of tubules within ten minutes and deposition of a mineral layer within six hours (Roveri *et al*., 2008).

(f) Nano Bioactive Glass

As previously mentioned in section 2.5.3.2, bioactive glasses are highly considered in remineralisation research because they release ions and form apatite layers (Forsback *et al*., 2004) but have proven to have long reactivity times limiting their use in dentistry as a remineralising agent. A study by Vollenweider *et al* (2007) investigated bioactive glass nanoparticles synthesised by flame spray synthesis. After artificial demineralisation with EDTA, dentine was treated with 20 – 50 nm bioactive glass nanoparticles and after treatment, an increase in mineral content of the dentine samples suggested mineralisation had occurred. However, the mechanical properties of the remineralised dentine samples were below that of sound dentine, suggesting that the deposited mineral was not attached to the collagen in a stable manner of that analogous to normal dentine. Nevertheless, the higher remineralisation rate induced by nano bioactive glass compared to micro-sized bioactive glass was evident, suggesting that the higher surface area of the nanoparticles allowed the faster dissolution of ions from the glass (Vollenweider *et al*., 2007).

(g) Silica Nanoparticles

Ideally, dentine remineralisation would consist of deep remineralisation of the collagen network. Although there are many remineralisation methods that have been effective at creating surface layers of mineral, there appears to be a lack of strategies that can remineralise beneath the surface. However, Besinis *et al* pioneered a novel remineralisation strategy for dentine involving the infiltration of colloidal silica nanoparticle solutions within the dentine matrix (Besinis *et al*., 2012, Besinis *et al*., 2014b). The colloidal suspension of the nanoparticles allows them to penetrate between the collagen fibres without surface precipitation (Forsback *et al*., 2004, Vollenweider *et al*.,

2007). Once the particles infiltrate the dentine collagen they act as a scaffold for subsequent remineralisation. So far, research has been *in vitro* proof of principle studies.

Electron microscopy images of the Ludox® silica nanoparticles that Besinis *et al* investigated are shown in Figure 2.15. They have a diameter of approximately 15 nm and are able to pass through the collagen inter-fibrillar spaces, infiltrating deeply into the demineralised dentine, assisted by the colloidal properties of the solution. The silica nanoparticles are commercially available either with a positive surface charge (coated with 5 nm Al_2O_3) or a negative surface charge (Besinis *et al*., 2012).

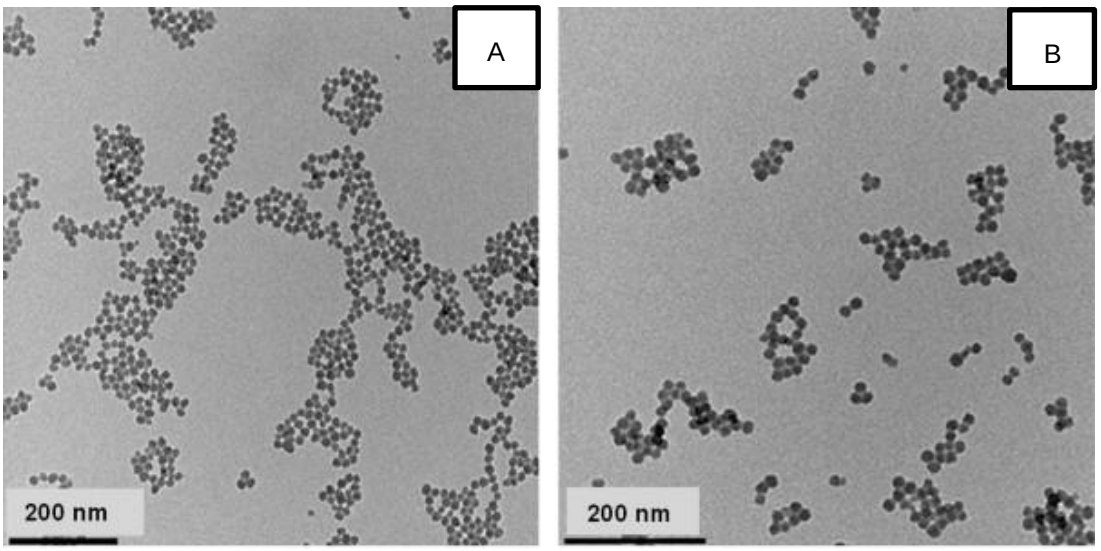

Figure 2.15: TEM images of silica nanoparticles: (A) positively charged nanoparticles; (B) negatively charged nanoparticles. With permission from Besinis *et al*., 2012.

Besinis *et al* used a range of characterisation techniques to verify that the nanoparticles were dispersed within the collagen network after infiltration, including TEM, as shown in Figure 2.16.

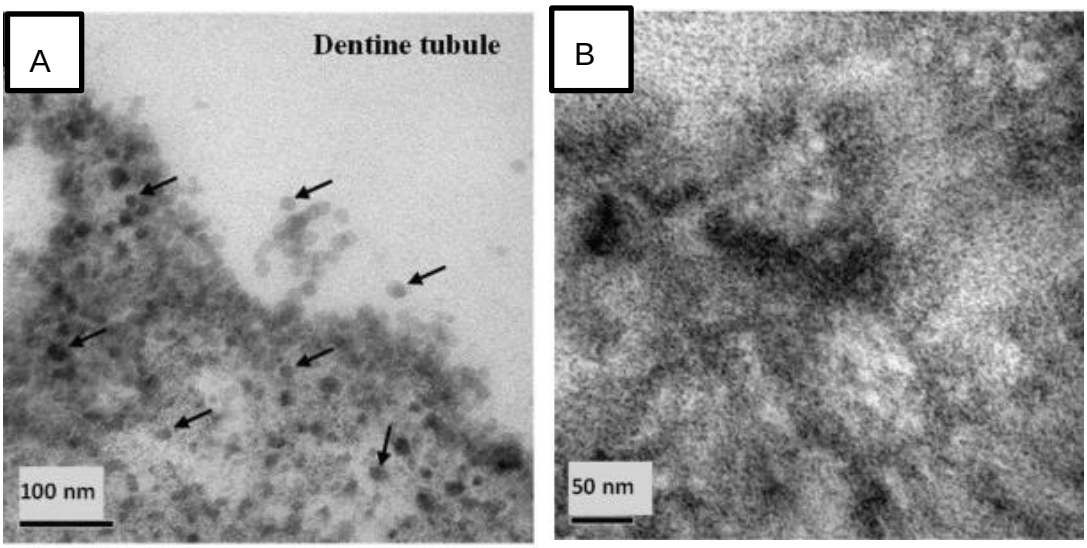

Figure 2.16: TEM images of (A) negatively silica particles within the collagen matrix (B) sol-gel hydroxyapatite with sodium hexametaphosphate (SHMP) within the collagen matrix. With permission from Besinis *et al*., 2012 (Besinis et al., 2012).

2.6 Summary

Caries is an ongoing dental health concern and acid erosion is becoming more prevalent. It is likely that dietary habits are responsible and that current prevention methods are inadequate, with reliable instruments for the removal of demineralised areas often too non-specific, removing healthy areas of the tooth. With life expectancy on the increase, there is more demand to maintain teeth for as long as possible. Teeth are more likely to be retained if restorative dentistry takes a more conservative approach, whereby less of the tooth is removed during cavity preparation and smaller restoratives are placed.

However, this requires a remineralisation strategy for the partially demineralised areas; current remineralisation treatments being insufficient. Although there is an abundance of research on novel strategies, they tend to focus on enamel and mainly rely on surface apatite precipitation. The remineralisation of dentine and its complex organic structure, of collagen and NCPs, is less understood. Surface remineralisation for dentine is inadequate as it prevents remineralisation beneath the surface and it is imperative that dentine remineralisation occurs throughout the collagen matrix. This undoubtedly requires nanotechnology. Nanoparticles are more likely to be

able to insert between collagen fibres and penetrate through the organic structure, which has already begun to be investigated by Besinis *et al* within The School of Clinical Dentistry, The University of Sheffield (Besinis *et al*., 2012, Besinis *et al*., 2014b). However, more knowledge is needed; for example, the interaction between the nanoparticles and the dentine organic structure should be explored, as well as the mechanism of how the nanoparticles enable nucleation of hydroxyapatite.

Furthermore, dentine has many NCPs which are known to be crucial for remineralisation and should definitely be considered as part of a remineralisation strategy. There are investigations exploring DMP-1 analogues, but these studies are in very early stages. Moreover, DPP is the most abundant NCP in dentine and is likely to have the biggest effect on remineralisation, but knowledge of DPP, including its location within dentine, is very limited.

Finally, all dentine remineralisation studies are dependent upon a demineralised dentine model for research purposes. Bacterial, gastric and dietary acids all demineralise teeth, but in order to create demineralised teeth models, demineralising agents such as laboratory acids, are used along with chemical fixation of the model, to prepare them for certain characterisation techniques. However, there is no ubiquitous standard, making it difficult to make comparisons between studies. The effect of these different acids and fixatives on the dentine structure and composition has been under investigated and it is assumed that they all work in the same way. In particular, the organic integrity of dentine is rarely considered, even though it is universally recognised as being crucial for effective remineralisation to occur.

3. Aims and Objectives

To summarise, Chapter 2 demonstrated that demineralisation represents an important deleterious change in tooth tissue, and therefore biomaterial systems that could reverse this process would have potential therapeutic value in dentistry. While recent studies have provided positive data, several challenges related to nanomaterial penetration and test methodology remain to be overcome before real clinical progress can be made. The aim of this PhD programme was therefore to investigate the development of a remineralisation process for dentine using silica nanoparticles, ideally describing an optimised method that would be effective at clinically relevant depths beneath the tissue surface and where necessary improving upon current *in vitro* models. The specific objectives of this research project may be summarised thus:

Objective 1: Characterisation of Demineralised Dentine

- To compare the effects of a range of low molecular weight acids of bacterial, gastric and dietary origin on the mineral content and collagen integrity or denaturation in dentine.
- To use immunohistochemistry to localise dentine phosphoprotein (DPP) in demineralised dentine.

Objective 2: Interaction between Nanoparticles and Collagen

- To assess the infiltration of colloidal suspensions of silica nanoparticles within a collagen network.
- To investigate whether silica nanoparticles adhere to the surface of collagen type I/III fibres.
- To discover whether adherence is affected by surface charges, exposure to acidic environments, and exposure to a chemical fixative.

Objective 3: Remineralisation of Dentine

- To investigate the interaction between negatively charged silica nanoparticles and calcium ions.

- To explore whether silica nanoparticles can increase mineral levels beneath the dentine surface.
- To investigate different infiltration times and different concentrations of silica solutions.

On completion of this research, the new knowledge created will make clinical progress more likely for the benefit of patients. In addition, the methods developed and optimised in the course of this research will be applicable to other studies directed towards understanding tooth mineral and tooth tissue-mineral interaction.

4. Materials and Methods

4.1 Collection of Teeth

This study received ethical approval from the National Health Service (NHS) Health Research Authority (HRA) for the collection of human teeth. The study code is STH16442 and approval was given by the London City Road and Hampstead Research Ethical Committee with reference number 12/L0/1189. Ethical approval was granted on the basis that teeth were collected from Charles Clifford Dental Hospital (Sheffield, UK) with full anonymity. Patient information sheets and consent forms were applicable for a range of participants: children 6 – 10 years, children 11 – 16 years and adults (16 years and over). After extraction, the teeth were immediately stored in 30 ml universal containers with a flow seal cap (Sigma Aldrich, Poole, UK) containing 20 ml 0.1 % thymol (Alfa Aesar, Massachusetts, USA) at 4 °C to prevent any microbial growth. All experiments in this project involved healthy (non-carious), premolars (from adolescents 12 – 18 years) that were extracted for orthodontic purposes. Other teeth (carious, primary, etc.) were used for practice only and were not used to generate any results presented in this thesis.

4.2 Preparation of Dentine Blocks

Dentine blocks (n = 369) were obtained from coronal dentine in extracted, caries-free human premolars. All teeth were used within 1 month of extraction. A slow speed carborundum disc blade was used to section 2 mm from the root apex. The pulpal tissue was then removed and the teeth were cleaned using a toothbrush and water. The enamel was removed (see Figure 4.1) by grinding with silicon carbide p500 papers (grit size 30.2 μm), without a lubricant, on a grinder-polisher wheel at 100 rpm (Buehler Metaserv, Dusseldorf, Germany). Specimens were then mounted onto a sectioning jig using superglue (Loctite®, Henkel, USA) and impression compound (Kerr, Bioggio, Switzerland). A slow speed precision blade saw (Buehler, Dusseldorf, Germany) with a Buehler 11-4276 diamond wafering blade

(diameter 152 mm and thickness 0.5 mm), as displayed on Figure 4.2, was used to section the dentine blocks. By rotating the jig at 90°, longitudinal and transverse cuts were achieved to cut dentine sections with dimensions of 6 mm x 4 mm x 2 mm, see Figure 4.3 and Figure 4.4. The blocks were placed in a sonicator (Grant Instruments, Cambridge, UK) for 5 minutes to remove any cutting debris. In this project, human coronal dentine was used unless otherwise stated.

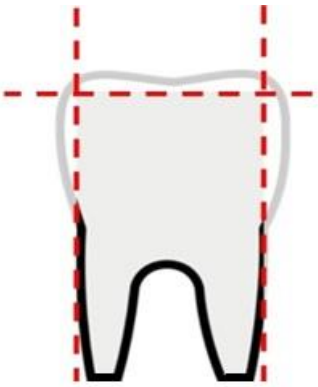

Figure 4.1: A diagram to show the where enamel was removed for specimen preparation using a grinder-polisher.

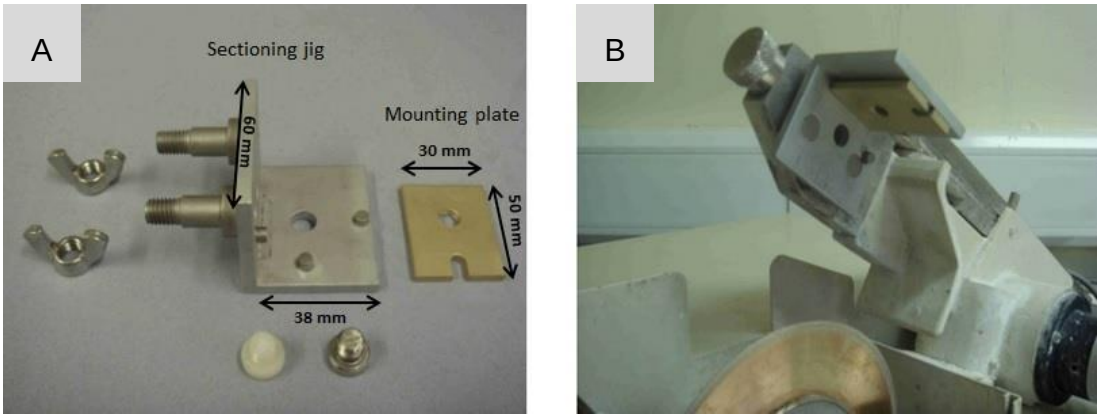

Figure 4.2: (a) A photograph to show the sectioning jig, mounting plate, and components; (b) a photograph to show the sectioning jig and mounting plate loaded onto the diamond blade saw

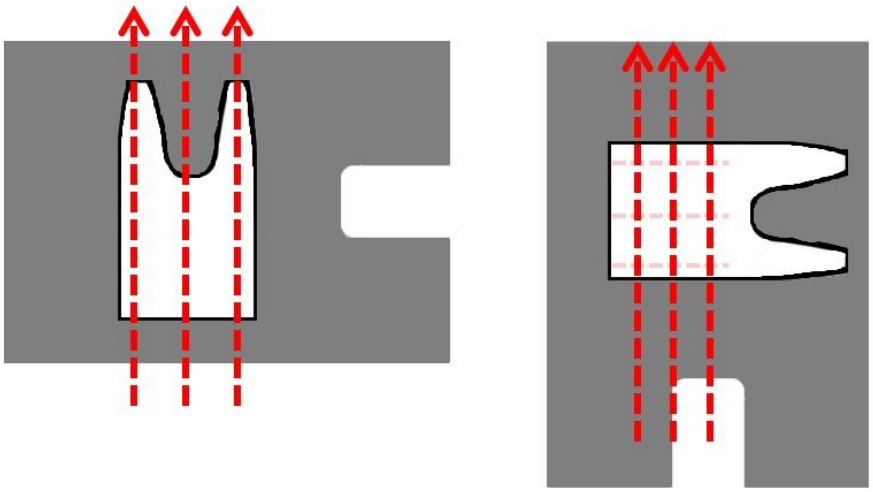

Figure 4.3: A diagram to show the longitudinal and transverse cuts made to the tooth whilst on the mounting plate.

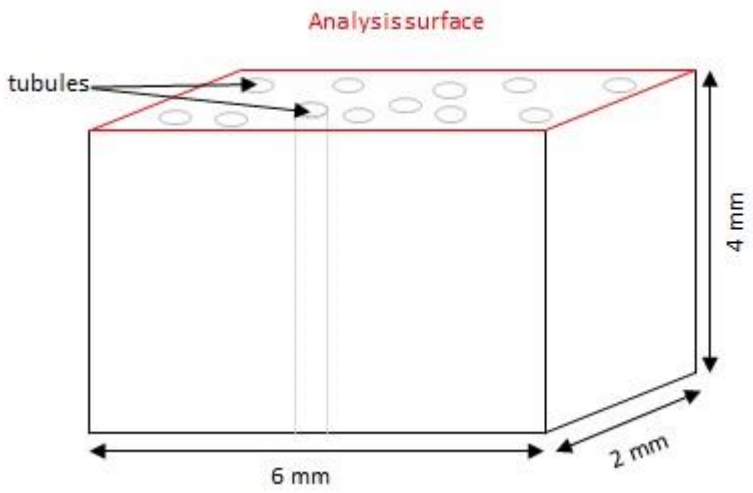

Figure 4.4: A diagram to show the dimensions and surface of analysis of the dentine blocks

4.2.1 Demineralisation of Dentine

Dentine blocks were first fixed by immersion in 3 % glutaraldehyde (Sigma Aldrich, Poole, UK) in 0.1 M cacodylate buffer (Sigma Aldrich, Poole, UK) at 4 °C for 24 hours. The reason why blocks were fixed prior to demineralisation was because previous work in the research group had shown that non-fixed specimens did not retain their structure, therefore preventing analysis of mineral content and collagen integrity. Unfortunately, fixing the samples prior

to demineralisation did not replicate *in vivo* conditions but since all samples were demineralised using the same protocol, within study comparisons were able to be made.

After fixation, the specimens were washed with 0.1 M cacodylate buffer (2 x 2 minutes) and distilled water (2 x 2 minutes) to remove any remaining fixative. Specimens were blot dried and nail varnish (Max Factor, Cincinnati, USA) was painted onto five surfaces so that only one surface was exposed to the demineralising agent. As described later in 5.3.1, specimens were then divided into 8 groups, according to the demineralising agent used:

(1) distilled water (control)
(2) lactic acid (caries acid)
(3) acetic acid (dietary acid)
(4) phosphoric acid (dietary acid)
(5) citric acid (dietary acid)
(6) formic acid (laboratory acid)
(7) EDTA (laboratory acid)
(8) hydrochloric acid (gastric acid).

All demineralising agents were obtained from Sigma Aldrich, Poole, UK. Each specimen was immersed in 10 ml demineralising solution for up to 14 days and refreshed every 24 hours. Demineralisation for all groups was terminated by removing the specimens from the acid solutions and washing in distilled water (2 x 2 minutes) and 0.1 M cacodylate buffer (2 x 2 minutes) before being stored in 0.1 M cacodylate buffer at 4 °C.

4.3 Collagen Membranes

4.3.1 Origin of the Membranes

Thirty collagen membranes (25 x 40 mm) consisting of type I and III collagen fibres (Bio-Gide®, Geistlich Sons Limited, Manchester, UK) were used to analyse the interaction between collagen and silica nanoparticles (see section 6.3.3). The highly purified natural porcine collagen membranes are usually used to cover the bone replacement material in maxillofacial surgery. The membranes were purified by the manufacturer so that all non-

collagenous proteins and enzymes were removed, leaving behind only mammalian collagen type I and III. The details regarding the preparation and purification of the Biogide® collagen membranes are provided in Appendix 1 (Section 12.1).

4.3.2 Preparation of the membrane discs

For this project, 6 collagen discs (1 mm thickness) were attained from each membrane using a 10 mm diameter cork borer, as shown in Figure 4.5 (180 discs created in total). Membrane discs were stored in a desiccator at room temperature until use.

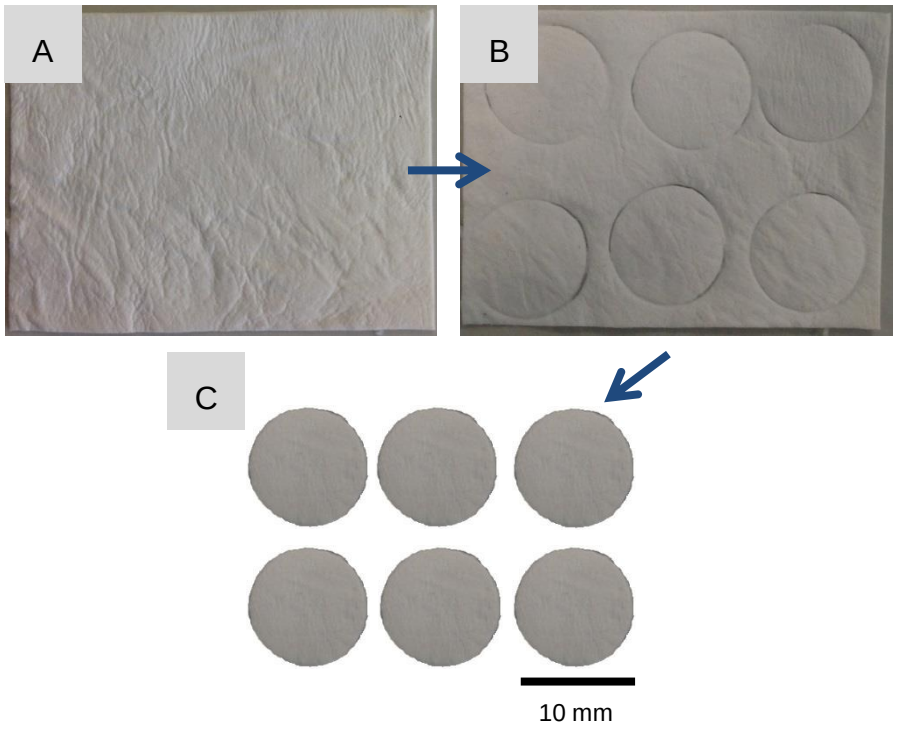

Figure 4.5: A diagram to illustrate the preparation of the collagen Biogide® membrane discs; (a) collagen membrane (b) collagen membrane after imprint with cork borer, (c) collagen discs

4.4 Silica Nanoparticles

Ludox® colloidal silica was obtained from Grace Materials Technologies (Worms, Germany). For the purposes of this study, two types of silica were obtained; electronegative silica solution (Ludox® HS) and electropositive silica solution (Ludox® CL) which contained silica particles coated with a

layer of aluminium oxide [Al_2O_3] (see section 6.3.1). The specifications of the silica solutions are shown in Table 4.1

Specifications	Ludox® CL	Ludox® HS
Lot number	420883	420824
Composition	$SiO_2 + Al_2O_3$	SiO_2
Particle size (nm)	12	12
Surface area m^2/g	230	220
Particle Surface Charge	positive	negative
Concentration (wt. %)	30	30
pH (25 °C)	4.5	9.8
Stabilising counter ion	chloride	sodium
Density (g/ml at 25 °C)	1.23	1.21

Table 4.1: Properties of Ludox® colloidal silica solutions

4.4.1 Infiltration of Nanoparticles through Collagen Membranes

The author would like to thank Dr Naeima Betamar, a visiting student from Benghazi University, Libya who assisted with the infiltration studies described in this section and Professor David Mowbray, Professor of Physics at The University of Sheffield for his help with calculating the pressure along the syringe.

Collagen discs (shown in Figure 4.5) were dried in a desiccator and weighed accurately to 4 decimal places using a digital balance (AE50, Mettler-Toledro, Uster Switzerland). Specimens were divided into 9 groups (n = 20 per group), details are shown in Table 4.2.

Table 4.2: Collagen membrane experimental groups

Group	Immersed in:	Duration (hours)	Temperature (°C)
1	3 % glutaraldehyde 0.1 M cacodylate buffer	24	4
2	no treatment	48	4
3	lactic acid (pH 2)	48	37
4	acetic acid (pH 2	48	37
5	phosphoric acid (pH 2)	48	37
6	citric acid (pH 2)	48	37
7	formic acid (pH 2)	48	37
8	EDTA (pH 7)	48	37
9	hydrochloric acid (pH 2)	48	37

Each group was further divided into two subgroups (n = 10):
(a) infiltrated with Ludox® CL 1 wt. % silica solution (positively charged)
(b) infiltrated with Ludox® HS 1 wt. % silica solution (negatively charged)

Specimens were secured to a syringe filter on the exit duct of a PHD 2000 22/2000 series syringe pump (Havard Apparatus, Holliston, USA) with 50 lb force. A 10 ml solution of colloidal silica particles was infiltrated through the collagen membranes with a flow rate of 0.05 ml per minute (pressure 349999.5 $x10^{-5}$ Pa) and then immediately flushed with 20 ml water with a flow rate of 1 ml per minute (pressure 349998.2 $x10^{-5}$ Pa) (Mowbray 2016), to dislodge any non-adhered particles. Specimens were left to air dry for 48 hours. Once dried, specimens were weighed again and stored in a desiccator. Figure 4.6 illustrates the key stages of the protocol, this method is later referred to in section 6.3.3.

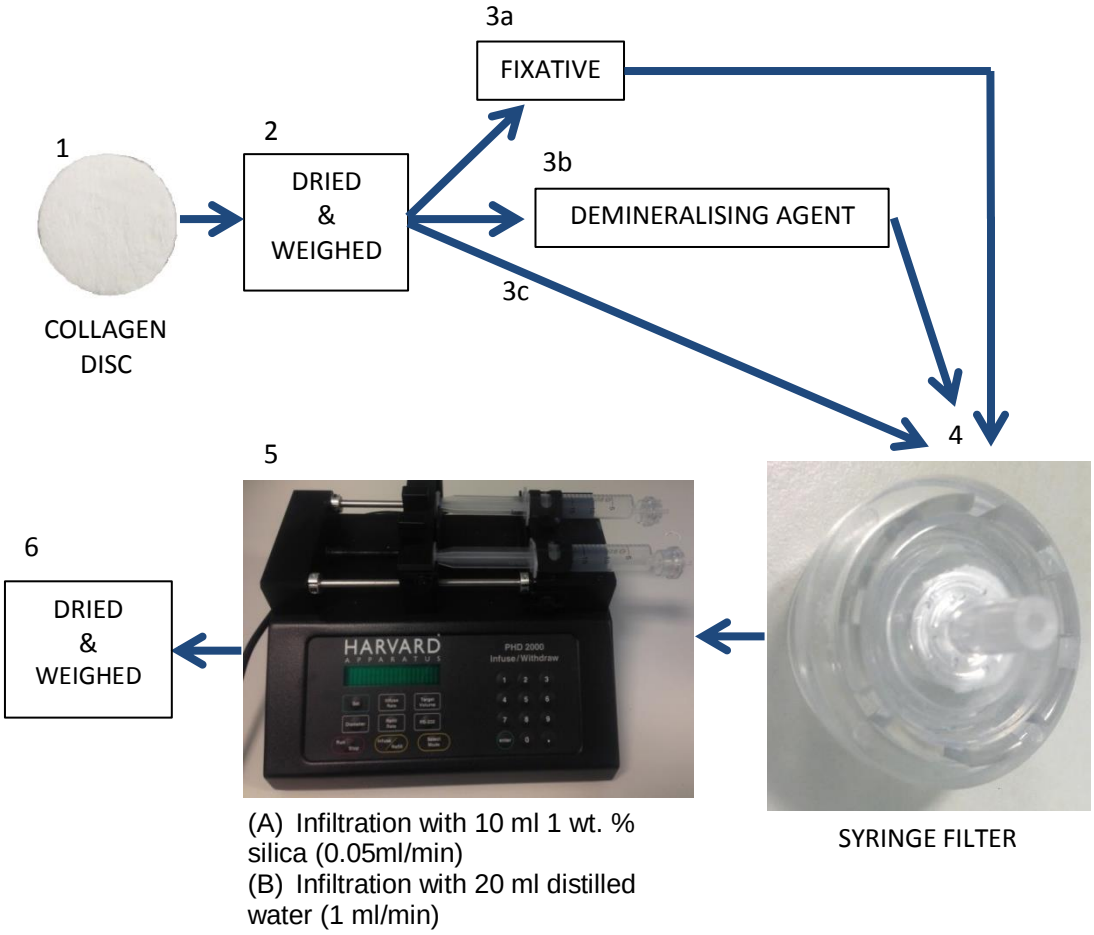

Figure 4.6: Diagram to outline the 6 main stages in the collagen infiltration experiment. (1) collagen discs were prepared as explained in section 4.3, (2) discs were dried and weighed, (3) discs were immersed in either (a) fixative, (b) demineralising agent or (c) no immersion, (4) discs were placed into a syringe filter, (5) the syringe filter was placed onto syringes on a syringe pump for infiltration with silica and water and (6) discs were dried and weighed.

4.5 Immunohistochemical Localisation of Dentine Phosphoprotein

All immunohistochemistry experimentation was undertaken with supervision from Dr Lynne Bingle, School of Clinical Dentistry, The University of Sheffield. Immunohistochemistry was used to monitor the presence of dentine phosphoprotein (DPP) in demineralised dentine specimens (as covered in section 5.3.4). A polyclonal antibody against DPP was produced by Eurogentec (Leige, Belgium).

Teeth were demineralised in EDTA as explained in 4.2.1 and immersed into xylene (Fisher Scientific, Loughborough, UK) before being embedded in paraffin wax. Fifty sections of 4 µm depth were cut from demineralised tooth specimens using a Leica EM UC6 ultramicrotome (Wetzlar, Germany) and placed onto 3-Aminopropyltriethoxysilane (APES) coated glass slides (Menzel-Glaser, Germany).

Slides were washed twice in xylene for 5 minutes before being immersed into pure ethanol (Fisher Scientific, Loughborough, UK) for 2 x 5 minutes. Enzymatic activity was quenched through the application of 3 % hydrogen peroxide (Sigma Aldrich, Poole, UK) for 20 minutes and then the slides were washed with phosphate buffered saline (PBS) (Sigma Aldrich, Poole, UK).

The next step was to undertake antigen retrieval, 3 different protocols were tested (all constituents from Sigma Aldrich, Poole, UK):

(1) Antigen retrieval with citrate: slides were immersed in 0.01 M sodium citrate solution and heated in a microwave for 8 minutes.

> (0.01 M sodium citrate solution = 1.18 g tri-sodium citrate dihydrate mixed with 400 ml distilled water).

(2) Antigen retrieval with proteinase K: slides were covered with proteinase k solution for 15 minutes at 37 °C and cooled for 10 minutes.

> (Proteinase K solution = 1 ml proteinase K stock solution (0.008 g proteinase K mixed with 10 ml tris-EDTA buffer pH 8 and 10 ml glycerol) mixed well with 19 ml tris-EDTA buffer pH 8).

(3) Antigen retrieval with trypsin: slides were covered with 0.05 % trypsin solution for 15 minutes at 37 °C and cooled for 10 minutes.

> (0.05 % trypsin solution = 1 ml trypsin stock solution (50 mg trypsin mixed with 10 ml distilled water) mixed with 1 ml 1 % calcium chloride solution (0.1 g calcium chloride with 10 ml distilled water) and 8 ml distilled water, adjusted to pH 7.8 with sodium hydroxide.

After another wash in PBS, all sections were left at room temperature whilst blocked with 1 % serum solution (Vector Laboratories, UK) to inhibit any non-specific binding. Following 30 minutes of incubation, the serum solution was

then removed from all slides (except from the non-specific controls). The control slides ensured that the secondary antibody and ABC (Avid and Biotinylated macromolecular Complex) was only binding to primary antibody labelled DPP. Slides were then coated with the primary antibody and left at 4 °C overnight.

The next day, all sections were washed in PBS for 2 x 5 minutes. The secondary antibody (Vector Laboratories, UK) was pipetted onto all of the slides and left for 30 minutes. During this time, the ABC complex (Vector laboratories, UK) was prepared according to manufacturer's instructions. After a further wash in PBS, all slides were covered with the ABC reagent and left for 30 minutes. In the meantime, the diaminobenzidine tetrahyrochloride (DAB) substrate solution (Vector Laboratories, UK) was prepared according to manufacturer's instructions. All specimens were washed in PBS before being subjected to the DAB solution for 5 minutes and then rinsed in distilled water. Slides were then counterstained with haematoxylin, cleared in xylene and mounted in DPX (Distrene, Plasticiser and Xylene) mounting medium (Fisher Scientific, Loughborough, UK).

4.5.1 Haematoxylin and Eosin Stain

Additional sections were stained with haematoxylin and eosin (H&E) for general histological analysis (see section 5.3.4). Sections were rinsed with distilled water before being covered in haematoxylin stain (Sigma Aldrich, Poole, UK) for 4 minutes and then rinsed with tap water. Sections were covered with 0.3 % acid alcohol (hydrochloric acid and ethanol, Sigma Aldrich, Poole, UK) for 2 seconds before being rinsed under water again. All sections were immersed into Scott's tap water (Sigma Aldrich, Poole, UK) and then rinsed under tap water. Sections were then subjected to eosin staining (Sigma Aldrich, Poole, UK) for 2 minutes, before being cleared in xylene and mounted in DPX mounting medium.

4.5.2 Collagen Staining

Additional sections were stained with Van Gieson to locate collagen (see section 5.3.4). Sections were rinsed with distilled water before undergoing 5 minute incubation with Celestin Blue stain (5 % ammonium ferric sulphate

and Celestin Blue (Sigma Aldrich, Poole, UK)). After another rinse in distilled water, sections were subjected to 5 minute incubation with haematoxylin before being rinsed with tap water for 5 minutes. Sections were then stained with Curtis stain (picric acid, ponceau S and acetic acid, (Sigma Aldrich, Poole, UK)) for 5 minutes, cleared in xylene and mounted in DPX mounting medium.

4.6 Remineralisation

4.6.1 Remineralising Solution

Constituents of the remineralisation solution (Fowler *et al*., 2006) added to distilled water:

(a) 0.2 mM magnesium chloride (VWR International Ltd, Lutterworth, UK)
(b) 1mM calcium chloride dihydrate (VWR International Ltd, Lutterworth, UK)
(c) 4mM potassium dihydrogen orthophosphate (VWR International Ltd, Lutterworth, UK)
(d) 20mM HEPES (N-2Hydroxyethylpiperazine-N'-ethanethesulphonic acid) (MP Biomedicals Inc, Santa Ana, USA)
(e) 16mM potassium chloride (Sigma Aldrich, Poole, UK)
(f) 4.5mM ammonium chloride (Sigma Aldrich, Poole, UK)

The solution was then adjusted to pH 7 with 1M potassium hydroxide (Sigma Aldrich, Poole, UK) using a PH211 microprocessor pH probe (Hanna Instruments, Leighton Buzzard, UK).

4.6.2 Infiltration of Nanoparticles through Dentine

One-hundred dentine blocks were split into 4 demineralisation groups (EDTA, lactic acid, hydrochloric acid, citric acid) and demineralised for 7 days as per protocol in 4.2.1, leaving 25 specimens per group. Each group of specimens was then further divided into five infiltration groups of n = 5: (1) infiltration with 1 wt. % silica nanoparticles for 1 minute; (2) infiltration with 1 wt. % silica nanoparticles for 24 hours; (3) infiltration with 0.1 wt. % silica

nanoparticles for 1 minute; (4) infiltration with 0.1 wt. % silica nanoparticles for 24 hours; (5) control, no infiltration.

The infiltration method consisted of immersing the blocks into 1 ml of either 0.1 wt. % or 1 wt. % Ludox® HS silica solutions under constant stirring at 200 rpm for either 1 minute or 24 hours, in 24-well plates (Sigma Aldrich, Poole, UK). Following infiltration, specimens were briefly rinsed in distilled water for 10 seconds; (see section 7.3.1 for more information).

4.6.3 Remineralisation of Dentine

The dentine blocks (infiltrated and control, as detailed in the previous section) were immersed in 20 ml of remineralisation solution (constituents in 4.6.1) in 30 ml universal pots (Sigma Aldrich, Poole, UK). Samples were removed from the remineralising solution after 4 weeks and were examined for signs of remineralisation, as discussed in section 7.3.3.

4.7 Characterisation Techniques

4.7.1 Micro-hardness Analysis

Dentine blocks (n = 105) were divided into the 7 demineralisation groups, giving 15 dentine blocks per group. These 15 blocks were further divided into the 5 demineralisation periods: 0 (control), 1, 2, 7 and 14 days. This allowed each variable to be tested in triplicate. Dentine blocks were demineralised according to their demineralisation group and following the protocol outlined in section 4.2.1.

Sample preparation involved embedding the dentine blocks into Kleer-set FF resin (Metprep, Coventry, UK) and gently grinding the surface until it was level using wet silicon carbide p1200 papers (grit size 15.3 μm) and p2400 papers (grit size 6.5 μm), without a lubricant, on a grinder-polisher wheel at 100 rpm (Buehler Metaserv, Dusseldorf, Germany). Specimens were stored in cacodylate buffer until use.

Micro-hardness analysis of the dentine surface was performed using a Mitutyo HN810 instrument (Hampshire, UK) with a Vickers diamond indenter. Vickers testing was chosen because it is less sensitive to surface conditions than other types e.g. the Knoop test and the impression produced by the

Vickers indenter is clearer than other hardness methods e.g. Brinell indenter and therefore, it was thought to be the most suitable hardness test for this project.

The Vickers hardness test consists of a diamond pyramid indentor (136° angle between the opposite faces) with a square base. The size of the indent is determined by measuring the two diagonals of the square indent. A load of 300 g was applied to the specimens with a dwell time of 20 seconds. Micro-hardness was expressed in terms of the Vickers Hardness Number (VHN), (the VHN is the load divided by the surface area of the indentation). The VHN of a specimen at each time point was based on the mean of 8 indents, as shown on Figure 4.7. Due to the heterogeneous surface of dentine composed of voids from tubule openings and areas of intra-tubular collagen matrix, the hardness of each specimen was measured at 8 separate points on the surface. The minimum distance between indentations was larger than 100 μm and the outer 0.5 mm of each sample was not tested, also shown on Figure 4.7. The maximum indentation depth was 2 μm. In order to optically measure the indentation, the edges needed to be sharp and uniform.

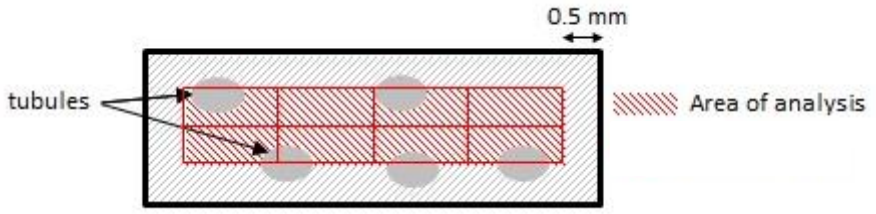

Figure 4.7: A diagram to show the occlusal surface of the dentine blocks. The area of analysis is highlighted in red, also indicating the 8 indentation areas.

4.7.2 Energy Dispersive Spectroscopy

(a) Dentine specimens

Dentine blocks were embedded in Kleer-set FF resin (Metprep, Coventry, UK) and each surface was ground gently using wet silicon carbide p1200 papers (grit size 15.3 μm) and p2400 papers (grit size 6.5 μm), without a lubricant, on a grinder-polisher wheel at 100 rpm (Buehler Metaserv, Dusseldorf, Germany). The blocks were subsequently carbon coated (Emitech, Molfetta, Italy) to enable energy dispersive spectroscopy (EDS) analysis (Jeol 6400, Massachusetts, USA) with an accelerating voltage of 20

kV and spot size 3. The signal was collected from a scanned area (100 μm^2). Scan parameters were as follows: magnification set at 512x368, scan speed set at “medium” and number of frames set at 1; spectrum conditions were as follows: process time set to 35, spectrum range 0-20 keV and number of channels set to 2K. Element peak intensities were quantified against a cobalt standard as the base for percentage; x ray peak Kα, 6.931 keV and density 8.86 g/cm^3. The cobalt standard was loaded into the corner of the sample holder, secured in place with a grub screw and then checked to make sure it was flush with the surface of the holder. The dentine sample was loaded and checked to make sure it was the same height as the surface of the sample holder to ensure accuracy. The microscope was focused on the cobalt surface and magnification increased to 20,000x before the spectrum was acquired. The microscope was then focused onto the sample surface and a spectrum was acquired. All EDS analysis included measurements of a 100 μm^2 inter-tubular area within the 4 different quadrants of surface, as illustrated below on Figure 4.8. Calibration using the cobalt standard was repeated after every other spectrum acquisition. Errors can be introduced by the structure of the sample surface as the EDS software assumes that the samples are flat, however, dentine cannot be polished to a mirror surface unlike other samples such as metals and therefore, although the peak intensities were quantified against a cobalt standard, the data obtained will only be considered qualitatively. There were 10 peaks in total identified in dentine (oxygen K, nitrogen K, calcium K, phosphorous K, silica K, sodium K, magnesium K, fluoride K, sulphur K, aluminium K) with additional peaks of potassium K and chlorine K in dentine which had been immersed in a remineralising solution, however, only data from the elements of interest (either calcium, phosphorous or silica) are displayed in this thesis. The relative wt.% did not take into consideration carbon, as carbon was used to coat the specimens and the instrument was set up to automatically remove carbon from the elements identified and their relative wt.%.

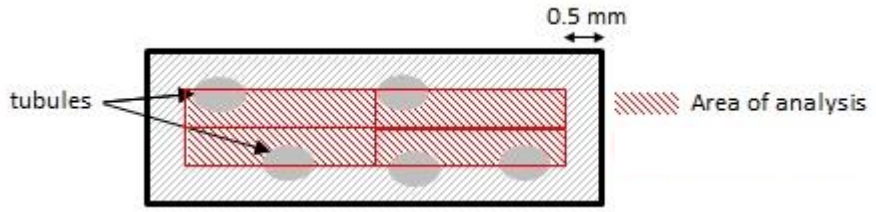

Figure 4.8: A diagram to show the area of analysis for EDS on the demineralised dentine specimens. The area of analysis is highlighted in red to show where the 4 measurements were taken.

(b) Collagen membranes

Collagen membranes were infiltrated with silica nanoparticles, outlined in sections 4.4.1. They were then analysed in triplicate using EDS. The membranes were attached to aluminium pin stubs (Agar Scientific, Essex, UK) using carbon sticky tabs (Agar Scientific, Essex, UK) and then they were carbon coated (Emitech, Molfetta, Italy) before analysis with EDS as described above in section 4.7.2 (a). The peaks identified included silica K, oxygen K and nitrogen K but only the silica results are displayed as this is the element of interest.

4.7.3 X-ray Fluorescence

X-ray fluorescence (XRF) was utilised for elemental analysis of dentine specimens, as explained in 5.3.2. Preparation of the samples for XRF first involved removing the nail varnish from the surfaces of the specimen using acetone, before grinding the specimens into a powder using a mortar and pestle. Next, the specimen (ground dentine) was fused with 8 g lithium tetraborate by heating to 1200 °C to produce a glass disc. The glass disc was then placed into the Phillips PW 2440 XRF instrument for analysis. All XRF analysis was undertaken by Robert Burton (XRF Technician) at Sheffield Hallam University. Elements identified included oxygen, nitrogen, calcium, phosphorous, silica, sodium, magnesium, fluoride, sulphur, aluminium however, only calcium (Ca) and phosphorous (P) results are displayed as these are the elements of interest.

4.7.4 X-ray Diffraction

Phase composition of dentine specimens (explained in section 5.3.2) was determined using a STOE STADI P diffractometer (Darmstadt, Germany). Dentine blocks from each demineralisation group were air-dried for 24 hours and then ground into a powder using a mortar and pestle. Each powder (1 g) was placed into aluminium sample holders within the x-ray diffractometer. Scan speed was set to 2° per minute measured within a 2Θ range of 5° - 80° at every 0.02°, using Cu Kα radiation with wavelength of λ=0.15406 nm generated at 40 kV and 40 mA. Identification of phases was carried out by comparing with international centre for diffraction data (ICDD) cards from the

powder diffraction files (PDF) database and searching for any phase containing calcium or phosphate using WinX^{POW} software.

4.7.5 Phosphate Assay

Phosphate levels in demineralised dentine specimens were quantified using a phosphate assay kit from Abcam® (Cambridge, UK) which consisted of a phosphate reagent and 10 mM phosphate standard, also discussed in section 5.3.2. Dentine specimens were demineralised (as described in section 4.2.1) and the demineralising solutions containing dissolved phosphate were analysed at days 0, 1, 2, 7 and 14. Samples were analysed in triplicate. Each demineralising solution (100 µl) was added to a 96 well plate and combined with 100 µl of distilled water. The phosphate standard was diluted with distilled water to 0, 1, 2, 3, 4 and 5 nmol and added to additional wells. Next, 30 µl of phosphate reagent was added to all sample and standard wells, mixed and then incubated at room temperature for 30 minutes. The absorbance was read at 650 nm using a spectrophotometer (Tecan, Switzerland). Phosphate concentration was determined by the following equation:

Equation 4.1:

$$\text{phosphate concentration} = \frac{\text{sample amount (nmol) from standard curve}}{\text{sample volume (undiluted) added to the well}}$$

4.7.6 Scanning Electron Microscopy

(a) Preparation of dentine blocks and collagen membranes

Scanning electron microscopy (SEM) was used to observe the surface microstructure of the dentine blocks (discussed in section 5.3.3) and Biogide collagen membranes (discussed in section 6.3.3). Preparation involved staining the specimens with 0.1 % osmium tetroxide (Sigma Aldrich, Poole, UK) for 1 hour prior to dehydrating the blocks by immersion into graded ethanol solutions and hexamethyldisilazane (HMDS) (Sigma Aldrich, Poole, UK) using the following protocol:

(1) 50 % ethanol for 20 minutes
(2) 75 % ethanol for 20 minutes
(3) 95 % ethanol for 30 minutes
(4) 100 % ethanol for 60 minutes
(5) 50 % ethanol and 50 % HMDS for 30 minutes
(6) 100 % HMDS for 20 minutes

The specimens were then air-dried in a fume cupboard overnight. Once dehydrated, the blocks were attached to aluminium pin stubs (Agar Scientific, Essex, UK) using carbon sticky tabs (Agar Scientific, Essex, UK), coated with silver dag (Agar Scientific, Essex, UK) and then gold spluttered using an EM SCOPE 500 (Quorum Technologies, Sussex, UK). Specimens were analysed using an Inspect F SEM (FEI™, Oregon, USA).

4.7.7 Hydroxyproline Assay

The hydroxyproline (Hyp) assay was performed on demineralised dentine blocks from each demineralisation group in order to quantify the amount of denatured collagen (see section 5.3.3). The demineralised dentine samples were transferred into 1 ml PBS at pH 7 prior to being incubated at 37 °C with gentle shaking for 24 hours in 20 mg trypsin (treated with L-1-tosylamide-2-phenylethyl chloromethyl ketone, (TPCK)) (Sigma Aldrich, Poole, UK) in 1 ml PBS at pH 8. Dentine blocks were then rinsed with 1 ml PBS pH 8. Each sample solution (100 µl) was transferred to a pressure-tight vial and incubated at 120 °C for 3 hours with 100 µl of 12 N hydrochloric acid (Sigma Aldrich, Poole, UK). Activated charcoal (5 mg) (Sigma Aldrich, Poole, UK) was stirred into the solutions before undergoing centrifugation at 13000 x g (Sigma Aldrich, Poole, UK) for 2 minutes. Each supernatant (10 µl) was added to a 96 well plate (Greiner BIO-One, Stonehouse, UK) and the quantity of Hyp was measured per mass of sample, against Hyp standards, using a spectrophotometer at 560 nm (Tecan, Switzerland).

4.7.8 Transmission Electron Microscopy

Silica nanoparticle solutions were diluted to 1 wt. % using distilled water, pipetted onto copper grids (Agar Scientific, Essex, UK) and left to dry overnight. TEM images were acquired using a Tecnai Spirit G2 120KV (FEI™, Oregon, USA).

4.7.9 Dynamic Light Scattering

All dynamic light scattering (DLS) experiments were undertaken using a Zeta Sizer NanoZS instrument (Malvern Instruments Ltd, Malvern, UK).

(a) Measuring particle size

Glass cuvettes, 3.5 ml, (Malvern Instruments Ltd, Malvern, UK) were washed with filtered distilled water and wiped with a fibre tissue. Silica solutions (1 wt. %) were added to individual cuvettes and sonicated (Grant Instruments, Cambridge, UK) for 20 seconds before being placed into the instrument. Three measurements were taken per sample at 25 °C and each sample was measured in triplicate; see section 6.3.1 for more information.

(b) Measuring zeta potential

Five drops of 1 wt. % silica solution were added to 20 ml potassium chloride (KCl) (Sigma Aldrich, Poole, UK) in a glass vial. After mixing well, a syringe was used to fill a capillary zeta cell cuvette (Malvern Instruments Ltd, Malvern, UK) with the KCl solution; see section 7.3.2 for more information.

4.8 Statistical Analysis

All data was checked for normality using the Shapiro–Wilk test. If normally distributed, then a one-way analysis of variance (ANOVA) with Tukey's multiple comparison post-test was used to determine the significance between groups ($p < 0.05$). If not normally distributed then a Kruskal-Wallis H Test was used and Dunn's (1964) post hoc analysis with Bonferonnii adjustment was performed to determine the significance between groups ($p < 0.05$).

All statistical analysis was performed using the SPSS 20.0.0 statistical software package (IBM, Chicago, USA).

All graphs display standard deviation in the error bars.

5. Characterisation of Demineralised Dentine

5.1 Introduction

All *in vitro* dentine remineralisation studies require the use of a reliable and predictable analogue demineralised dentine model. As discussed in section 2.3, dentine is a complex structure composed of (i) mineral, (ii) collagen and (iii) non-collagenous proteins (NCPs), all of which play an essential role in mineralisation. Before a remineralisation strategy can be investigated it is crucial to assess the integrity of these components in the demineralised dentine model.

(i) Dentine Mineral

There are many techniques available to observe the levels of calcium (Ca) and phosphate (PO_4) in demineralised dentine and these are often used to assess the levels of mineralisation. Tay and Pashley have suggested that remnant intra-fibrillar dentine hydroxyapatite present after partial demineralisation may act as sites for future hydroxyapatite nucleation and regrowth; thus, facilitating remineralisation (Tay and Pashley, 2008, Tay and Pashley, 2009). This proposes that mineral crystallites must already be absorbed onto collagen fibres for successful remineralisation to occur. Therefore, the remaining mineral content in demineralised dentine models should be measured prior to remineralisation experiments.

(ii) Dentine Collagen

Clinical evidence indicates that in severe caries, the collagen matrix is denatured to the extent that it cannot function correctly, however, the dentine collagen matrix remains practically unaffected in early stages of carious lesions (Deyhle *et al*., 2011); thus potentially enabling future remineralisation strategies of caries-affected dentine.

There are currently two proposed mechanisms for the denaturation of dentine collagen: (i) bacterial proteases and (ii) matrix metalloproteinases (MMPs)/cathepsins, (see section 2.4.3.1 for more details).

Notwithstanding, to date there have been no studies to determine whether this process of denaturation of the dentine collagen occurs in the same manner in artificially demineralised dentine models. Zhang *et al* have suggested that high hydrogen ion concentrations from acidic solutions may alter the tertiary structure of proteins by the protonation of amino groups and ionisation of carboxyl groups (Zhang *et al*., 1998); but it is unknown if this applies to dentine collagen. All dentine remineralisation strategies have to be developed and tested in *in vitro* models and mostly require an intact dentine collagen fibres network for successful remineralisation to occur (Besinis *et al*., 2014b, Gower, 2008, Olszta *et al*., 2003a, Olszta *et al*., 2003b, Forsback *et al*., 2004, Vollenweider *et al*., 2007). Thus, it is very important to know whether demineralised dentine models have a denatured collagen matrix, akin to that of the *in vivo* situation.

(iii) Non-collagenous Proteins

As discussed in section 2.3.2, non-collagenous proteins (NCPs) play a crucial role in dentine remineralisation (Qin *et al*., 2004, Qin *et al*., 2007, Stetlerstevenson and Veis, 1986, Goldberg and Boskey, 1996). The exact mechanisms are uncertain but it is speculated that they may act as regulators or nucleators and there is strong evidence that dentine phosphoprotein (DPP), the most abundant NCP in dentine, is involved in mineralisation (Butler *et al*., 1979, Takagi *et al*., 1986). Therefore, confirmation of the presence of DPP following demineralisation should be considered to be a pre-requisite for subsequent remineralisation strategies.

5.1.1 Demineralising Agents

There are many acids of different origins (bacterial, gastric and dietary) that can cause demineralisation during caries or erosion. Currently, there is a presumption that the various acids must affect dentine in a similar manner and hence, it has become standard to use laboratory acids such as formic acid (Eggert and Germain, 1979, Besinis *et al*., 2014b, Kim *et al*., 2011) or chelating agents such as ethylenediaminetetraacetic acid (EDTA) (Habelitz *et al*., 2002, Wang and Spencer, 2002) to produce demineralised dentine

models for use in *in vitro* remineralisation studies. However, early work with enamel, by Featherstone *et al* (2006), indicated that the type of acid (strong or weak) and the pH of the working solution are significant when considering the extent of enamel demineralisation (Featherstone and Lussi, 2006). Also, Hughes *et al* (2000) indicated that at a low pH, lactic acid (produced by bacteria during caries) may have a more erosive affect than citric acid (a common dietary acid e.g. orange juice) (Hughes *et al,* 2000). However, this work has not been conducted in human dentine, thus the effect of different acids on the organic and inorganic human dentine is unknown. Additional acids such as hydrochloric acid, found in gastric fluids and causes erosion during regurgitation or vomiting, and acetic acid, a dietary acid found in vinegar, should also be considered. In order to investigate this further, the properties of the acids and their demineralising mechanisms must be fully understood.

5.1.2 Dissociation of Strong and Weak Acids

The reason why different acids may have different demineralising abilities may be explained by the mechanism by which an acid can cause demineralisation. First, the chemistry of weak and strong acids and their ability to dissociate into hydrogen ions (H^+) must be explored. Equation 5.1 shows the formula for the acid dissociation constant (Ka) of an acid in a solution where [HA] represents the acid in an associated state.

$$\mathrm{Ka} = \frac{[\mathrm{A^-}][\mathrm{H^+}]}{[\mathrm{HA}]} \qquad \text{(Equation 5.1)}$$

Strong acids, such as hydrochloric acid, have a high Ka value. As implied by equation 5.1, this means that strong acids are more likely to dissociate. In fact, once immersed into water, strong acids irreversibly dissociate fully, as shown in equation 5.2.

$$[\mathrm{HA}] \rightarrow [\mathrm{A^+}][\mathrm{H^-}] \qquad \text{(Equation 5.2)}$$

Conversely, weak acids, such as lactic acid (involved in caries), have a low Ka value and therefore, less dissociation. In fact, weak acids are always in equilibrium between association and dissociation as shown in equation 5.3.

$$[HA] \rightleftharpoons [A^+][H^-] \qquad \text{(Equation 5.3)}$$

However, the most useful way of measuring an acid's dissociation ability is not to use the Ka value but instead to use pKa which is explained in equation 5.4.

$$pKa = -\log_{10}Ka \qquad \text{(Equation 5.4)}$$

Consequently, strong acids have a high Ka value but a low pKa value and weak acids have a low Ka value but a high pKa value.

As shown in equation 5.3, the dissociation of weak acids is always in equilibrium. Figure 5.1 demonstrates the effect of pH on this dissociation. When the pH of the solution is the same as the pKa value of the acid, the acid is 50 % dissociated and 50 % associated.

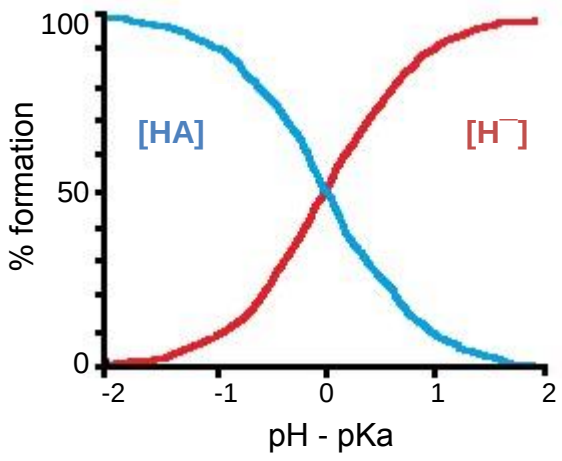

Figure 5.1: Graph to demonstrate the dissociation of weak acids depending on pH value

If the pH of the solution is lowered then the equilibrium (equation 5.3) will shift to the left to become less dissociated. According to Figure 5.1, phosphoric acid (dietary acid) has a pKa value of approximately 2; therefore, at pH 2 it will be 50 % dissociated. However, lactic acid (pKa approximately 4) will be less dissociated at pH 2, according to the theory shown in Figure 5.1.

5.1.3 Mechanisms of Acid Demineralisation

As reviewed in 2006 by Featherstone and Lussi (Featherstone and Lussi, 2006), there are two ways by which acids are believed to cause demineralisation of dentine hydroxyapatite:

(1) Dissociated hydrogen ions (H^+)
(2) Anions (A^-) binding to calcium

(1) Dissociated Hydrogen Ions

As acids dissociate in solution they release hydrogen ions. These hydrogen ions attack the mineral crystal by joining with the carbonate or phosphate ion, causing the surface of the crystal to be etched away. Hydrochloric acid is a strong acid and dissociates fully into hydrogen and chloride ions. The release of these hydrogen ions directly dissolves the mineral surface quickly; whereas chloride ions do not participate in demineralisation. This mechanism also explains the erosion caused by acetic acid (dietary acid) as reported by Lussi *et al* (Lussi *et al*., 1993).

(2) Anions binding to calcium

Demineralisation can also occur from the presence of anions in a mechanism known as chelation; whereby anions (such as citrate from citric acid) combine and remove calcium from the surface of the crystal. Every anion has a unique level of calcium complexation, this is dependent upon the structure of the molecule and how easily it can draw the calcium ion towards it. The strength of this interaction is measured by the stability constant (K). The higher the K value, the stronger the bond to calcium. A stronger bond

means that the anion has more of an ability to pull calcium ions from the crystal into the solution (Featherstone and Lussi, 2006).

The demineralisation of dentine hydroxyapatite is believed to be a combination of both mechanisms. Some demineralising agents such as hydrochloric acid (gastric acid) only cause demineralisation by using mechanism (1) whereas some such as EDTA (laboratory acid) cause demineralisation by mechanism (2). Additionally, some acids such as citric acid (dietary acid) can cause demineralisation by using both mechanisms.

5.2 Aims and Objectives

The aim of this study was to compare the effects of a range of bacterial (lactic), dietary (citric, acetic and phosphoric), gastric (hydrochloric) and laboratory (EDTA and formic) demineralising agents on the integrity of the organic and inorganic components of dentine. The specific objectives were:

1) To create a variety of demineralised dentine models using acids associated with bacterial, dietary, gastric or laboratory-induced demineralisation as well as the chelating agent, EDTA.
2) To use micro-hardness, energy dispersive spectroscopy (EDS), x-ray fluorescence (XRF) and a phosphate assay in an attempt to observe the mineral levels in the demineralised dentine models.
3) To determine whether the different demineralising agents have a different effect on the mineral levels.
4) To analyse the remnant dentine mineral using x-ray diffraction (XRD) to explore whether other calcium phosphate phases exist during demineralisation.
5) To use scanning electron microscopy (SEM) and a hydroxyproline (Hyp) assay to determine whether the demineralising agents cause the dentine collagen to become denatured.
6) To determine whether the different demineralising agents effect the collagen structure differently.
7) To assess whether the effects of the demineralising agents are related to their specific properties e.g. pKa and logKCa values.

8) To determine which demineralising mechanism is more effective: (i) dissociated hydrogen ions binding to phosphate or (ii) anions binding to calcium (chelation).
9) To localise DPP in the demineralised dentine models, using immunohistochemistry, and to use Van Gieson and H&E stains for general histology assessment.

5.3 Materials and Methods

5.3.1 Demineralising Agents

For this study, seven demineralising agents involved in caries, erosion or artificial demineralised dentine models were investigated. Dentine blocks were prepared as described in section 4.2 and demineralised as described in section 4.2.1. The key properties of the seven demineralising agents are summarised in Table 5.1.

Table 5.1: Key properties of the 7 demineralising agents, including their pKa values and logKCa values

Demineralising agents	Molecular formula	Type of acid	pH*	Concentration	pKa	logKCa	Mechanisms of demineralisation
Lactic acid (caries)	$C_3H_6O_3$	Weak	2	0.1 M	3.86	1.45	Hydrogen ions & Anions
Hydrochloric acid (intrinsic erosion)	HCl	Strong	2	0.01 M	-9.30	N/A	Hydrogen ions
Citric acid (extrinsic erosion)	$C_6H_8O_7$	Weak	2	0.1 M	3.13	1.10	Hydrogen ions & Anions
Acetic acid (extrinsic erosion)	$C_2H_4O_2$	Weak	2	4 M	4.76	1.18	Hydrogen ions & Anions
Phosphoric acid (extrinsic erosion & etching)	H_3PO_4	Weak	2	0.02 M	2.15	1.40	Hydrogen ions & Anions
Formic acid (artificial caries models)	CH_2O_2	Weak	2	0.5 M	3.75	0.53	Hydrogen ions & Anions
EDTA (artificial caries models)	$C_{10}H_{16}N_2O_8$	Weak	7	0.5 M	N/A	10.70	Anions

* recording of pH on the pH probe is accurate to ±0.3

5.3.2 Analysis of Mineral Content

(a) Micro-hardness

Micro-hardness was used to measure the mineral content of the demineralised dentine models at baseline (day 0, before demineralisation) and at day 1, 2, 7 and 14 of demineralisation. Details of the methodology are described in section 4.7.1

(b) Energy dispersive spectroscopy

The presence of calcium (Ca Kα, 3.692 keV) and phosphorous (P Kα, 2.010 keV) was measured using EDS. Section 4.7.2 outlines the details of EDS methodology. Twenty-four dentine blocks were randomly assigned to the 7 demineralisation groups or control group (see section 4.2.1), allowing 3 blocks per group. From each specimen, 4 measurements were taken (1 in each quadrant as displayed in Figure 4.8) each measurement covering a 100μm^2 inter-tubular area. Only calcium (Ca) and phosphorous (P) results are displayed in this thesis as these are the only elements of interest.

(c) X-ray fluorescence

X ray fluorescence (XRF) was used to look at the levels of calcium (Ca) and phosphorous (P) in the dentine specimens. Eight dentine specimens were randomly assigned to a demineralisation group or control group (as outlined in section 4.2.1) and were prepared for XRF, preparation for XRF is described in section 4.7.3. Only calcium (Ca) and phosphorous (P) results are displayed in this thesis as these are the only elements of interest.

(d) Phosphate assay

A phosphate assay was used to measure the release of phosphate from the dentine specimens. Twelve dentine blocks were randomly assigned to a demineralisation group: hydrochloric acid, lactic acid, citric acid or EDTA (n = 3 per group). See section 4.7.5 for methodology details.

(e) X-ray diffraction

XRD was employed to analyse the residual mineral content. Dentine specimens (24 in total) were randomly assigned to one of the 7

demineralisation groups or the control group; allowing 3 different dentine blocks to be analysed per group. The detailed protocols for XRD can be found in section 4.7.4.

5.3.3 Analysis of Collagen Integrity

(a) Scanning Electron Microscopy

SEM was employed to observe the collagen structure in the demineralised dentine models (n = 3), as described in section 4.7.6. Initial imaging was undertaken to develop a suitable protocol, this involved examining four different areas of the specimen at a range of magnifications. It was noticed that at low magnification (1000x and 3000x) there was little difference between the areas of the specimen. However, at high magnification (60000x and 80000x) the sample varied from area to area; see Figure 5.2.

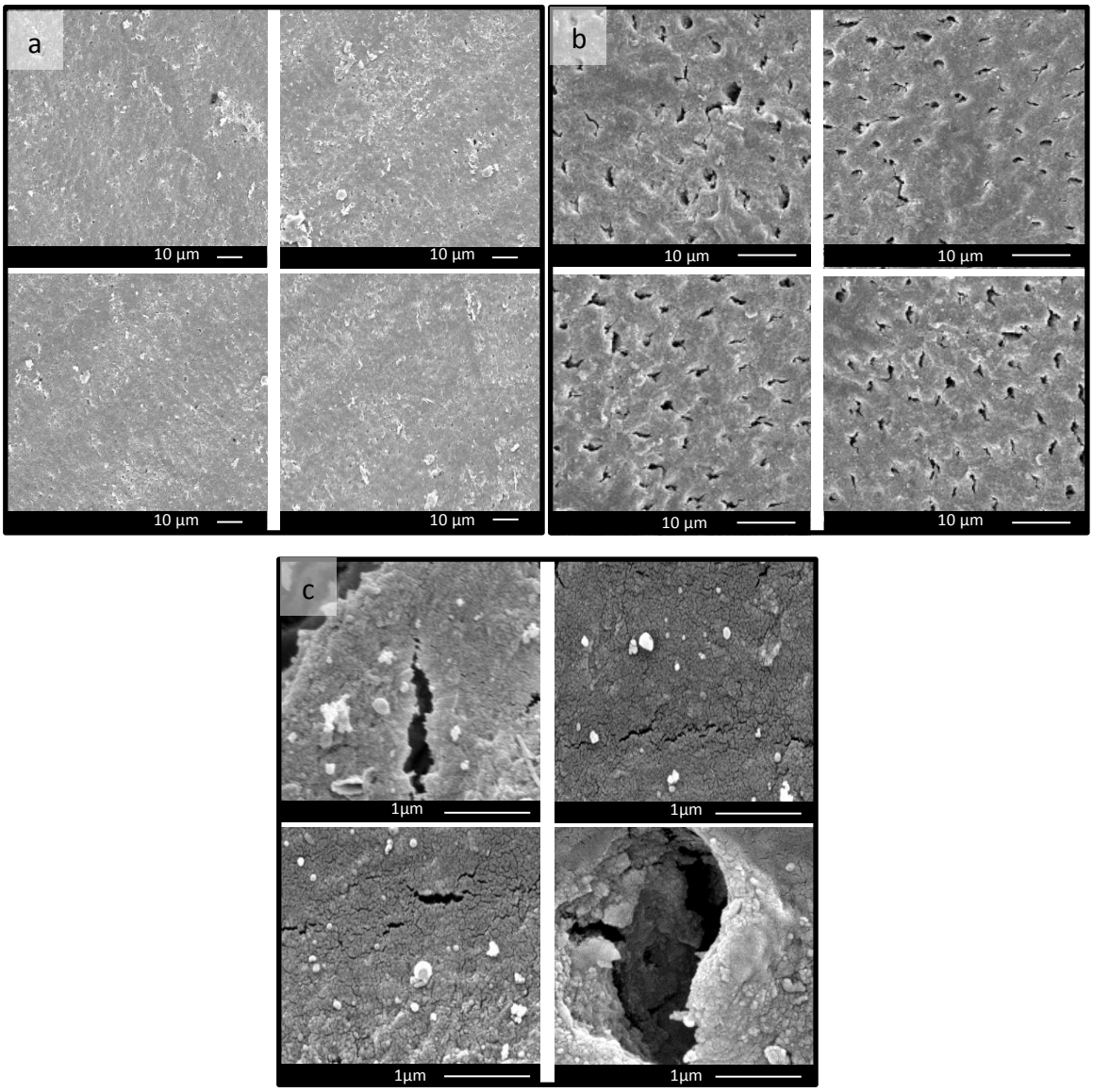

Figure 5.2: SEM images of non-demineralised dentine taken at four areas of a specimen at various magnifications: (a) 1000x magnification, (b) 3000x magnification and (c) 80000x magnification.

Therefore, a protocol was developed for SEM imaging that involved looking at 4 areas of the specimen at the following magnifications: 1000x, 3000x, 10000x, 20000x, 40000x and 80000x. On each sample, 4 images were taken at each magnification; at high magnifications it involved taking 2 images of intra-tubular dentine and 2 images of inter-tubular dentine. When measuring collagen fibre dimensions measurements were taken from 5 areas on 3 high magnification (80000x) images. Measurements were taken with ImageJ software version 1.46. This is explained in Figure 5.3 using a non-demineralised (control) specimen viewed at 80000x magnification as an example.

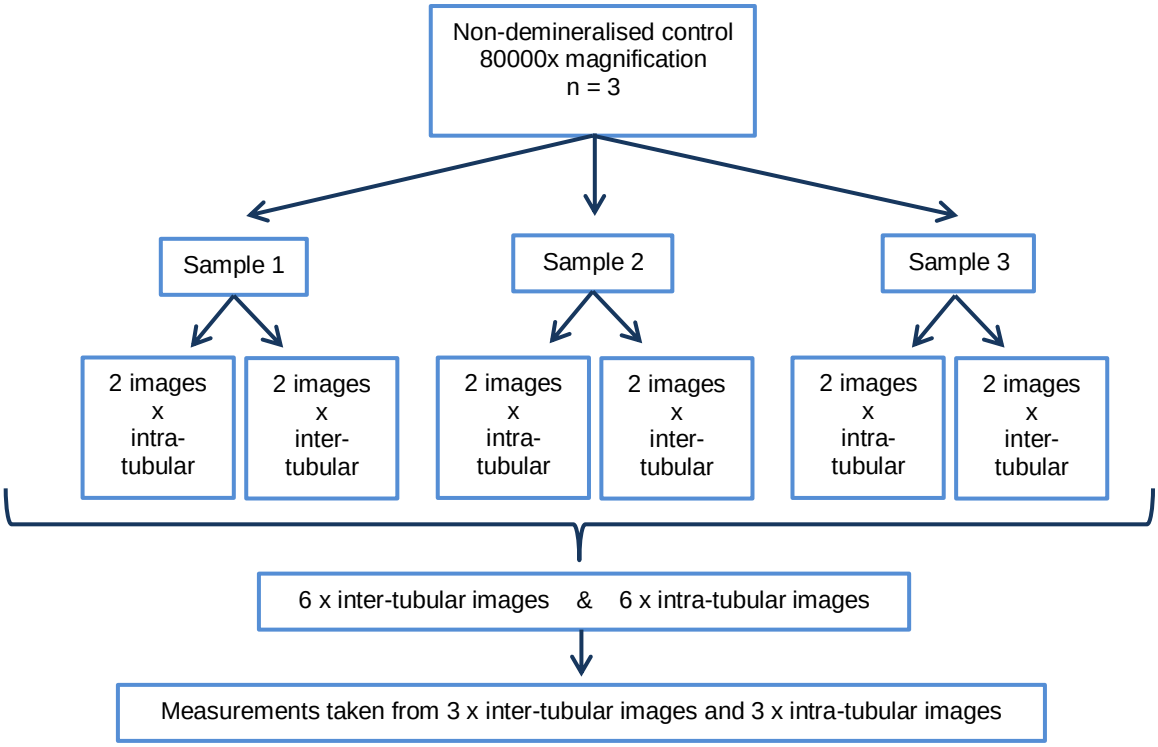

Figure 5.3: A diagram to help explain how many images and measurements were taken per specimen.

(b) Hydroxyproline Assay

The Hyp assay was used to quantify any denatured collagen (n = 3). The detailed protocol for this experiment is explained in the Materials and Methods Chapter; section 4.7.7.

5.3.4 Immunohistochemical Localisation of Dentine Phosphoprotein

Sections, 4 μm thick, were produced from demineralised teeth and stained with haematoxylin and eosin (H&E) and Van Gieson (protocols in sections 4.5.1 and 4.5.2, respectively) to observe the structure of the specimens. Sections were then processed for immunohistochemical localisation of dentine phosphoprotein (DPP) (protocol in Materials and Methods Chapter, section 4.5).

5.4 Results

5.4.1 Micro-hardness Results

The Vickers hardness normalised results are displayed in Figure 5.4, showing that the surface hardness of every specimen decreased over the 14 day period. By day 1, there was little difference in surface hardness between each specimen (Vickers hardness numbers (VHN) ranged from 41 – 48) but by day 2 the hardness values were more diverse; ranging from 33 (lactic acid treated specimen) to 47 (EDTA treated specimen). From day 1 the specimens began to develop different trends; the surface hardness of the lactic acid specimen declined rapidly whilst the surface hardness of the other specimens appear to have decreased less considerably. After 14 days, the surface hardness of lactic acid specimens had decreased by approximately 80 % yet the surface hardness of citric acid specimens had only decreased by approximately 40 %, from baseline values. The depth of the indents ranged from approximately 100 μm to 200 μm.

Figure 5.5 displays the Vickers hardness values of the specimens after 7 days in the demineralising agents. There was a significant difference ($p < 0.05$) between lactic acid treated specimens compared with: hydrochloric acid treated specimens, acetic acid treated specimens and citric acid treated specimens. There was also significant difference ($p < 0.05$) between citric acid treated specimens compared with: lactic acid, phosphoric acid, EDTA and formic acid treated specimens.

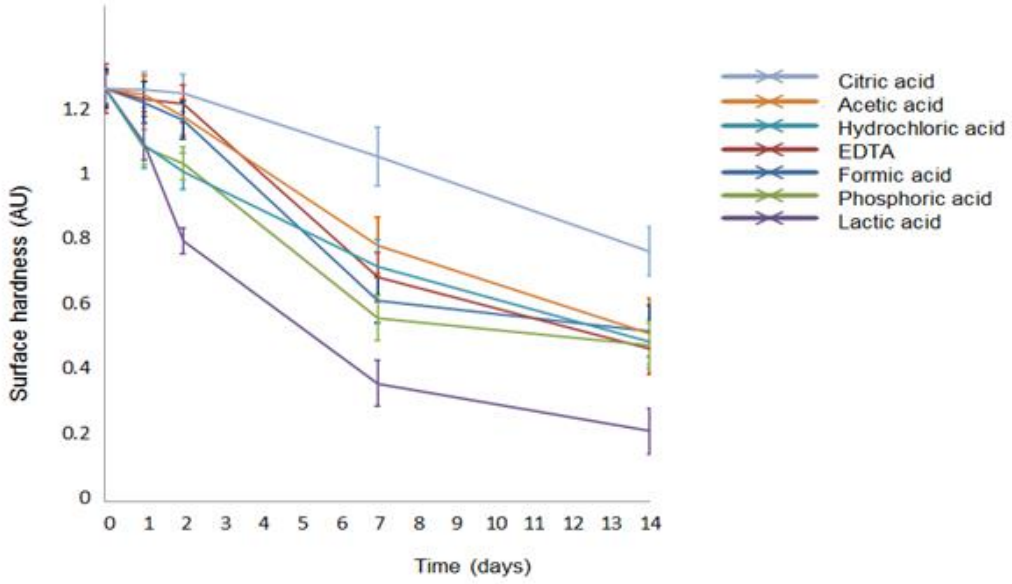

Figure 5.4: Normalised values for micro-hardness of dentine specimens following demineralisation with various acids from day 0 – day 14.

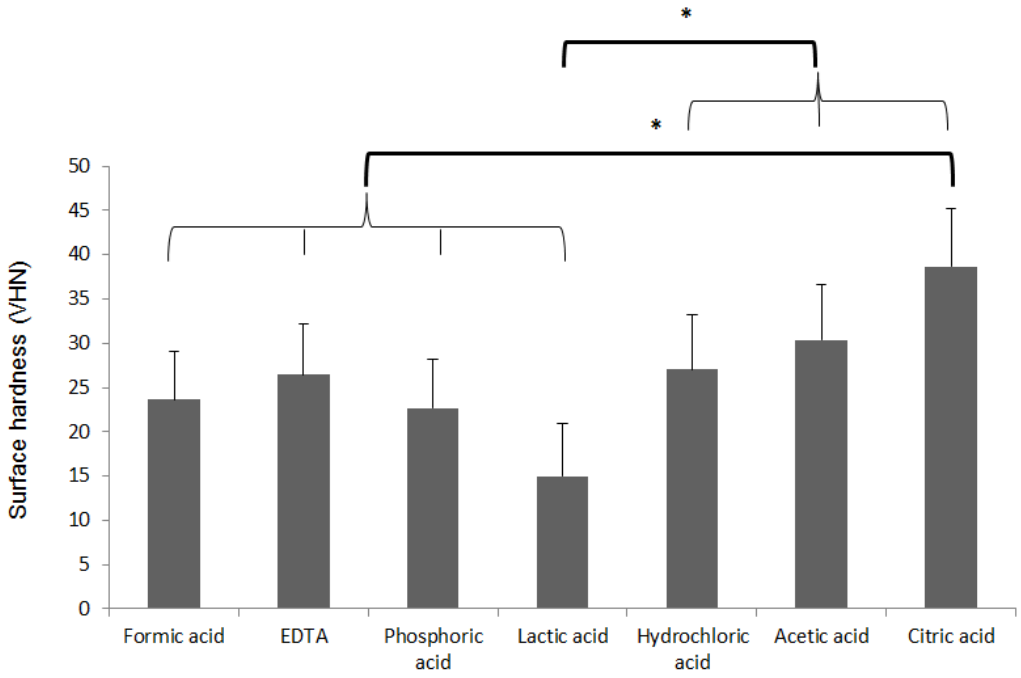

Figure 5.5: Vickers micro-hardness (VHN) after 7 days in the demineralising agents. There are no significant differences between variables unless indicated. * = Significant differences ($p < 0.05$) using one-way ANOVA and Tukey post-hoc analysis. The bold brackets compare the variables that are significantly different from one another. The thin braces group variables together where they are all significantly different from another variable. For example, hydrochloric acid, acetic acid and citric acid are not significantly different from one another but are significantly different from lactic acid.

5.4.2 Energy Dispersive Spectroscopy and X-ray Fluorescence Results

Energy dispersive spectroscopy (EDS) and x-ray fluorescence (XRF) analysis was undertaken at day 7 of demineralisation. As mentioned in section 4.7.2, although a cobalt standard was used to calculate the levels of Ca and P in the area of the sample hit by the beam, the EDS results can only be considered to be qualitative, as the surfaces of the dentine were not mirror flat. Figures 5.6 and 5.7 display the EDS results showing the presence of calcium (Ca) and phosphorous (P) in the dentine specimens. The relative levels of Ca and P in the control samples were approximately 25 wt. % and 15 wt. %, respectively; but these values were reduced in all demineralised specimens.

There was a significant difference in relative Ca levels between the control specimens and all other specimen groups except for the citric acid treated specimens. In fact, on average, the relative perentages of calcium measured in the citric acid treated specimens was approximately 10 % less than the control group, whereas the lactic acid treated specimens and EDTA treated specimens had approximately 50 % less calcium.

The biggest change in relative P levels was observed by lactic acid with approximately 50 % less P than the control specimens, however, there was no significant difference between the lactic acid treated specimens compared with the specimens demineralised with either phosphoric acid, EDTA, hydrochloric acid or acetic acid. Similarly to the Ca results, citric acid appeared to have the least effect on relative P levels by only showing approximately 10 % less P than the control, however, the citric acid treated specimens only showed a significant difference compared with lactic acid and hydrochloric acid treated specimens. Specimens demineralised with citric acid, formic acid and phosphoric acid all showed no significant difference compared with the control specimen.

Figure 5.8 displays the XRF results which also indicated a reduction in Ca and P levels following demineralistion. Similarly to EDS, XRF results also appear to demonstrate that citric acid caused the least change in Ca and P

content. However, as XRF analysis was only performed once per sample, statistical analysis could not be performed. Therefore, the results of the XRF analysis could only be considered to give a probable indication of Ca and P level trends rather than being able to draw absolute conclusions.

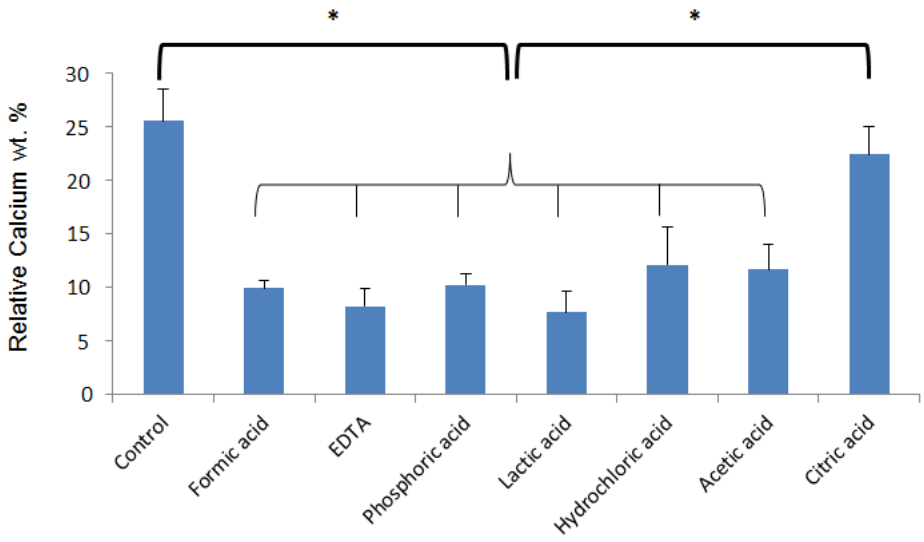

Figure 5.6: Relative calcium wt.% (Kα; 3.692 keV) using EDS of dentine after 7 days in the demineralising agents.

* = Significant differences ($p < 0.05$) using one-way ANOVA and Tukey post-hoc analysis. There are no significant differences between variables unless indicated. The bold brackets compare the variables that are significantly different from one another. The thin braces group variables together where they are all significantly different from another variable. For example, formic acid, EDTA, phosphoric acid, lactic acid, hydrochloric acid and acetic acid are not significantly different from one another but are all significantly different from the control and the citric acid variable.

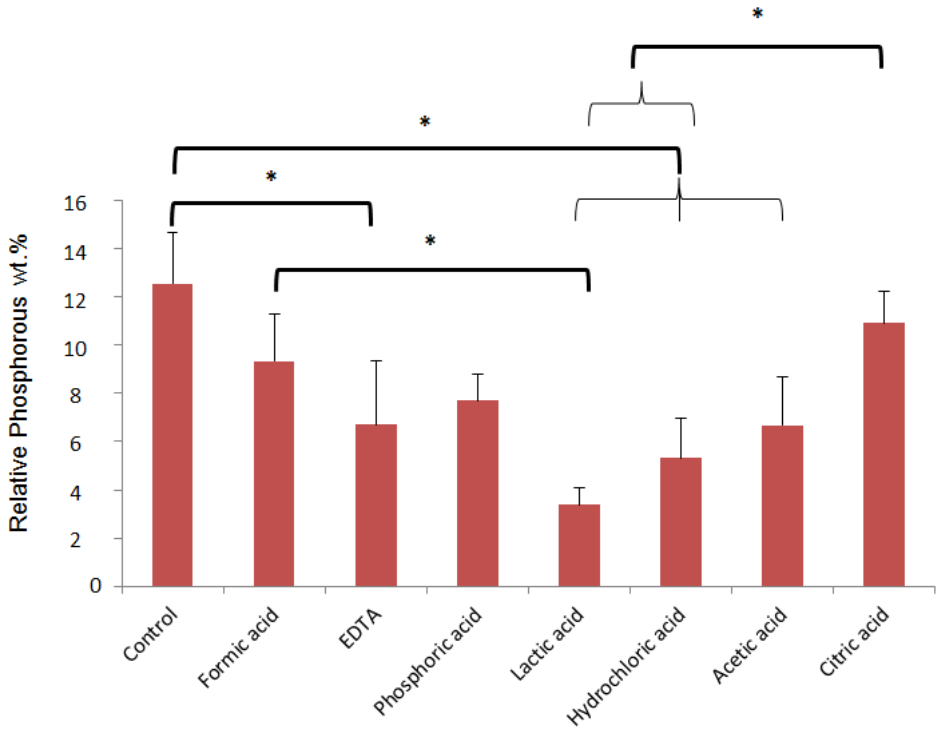

Figure 5.7: Relative phosphorous wt.% (Kα; 2.1010 keV) using EDS of dentine after 7 days in the demineralising agents.

There are no significant differences between variables unless indicated.

* = Significant differences ($p < 0.05$) using one-way ANOVA and Tukey post-hoc analysis. The bold brackets compare the variables that are significantly different from one another. The thin braces group variables together where they are all significantly different from another variable. For example, lactic acid, hydrochloric acid and acetic acid are not significantly different from one another but are all significantly different from the control, and the control is also significantly different from the EDTA variable.

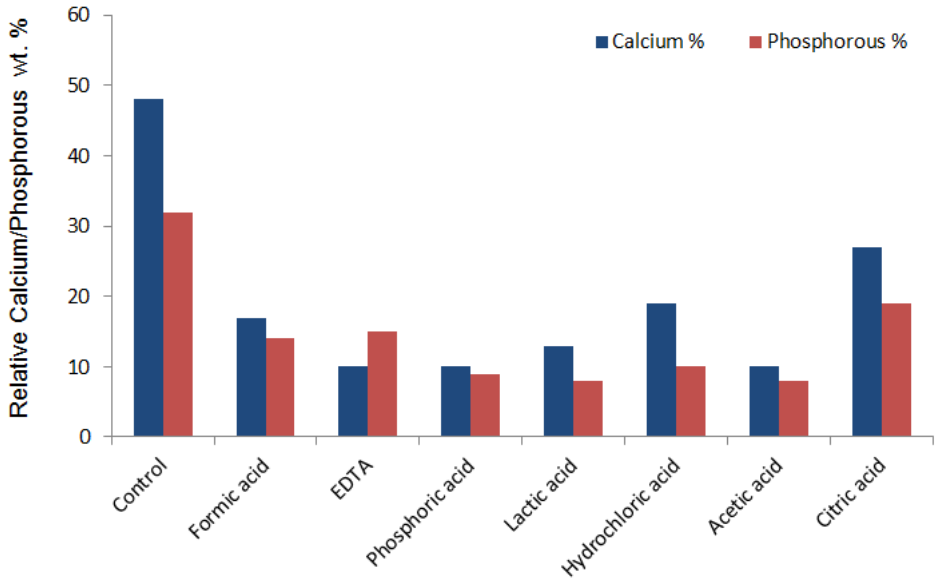

Figure 5.8: XRF results displaying the levels of calcium and phosphorous in the various demineralised dentine specimens. There is no statistical analysis because n = 1.

5.4.3 Phosphate Assay Results

The phosphate released from dentine specimens treated with lactic acid, hydrochloric acid, citric acid and EDTA was measured, from day 1 to day 14, using a phosphate assay (Figure 5.9). Figure 5.9 displays the accumulated loss of phosphate from the dentine specimens. All specimens released phosphate every day throughout the 14 day period, lactic acid released a total of 12 nmol per mg phosphate, whereas EDTA only released approximately 4 nmol. Figure 5.10 shows the phosphate release at day 7, all demineralised groups were significantly different compared to the control group.

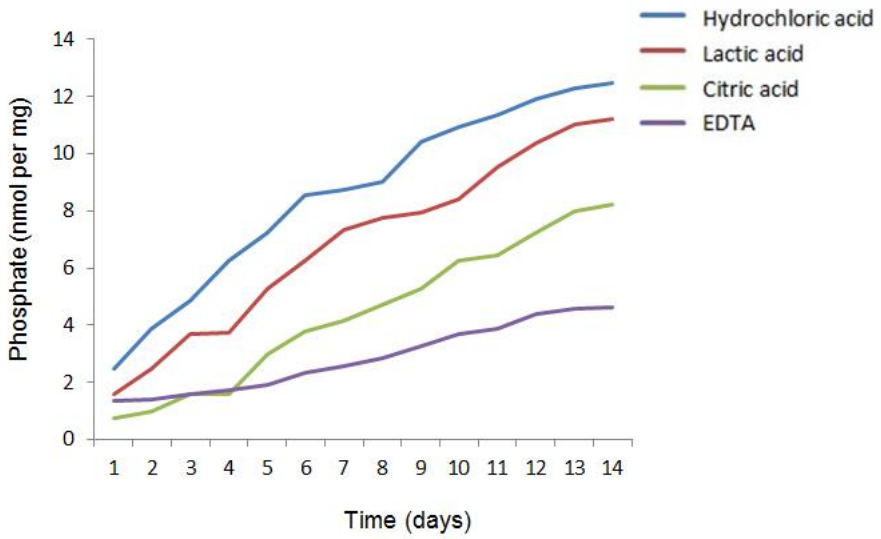

Figure 5.9: Phosphate release (nmol) from demineralised dentine specimens over a 14 day period (mean values n = 3)

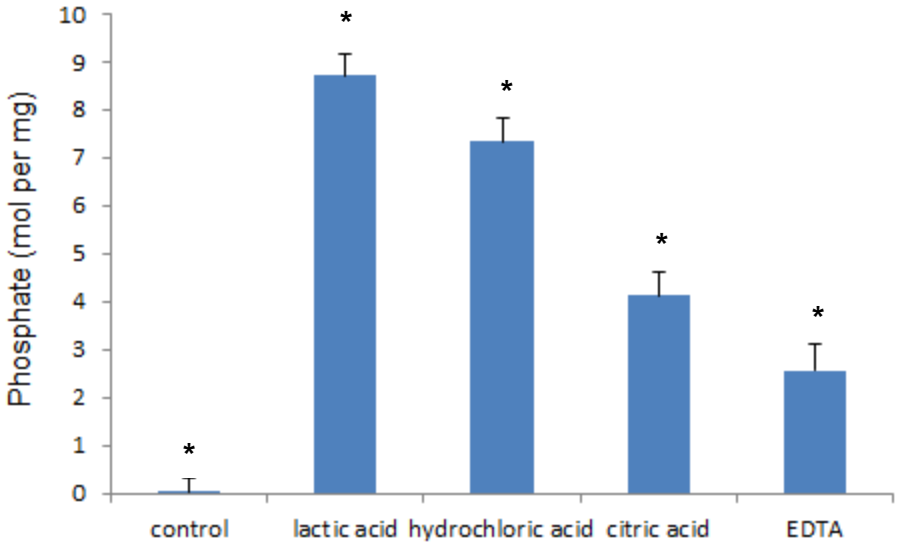

Figure 5.10: Phosphate assay results displaying the phosphate release (nmol) from demineralised dentine specimens after 7 days in a demineralising agent. * = Significant difference ($p < 0.05$) by one way anova and Tukey's post hoc analysis. All variables show significant difference between one another.

5.4.4 X-ray Diffraction Results

Figures 5.11 – 5.18 display the x-ray diffraction (XRD) spectra for dentine treated with water (control), citric acid, formic acid, hydrochloric acid, acetic acid, lactic acid, EDTA and phosphoric acid for 7 days. The red peaks displayed were the hydroxyapatite standard from the International Centre for Diffraction Data (ICDD) database. The blue spectra were representative of the individual specimens. Figure 5.11 shows the non-demineralised dentine specimen (control) aligning with the hydroxyapatite standard. The XRD spectra for dentine treated with citric acid (Figure 5.12), formic acid (Figure 5.13) and hydrochloric acid (Figure 5.14) also aligned with the hydroxyapatite standard but with a lower intensity. The spectra for dentine treated with acetic acid (Figure 5.15), lactic acid (Figure 5.16) and EDTA (Figure 5.17) have peaks that aligned with the hydroxyapatite peaks but they appear to be low in intensity. These specimens appear to be semicrystalline because some of their peaks, (labelled #), resemble that which would be expected of an amorphous phase. Following treatment with phosphoric acid (Figure 5.18) the blue spectrum had few, extremely low intensity, peaks making it difficult to align with the standard. All XRD spectra also show a systematic shift of peaks; although this does not affect any results or conclusions drawn, it is most likely due to a setting on the XRD instrument.

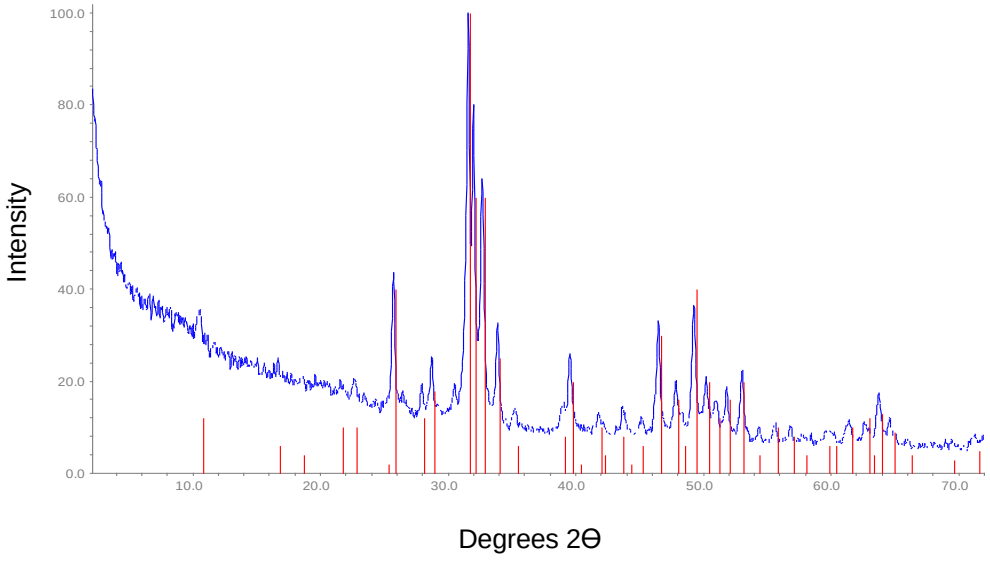

Figure 5.11: XRD spectrum for non-demineralised dentine specimen (control) shown in blue. The red peaks are the hydroxyapatite standard (ICDD card 9-432)

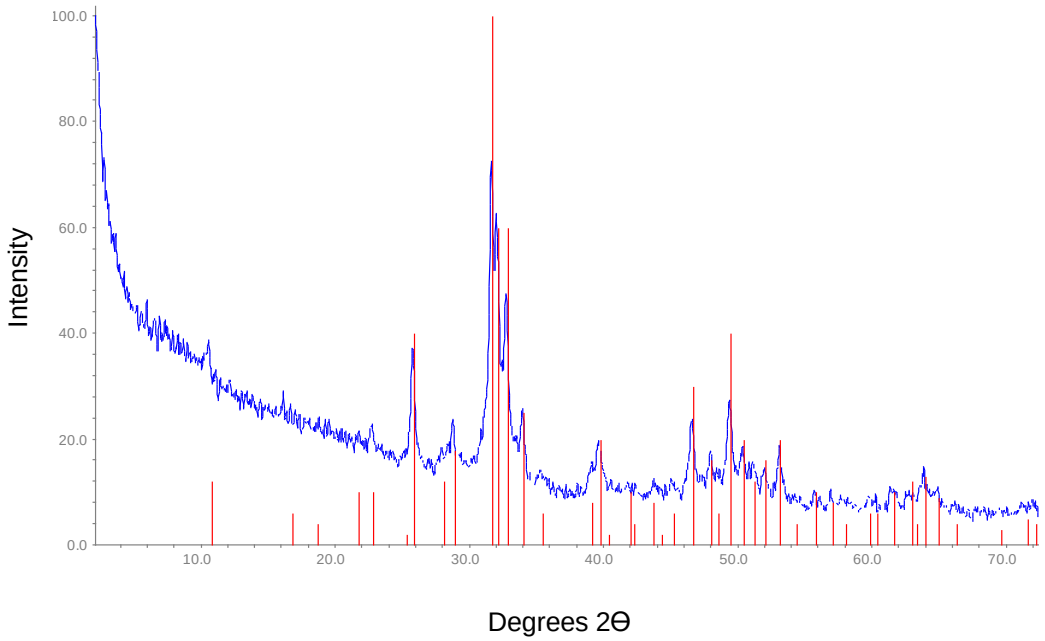

Figure 5.12: XRD spectrum for dentine demineralised for 7 days with citric acid shown in blue. The red peaks are the hydroxyapatite standard (ICDD card 9-432)

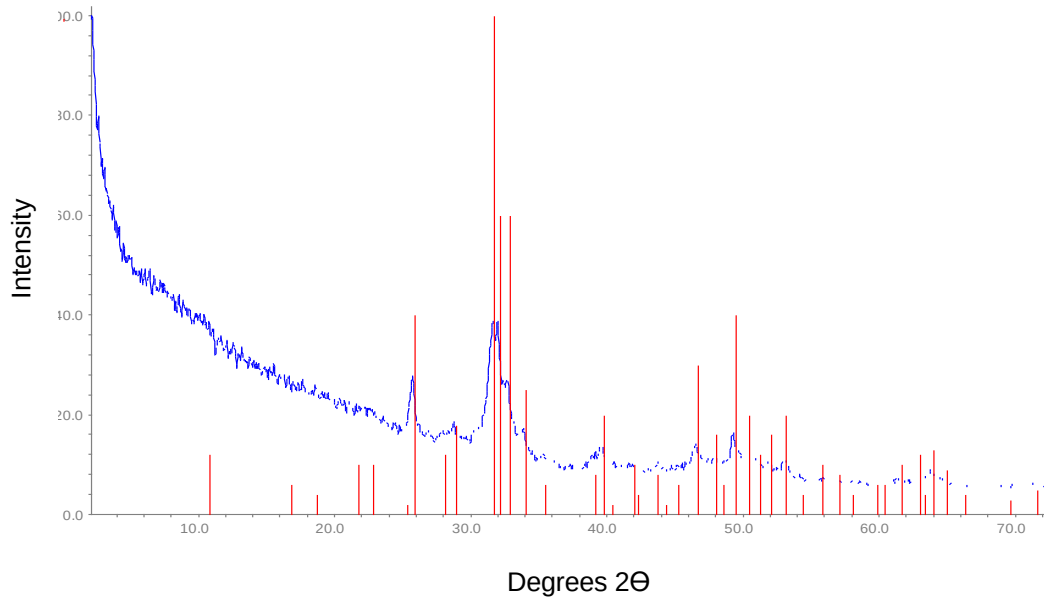

Figure 5.13: XRD spectrum for dentine demineralised for 7 days with hydrochloric acid shown in blue. The red peaks are the hydroxyapatite standard (ICDD card 9-432)

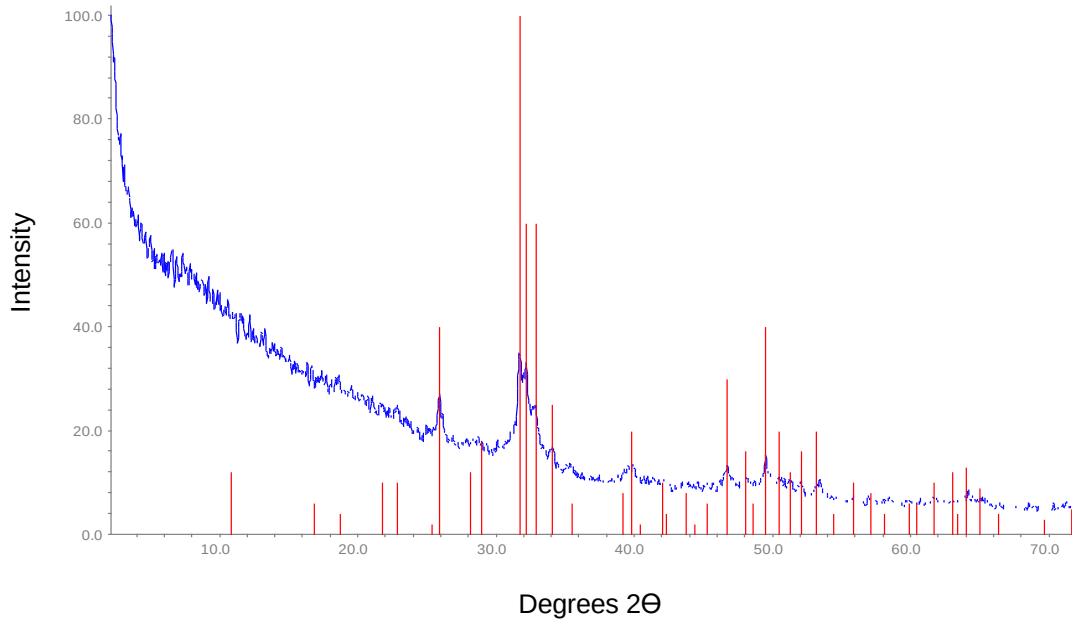

Figure 5.14: XRD spectrum for dentine demineralised for 7 days with formic acid shown in blue. The red peaks are the hydroxyapatite standard (ICDD card 9-432)

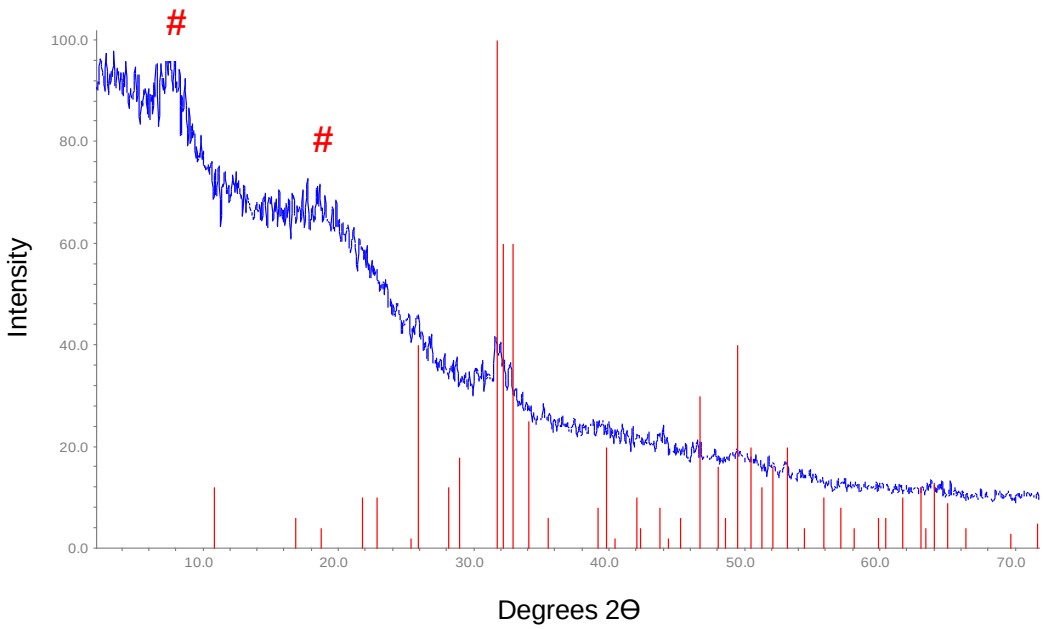

Figure 5.15: XRD spectrum for dentine demineralised for 7 days with acetic acid shown in blue. The red peaks are the hydroxyapatite standard (ICDD card 9-432)

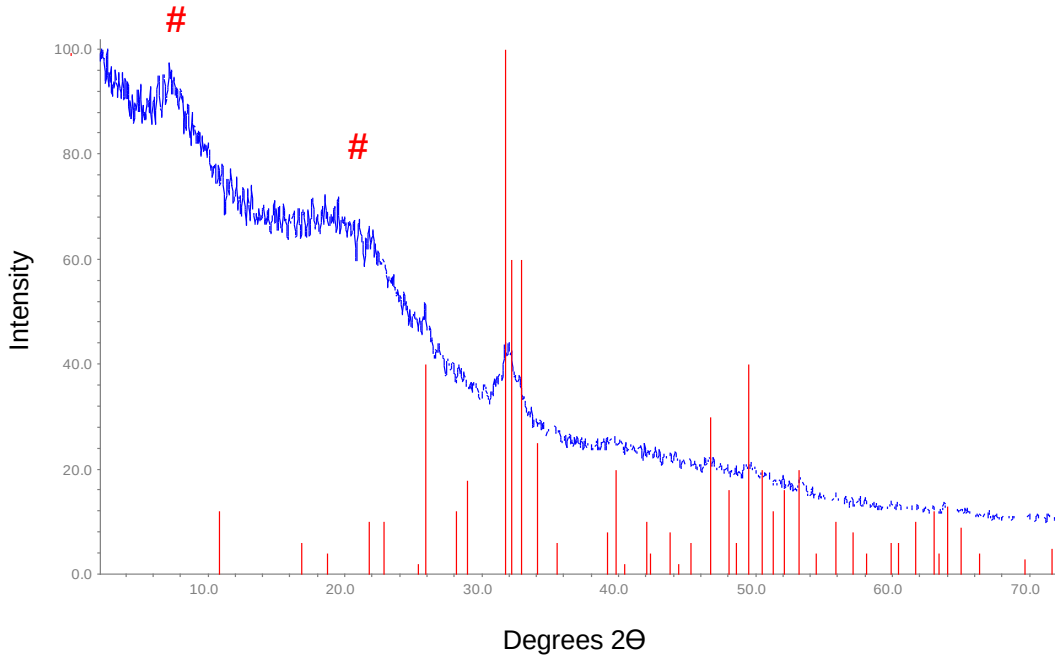

Figure 5.16: XRD spectrum for dentine demineralised for 7 days with lactic acid shown in blue. The red peaks are the hydroxyapatite standard (ICDD card 9-432)

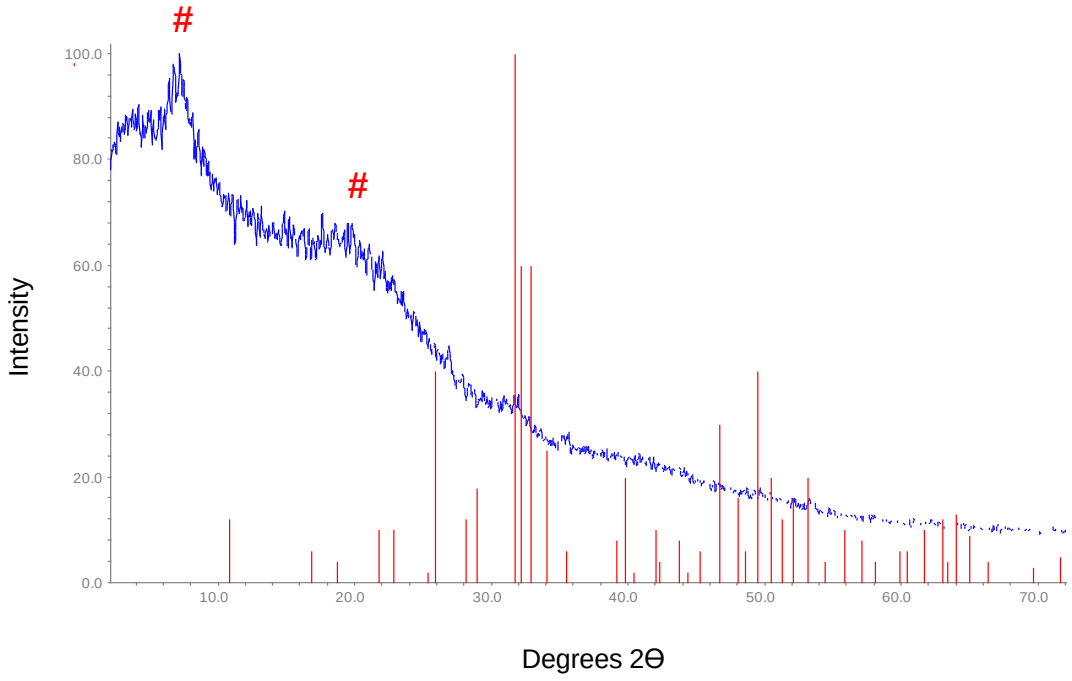

Figure 5.17: XRD spectrum for dentine demineralised for 7 day with EDTA shown in blue. The red peaks are the hydroxyapatite standard (ICDD card 9-432). # = amorphous peaks.

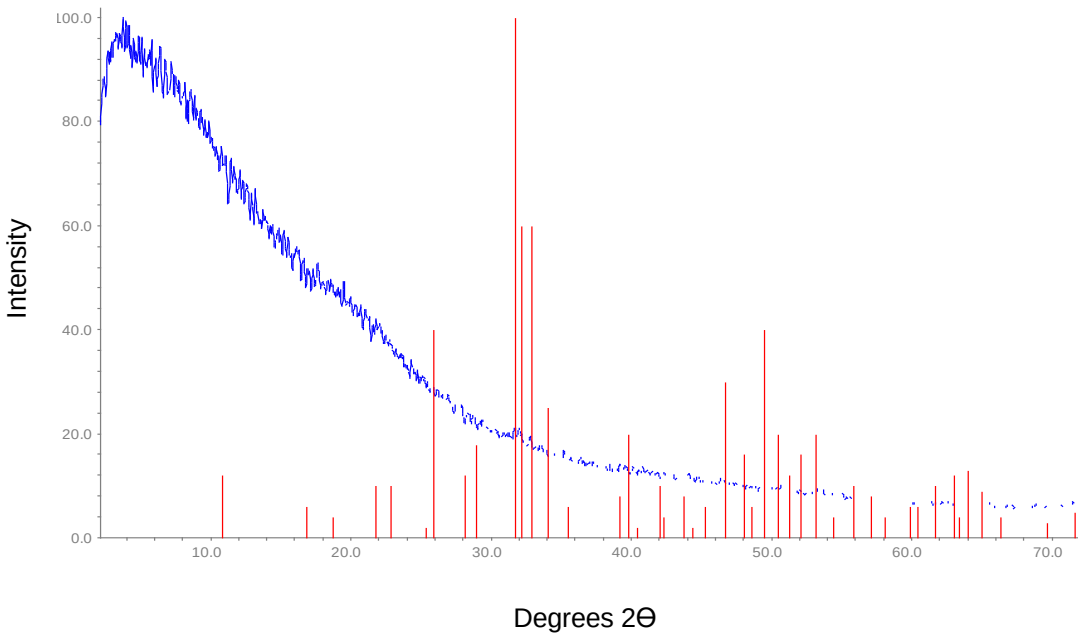

Figure 5.18: XRD spectrum for dentine demineralised for 7 days with phosphoric acid shown in blue. The red peaks are the hydroxyapatite standard (ICDD card 9-432).

5.4.5 Scanning Electron Microscopy Results

Scanning Electron Microscopy (SEM) was used to observe the collagen structure of the dentine surfaces following a 7 day exposure to the various demineralising agents and non-demineralised control specimens (Figures 5.19 – 5.26). There are three images (a, b and c) displayed for each specimen. Image (a) is a low magnification image to show the geometry and microstructure of the sample surface, images (b) and (c) show a dentine tubule. Focus was on the tubules as this enabled the inner surface to be examined.

Figure 5.19 (non-demineralised): Tubules appear exposed but the collagen fibre network seems masked with mineral, no individual fibres are visible.

Figure 5.20 (demineralised with formic acid pH 2): The intra-tubular dentine appears to be demineralised and the collagen fibres appear exposed. The collagen network within the tubules appears to be collapsed. There seems to be some residual mineral left within the inter-tubular dentine.

Figure 5.21 (demineralised with EDTA pH 7): The intra-tubular dentine appears demineralised and the collagen fibres appear exposed. The collagen network seems to have maintained its 3D structure.

Figure 5.22 (demineralised with phosphoric acid pH 2): Although dentine treated with phosphoric acid appears to have exposed the intra-tubular collagen network, the matrix appears condensed. Collagen fibres appear exposed in the inter-tubular regions.

Figure 5.23 (demineralised with lactic acid pH 2): The collagen network appears to be condensed. There seems to be residual mineral on the inter-tubular dentine surface. The SEM images also display the banding patterns on the individual collagen fibres.

Figure 5.24 (demineralised with hydrochloric acid pH 2): Some areas of the inter-tubular dentine appear to have exposed collagen network. Collagen banding patterns can be observed on the high magnification image.

Figure 5.25 (demineralised with acetic acid pH 2): Acetic acid resulted in residual mineral appearing to be present on the inter-tubular collagen but the intra-tubular dentine appears to be exposed.

Figure 5.26 (demineralised with citric acid pH 2): Citric acid seems to have a unique effect on dentine collagen. There appears to be a smear layer of mineral on the surface. The tubules seem to be exposed but the inter-tubular collagen and individual fibres do not seem to be visible.

The collagen fibre widths are presented in Table 5.2. The average width of the collagen fibres ranged from approximately 20 nm to 177 nm.

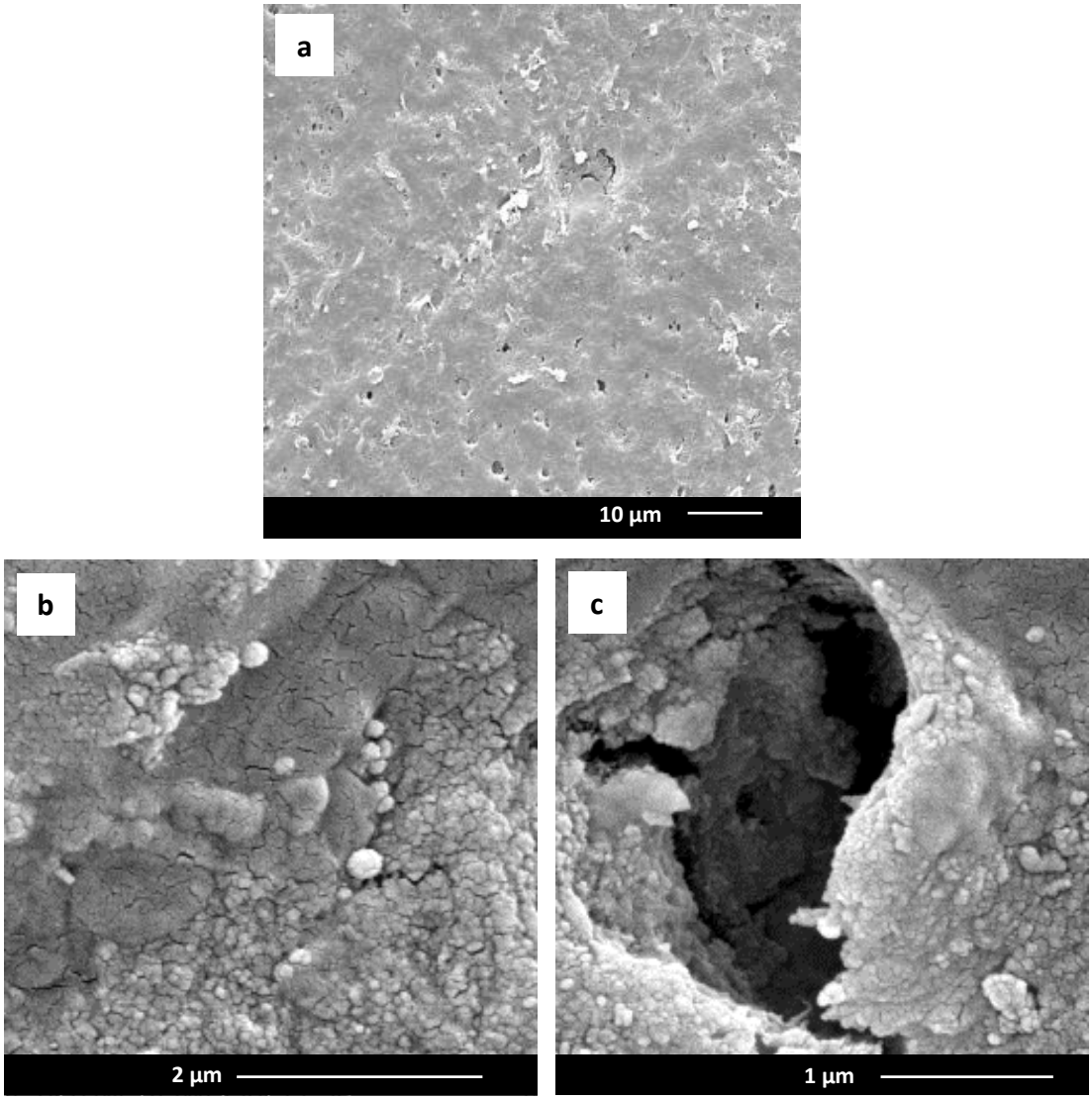

Figure 5.19: SEM micrographs of a dentine specimen not demineralised. Images taken at (a) 4000x; (b) 60000x; (c) 80000x magnifications.

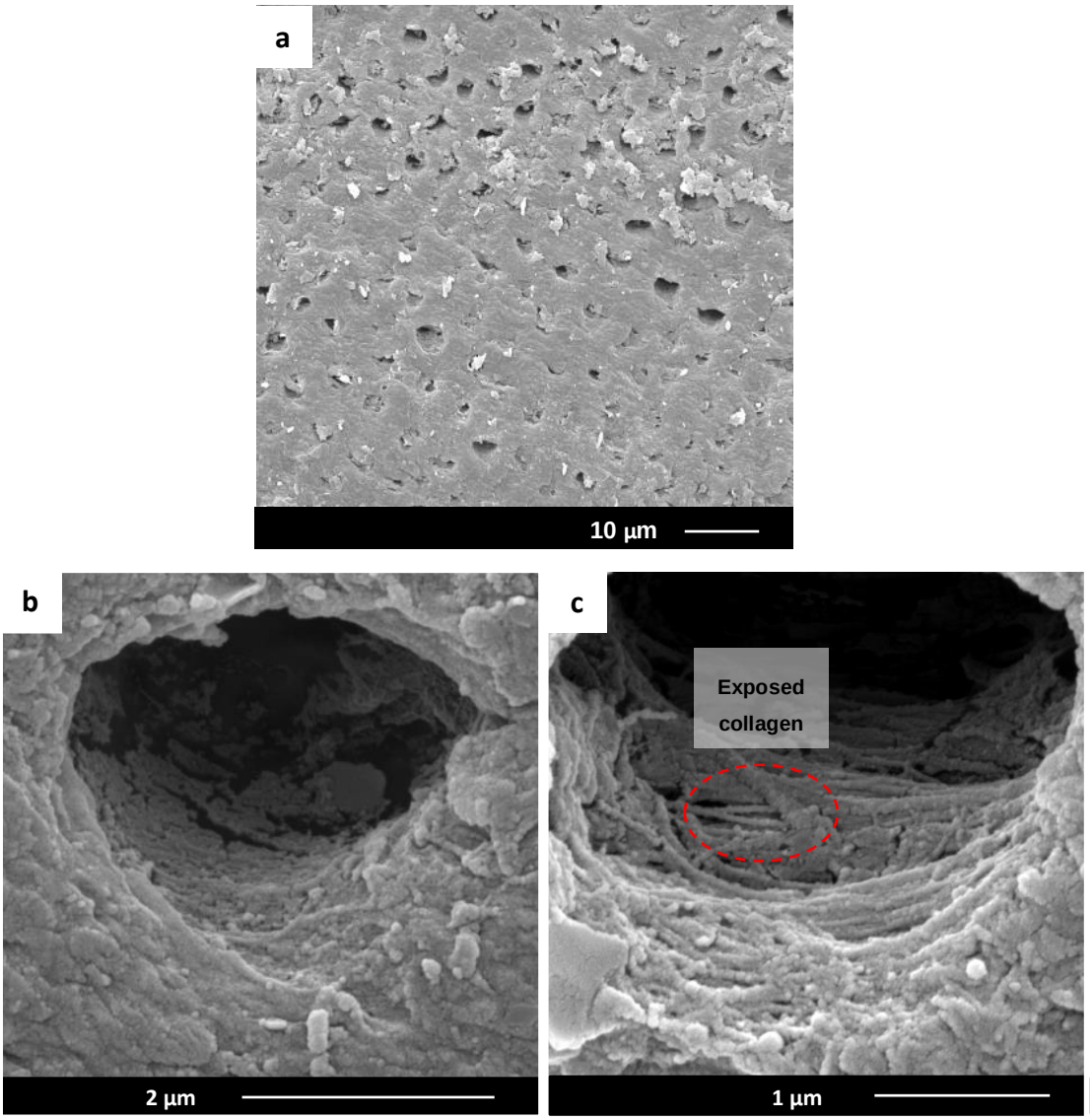

Figure 5.20: SEM micrographs of a dentine specimen after demineralisation with formic acid pH 2 for 7 days. Images taken at (a) 4000x; (b) 60000x; (c) 80000x magnifications.

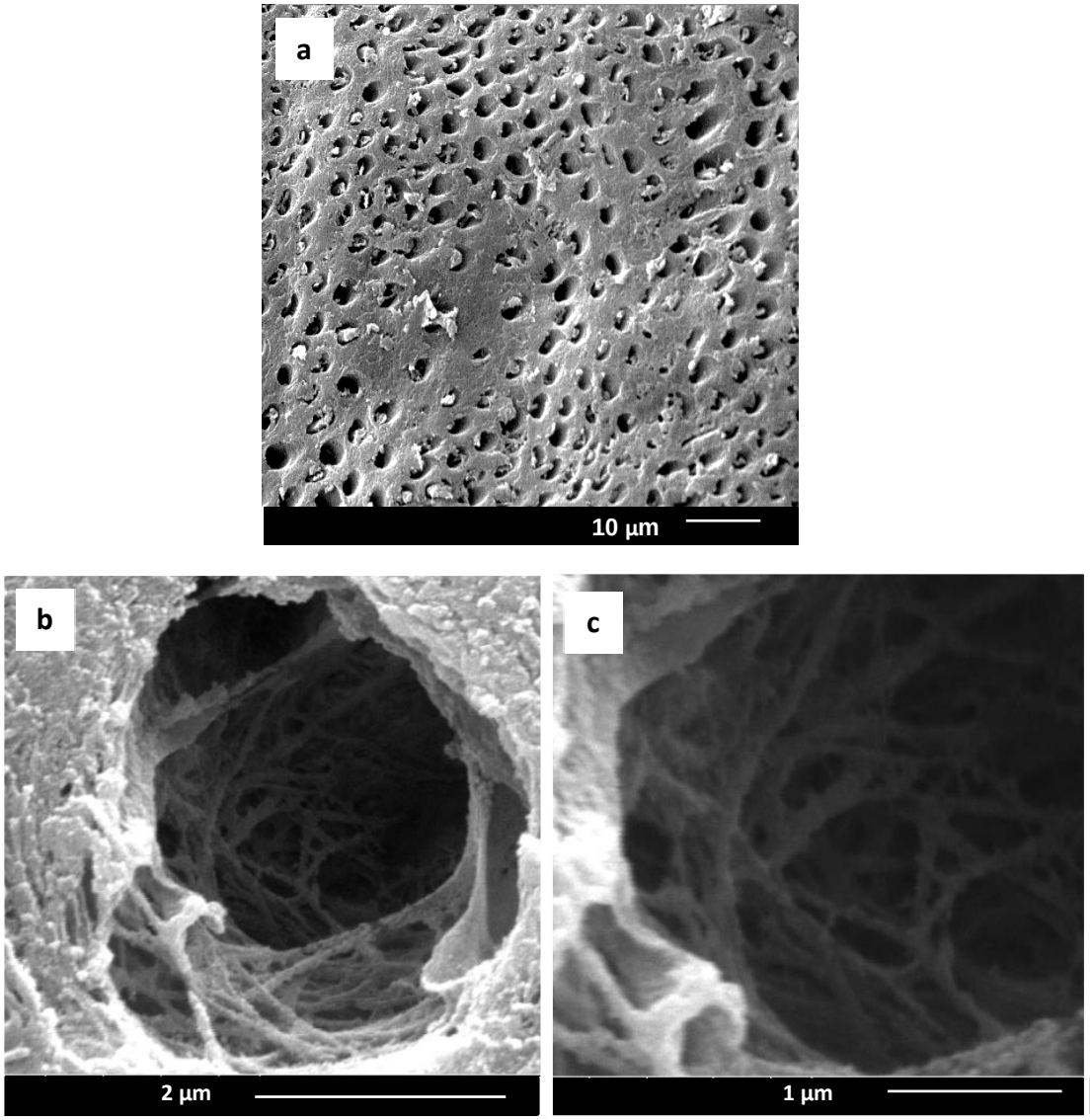

Figure 5.21: SEM micrographs of a dentine specimen after demineralisation with EDTA pH 7 for 7 days. Images taken at (a) 4000x; (b) 60000x; (c) 80000x magnifications.

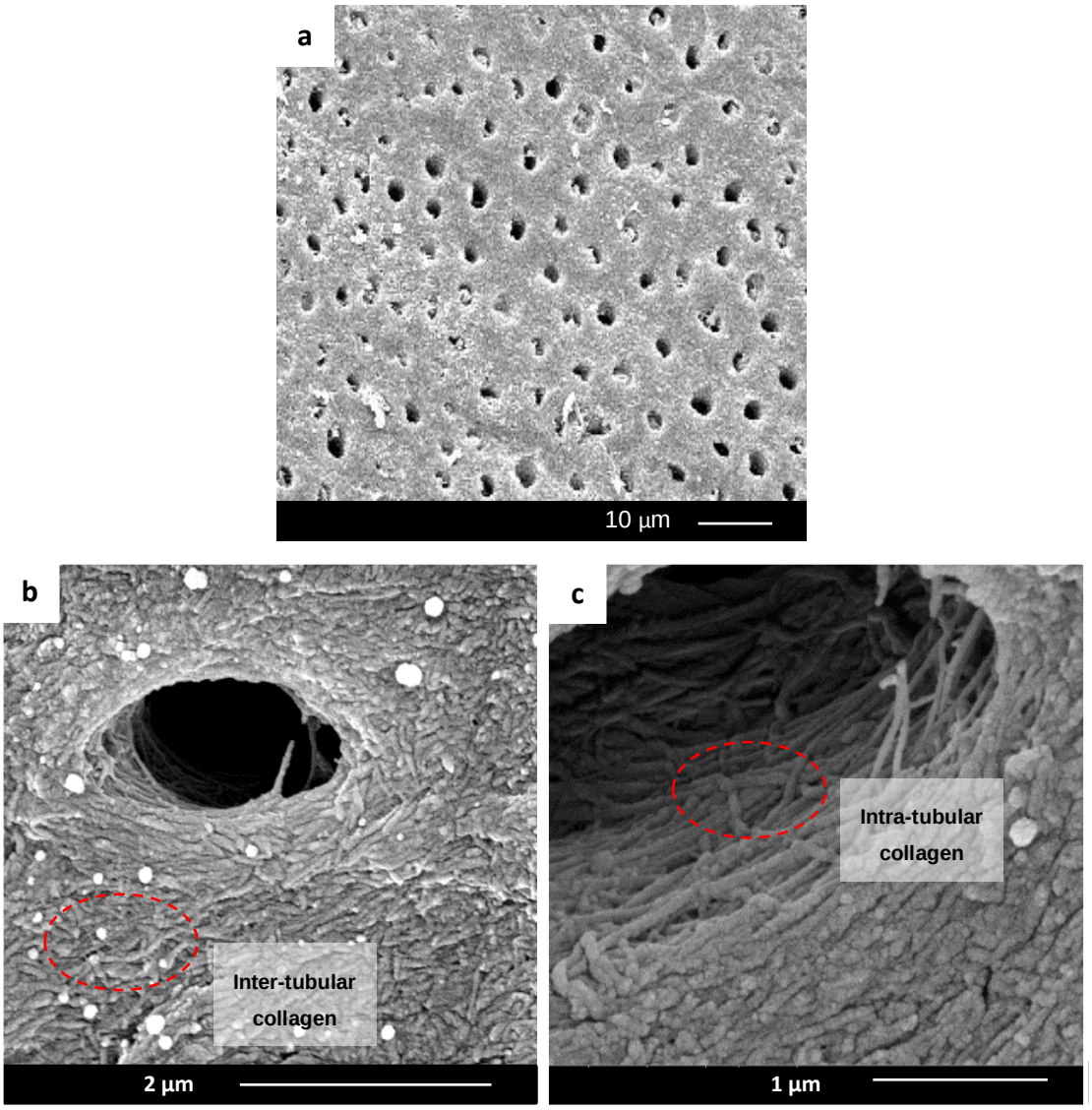

Figure 5.22: SEM micrographs of a dentine specimen after demineralisation with phosphoric acid pH 2 for 7 days. Images taken at (a) 4000x; (b) 60000x; (c) 80000x magnifications.

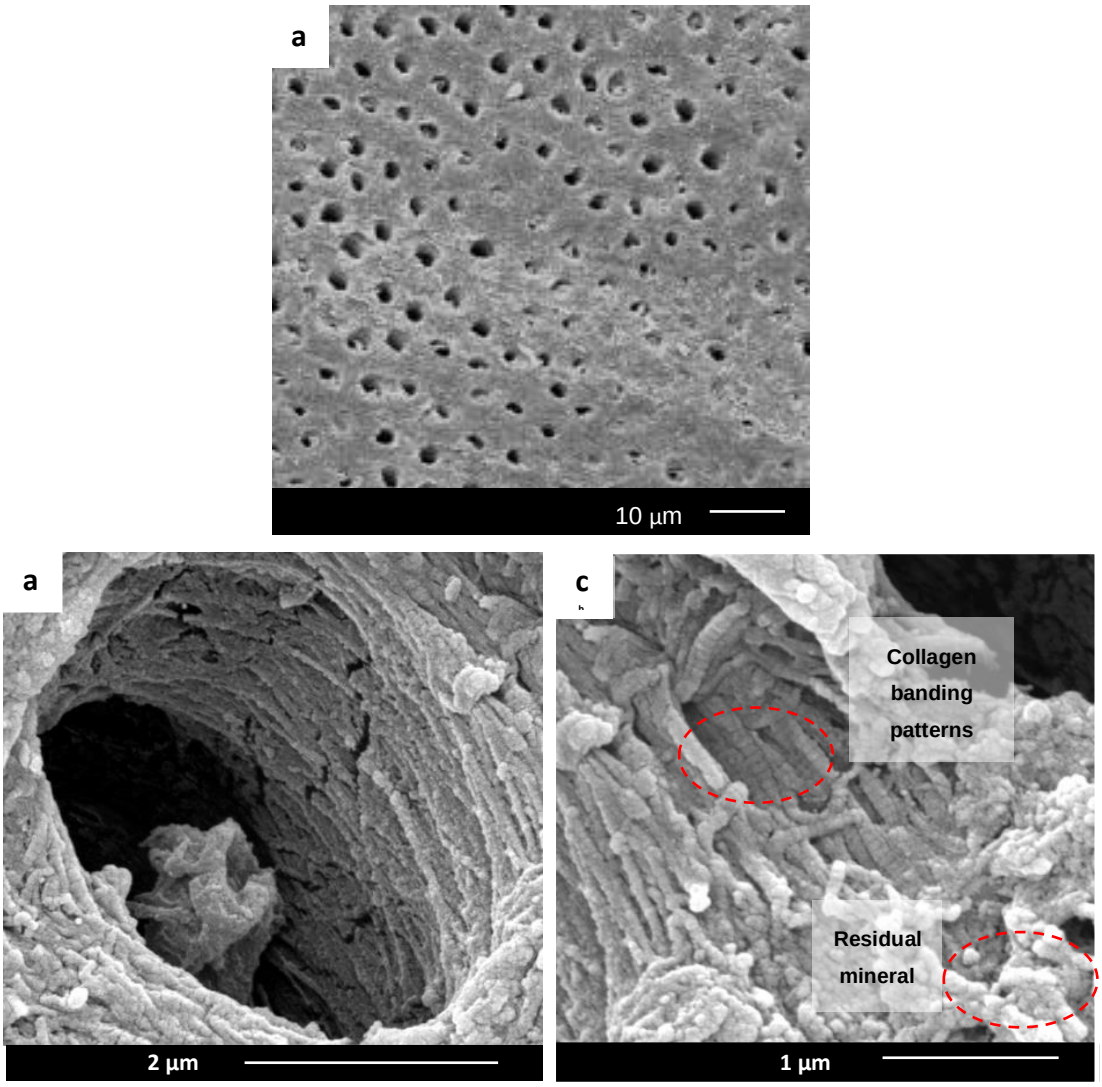

Figure 5.23: SEM micrographs of a dentine specimen after demineralisation with lactic acid pH 2 for 7 days. Images taken at (a) 4000x; (b) 60000x; (c) 80000x magnifications.

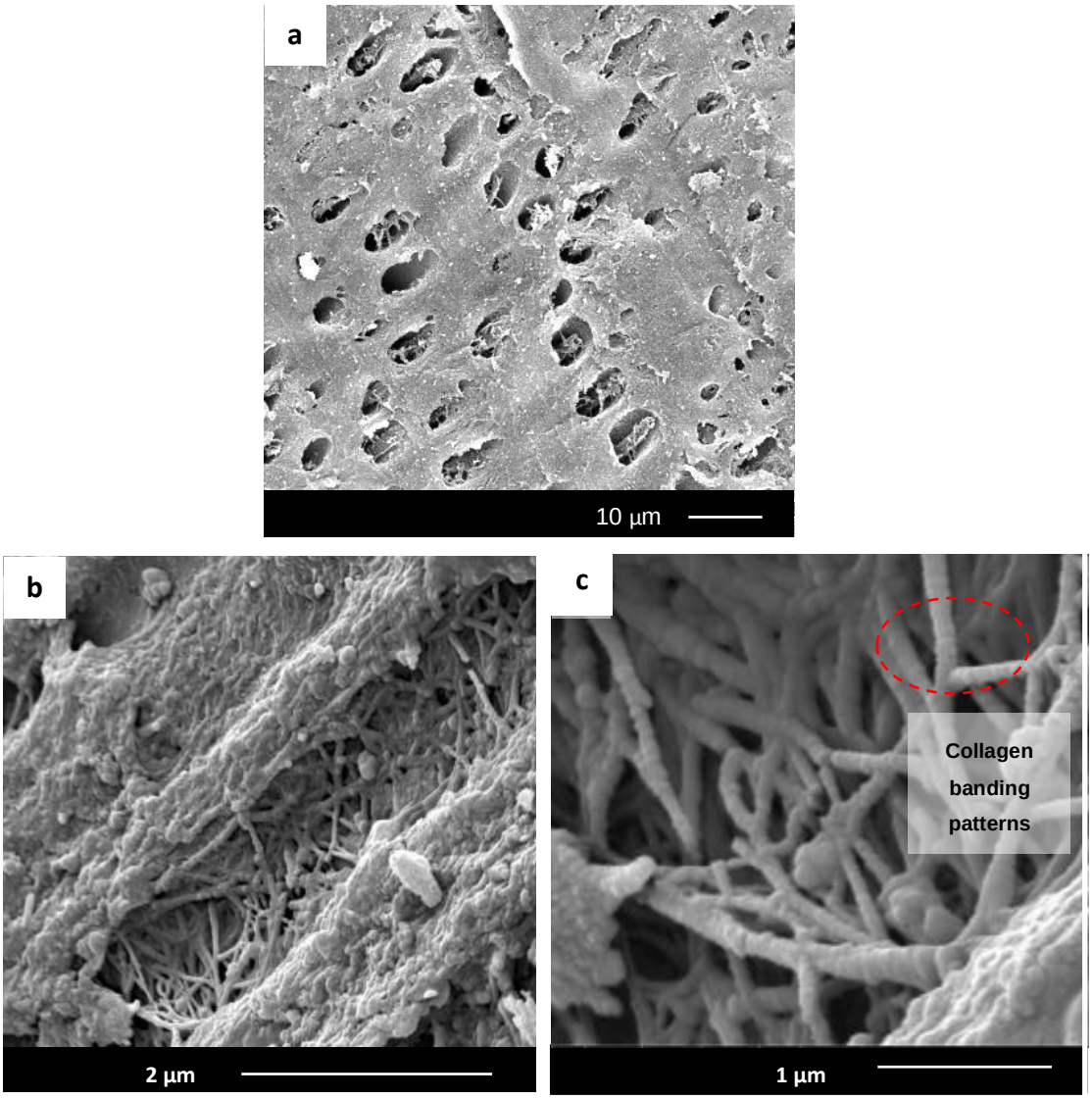

Figure 5.24: SEM micrographs of a dentine specimen after demineralisation with hydrochloric acid pH 2 for 7 days. Images taken at (a) 4000x; (b) 60000x; (c) 80000x magnifications.

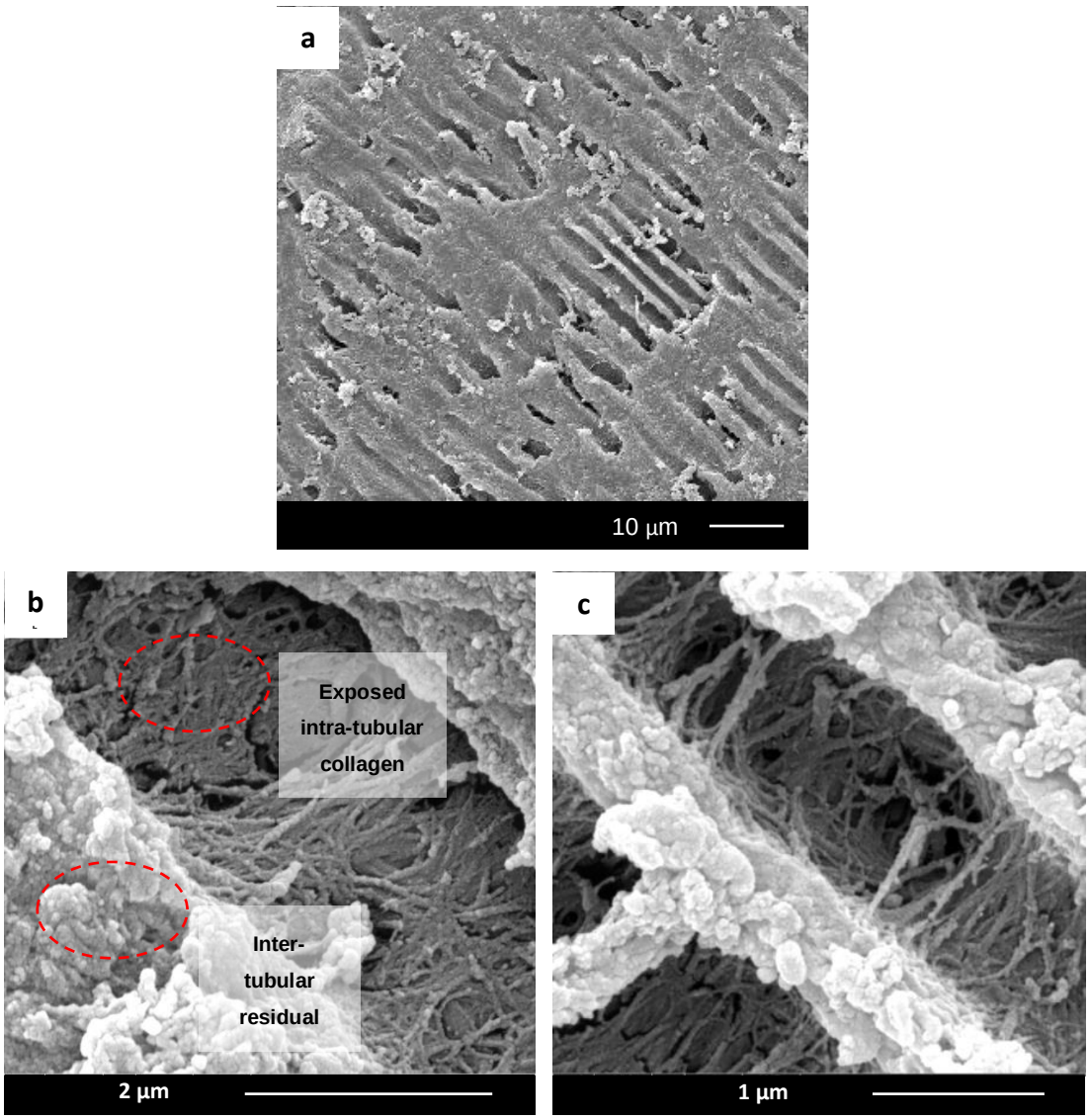

Figure 5.25: SEM micrographs of a dentine specimen after demineralisation with acetic acid pH 2 for 7 days. Images taken at (a) 4000x; (b) 60000x; (c) 80000x magnifications.

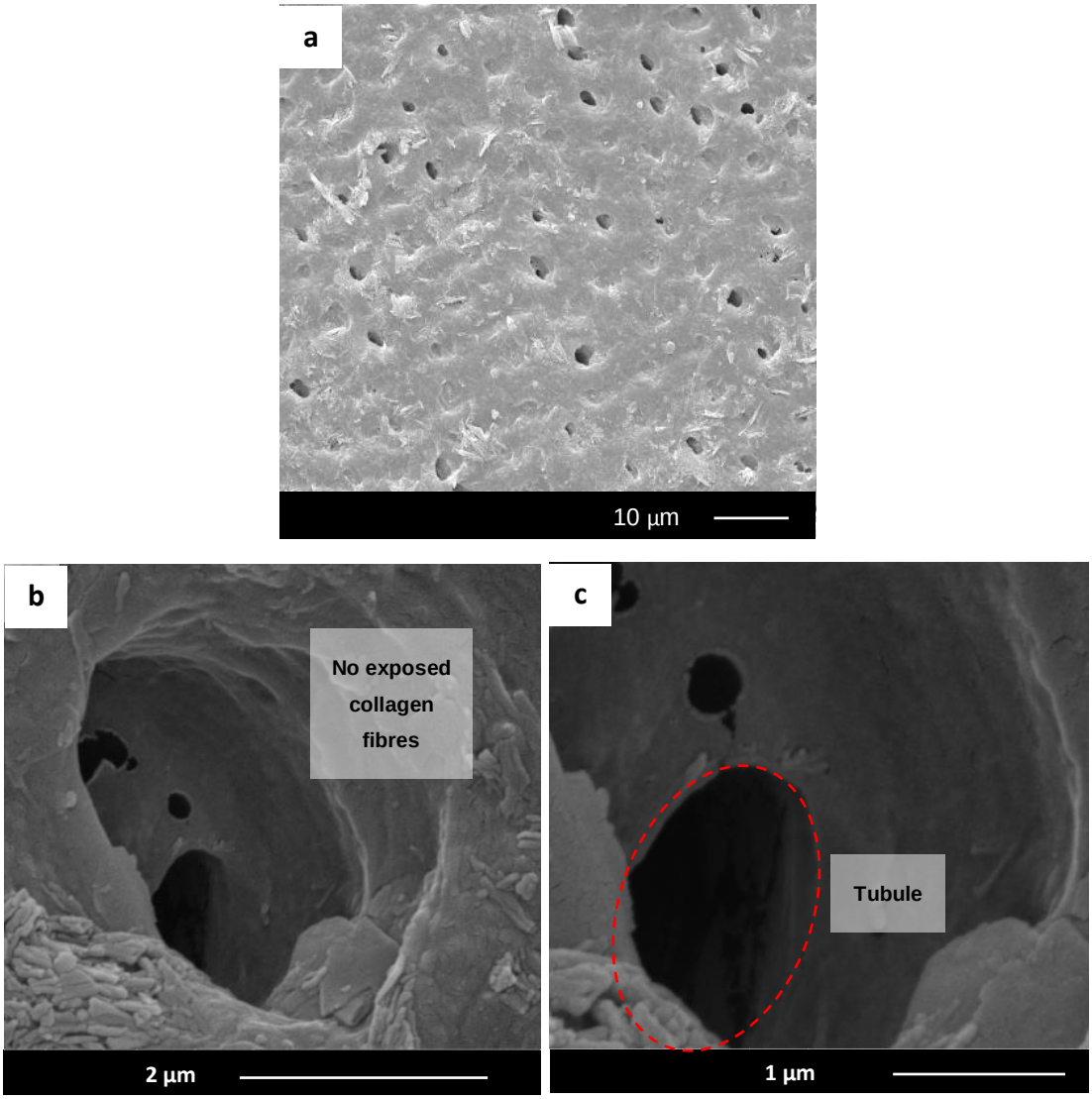

Figure 5.26: SEM micrographs of a dentine specimen after demineralisation with citric acid pH 2 for 7 days. Images taken at (a) 4000x; (b) 60000x; (c) 80000x magnifications.

Table 5.2: Measurements taken from SEM images of dentine at 80000x magnification (n = 15). Measurements could not be taken from the citric acid sample because individual collagen fibres could not be identified. Statistical analysis was performed using one way ANOVA with Tukey's post hoc analysis*.

Specimen type:	**Collagen fibre width (mean average)**	**Standard deviation**	**Significant difference ($p < 0.05$) with:**
Formic acid	54.21	20.40	EDTA, hydrochloric acid
EDTA	102.30	38.21	formic acid, acetic acid, phosphoric acid
Phosphoric acid	68.60	15.20	EDTA, hydrochloric acid
Lactic acid	77.00	18.09	Acetic acid
Hydrochloric acid	104.44	20.98	formic acid, acetic acid and phosphoric acid
Acetic acid	45.70	10.78	EDTA, hydrochloric acid, lactic acid
Citric acid	**N/A**	**N/A**	

** There is significant difference with EDTA results compared with formic acid, acetic acid and phosphoric acid specimens. There is significant difference with hydrochloric acid specimens compared with formic acid, acetic acid and phosphoric acid specimens. There is also significant difference between acetic acid and lactic acid specimens.*

All SEM images were taken of the occlusal surface of the dentine blocks. Although tubules run from the enamel edge to the pulp edge, their sigmoidal shape means that the SEM images often showed tubules at various orientations. The diameter of tubules increases from the enamel edge to the pulp edge. This variation is explained in further detail in Discussion section 5.5.1.1.

5.4.6 Hydroxyproline Results

The results from the hydroxyproline (Hyp) assay are shown in Figure 5.27. The results suggest that all of the acids caused some collagen to be denatured. A one-way ANOVA statistical test with Tukey's post-hoc analysis

was used to demonstrate that there was a significant difference between all the groups except between EDTA treated and acetic acid treated specimens. All demineralised specimens differed significantly from the control group.

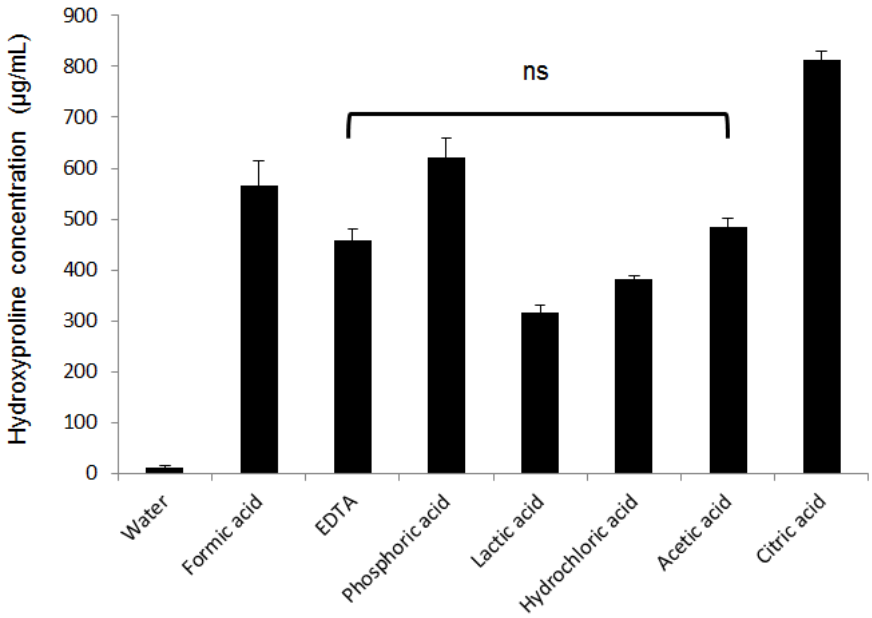

Figure 5.27: Hyp assay showing quantity of denatured collagen from dentine specimens following 7 days in each demineralising agent. Statistical analysis using one way ANOVA with Tukey's post hoc analysis; unless specified, all group combinations showed significant difference ($p < 0.05$). ns = no significance

5.4.7 Haematoxylin and Eosin Histology Results

Figure 5.28 displays EDTA treated tooth sections following staining with haematoxylin and eosin (H&E). The dentine and pulp are stained with eosin (in pink) and the tubular structure is clear. Nuclei are also clearly visible as they became stained with haematoxylin; noticeable as purple spheres, more prevalent at the pulp edges.

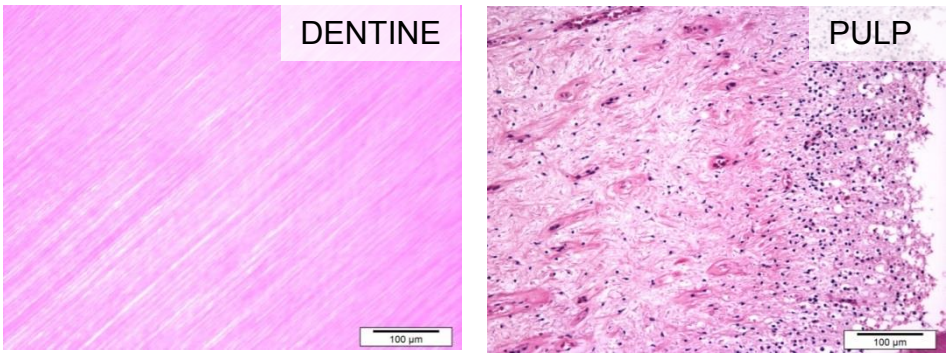

Figure 5.28: Light micrographs of H&E stained sections of EDTA-treated tooth specimens

5.4.8 Van Gieson Histology Results

Figure 5.29 shows EDTA treated tooth sections following the Van Gieson stain. Collagen is displayed in red and is visible throughout the dentine and less densely in the pulp. There are areas of brown/black staining within the pulp which is representative of nuclei/cytoplasmic material.

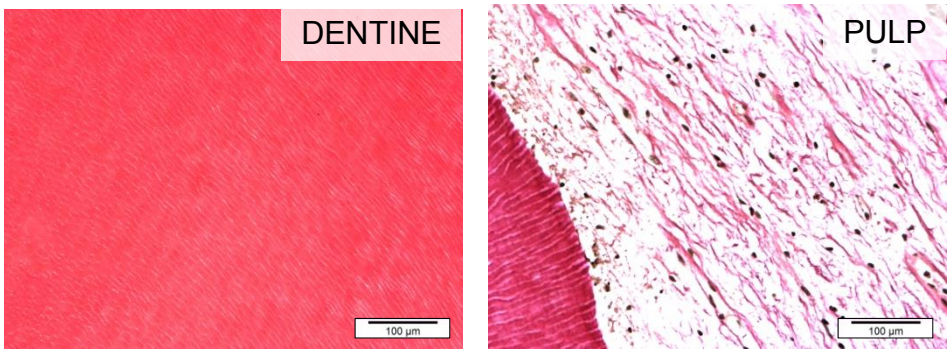

Figure 5.29: Light micrographs of Van Gieson stained sections of EDTA-treated tooth specimens

5.4.9 Dentine Phosphoprotein Immunohistochemistry Results

Figures 5.30 – 5.32 are light micrographs of EDTA treated tooth sections following immunohistochemistry with a dentine phosphoprotein (DPP) antibody at a range of concentrations from 1:50 to 1:1000. The controls were not subjected to specific DPP staining. Three different antigen retrieval protocols were tested: sodium citrate (Figure 5.30), proteinase K (Figure 5.31) and trypsin (Figure 5.32). Regardless of the DPP concentration or type

of antigen retrieval used, there is non-specific staining noticeable in every specimen. There is no obvious specific staining of DPP in any of the specimens.

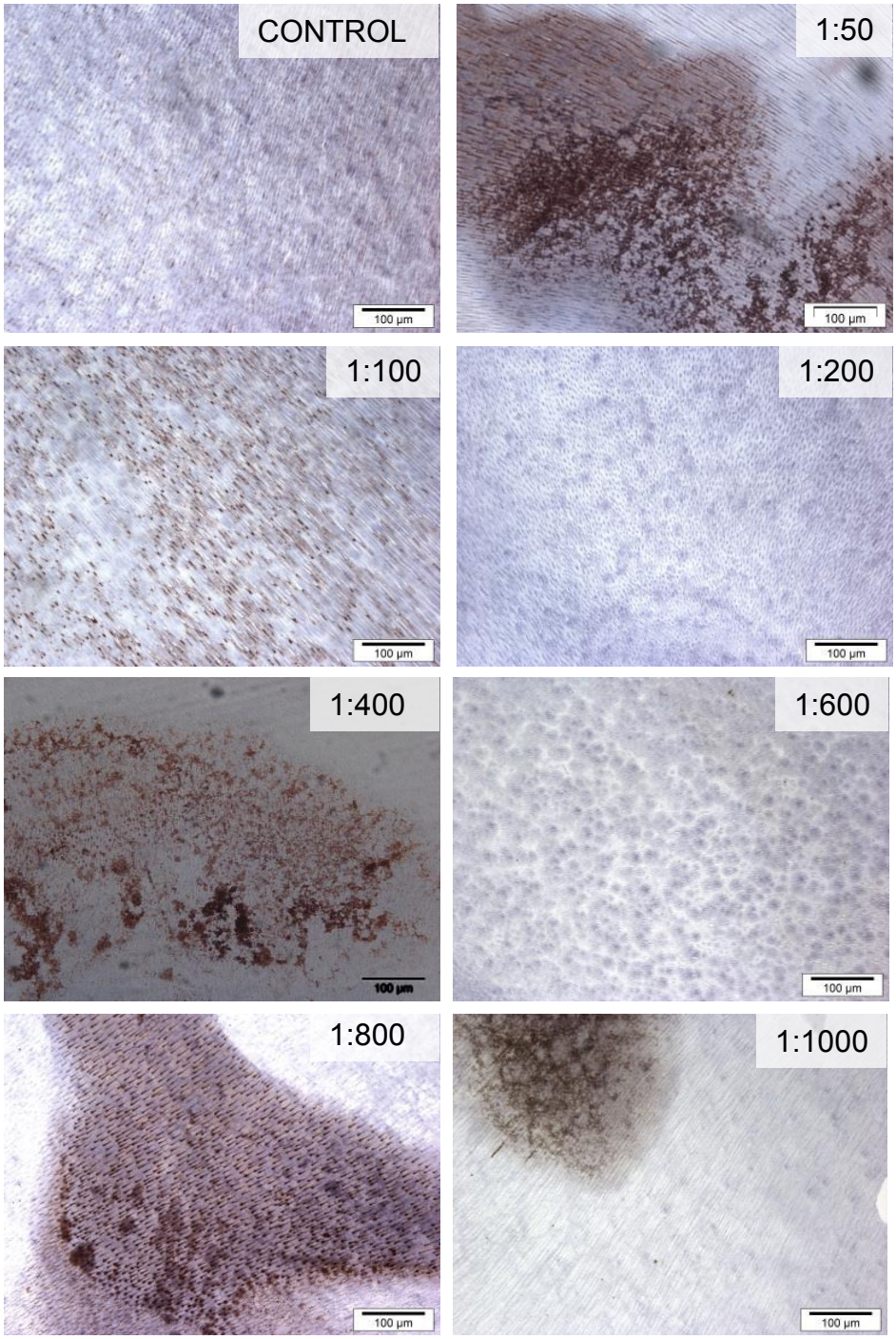

Figure 5.30: Light micrographs of EDTA-treated tooth sections following immunohistochemistry with various concentrations of DPP, except for the control. Antigen retrieval using sodium citrate.

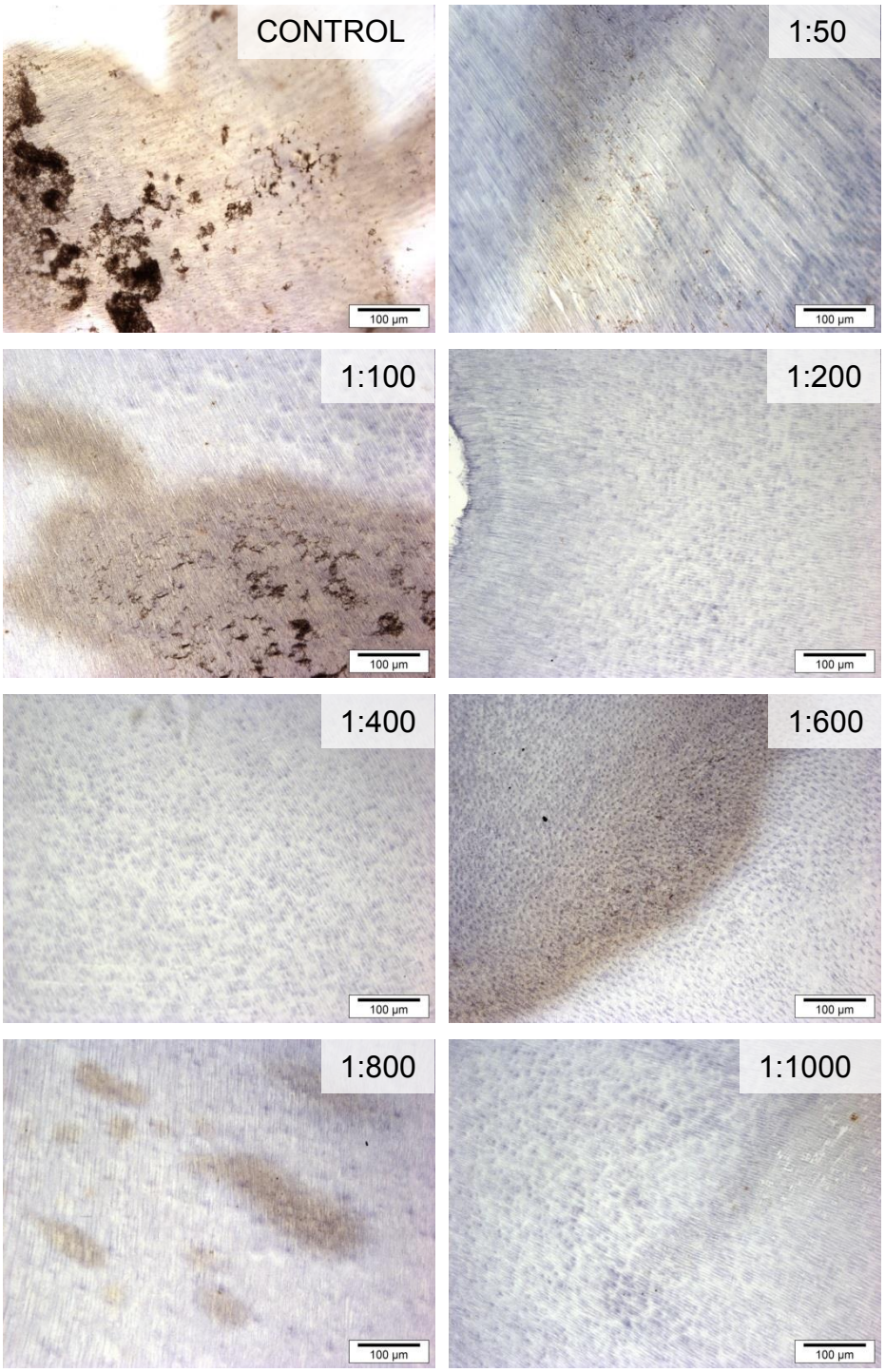

Figure 5.31: Light micrographs of EDTA-treated tooth sections following immunohistochemistry with various concentrations of DPP, except for the control. Antigen retrieval using proteinase K.

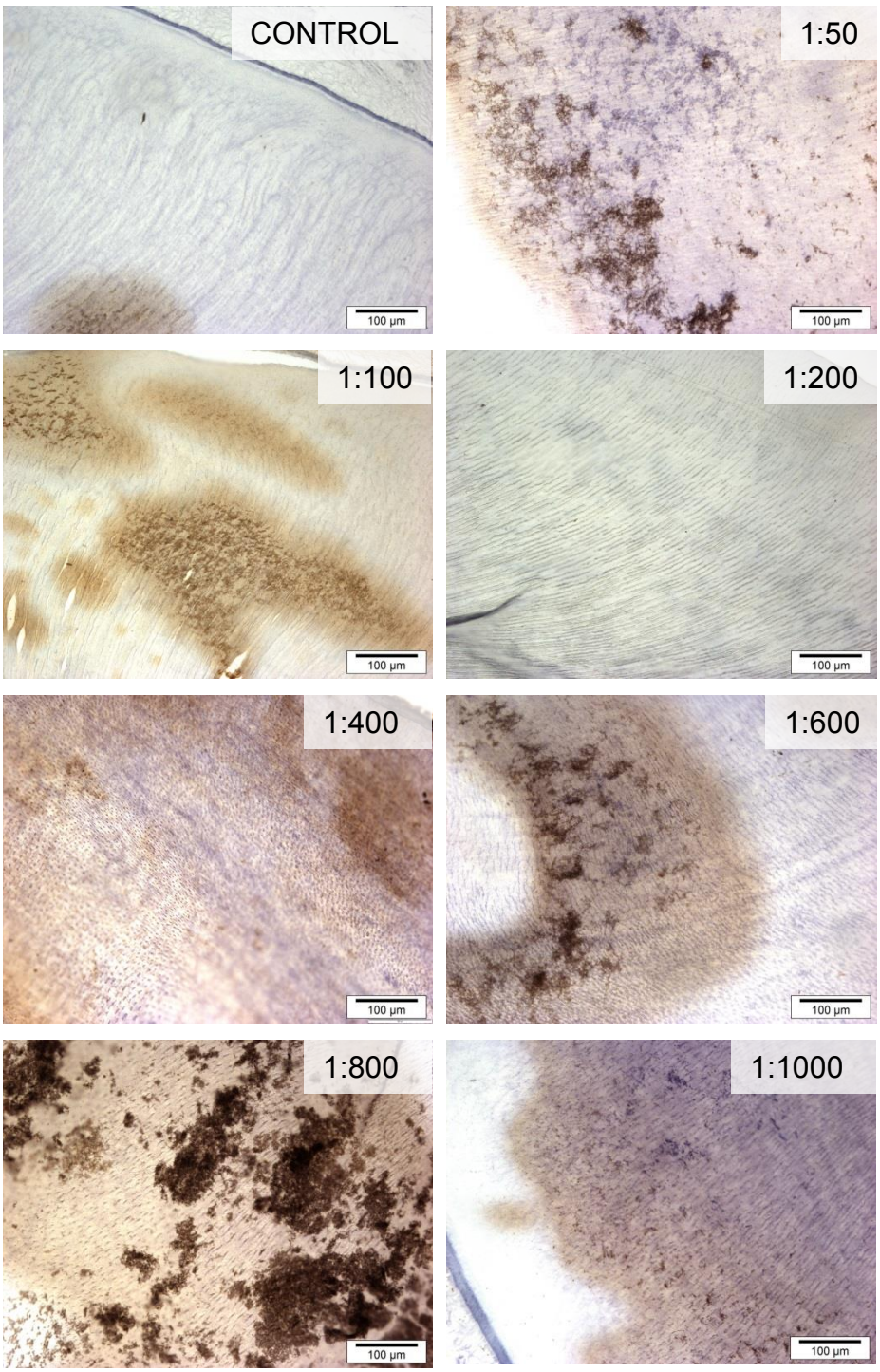

Figure 5.32: Light micrographs of EDTA-treated tooth sections following immunohistochemistry with various concentrations of DPP, except for the control. Antigen retrieval using trypsin.

5.5 Discussion

Bacterial acids (e.g. lactic acid), dietary acids (e.g. citric acid, phosphoric acid and acetic acid) and gastric acids (e.g. hydrochloric acid) all demineralise the tooth surface during caries or acid-erosion. *In vitro* remineralisation studies require artificially demineralised dentine models which are often created using laboratory agents, such as formic acid or EDTA, under the presumption that that these have the same effect on the dentine as the erosive and caries related acids.

The aim of this study was to test this preconception by characterising and comparing the effects of these acids on the organic and inorganic components of dentine. This was a proof of principle study to determine the effects of various acids. As such, the study did not consider other components, including naturally or artificially added buffers, plaque or saliva; this is also in line with laboratory dentine remineralisation models that will normally use acids in a pure form (Besinis *et al*., 2014b, Kim *et al*., 2011, Wang and Spencer, 2002).

5.5.1 Limitations

5.5.1.1 Dentine specimens

Due to the highly heterogeneous structure of dentine, no two dentine specimens are the same regardless of whether they are from the same individual or even the same tooth. Several researchers have shown that dentine is an anisotropic biological material (Marshall *et al*., 1997, Wang and Weiner, 1998) suggesting that the structure and composition of the dentine blocks is likely to differ in mineral content, tubules (number of, diameter, orientation) and collagen network (width of fibres, number of fibres, density). Consequently, when Pashley *et al* were investigating dentine permeability, they found that it differed significantly across dentine blocks of the same thickness and dimensions, indicating inherent differences and concluding that it was impossible to have a range of uniform dentine blocks or more than one block of the same structure (Pashley *et al*., 1987).

To minimise the impact of this variance, all dentine blocks in this project were obtained from permanent premolars to eliminate variance between different types of teeth and between primary and permanent dentition. Also, all teeth were extracted from individuals of an adolescent age (12 to 18 years) to reduce the variance in dentine structure from different age groups. All surface analysis was undertaken on the occlusal surface of the dentine blocks to try and reduce the variability from the orientation of the tubules. Furthermore, all dentine blocks were sectioned from the crown area of the tooth to reduce the variability between crown dentine and root dentine.

It should be noted that this investigation did not consider the differences between coronal dentine near the enamel (with tubules widths approximately 1μm wide) and coronal dentine near the pulp (with tubules widths up to 2.5 μm), and this was due to the limited total number of teeth available for this research project. Researchers have also discussed that tubule orientation differs throughout dentine and the only way to overcome this is to analyse many different points over a large surface area of dentine, covering at least 3 mm, to allow averaging of the regional differences (Pashley *et al*., 1987, Garberoglio and Brannstrom, 1976). The sigmoidal shape of tubules and the varation in the tubule diameters also explains the variation in tubule diameter and shape (oval or circle) shown in Figures 5.19 – 5.26).

During this investigation, the same specimen was never used more than once, therefore, every experiment used new dentine blocks. This was because the sample preparation varied for each experiment, for example, some specimens were carbon coated, some gold coated, some ground into a powder and some embedded into a resin. This produced further variability between experiments. It is clear from looking at the standard deviations in Table 5.2, which shows the average collagen fibre widths of dentine specimens, that all of the dentine blocks, even those from the same experimental group, had a large range of collagen widths (20 nm to 177 nm). However, taking the heterogeneous structure of dentine into consideration, and the fact that different samples were used, this thesis has also shown that, with an appropriate and precise protocol, reliable results with relatively

small standard deviations can still be achieved, as demonstrated with the Hydroxyproline (Hyp) assay results (Figure 5.27).

The variance in dentine specimens (between specimens and within a specimen) is likely to have affected the micro-hardness results considerably. Hardness is considered as the resistance to an indentation; the harder the material, the greater the resistance and therefore, is related to the mechanical properties of a material. Certain factors, in this case the microstructure of dentine, can influence the hardness results. For example, there have been many different values reported for dentine hardness at baseline (Pashley *et al*., 1985) presumably due to the variability of the dentine microstructure. There are also reports that dentine hardness may be relative to tubule density; with dentine hardness decreasing when tubule density increases (Craig and Peyton, 1958, Craig *et al*., 1959, Pashley *et al*., 1985), local composition of the tissue (i.e. mineral content), as well as the indentation parameters (Marshall *et al*., 2001). Although the indentation parameters were constant, the level of variability between dentine specimens and within the surface of one specimen means that no two indents will have occurred on exactly the same surface with the same properties.

The depth of the indents from micro-hardness analysis was up to 200 μm beneath the surface. Another problem with the unpredictable composition and structure of dentine is that it is unknown what is underneath the surface, for example, whether there is a dense network of collagen fibres, any tubules (and their size and orientation) and mineral content. Therefore, the details of the composition, properties and structure of the surface that the indenter reaches, is unknown.

There is also evidence to show that hardness is affected by the hydration state, fixing treatments and storage conditions of the teeth (Pugach *et al*., 2009). To eliminate variation from these aspects, all samples in this study underwent the same processing steps and storage conditions, validating within-study comparisons.

Generally, micro-hardness is associated with mineral content levels (Miyauchi *et al*., 1978) but with specimens as variable as dentine this can

sometimes lead to non-linear relationships between micro hardness and mineral content (Kinney *et al*., 2003, Landis, 1995). Therefore, it would not be appropriate to use micro-hardness results alone to quantify mineral levels. However, micro-hardness can be used as an indicator of general trends in mineral levels, and in this study was used alongside other techniques.

Ultimately, the non-uniform and heterogeneous nature of dentine will have affected all experiments in this thesis. To limit these effects, an effort was made to measure as many areas of the dentine surface as possible, as advised by Pashley *et al* (Pashley *et al*., 1987).

5.5.1.2 Fixing of the Specimens

The use of a fixative is essential before analysis with an SEM or EDS but is not essential before analysis using micro-hardness or the Hyp assay, for example. To remain consistent and to reduce the already high levels of variance between specimens, it was decided that all specimens would be chemically fixed.

All of the dentine specimens were fixed prior to the demineralisation stage, as was the case with a 2002 study looking at the effects of a fluoride varnish (Schmit *et al*., 2002). Previous experimental work in the research group showed that it was essential to fix the dentine blocks prior to demineralisation in order to retain the ultrastructure enough to allow analysis using techniques such as SEM (Besinis, 2010). Hence during analysis of the results consideration had to be given to the fact that the dentine specimens were fixed prior to demineralisation and therefore the results did not replicate the *in vivo* process. It is possible, but not tested in this work; that the results would have been different if the fixative had been applied after demineralisation rather than prior to demineralisation. As discussed in section 6.1.2.2, glutaraldehyde is a highly effective fixative and it is possible that it could help to resist the effect of the demineralising agents by cross linking the collagen and preventing degradation.

5.5.1.3. Dentine surface damage

Dentine blocks were cut from extracted molars using a diamond wafering blade, as described in section 4.2. Cutting dentine blocks creates a smear layer denatured cutting debris, first reported by Eick *et al* (Eick *et al*., 1970). Although the dentine blocks in this study were treated to ultrasonic cleaning in an attempt to reduce any cutting debris, a smear layer could still be observed on Figure 5.19 (a), an SEM micrograph of non-demineralised dentine where the tubules were concealed (by the smear layer). The depth of the smear layer shown on Figure 5.19 (a) is unknown, but it is exected to be deeper than the average smear layer (1 – 2 μm) produced from traditional dental burs. The presence of a smear layer can make it difficult to analyse the true surface of the specimen. The exact components of the smear layer are unknown but it is speculated to be a combination of both organic and inorganic matter (Goldman *et al*., 1981). Studies have shown that as little as 5 seconds immersion in an acid may remove the smear layer from dentine (Pashley *et al*., 1981) and so it can be assumed that the smear layer was probably removed during demineralisation, as suggested by the SEM analysis where all demineralised dentine specimens had exposed tubules. The implication of a smear layer on the non-demineralised dentine may mean that the results from the mineral content analysis may have been effected.

Furthermore, the smear layer may act as a protective layer on the dentine and consequently, it may have limited the degree of demineralisation. Cameron (1983) and Mader *et al* (1984) were the first to discuss that the smear layer may also involve debris being forced up to 40 μm into the tubules (Mader *et al*., 1984, Cameron, 1983, Moodnik *et al*., 1976). The depth of this packing is probably dependant on the type of cutting instrument used and the studies discussed have looked at the effects of hand drills and burs.

5.5.1.4 Specimen polishing

The inability to have a homogenous and perfectly polished dentine surface affects the ability to obtain accurate results for micro-hardness and EDS analysis. EDS is commonly used for assessment of calcium and

phosphorous in dentine (Itota *et al*., 2006, Arnold *et al*., 2001, Besinis *et al*., 2012, Earl *et al*., 2011, Petrou *et al*., 2009, Besinis *et al*., 2014b) but the ideal EDS sample preparation involves polishing the samples so that surface roughness does not affect the results (a rough surface can cause the direction of the emitted x-rays to deviate, leading to variability in results).

Dentine blocks will never have a perfectly smooth and homogenous surface meaning that results cannot be considered quantitative. However, micro-hardness and EDS results can still be used qualitatively and in conjunction with other techniques, to indicate trends in mineral levels. It is difficult to estimate the interaction volume due to the wide variety of constituents and the large variability in local density.

5.5.1.5 Specimen grinding

X-ray fluorescence (XRF) and x-ray diffraction (XRD) also first required grinding the dentine specimens into a powder. Both of these techniques are most accurate when the powder is homogenous with an even particle size. Due to the heterogeneous nature of dentine and the difficulty in grinding demineralised dentine (composed of organic matter) into a fine powder, meant that it was unlikely that all particles sizes were the same. To overcome these differences, more repeats and in the case of XRF, statistical analysis, would have been necessary in order to monitor the impact of these inhomogeneities.

5.5.1.6 Specimen dehydration

For hardness analysis, specimens were not dehydrated and were only removed from the cacodylate buffer during the indentation process. For EDS and SEM analysis, dehydration is essential (a) for the carbon/gold coating and (b) for the vacuum conditions of the SEM/EDS instrument.

SEM analysis is a highly regarded technique for the analysis of dentine collagen that has been used a copious amount of times in previous studies (Arnold *et al*., 2001, Banerjee *et al*., 2000a, Breschi *et al*., 2002, Earl *et al*., 2011, Gjorgievska and Nicholson, 2010, Vanmeerbeek *et al*., 1993).

Previous studies have shown that demineralised dentine may shrink up to 66 % in volume when dehydrated in air (Carvalho *et al*., 1996) therefore this study made an effort to reduce the effects of dehydration by using a gentle dehydration protocol that avoided air-drying (see section 4.1 for details). It could be assumed that any collapse in the collagen network was mainly due to the effect of the demineralising agent and not the dehydration protocol.

5.5.2 The Effects on Mineral Content

Analysing the mineral content of the various demineralised dentine models led to the following findings: (1) each demineralising agent appeared to affect the mineral content differently; (2) the results did not verify which demineralising mechanism was most effective and (3) XRD results suggested that hydroxyapatite was the only calcium phosphate phase present in dentine during demineralisation; all of which are described in more detail below.

(1) Each demineralising agent appeared to affect the mineral content differently

Micro-hardness, XRF, EDS and a phosphate assay were all used to measure the mineral levels in dentine before and after demineralisation. All of experiments showed a reduction in mineral content in all of the specimens resulting in partially demineralised dentine.

The micro-hardness results in this thesis appeared to replicate findings from other publications. For example, this study indicated that all specimens had a baseline hardness between 46 and 52 VHN (average 49 VHN), which was compared to previous studies suggesting sound dentine has a hardness value of 50 VHN (Gutiérrez-Salazar and Reyes-Gasga, 2003).

The main drawback of micro-hardness testing was the need to optically measure the indent size (refer to 4.7.1 for details). This required the test point to be mirror flat to view the indents clearly in order to make an accurate measurement. As dentine becomes demineralised it becomes softer and the measurement of the indents can become difficult because the edges of the indent become less clear. There was one specimen that had been treated

with lactic acid for 14 days which had two indents that were impossible to measure. Therefore, the 14 day data for this variable had a lower n number and, for this reason, it was decided to analyse the data from day 7 (Figure 5.5) and to perform EDS and XRF analysis at day 7 as well.

Each analysis demonstrated that all of the demineralising agents reduced the mineral content, but some were more effective than others. In particular, all techniques suggested that lactic acid caused the most demineralisation. This complies with previous research undertaken by Moore *et al* (1956) where they showed that lactic acid, produced by oral bacteria, is the most powerful demineralising agent, causing the greatest loss of mineral (Moore *et al*., 1956). However, in 1981 it was suggested that oral bacteria can also produce some quantities of acetic acid and it was speculated to be just as damaging to the tooth surface (Featherstone and Rodgers, 1981). This has since never been verified. The results from this current study did not verify whether acetic acid was as damaging to mineral content as lactic acid. Although EDS data (Figures 5.6 and 5.7) did not show any significant difference in relative calcium or phosphorous levels between specimens treated with the two acids, micro-hardness results (Figure 5.5) showed significant difference comparing acetic acid and lactic acid, with lactic acid being one of the most effective demineralising agents and acetic acid appeared to be one of the least effective demineralising agents.

The micro-hardness results (Figure 5.4 and Figure 5.5) also suggest that citric acid was the least effective demineralising agent because it appears to have caused the least reduction in hardness. The citric acid treated specimens were significantly different compared with all other specimens except for the acetic acid and hydrochloric acid treated specimens. Once more, according to the EDS results (Figure 5.6) citric acid appears to have been the least effective at reducing relative calcium levels; all specimens had significantly less calcium compared to the citric acid treated specimen which only shows no significant difference compared with the control specimen.

Table 5.1 displays that citric acid is a weak acid (pKa 3.13) with a chelating ability (LogKCa 1.10) which would suggest that it would be effective at

removing calcium from the dentine, therefore, it was surprising that the relative calcium levels had not dropped significantly (EDS data, Figure 5.6). All the other acids resulted in a range of values that were all significantly lower than the control and citric acid treated specimens. However, there was no significant difference between each of them. The EDS data showing the relative phosphorous levels (Figure 5.7) also showed no significant difference between the citric acid specimens and the control specimens, suggesting that citric acid was not very effective at removing neither the calcium nor the phosphorous.

The phosphate assay (Figure 5.10) showed that lactic acid, hydrochloric acid, citric acid and EDTA all caused phosphate to be released from the dentine specimens and they all showed a significant difference compared to the control and to each other, showing that they all caused demineralisation but not at the same rate. The phosphate assay was the only analysis that did not show that citric acid was the least effective, instead, EDTA appeared to have the least effect on phosphate release, which would be as expected due to its chelating ability (LogKCa 10.70) and lack of ability to dissociate into hydrogen ions.

There was no statistical analysis for XRF data but visual assessment of the bar chart (Figure 5.8) showed similar findings to the EDS data, where the acids did not have the same effect as each other and that citric acid appeared to be the least effective. More XRF data would be required to enable statistical analysis and conclusions (see section 5.6; Future Work).

(2) The results do not verify which demineralising mechanism is the most effective

There are two mechanisms that cause demineralisation, one is the dissociation of hydrogen ions and the other is the chelation of calcium ions. Part of this investigation was to determine whether one mechanism was more effective than the other.

Hydrochloric acid was the only strong acid investigated. As illustrated in Table 5.1, because hydrochloric acid is a strong acid it has an extremely low

pKa value of -9.30 and fully dissociates into hydrogen ions when in solution. Regarding all of the demineralising agents assessed in this study, hydrochloric acid had the strongest ability to cause demineralisation by this dissociated hydrogen ion mechanism. Furthermore, as also illustrated in Table 5.1, hydrochloric acid does not have a logKCa value because it does not have the ability to chelate calcium ions. It can only cause demineralisation through its ability to dissociate into hydrogen ions.

On the other hand, EDTA was the only demineralising agent that was investigated which can only cause demineralisation by the chelation mechanism. As illustrated on Table 5.1, EDTA has a logKCa value of 10.70 and does not have a pKa value because it does not dissociate into hydrogen ions. Regarding all of the demineralising agents used in this study, EDTA had the strongest ability to cause demineralisation via the chelation mechanism because it had the highest logKCa value. As shown on Table 5.1, all of the other demineralising agents had the ability to cause demineralisation by both mechanisms.

Due to the way that the different mechanisms work, it could be assumed that a chelating agent (EDTA) would cause the biggest reduction in calcium ions and that a strong acid (hydrochloric acid) would cause the biggest reduction in phosphorous. However, according to the data analysed from micro-hardness and EDS (Figures 5.5 – 5.7), there was no significant difference in calcium or phosphorous content between specimens treated with EDTA and specimens treated with hydrochloric acid. It was only the phosphate assay, displayed on Figure 5.10 that showed hydrochloric acid causing more phosphate to be released from dentine and EDTA causing the least. On the whole, from the experiments presented in this thesis, the most effective type of demineralising agent (whether it be a chelating agent or strong acid) could not be verified.

Based on the dissociation of hydrogen ions mechanism, it would be expected that hydrochloric acid (pKa -9.30) would be the most effective followed by phosphoric acid (pKa 2.15) and citric acid (pKa 3.13) but this trend was not observed in any of the experiments. Based on the chelating mechanism, it

would be expected that EDTA (LogKCa 10.70) would be the most effective, followed by lactic acid (LogKCa 3.86) and then phosphoric acid (LogKCa 1.40). The only time this trend may be implied was in the EDS calcium results (Figure 5.6) where lactic acid and EDTA showed the biggest reduction in calcium. However, this cannot be statistically verified as they were not significantly different to any other variables except citric acid (and the control).

There were five demineralising agents that were all able to cause demineralisation using both mechanisms (see Table 5.1) but there was no evidence from this thesis to suggest that this allowed more demineralisation to occur. If both mechanisms are equally effective, then in theory, phosphoric acid and lactic acid should be the most effective demineralising agents because they have the strongest ability to use both mechanisms effectively, but again, this was not verified during the experiments.

(3) XRD results suggest that hydroxyapatite was the only calcium phosphate phase present in dentine during demineralisation

XRD is frequently used to characterise calcium phosphate substances, in particular hydroxyapatite, and is considered a reliable analysis tool if the sample preparation is adequate (Karlinsey *et al*., 2010a, Liu *et al*., 2002, Panda *et al*., 2003).

Although all of the specimens matched the hydroxyapatite standard, the specimens treated with citric acid appeared to match the peaks the most accurately (Figure 5.12). This suggests that after demineralisation with citric acid, there was sufficient hydroxyapatite left in the specimens resulting in peak intensities similar to that of the control sample. As with the EDS results (Figures 5.6 and 5.7) where there was no significant difference between citric acid specimens and control specimens. Other specimens, such as formic acid shown in Figure 5.14 had a much lower intensity presumably because there was less mineral content in the specimen.

As dentine specimens became more demineralised, they were mainly composed of organic matter, and furthermore, they became more difficult to

grind into a powder which was essential for acquisition of accurate XRD spectra. Some spectra demonstrated a very amorphous nature (acetic acid, lactic acid and EDTA, Figures 5.15 – 5.17) which is indicative of lower mineral content.

5.5.3 The Effects on Collagen Integrity

Characterising the collagen integrity of the various demineralised dentine models led to the following findings: (1) each demineralising agent appeared to affect the collagen structure and there were signs of collagen denaturation; (2) there was no obvious correlation between demineralising mechanism and effect on collagen integrity and; (3) citric acid appeared to have had a unique effect on collagen integrity; all of which are described in more detail below.

(1) Each demineralising agent affected the collagen structure and there were signs of collagen denaturation

As described in the Literature Review section 2.4.3.1, it has been suggested that collagen may be degraded by unspecific bacterial proteases during caries (Kleter *et al*., 1998, Tjaderhane *et al*., 1998). However, it is not clear whether collagen is denatured in the absence of bacteria, e.g. in erosion or artificially demineralised models. Some studies have suggested that acids have an indirect denaturing ability because they can activate matrix metalloproteinases (MMPs) which can lead to collagen degradation. In addition, Zhang *et al* have suggested that a low pH solution may denature proteins (Zhang *et al*., 1998) but there is little evidence as to whether this applies to collagen, especially because helical collagen has such high rigidity and strength (Ramachan.G and Sasisekharan, 1961). Presumably, the reason why there has been little evidence of collagen denaturation in demineralised dentine models is because collagen is very difficult to characterise (refer to section 2.4.3.1).

Hyp is a non-proteinogenic amino acid found in collagen and the Hyp assay is a standard experiment in biochemical studies (Bornstein and Sage, 1980). It has recently been used to quantify collagen degradation in studies exploring the use of chlorhexidine as an MMP-inhibitor (Carrilho *et al*., 2009). As explained in section 4.7.7, the demineralised dentine specimens were digested with trypsin, which only digests denatured collagen. The undigested (native) collagen was removed and the quantity of Hyp in the remaining denatured collagen samples was analysed. Hyp is limited to collagen and

therefore, the quantity of Hyp can equate to the quantity of denatured collagen.

The Hyp assay results (Figure 5.27) suggest that collagen became denatured in demineralised dentine, without the presence of bacteria. All demineralised specimens were significantly different from the control specimen. This is a novel addition to the field although the exact mechanism of the denaturation is still unclear. It may have been due to the activation of MMPs or possibly due to low pH levels directly effecting the collagen fibres (Zhang *et al*., 1998) by breaking the ultrastructure of the collagen fibrils. Previous investigations have suggested that chelating agents may inhibit the degradation of collagen because they sequester calcium and zinc ions which prevent the activation of MMPs (Tezvergil-Mutluay *et al*., 2010, Hannas *et al*., 2007). However, results from this study have shown that chelating agents also seemed to cause collagen to become denatured and there appeared to be no clear relationship between the type of demineralising mechanism and the extent of damage to the collagen.

SEM analysis showed that collagen fibre banding patterns were present on specimens after treatment with lactic acid or hydrochloric acid. Collagen banding patterns are formed from the assembly of collagen on a macromolecular level, so it can be indicative of native, un-denatured dentine and it is possible that denatured collagen would lose its banding patterns due to breakage of the bonds within the banding pattern. The fact that banding patterns were present on specimens, but the Hyp assay showed signs of denaturation, suggests that the dentine specimens contained a combination of denatured and non-denatured collagen. Another theory is that collagen denaturation may occur within smaller bonds (not within the banding patterns) and that collagen may be denatured whilst maintaining its cross links and banding patterns. This could have been facilitated by the use of glutaraldehyde which stabilises the cross links.

(2) There was no obvious correlation between demineralising mechanism and effect on collagen integrity

Although all of the demineralising agents had a different effect on the collagen, they all caused some degree of denaturation. There appeared to be no correlation between the mechanism of demineralisation and the integrity of the collagen. For example, there was no evidence to suggest that weak acids, strong acids or chelating agents were more destructive to the collagen than others.

The Hyp assay showed that there was no significant difference comparing EDTA and acetic acid, which are both strong chelating agents, but there were no obvious trends in the Hyp assay to suggest either strong acids or chelating agents caused the most denatured collagen. Previous studies have implied that EDTA preserves biological structure but the Hyp assay did not show that EDTA retained the native collagen more than the other demineralising agents (Vanmeerbeek *et al*., 1993, Tay *et al*., 1996).

In addition, SEM analysis did not show any relationship between demineralising mechanism and collagen integrity. Only dentine demineralised with lactic acid or hydrochloric acid showed fibres with banding patterns. However, these two acids did not cause demineralisation in the same way, as displayed on Table 5.1; the properties of the acids differ, lactic acid being a weak acid with chelating ability. The collagen fibre widths, displayed in Table 5.2, also did not show any relationship between the fibre sizes and type of demineralising agent.

(3) Citric acid appears to have had a unique effect on collagen integrity

The behaviour of citric acid during this study was particularly interesting due to its contrasting effects on mineral content and collagen integrity. The Hyp assay showed high levels of denaturation after citric acid and the SEM images (Figure 5.26) showed that the dentine collagen network had completely altered in structure; it was the only demineralising agent that resulted in dentine where individual collagen fibres were not identifiable. One theory is that the citric acid did not remove mineral effectively from the

collagen surface, making it difficult to identify the collagen network. However, the SEM images of citric acid specimens appeared different from the control specimens, as the surface appeared smoother and did not resemble a mineral layer. An alternative explanation is that citric acid caused demineralisation, exposed the collagen network to be vulnerable to denaturation, and then precipitated the mineral back onto the dentine surface, creating a surface that was a combination of denatured collagen and re-precipitated mineral. This may further explain why citric acid resulted in a dentine surface with a high hardness value and preserved high levels of calcium and phosphorous; but there is no scientific evidence or reports to confirm this theory.

5.5.4 Immunohistochemical Localisation of Dentine Phosphoprotein

There is a body of evidence that suggests dentine phosphoprotein (DPP) is involved in initiating and modulating dentine hydroxyapatite formation (Linde and Lussi, 1989, Lussi and Linde, 1993, Linde, 1989, Zhang *et al*., 2001, Feng *et al*., 2002). The aim of this experiment was to discover whether DPP is present in dentine following demineralisation.

Van Gieson and haematoxylin and eosin (H&E) staining were performed on tooth sections for general histological assessment and to verify that the specimen preparation required for immunohistochemistry (protocol in section 4.5) had not altered the tooth structure. The stains confirmed that native collagen with a tubular structure was maintained in the dentine and nuclei and other cellular material were present in the pulp (see Figure 5.28 and 5.29).

However, immunohistochemical localisation of DPP on the tooth sections was ineffective (Figures 5.30 – 5.32). The major challenge with this experiment was to develop a successful protocol because immunohistochemistry with a DPP antibody has never been reported before, so there is no standard protocol available. The initial aim was to examine tooth sections that had been demineralised with a variety of demineralising agents. However, whilst the protocol was being developed it was decided to

only use EDTA as the demineralising agent because it is reported to be the gentlest agent for immunohistochemistry experiments (Hillemann and Lee, 1953).

The first challenge was to maintain the tooth sections on the slides throughout the experiment. Initially, general microscopic glass slides (VWR International, UK) were utilised but the tooth sections did not remain on the slides following the various washing stages of the protocol (Figure 5.33a). However, when APES-coated glass slides were used, which are manufactured to help prevent tissue loss, the sections adhered to the slides better, as demonstrated below in Figure 5.33b. Furthermore, if less agitation was used during the PBS washing stages, then the tooth sections were able to better remain on the APES-coated slides (Figure 5.33c).

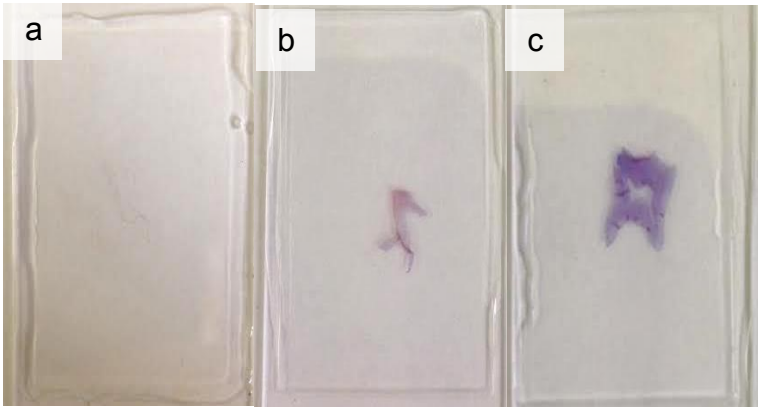

Figure 5.33: Slides mounted with EDTA-treated tooth sections after immunohistochemistry with DPP: (a) on a glass slide, (b) on an APES-coated slide, (c) on an APES-coated slide with decreased PBS washes

The next challenge was to develop a protocol for immunohistochemistry with DPP. It was decided to use a protocol similar to that used for immunohistochemistry with a dentine sialoprotein (DSP) antibody (Yuan *et al*., 2009, Alvares *et al*., 2006, Zhu *et al*., 2012). This involved the use of heat-induced sodium citrate for antigen retrieval, an essential step in immunohistochemistry protocols because it reverses the cross-links caused by the fixation step, which allows the desired protein (DPP) to become exposed (Shi *et al*., 1991). Dilutions of DPP from 1:50 to 1:1000 were explored to find its optimum concentration.

No specific staining was witnessed on any of the specimens (Figure 5.30), regardless of the concentration of DPP used. There are many other antigen retrieval methods reported in literature, such as proteinase K and trypsin solutions that work by cleaving the proteins which may be masking the desired epitope (Shi *et al*., 2001). However, the use of proteinase K and trypsin as antigen retrieval methods was also unsuccessful (Figure 5.31 and 5.32).

As there appeared to be little difference between the 1:50 and 1:1000 DPP solutions, it can be assumed that the staining problem is not due to DPP concentrations. Furthermore, it is unlikely that background staining is due to non-specific binding of secondary antibodies to the tissue, because the tissue was treated with normal serum from the same species as the secondary antibodies. It has been reported in literature that the PBS washing steps in immunohistochemical procedures is essential to remove non-specific staining (Buchwalow and Böcker, 2010). Therefore, it is possible that the reduction in PBS washing, in order to maintain the specimens onto the slides, could have hindered the staining process. Furthermore, other antigen retrieval protocols with different incubation times may help to increase specific staining. In addition, some studies suggest that using tris-buffer saline (TBS) instead of PBS, is particularly successful when locating phosphoproteins (Burns *et al*., 2009).

5.5.5. Summary

A variety of partially demineralised dentine models were created using bacterial, dietary, gastric or laboratory acids. Numerous characterisation techniques were employed to compare the dentine mineral levels and collagen integrity of the various demineralised models, to determine whether the different demineralising agents affected the dentine differently. These results have suggested that there is no clear link between the type of demineralising agent and the effect on the organic and inorganic dentine. However, the results did show that it is important to characterise the dentine model, in terms of its inorganic and organic nature, before considering

remineralising strategies. The composition and structure (e.g. collagen integrity) of the demineralised dentine cannot be assumed.

The results from this study also showed that there is more to be learnt about the different techniques used to characterise dentine. As discussed in section 5.5.1**,** the non-uniform, heterogeneous structure of dentine makes it very difficult to analyse, especially as some techniques such as micro-hardness and EDS rely on a flat, polished, uniform surface to gain accurate results. Therefore, it may be more appropriate to focus on biochemical analysis (e.g. phosphate assay and Hyp assay), which do not rely on such specialied specimen preparation.

5.6 Future work

- Further characterisation techniques such as micro computed tomography (micro-CT), environmental SEM (ESEM) and atomic force microscopy (AFM) could be used to further characterise demineralised dentine. Micro-CT would allow the assessment of trabecular separation and bone percentage whilst also providing visual representations of the full specimens. AFM and ESEM would enable the dentine collagen network to be observed in a hydrated state which would prevent the collagen from collapsing and tensile strength testing would demonstrate the mechanical properties of the demineralised dentine. The possibility of measuring the mineral levels in the acid solutions, rather than the dentine specimens, should also be investigated as this would overcome the problem with dentine specimen preparation outlined in section 5.5.1. Furthermore, additional XRF repeats should be considered so that statistical analysis can be obtained.
- One of the main limitations of this study was the variability of the dentine specimens (see section 5.5.1.1). In order to limit this variability, more dentine specimens should be obtained in order to increase the number of repeats. This would also enable XRF analysis to be repeated to ensure that statistical analysis can be performed. Increase of sample number would also allow regional differences

within dentine to be examined. For example, to compare the analysis of dentine near the enamel with dentine near the pulp. Furthermore, the number of tubules in an area of the specimen surface could be quantified, to examine the difference between specimens and whether this affects the results. Obtaining more dentine specimens would also allow different sized dentine specimens, with large surface areas, to be examined, to ensure that more repeats within each sample can be achieved.

- This study used a Hyp assay to quantify denatured dentine collagen following treatment with a demineralising agent. The quantity of denatured collagen could also be measured following treatment with a chemical fixative or MMP inhibitors to explore whether these treatments help to preserve the collagen integrity.
- The study has so far only investigated demineralising agents at pH 2 (or pH 7 for EDTA) but this is not representative of the conditions in the oral cavity. The demineralising process could be more replicable for the natural caries/erosion processes by: a) combining many demineralising acids b) the addition of buffers or c) cycling with artificial saliva. Furthermore, the results indicate that pH is likely to influence the properties of the dentine specimens and therefore, a range of pH values could be explored. In particular, focus should be on citric acid because this had unique effects on mineral and collagen and is frequently found in many soft drink beverages that are associated with dietary dental erosion.
- The protocol for immunohistochemical localisation of DPP needs further exploration. TBS should be investigated instead of PBS to explore whether this improves the staining, and other antigen retrieval methods should be explored as well. A method for increasing buffer washes whilst maintaining the specimens on the slides should be investigated. In addition, other antibodies such as the commercially available DSP antibody should be tested to verify the protocol. Furthermore, once a successful protocol has been established, other

demineralising agents should be explored and the presence of DPP compared between different demineralised dentine models.

6. Interaction between Nanoparticles and Collagen

6.1 Introduction

As discussed in the Literature Review section 2.5.3, there are many successful remineralising strategies for demineralised dentine that involve deposition of apatite material onto the dentine surface whilst obstructing the exposed tubules. Much of this research focuses on the delivery of calcium to the demineralised dentine (Meyer-Lueckel *et al*., 2015, Vyavhare *et al*., 2015, Rezvani *et al*., 2015, Agrawal *et al*., 2015), the delivery of calcium in synergy with fluoride (Karlinsey *et al*., 2010b, Mathews *et al*., 2012) or the use of bioactive glasses (Burwell *et al*., 2009, Mitchell *et al*., 2011, Earl *et al*., 2011).

Despite these valuable contributions a challenge still remains to achieve sub-surface remineralisation deep into the dentine matrix, which requires consideration of the complex organic network of dentine. Some recent research, for example the use of polymer-induced liquid-precursors (PILPs) (Gower, 2008, Jee *et al*., 2010a, Thula *et al*., 2011) and self-assembling peptide scaffolds (SAPS) (Kirkham *et al*., 2007) have considered the importance of the collagen fibres during remineralisation but many of these studies are proof of principle investigations and are mainly focused on isolated collagen rather than dentine. Other remineralisation studies that have considered the organic components of dentine are those involving non-collagenous protein (NCP) analogues (Tay and Pashley, 2008, Gu *et al*., 2011) but once more, these studies are in very early stages and the remineralising potential is currently unknown.

In order to successfully achieve sub-surface remineralisation of dentine, there must be a strategy that involves infiltrating into the dentine collagen matrix, between the individual fibres. There has recently been an interest in nanotechnology as part of a dentine remineralising strategy, due to their small size and colloidal properties in solution (Mitchell *et al*., 2011, Curtis *et al*., 2010, Wu *et al*., 2012, Besinis *et al*., 2014b). Of particular interest is the research by Besinis *et al*; they have demonstrated the ability of colloidal silica nanoparticles to infiltrate between the collagen fibres to form a

nanoparticle-collagen scaffold that can then enable remineralisation (Besinis *et al*., 2014b). This strategy involves a three stage process: (1) deep infiltration of nanoparticles into the demineralised collagen matrix, (2) adhesion of the nanoparticles to the collagen fibres and (3) nucleation of mineral around the particles when exposed to a suitable ion-rich solution.

6.1.1 Silica Nanoparticles

Nanoparticles are small enough to infiltrate between collagen fibres and when in a colloidal suspension they enable dispersion deep into the dentine without agglomerating onto the surface. It has been demonstrated previously that successful infiltration is restricted to particle size and that nanoparticles less than 20 nm in diameter infiltrate much better into the dentine network (Besinis *et al*., 2012).

As discussed in section 2.5.3, Besinis *et al* demonstrated that silica nanoparticles could infiltrate within the collagen network but it was unclear whether the nanoparticles were adhering to the collagen fibres or simply became trapped within the matrix. It was speculated that the nanoparticles attached to the collagen fibres because during TEM analysis nanoparticles were mainly found in the inter-tubular space and only a few nanoparticles were residing within the tubules, suggesting that non-adhered particles would have been washed out during sample preparation. It would be desirable if the nanoparticles did adhere to the collagen fibres because this would ensure they do not become dislodged during subsequent immersion in ion-rich remineralising solutions.

This thesis continued the investigation of silica nanoparticles to infiltrate dentine and facilitate the remineralisation of dentine. Silica nanoparticles were chosen because they are available in a stable dispersion and remain in suspension for a long time, plus there is evidence that they can act as a mineral scaffold, as discussed. Furthermore, studies have also shown that silica nanoparticles may prevent bacterial adherence and proliferation (Cousins *et al*., 2007, Gaishun *et al*., 2002). Consequently, this suggests that they would make a suitable scaffold to facilitate dentine remineralisation.

6.1.2 Interaction between Nanoparticles and Collagen

To assess whether the nanoparticles adhere to collagen fibres it is beneficial to explore the relationship between the nanoparticles and pure collagen type I fibres, rather than dentine collagen. This allows specific interactions between collagen surfaces and nanoparticle surfaces to be examined and would also help to reduce the number of variables associated with a demineralised dentine model. Moreover, to further understand the interaction between the nanoparticles and the collagen, it is crucial to consider the implications of the *in vitro* experimental procedures which differ from the *in vivo* equivalent; for example the use of demineralising agents and chemical fixatives.

6.1.2.1 The Effects of Demineralising Agents

Besinis *et al* used 4 N formic acid to produce fully demineralised dentine models for their infiltration study (Besinis *et al*., 2014b, Besinis *et al*., 2012). In Chapter 5 it was demonstrated that formic acid exposes dentine fibres and completely opens up the collagen network making it ideal for nanoparticle infiltration. However, formic acid is not present during caries or erosion. In Chapter 5, it was also demonstrated that each demineralising agent had a different effect on the integrity of the dentine collagen. For example, citric acid damaged the collagen matrix in a way that resulted in no inter-fibrillar spaces and therefore, citric acid-treated dentine may be unlikely to allow infiltration of nanoparticles. Therefore, a variety of demineralising agents should be assessed to investigate how they affect nanoparticle infiltration. Moreover, it is unlikely that dentine would be fully demineralised *in vivo*, so partially-demineralised dentine models should also be considered for their infiltration abilities.

In addition, it has been suggested that the collagen fibre surfaces may be more positively charged following exposure to an acid (Stetlerstevenson and Veis, 1986). If this is correct, this suggests that the demineralising agents may affect the collagen fibre surfaces in a way which could influence the ability of the nanoparticles to adhere to the collagen.

6.1.2.2 The Effects of Chemical Fixation

Scanning electron microscopy (SEM), transmission electron microscopy (TEM) and energy dispersive spectroscopy (EDS) analysis are essential to assess the interaction of the nanoparticles within the collagen matrix, but they require chemically fixing the specimens which does not replicate *in vivo* conditions. Thus a big un-answered question that is fundamental to further *in vitro* experimentation is whether fixation, that is essential for *in vitro* analysis but does not occur *in vivo*, affects nanoparticle adherence. The sample preparation of demineralised dentine for use with these analytical techniques requires the specimens to be dehydrated and immersed in 3 % glutaraldehyde for 24 hours.

Chemical fixation stabilises a specimen by the introduction of covalent crosslinks to retain the ultrastructure. In 1963, glutaraldehyde was first identified as an effective crosslinking reagent for preserving cellular structure (Sabatini *et al*., 1963). Since then, no other fixative has exceeded it in its ability to crosslink and stabilise tissue proteins and therefore, it is the most widely used of all fixatives in electron microscopy studies (Hayat, 1981, Bullock, 1984).

Regarding collagen, the primary targets of glutaraldehyde seem to be lysyl and hydroxylysyl residues (Bowes and Cater, 1968) but the effects of glutaraldehyde on the secondary and tertiary conformations of collagen are uncertain. On a macroscopic scale, some changes can be seen in specimens; for example, collagen fibres exhibit increased mechanical, thermal and chemical stabilities (Gustavson, 1956).

Chapman *et al* used collagen as a test material to explore the effect of fixatives at a chemical level (Chapman *et al*., 1990). They fixed collagen with glutaraldehyde and then subjected the collagen to positive and negative staining and compared these to non-fixed controls. Glutaraldehyde appeared to have a drastic effect on the negative staining pattern of collagen (Grant *et al*., 1967, Bairati *et al*., 1972, Bruns and Gross, 1974). They discovered that:

(1) Glutaraldehyde reacts with lysyl and hydroxylysyl residues, modifying the charge distribution along the collagen molecule by removing the positive charges
(2) Glutaraldehyde induces polymeric crosslinks, unlike most fixatives which induce monomeric crosslinks (Korn *et al*., 1972)

Furthermore, it is thought that when glutaraldehyde attaches to a lysyl residue, it sterically inhibits negative stains from adhering to the arginine residue next to it. This indicates that when dentine is chemically fixed, it loses some of its positive charge which could affect the degree of adherence with negatively charged particles.

6.1.1.3 Surface Charge of the Nanoparticles

It is likely that surface charge affects the nanoparticle-collagen interaction. It has already suggested that both the effects of a demineralising agent and a chemical fixative may alter the surface charge of the collagen fibres.

Commercially available Ludox® silica nanoparticles were used as provided by the manufacturer and previously by Besinis *et al* (Besinis *et al*., 2012, Besinis *et al*., 2014b). They are available with a positive (Al_2O_3 coated) or a negative surface charge which makes them ideal for testing the effects of surface charge on adherence. The anionic silica particles exist as SiO^- and are in a solution with sodium counter ions (Na^+) whereas the cationic silica particles are modified by coating the surface with a layer of aluminium oxide. They exist as SiO_2 particles at the core but their surface is Al_2O_3, they are in a solution with chloride counter ions (Cl^-). The properties of these particles are shown in Table 4.1. Besinis *et al* used both types of silica in their infiltration studies and showed that they had similar results according to TEM and SEM (Besinis *et al*., 2012).

6.2 Aims and Objectives

The aim of this study was to investigate whether nanoparticles adhere to acid-affected collagen fibres and if so, is the adherence affected by: (a) demineralising agents, (b) chemical fixatives and (c) surface charge of the nanoparticles. The specific objectives were:

1) To obtain Ludox® silica nanoparticles and characterise them using TEM and dynamic light scattering (DLS)
2) To design a method to infiltrate collagen type I/III membranes with silica nanoparticles
3) To use SEM and EDS to verify the silica nanoparticles within the collagen membranes
4) To investigate whether the silica nanoparticles adhere to the collagen fibres by trying to dislodge the nanoparticles from the fibres with flushing water
5) To use positively (Al_2O_3 coated) and negatively charged silica nanoparticles to observe whether the surface charge affects adherence of nanoparticles
6) To compare fixed vs. non-fixed collagen membranes to investigate whether chemical fixation affects adherence of nanoparticles
7) To assess whether demineralising the collagen membranes with a variety of demineralising agents (used in chapter 5) affects adherence of nanoparticles
8) To explore a variety of nanoparticle solution concentrations and infiltration times

6.3 Materials and Methods

6.3.1 Silica Nanoparticles

Ludox® silica nanoparticles were obtained from Grace Materials Technologies (Worms, Germany) in a 30 wt. % concentration, with either an electronegative or an electropositive (Al_2O_3 coated) surface charge. The properties of the silica nanoparticle solutions are displayed in Table 4.1. In this study, the solutions were used in a 1 wt. % concentration. TEM and DLS were used to characterise the silica nanoparticles, the protocols are found in sections 4.7.8 and 4.7.9, respectively.

6.3.2 Collagen Membranes

Porcine collagen type I and III membranes (Biogide®, Geistlich Sons Limited, Manchester, UK) were used instead of dentine collagen because they enabled the direct interaction between nanoparticles and collagen to be

assessed; thus avoiding the confounding different patterns and levels of demineralisation found in dentine. The properties of the collagen membranes and the preparation of collagen discs are explained in section 4.3. SEM analysis was used to observe the structure of the collagen membranes (SEM protocol in section 4.7.6).

6.3.3 Infiltration of Nanoparticles through Collagen Membranes

A novel method was designed (outlined in section 4.4.1) to infiltrate the nanoparticles through the collagen membrane discs. Table 4.2 displays the range of experimental groups, each group was further divided into 2 sub-groups (n = 10). Details are outlined in section 4.4.1 and an overview of the methodology is illustrated in Figure 4.6.

(a) Scanning Electron Microscopy

Following the infiltration method, SEM was used to observe the nanoparticles on the collagen membranes (SEM protocol in section 4.7.6). Each variable was analysed in triplicate. To ensure that all areas of the surface were examined, at least 4 areas of the specimens were examined with the SEM, outlined below in Figure 6.1. Four images were taken at each magnification: 300x, 1000x, 4000x, 10000x, 20000x, 40000x and 80000x.

When measuring collagen fibre dimensions, measurements were taken from 5 areas on 3 high magnification (80000x) images. Measurements were taken with ImageJ software Version 1.46.

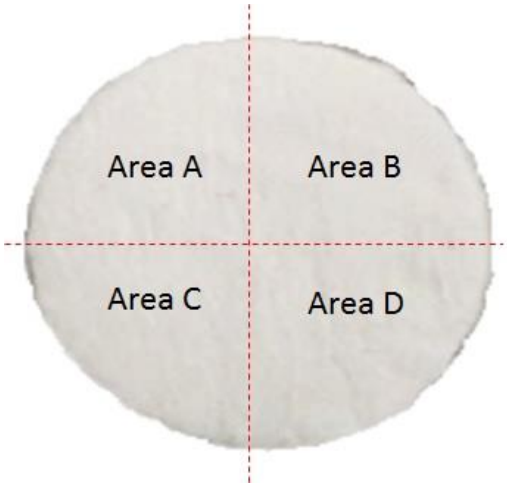

Figure 6.1 A diagram showing the 4 areas of a collagen membrane that are analysed using SEM and EDS

(b) Energy Dispersive Spectroscopy

Following the infiltration method, EDS was used to identify the nanoparticles (silica Kα, 1.740 keV) on the collagen surface, (EDS protocol is detailed in section 4.7.2 (b)). Three collagen membranes were analysed per group and to ensure that all areas of the surface were examined, EDS measurements each covering a 100μm^2 area were taken from each quadrant (A to D), as outlined in Figure 6.1.

6.4 Results

6.4.1 Characterisation of Silica

6.4.1.1 Transmission Electron Microscopy and Dynamic Light Scattering Results

Figure 6.2 displays TEM micrographs of (a) negatively charged silica nanoparticles and (b) positively charged (Al_2O_3 coated) silica nanoparticles. The particles have a diameter between 12 and 18 nm according to TEM and 20 – 25 nm according to dynamic light scattering (DLS).

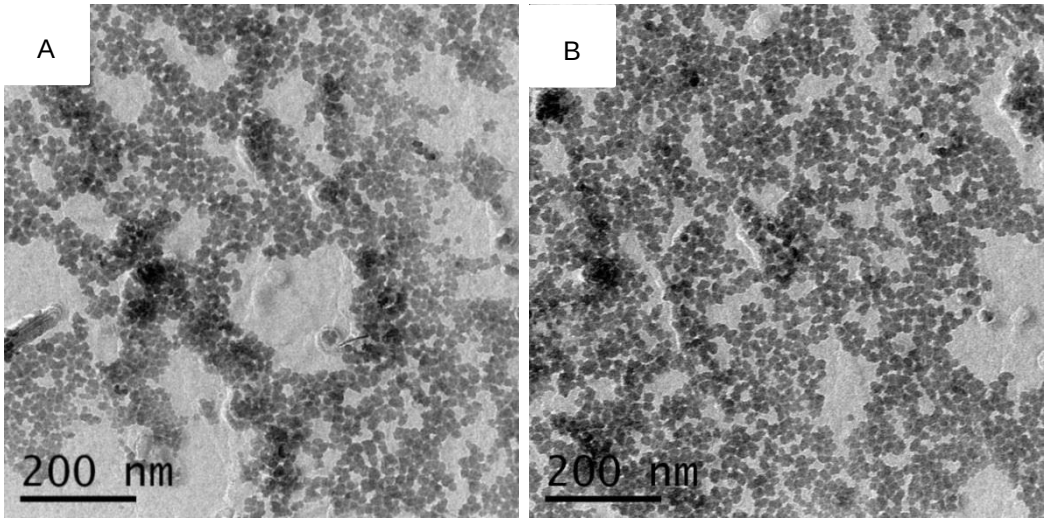

Figure 6.2 TEM images of silica nanoparticles (a) negatively charged and (b) positively charged (Al_2O_3 coated)

6.4.2 Infiltration of Collagen Membranes with Nanoparticles

6.4.2.1 Change in Collagen Mass

Figure 6.3 shows the increase in collagen membrane mass (mg) after infiltration with 1 wt. % silica nanoparticles. The orange bars are representative of specimens infiltrated with positively charged (Al_2O_3 coated) silica nanoparticles, whilst the blue bars are representative of specimens infiltrated with negatively charged silica nanoparticles (uncoated). The patterned bars (diagonal hash) are representative of specimens that were chemically fixed with glutaraldehyde prior to infiltration. The x-axis illustrates the 9 experimental groups i.e. the solutions of which the collagen discs were immersed in prior to infiltration.

The control group, consisting of collagen discs, were only immersed in water and were not infiltrated with silica, demonstrated no increase in mass. There was a significant difference between the control group compared with all of the other variables. This shows that there was always an increase in collagen mass (up to approximately 7 mg) when collagen discs were infiltrated with nanoparticles, regardless of the other factors (surface charge, fixative, acid treatment).

There was no significant difference when comparing fixed specimens with non-fixed specimens. Furthermore, there was no significant difference in collagen mass between the specimens that had been treated with an acid and specimens that had not been treated with an acid.

There was a significant difference ($p < 0.05$) in the increase of collagen mass between specimens that had been infiltrated with positively charged (Al_2O_3 coated) silica nanoparticles compared with those infiltrated with negatively charged silica nanoparticles, in both fixed and non-fixed groups, that were immersed in EDTA. However, there was no significant difference between the surface charge of the silica nanoparticles in any of the other experimental groups.

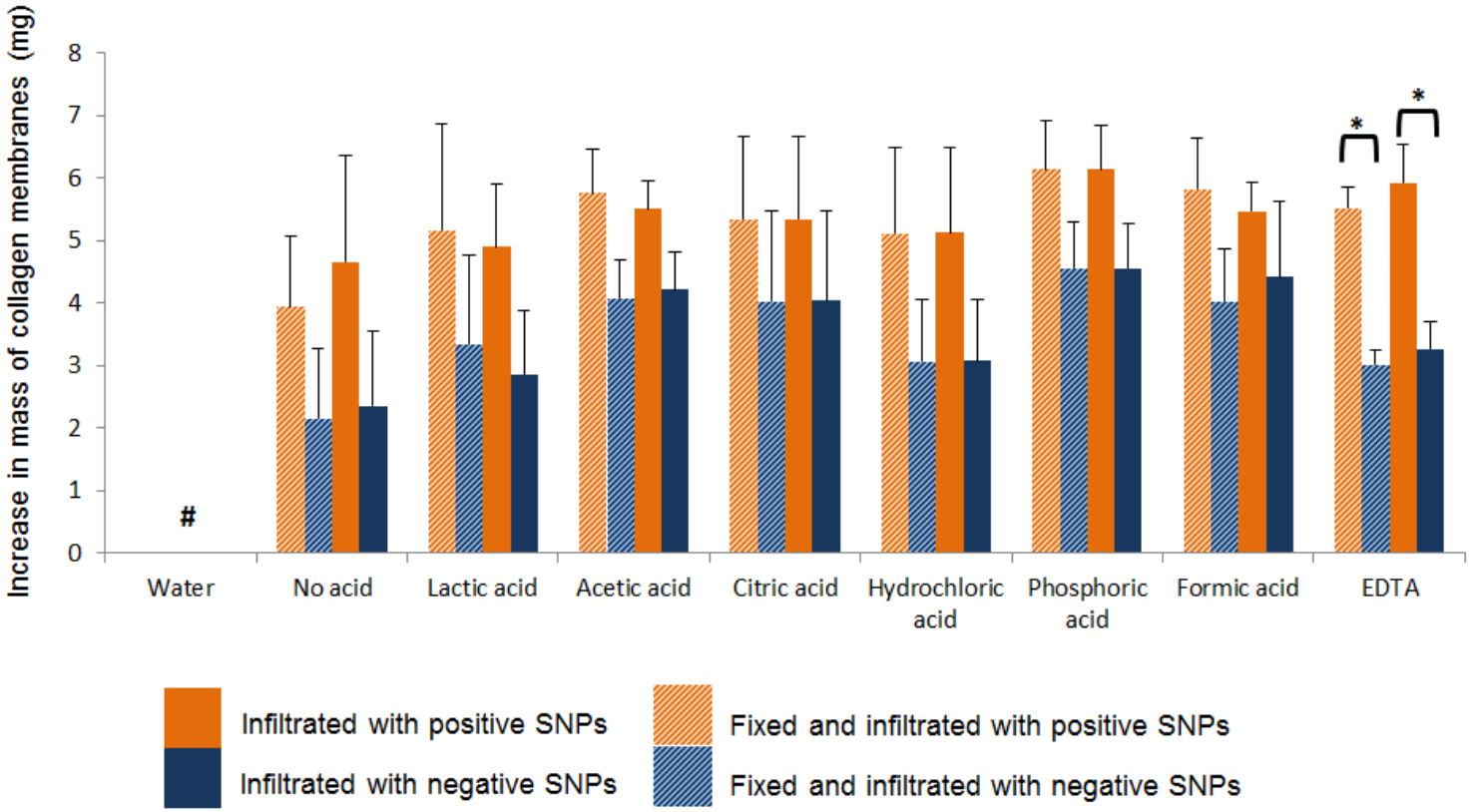

Figure 6.3: Average increase (mg) of collagen mass after infiltration with negatively charged or positively charged (Al_2O_3 coated) silica nanoparticles following following different treatments. Statistical analysis using one-way ANOVA and Tukey's post hoc analysis. There is no significant differences between groups unless indicated using the key below.

= this is significantly difference ($p < 0.05$) compared with all other groups
* = significant difference ($p < 0.05$) between with the variables underneath the bracket

SNP = silica nanoparticles

6.4.2.2 Scanning Electron Microscopy and Energy Dispersive Spectroscopy Results

(a) Fixed and non-fixed collagen membranes prior to infiltration

Figure 6.4 displays scanning electron microscopy (SEM) images of collagen membranes prior to infiltration. Figures 6.4 (a), (b) and (c) are non-fixed membranes and Figures 6.4 (d), (e) and (f) are membranes that were fixed with 3 % glutaraldehyde, taken at various magnifications. All collagen membranes displayed fibres of various dimensions; as displayed in Table 6.1, the mean average fibre width for fixed specimens was 125.60 nm and for non-fixed was 167.90 nm. Statistical analysis using the Kruskal-Wallis H Test with Dunn's 1964 post hoc analysis and Bonferroni adjustment revealed no significant difference between the collagen fibres in fixed specimens compared with non-fixed specimens. The collagen banding patterns of the fibres can be observed on the high magnification images: Figure 6.4 (c) and (f).

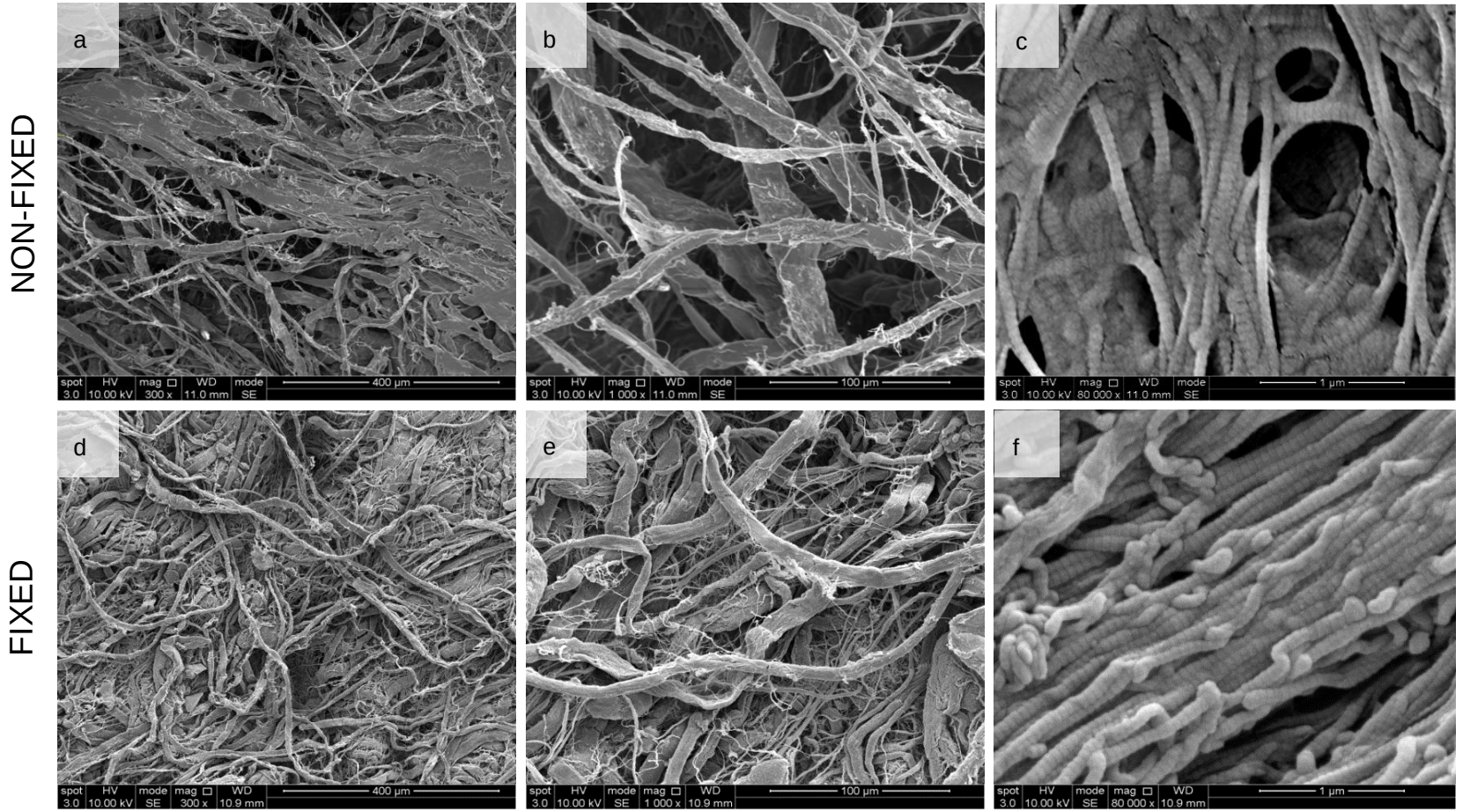

Figure 6.4: SEM micrographs of the Biogide® collagen membranes. Images a, b and c are non-fixed membranes and images d, e and f are chemically fixed collagen membranes. Images taken at 300x, 1000x and 80000x magnifications, respectively.

(b) Collagen membranes after infiltration with nanoparticles

Figure 6.5 displays the fixed and non-fixed collagen membranes following infiltration with either 1 wt. % positively charged (Al_2O_3 coated) silica nanoparticles or 1 wt. % negatively charged silica nanoparticles. At high magnification (80000x), silica nanoparticles could be observed on the collagen fibres. The nanoparticles appear to have been evenly dispersed throughout the membrane, but there were some small agglomerates of the negatively charged silica nanoparticles, as highlighted on Figures 6.5 (b) and (d). From visual assessment of the SEM images there appeared to be no difference between fixed and non-fixed specimens.

Energy dispersive spectroscopy (EDS) analysis was employed to verify that the nanoparticles observed by the SEM were that of silica. Figure 6.6 shows that there was a statistical significance difference of the relative levels of silica found on the collagen surface when comparing the control to the specimens infiltrated with either positively charged (Al_2O_3 coated) or negatively charged silica nanoparticles. There was no significant difference between membranes infiltrated with positively charged (Al_2O_3 coated) nanoparticles compared to membranes infiltrated with negatively charged nanoparticles.

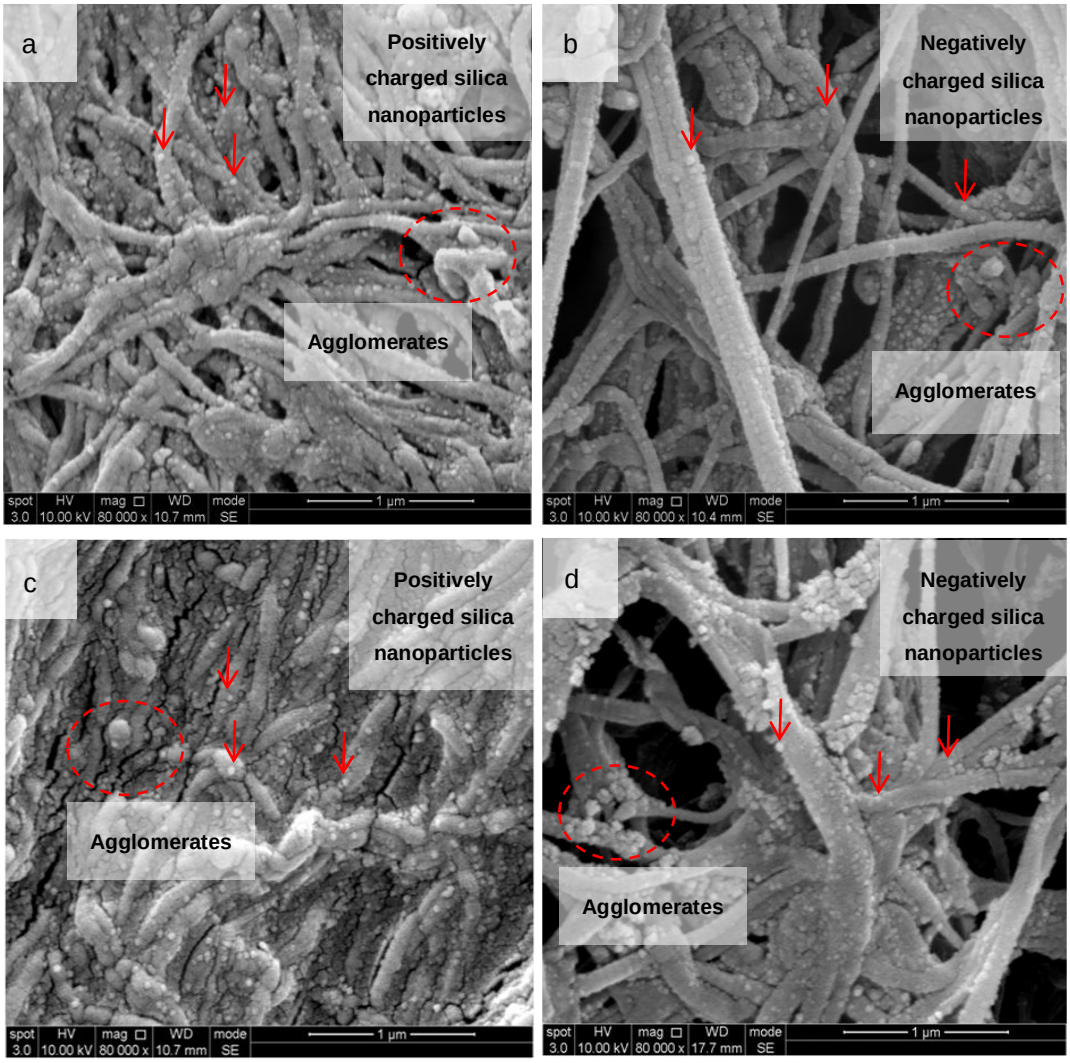

Figure 6.5: SEM micrographs of collagen membranes; (a) infiltrated with positively charged (Al_2O_3 coated) silica nanoparticles, (b) infiltrated with negatively charged silica nanoparticles, (c) fixed with glutaraldehyde and infiltrated with negatively charged silica nanoparticles, (d) fixed with glutaraldehyde and infiltrated with positively charged (Al_2O_3 coated) silica nanoparticles. All magnifications taken at 80000x.

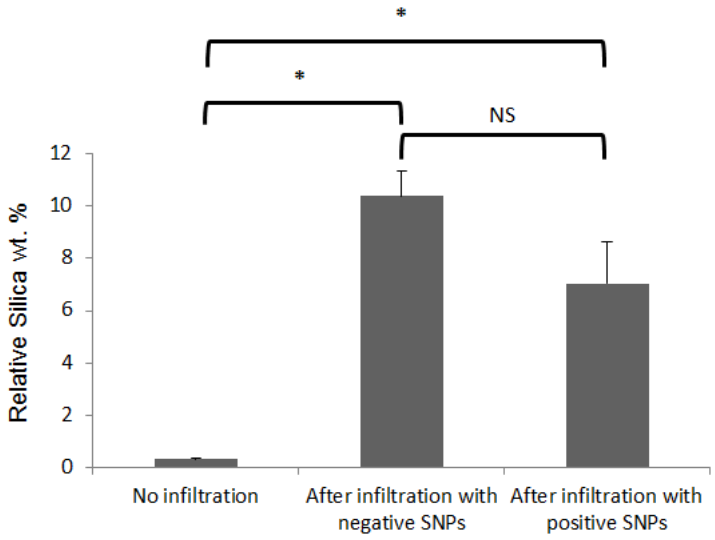

Figure 6.6: Relative silica wt. % (Kα 1.740 keV) using EDS of collagen membranes following: no infiltration, infiltration with negatively charged silica nanoparticles and infiltration with positively charged (Al_2O_3 coated) silica nanoparticles. Statistical analysis using one-way ANOVA and Tukey's test ; NS = no significance, * = P<0.05

(c) Collagen membranes after immersion in demineralising agents

Figure 6.7 and Figure 6.8 display SEM images of collagen membranes after 48 hours in (a) water, (b) lactic acid, (c) acetic acid, (d) citric acid, (e) hydrochloric acid, (f) phosphoric acid, (g) formic acid or (h) EDTA. All specimens displayed a collagen structure with a variety of fibre diameters, the mean average collagen fibre widths are displayed on Table 6.1. A Kruskal-Wallis H Test with Dunn's 1964 post hoc analysis and Bonferroni adjustment revealed that there was a statistical significant difference between the fixed specimens and the acetic acid group, but not between any other group combination.

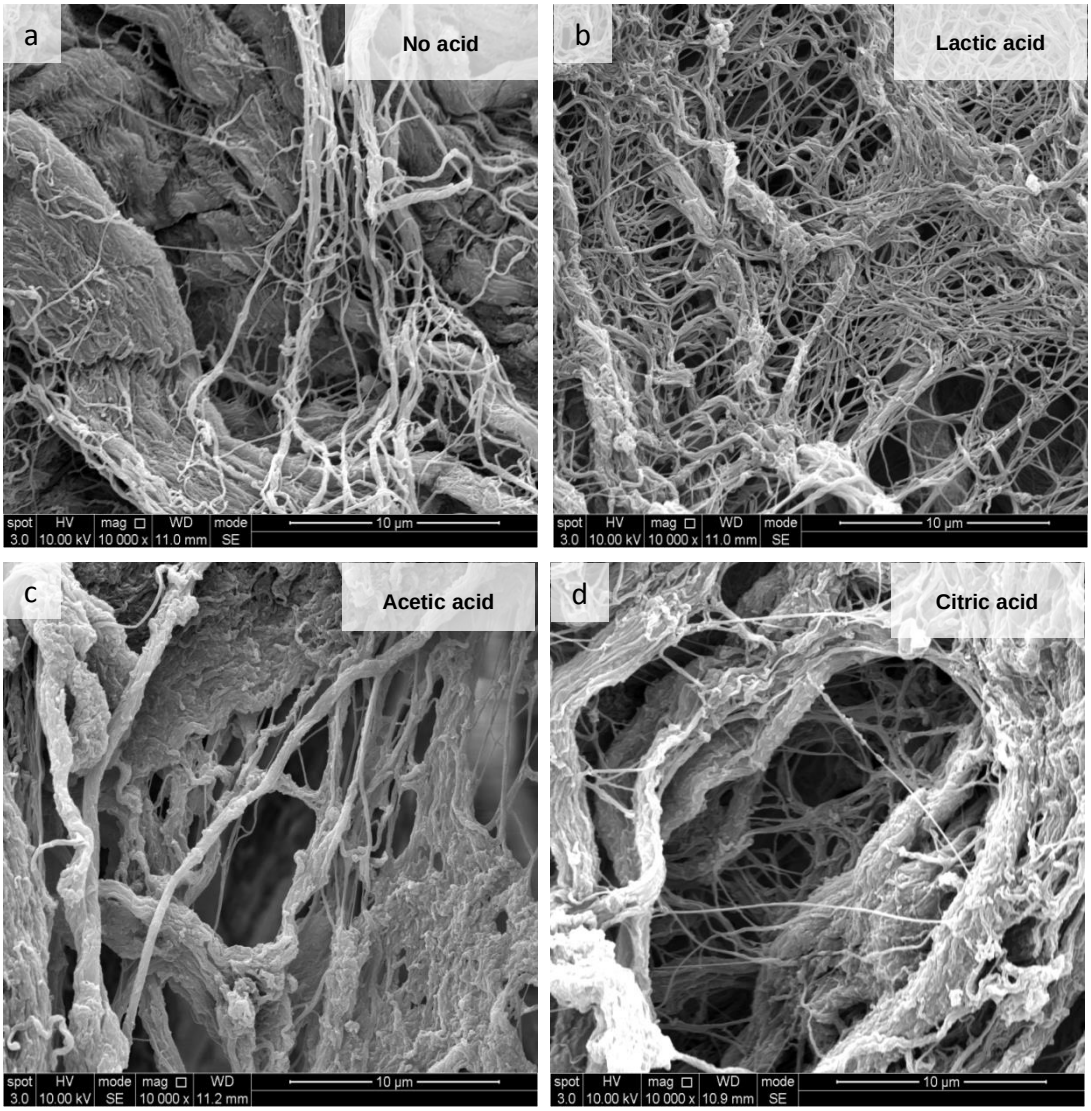

Figure 6.7: SEM micrographs taken at 10,000x magnification of collagen membranes after 48 hours immersed in (a) water, (b) lactic acid , (c) acetic acid, (d) citric acid.

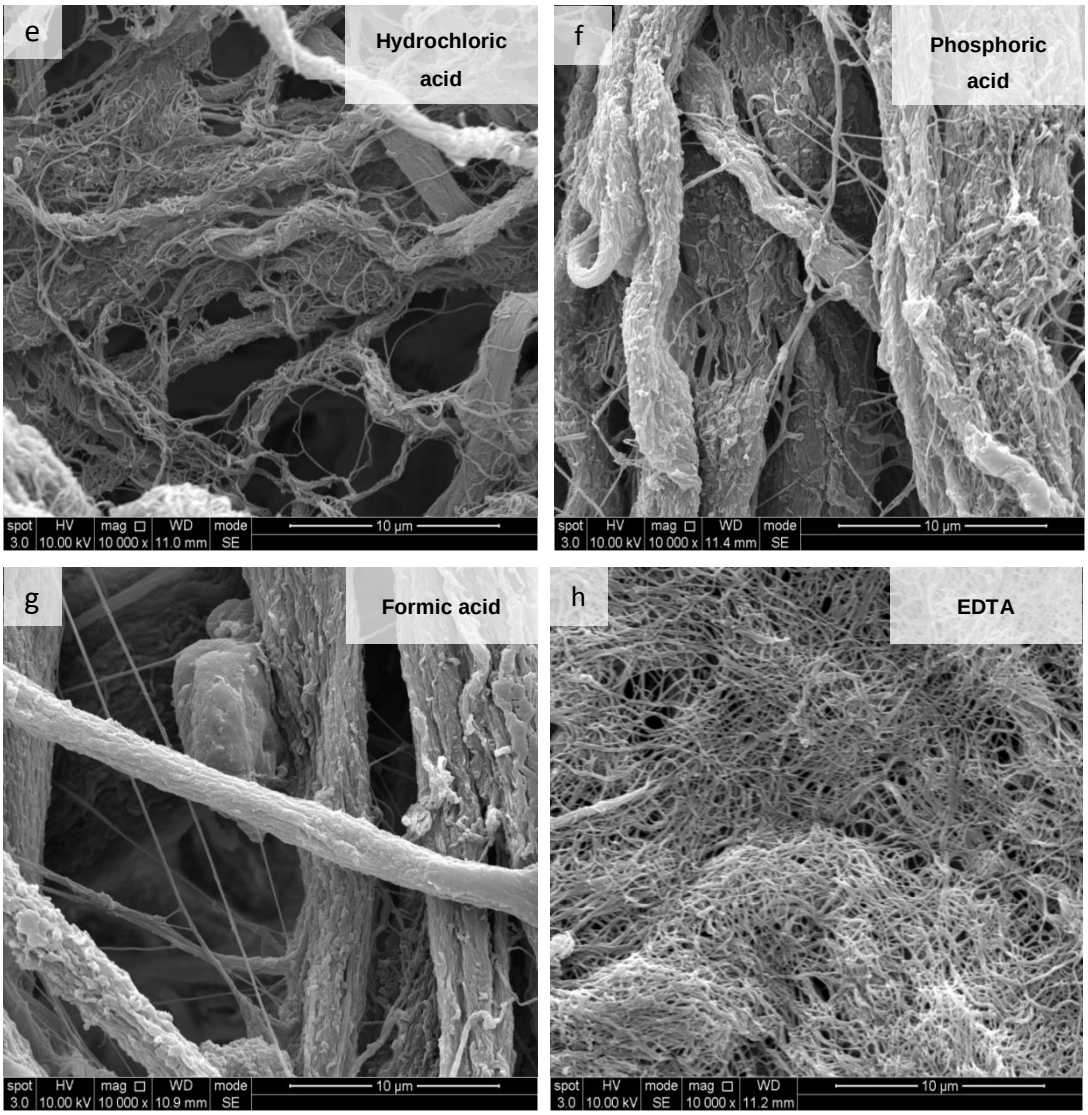

Figure 6.8: Continuing from Figure 6.7; SEM micrographs taken at 10,000x magnification of collagen membranes after 48 hours immersed in (e) hydrochloric acid, (f) phosphoric acid, (g) formic acid, (h) EDTA.

Table 6.1: Mean average collagen fibre widths of the BioGide specimens along with the standard deviations. Statistical analysis was performed using a Kruksal-Wallis H test and Dunn's (1964) post hoc analysis with a Bonferroni adjustment. * significant difference ($p < 0.05$) between the fixed specimen and the acetic acid specimen. SD = standard deviation.

Specimen type:	**Mean average collagen fibre width (nm)**	**SD**
Fixed	125.60*	119.94
Non-fixed	167.90	43.31
Formic acid	188.70	113.95
EDTA	178.90	49.06
Phosphoric acid	167.44	82.18
Lactic acid	220.73	119.94
Hydrochloric acid	136.94	45.97
Acetic acid	256.41*	144.41
Citric acid	190.40	77.29

(d) Acid-treated collagen membranes after infiltration with nanoparticles

High magnification (80000x) SEM analysis was used to observe the acid-treated collagen membranes following infiltration with positively (Al_2O_3 coated) or negatively charged silica nanoparticles (Figures 6.9 – 6.15). All acid-treated collagen membranes showed similar results: without infiltration, there were no nanoparticles present on the collagen fibres (micrograph (a)) but after infiltration with 1 wt. % positively charged (Al_2O_3 coated) (micrograph b) or 1 wt. % negatively charged silica nanoparticles

(micrograph c), the surface of the collagen fibres was covered in nanoparticles. This covering of nanoparticles created a layer that prevented individual collagen fibres from being observed and the number of nanoparticles visible seemed to be higher than on non-acid treated collagen. By visual assessment of the SEM images, there seemed to be no apparent difference between positively charged (Al_2O_3 coated) and negatively charged silica nanoparticles.

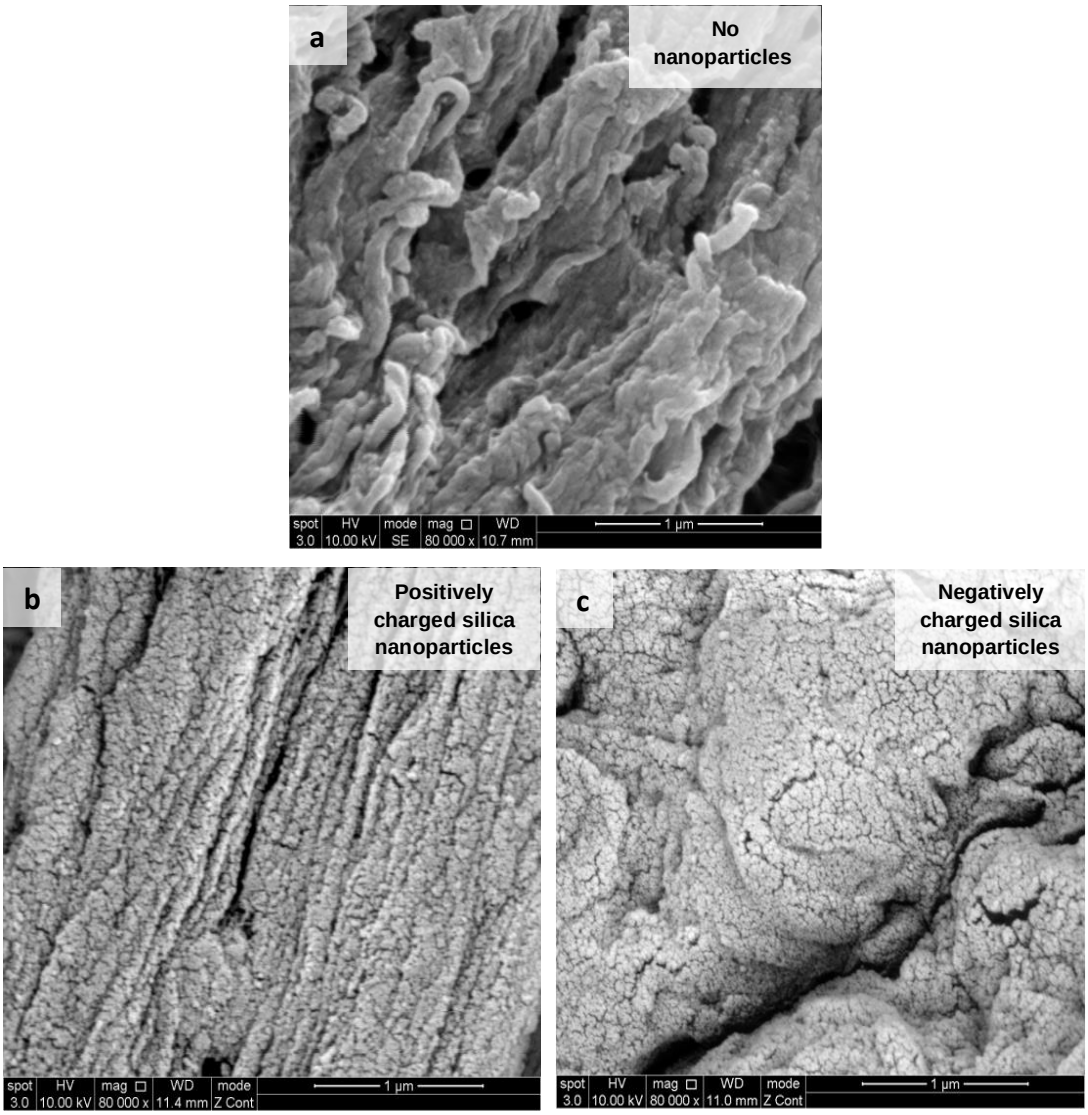

Figure 6.9: SEM micrographs of collagen membranes; (a) exposed to lactic acid for 48 hours; (b) followed by infiltration with positively charged (Al_2O_3 coated) silica nanoparticles; (c) Infiltration with negatively charged silica nanoparticles. All magnifications taken at 80000x.

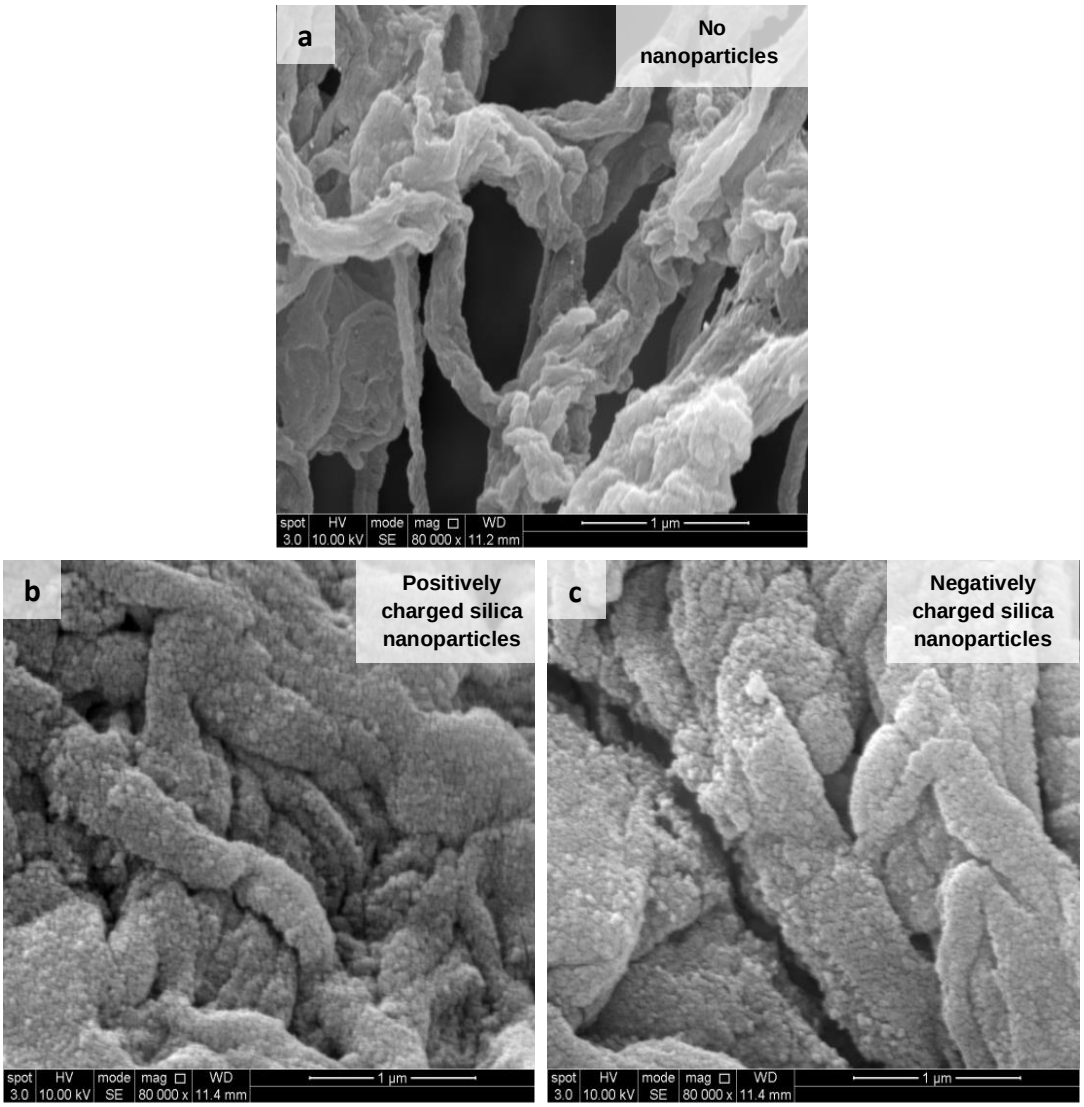

Figure 6.10: SEM micrographs of collagen membranes; (a) exposed to acetic acid for 48 hours; (b) followed by infiltration with positively charged (Al_2O_3 coated) silica nanoparticles; (c) Infiltration with negatively charged silica nanoparticles. All magnifications taken at 80000x.

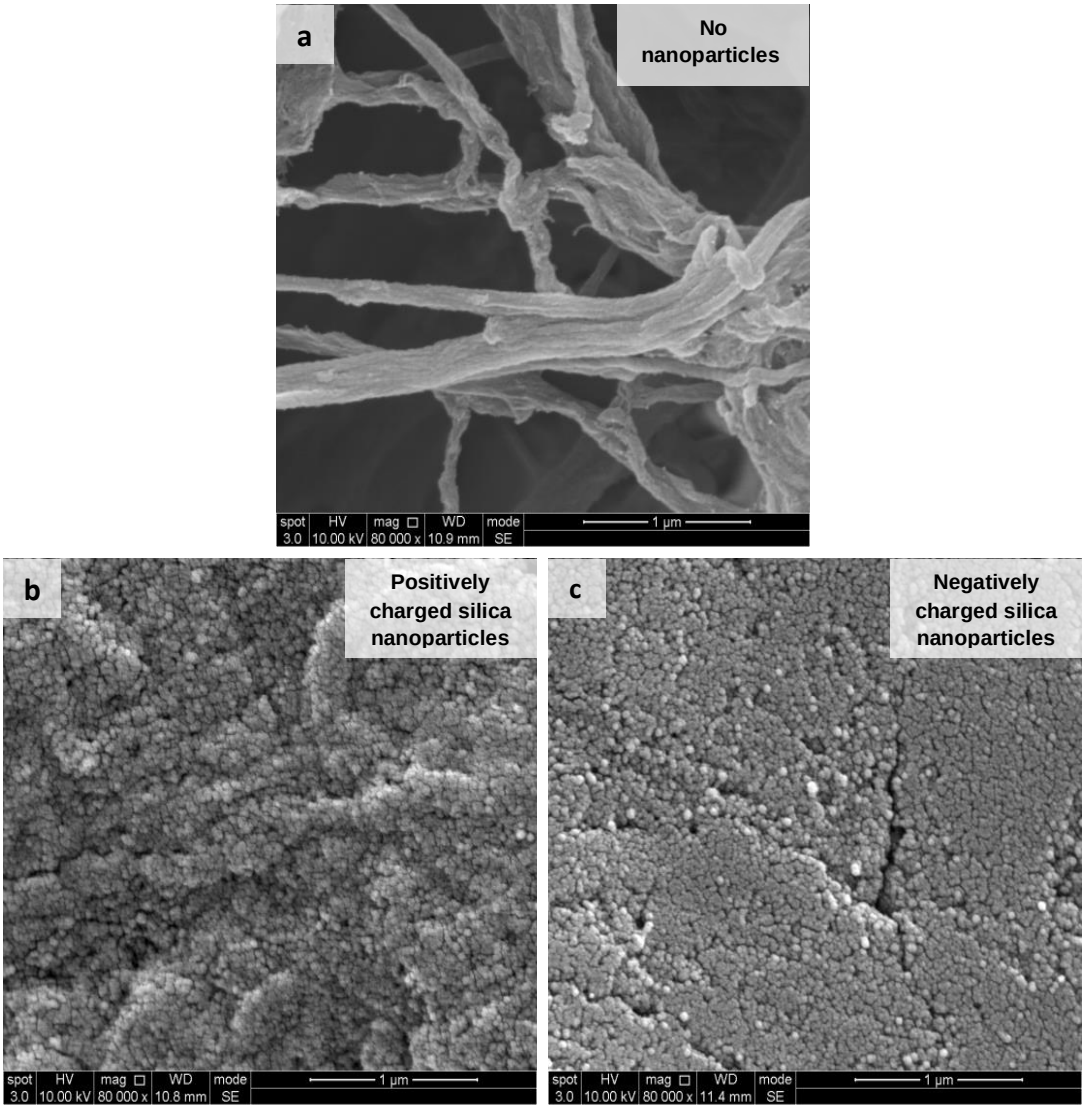

Figure 6.11: SEM micrographs of collagen membranes; (a) exposed to citric acid for 48 hours; (b) followed by infiltration with positively charged (Al_2O_3 coated) silica nanoparticles; (c) Infiltration with negatively silica nanoparticles. All images taken at 80000x magnification.

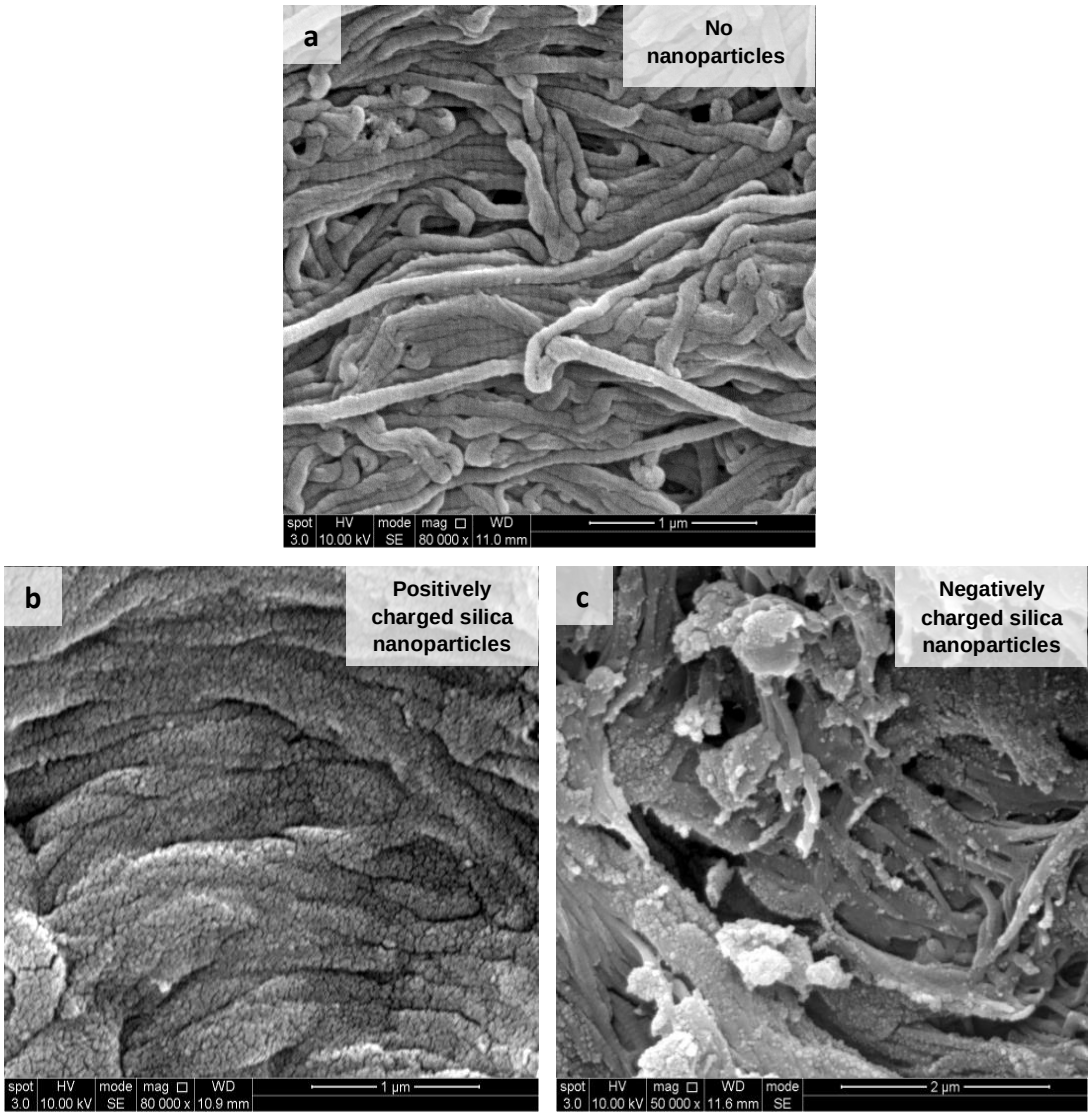

Figure 6.12: SEM micrographs of collagen membranes; (a) exposed to hydrochloric acid for 48 hours; (b) followed by infiltration with positively charged (Al_2O_3 coated) silica nanoparticles; (c) Infiltration with negatively charged silica nanoparticles. All images taken at 80000x magnification

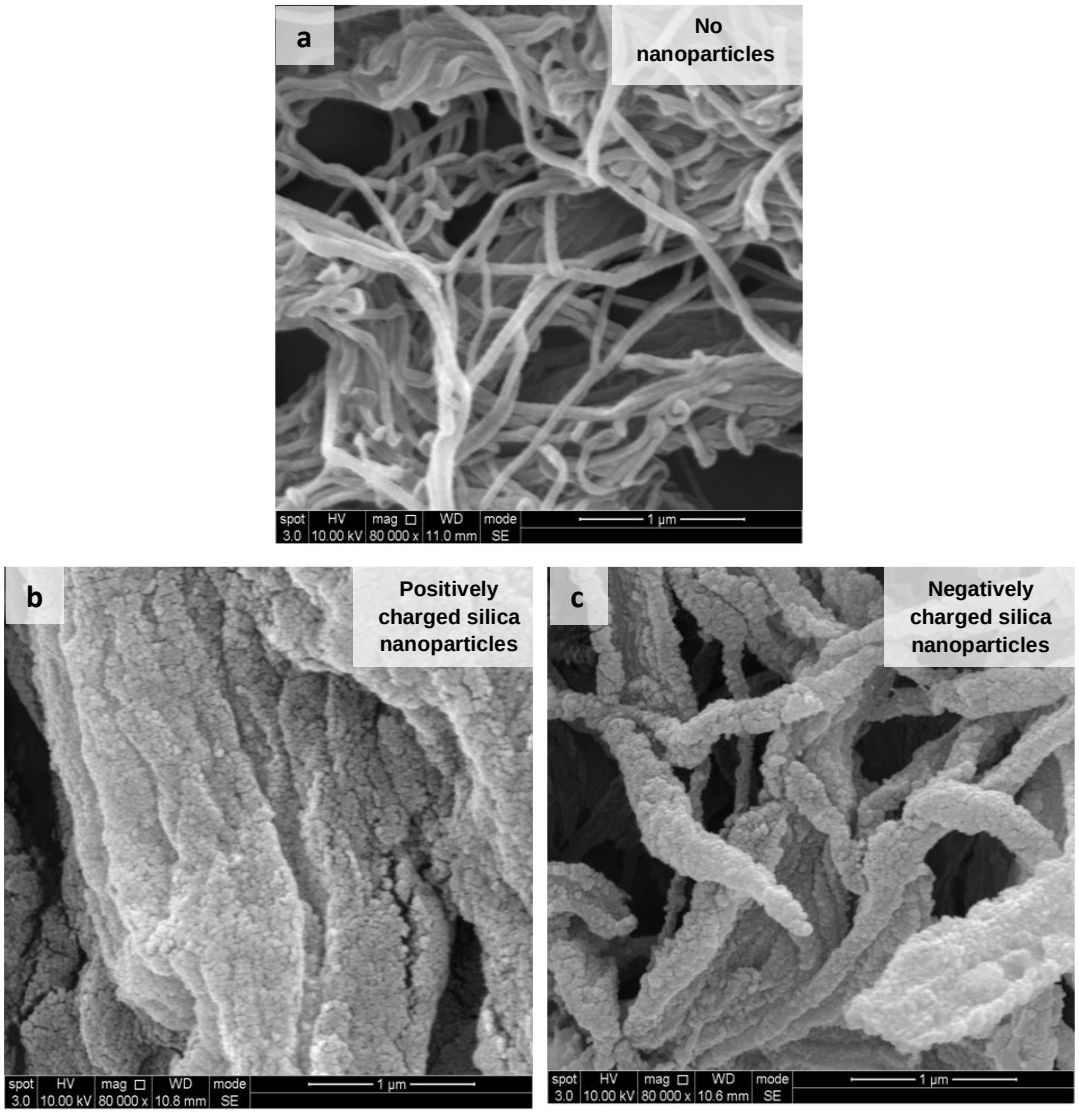

Figure 6.13: SEM micrographs of collagen membranes; (a) exposed to phosphoric acid for 48 hours; (b) followed by infiltration with positively charged (Al_2O_3 coated) silica nanoparticles; (c) Infiltration with negatively charged silica nanoparticles. All images taken at 80000x magnification.

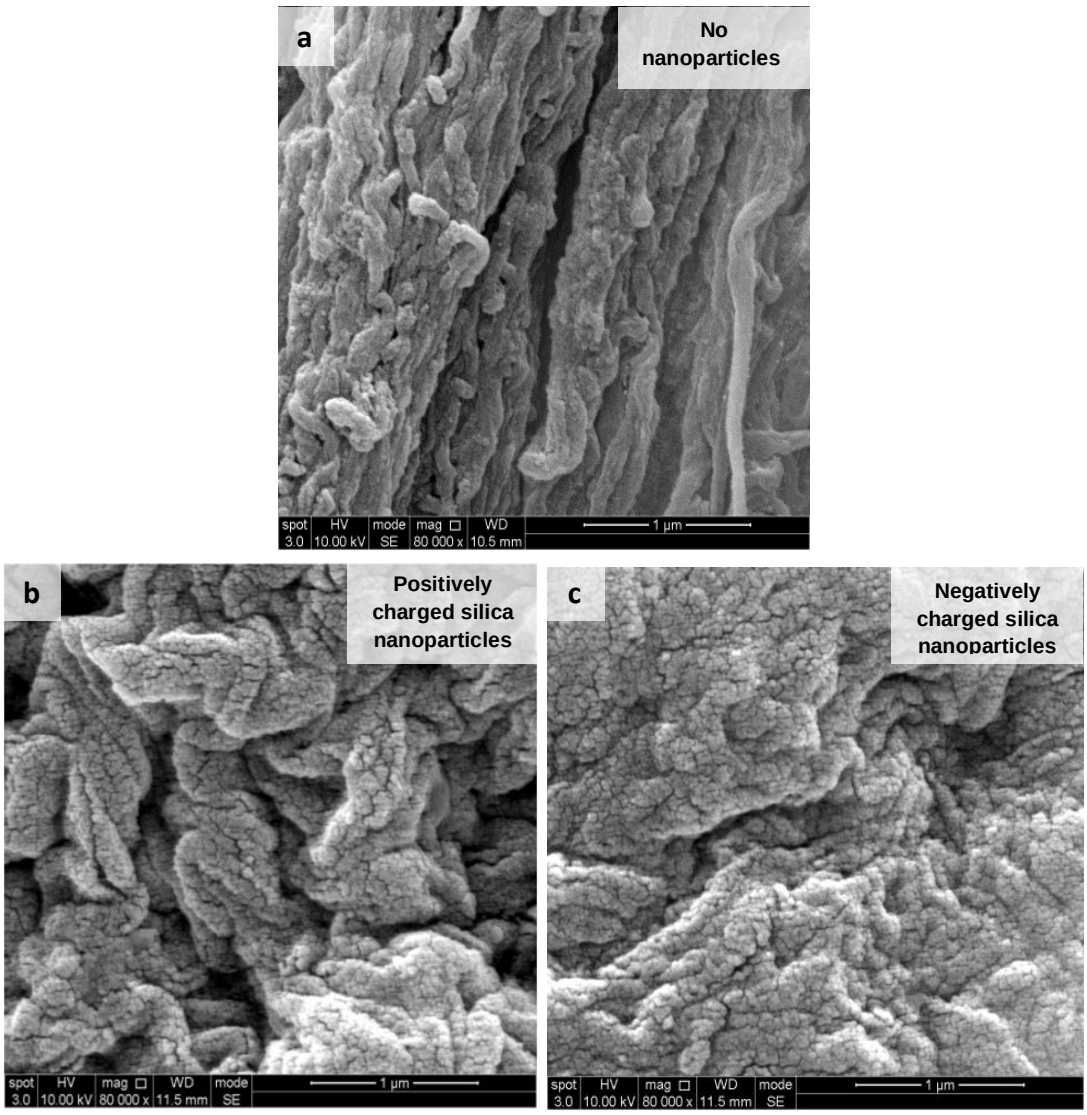

Figure 6.14: SEM micrographs of collagen membranes; (a) exposed to formic acid for 48 hours; (b) followed by infiltration with positively charged (Al_2O_3 coated) silica nanoparticles; (c) Infiltration with negatively charged silica nanoparticles. All images taken at 80000x magnification.

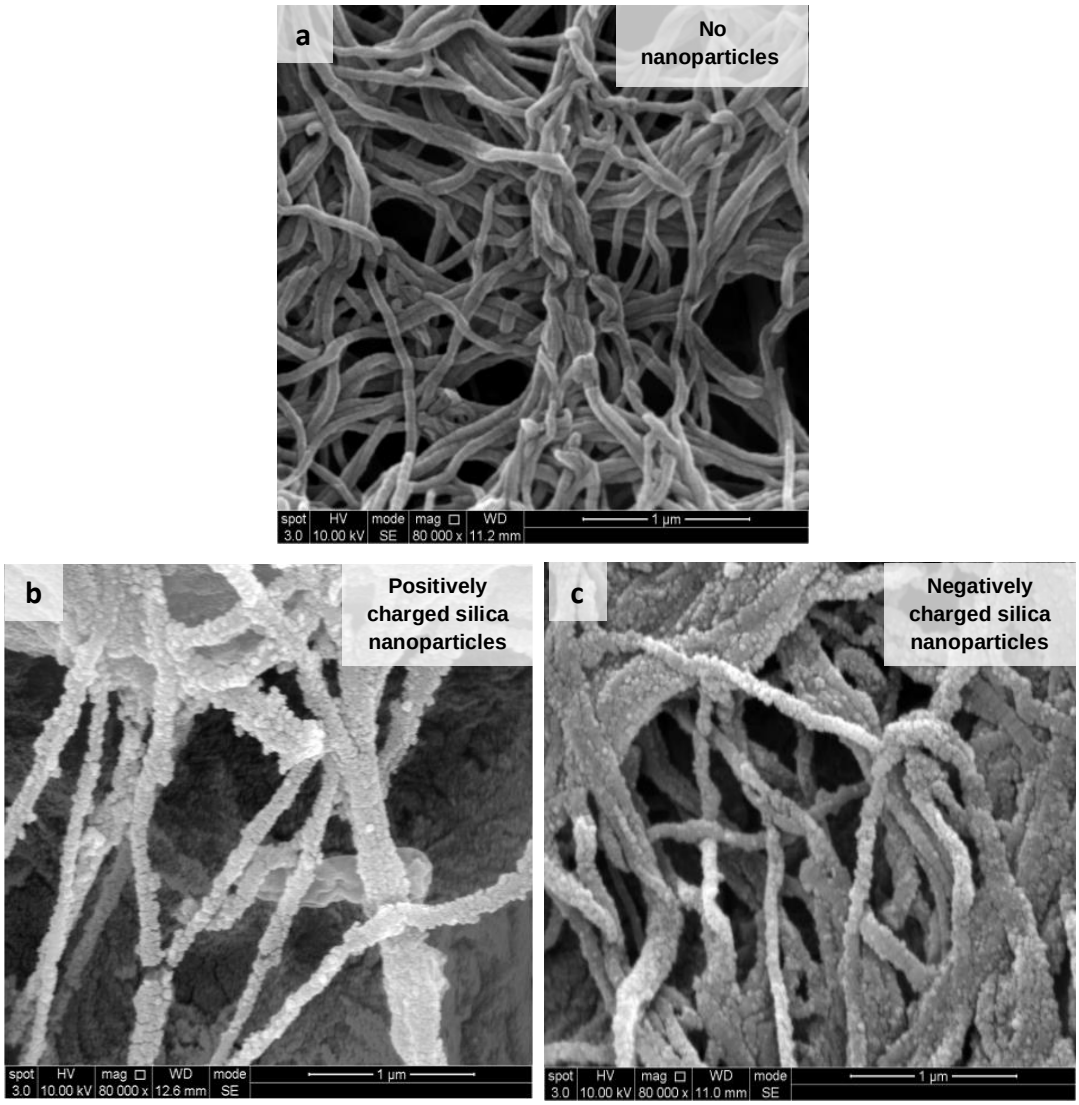

Figure 6.15: SEM micrographs of collagen membranes; (a) exposed to EDTA for 48 hours; (b) followed by infiltration with positively charged (Al_2O_3 coated) silica nanoparticles; (c) Infiltration with negatively charged silica nanoparticles. All images taken at 80000x magnification.

6.5 Discussion

It has previously been established that colloidal suspensions of nanoparticles are able to infiltrate through dentine specimens (Besinis *et al*. 2012) but it has not been clarified whether the nanoparticles adhere to the collagen fibres or merely become trapped between them. It would be beneficial if they adhere to the fibres so that they remain in place when exposed to a remineralising solution so that they can act as a mineral scaffold. Due to the complexity of the dentine composition, it is difficult to establish whether there is a direct interaction between nanoparticles and collagen fibres. To overcome this, porcine Biogide® membranes consisting of the same type of collagen as dentine (collagen I and III) were used to investigate whether the nanoparticles could adhere to collagen fibres. Using these membranes also allowed the implications of chemically fixing the collagen or exposing it to a range of demineralising agents to be examined.

6.5.1 Biogide® as an analogue for demineralised dentine collagen

In order to investigate the relationship between collagen fibres and silica nanoparticles, Biogide® collagen membranes (Geistlich Sons Limited, Manchester, UK) were used instead of dentine collagen. As explained in section 5.5.1.1, it is impossible to obtain two dentine specimens that are identical in terms of structure and content. As it would have been very difficult to test the many variations of nanoparticle solutions on a specimen as inhomogeneous as dentine, Biogide® collagen membranes were essential in order to have a more consistent model. There are many commercially available collagen membranes but Biogide® membranes were chosen because they were supplied in a non-fixed format, thus offering the opportunity to assess the effect of tissue fixing on particle adherence.

Biogide® collagen membranes are derived from the peritoneum membranes in pigs, meaning that they contain the same mammalian collagen type I and III that is found in human dentine; for example, collagen fibres from both sources consist of 3 polypeptide alpha chains coiled around each other to form the triple helix configuration consisting of the “GLY X Y” repeat (explained previously in section 2.3.3.1). Although porcine and human

collagen type I and III are the same, the substances accompanying the collagen may differ, hence, as described Appendix 1 (section 12.1), the porcine membranes were purified to remove any non-collagen substances, only leaving behind mammalian collagen type I and III.

The main difference between Biogide® collagen and dentine collagen is the collagen network structure (i.e. the positioning of the fibres). The dentine collagen network is interjected with tubules and contain a mineral phase which are not present in the Biogide® collagen membranes. Nonetheless, as this experiment did not focus on the movement or interaction of the nanoparticles through tubules specifically, the absence of tubules in the Biogide® membranes did not affect the ability to monitor the nanoparticle adherence. Furthermore, Biogide® collagen membranes are only 1 mm in depth, which could cause difficulty when monitoring the depth of infiltration of the nanoparticles. However, the purpose of this experiment was to assess the interaction between the nanoparticles and collagen fibres, and the depth of the membranes did not affect the conclusions drawn.

Although Biogide® collagen membranes do not perfectly replicate dentine microstructurally nor in depth and complexity (i.e. they do not contain an inorganic phase), they are composed of mammalian codrllagen type I and type III (the same as dentine collagen). For the purpose of this thesis, Biogide® membranes were an appropriate model for the proof of concept experiment because they are a simple yet experimentally similar model of human dentine collagen.

6.5.2 Silica nanoparticles appeared to infiltrate through the collagen membranes and adhere to the collagen fibres

A novel method using a syringe pump, illustrated in Figure 4.6, was designed to enable the infiltration rate to be controlled. As the optimum concentration of the silica nanoparticles was not known, a 1 wt. % solution was tested in an initial pilot study. SEM analysis of the collagen membranes infiltrated with 1 wt. % silica nanoparticles (Figure 6.5) displayed an even coating of nanoparticles on the collagen fibre network and henceforth, 1 wt. % solutions were used for the rest of the study. Ideally, a range of concentrations would

have been investigated. When comparing the SEM images of non-infiltrated specimens (Figure 6.4) with infiltrated specimens (Figure 6.5) it is clear that nanoparticles were present on the infiltrated Biogide® membranes. Visual assessment of these images suggests that the infiltration was even across the specimens but there were some areas of agglomeration.

To verify that the nanoparticles observed on Figure 6.5 were that of silica, EDS analysis (Figure 6.6) was employed. The EDS analysis on Figure 6.6 displays the percentage of the area of the sample analysed that is composed of silica. The amount of silica on both infiltrated specimens was significantly higher compared with the non-infiltrated control specimen.

Collagen membranes were weighed before being placed into a syringe filter on a syringe pump, they were then subjected to 10 ml of either positively charged (Al_2O_3 coated) or negatively charged silica solutions (at 1 wt. % concentration). Immediately following infiltration, the collagen membranes were flushed with 20 ml water at a fast flow rate, before being dried and re-weighed. The idea of the water flushing step was to dislodge any of the non-adhered nanoparticles from the membranes, leaving behind only the adhered nanoparticles. It can be assumed that any increase in mass was linked to the adherence of silica nanoparticles.

As shown in Figure 6.3, all collagen specimens except for the control had an increase in mass following infiltration with either of the silica solutions. The control was only subjected to the water flushing (no silica infiltration) and did not show a change in mass after being re-desiccated and weighed; this was also shown statistically.

To understand the chemistry between the silica nanoparticles and the collagen fibres and to identify the type of adherence mechanism, it is important to consider the effects on adherence of (a) nanoparticle surface charge, (b) glutaraldehyde fixative and (c) exposure to an acid.

(a) Effect of nanoparticle surface charge

Results from the syringe pump experiment (Figure 6.3) showed that there was a significant difference when comparing infiltration with positively (Al_2O_3

coated) and negatively charged nanoparticles in the specimens that had been pre-treated with EDTA. However, in regards to any of the other variable groups, there were no significant differences in infiltration between positively (Al_2O_3 coated) charged and negatively charged particles. SEM analysis (Figure 6.5 and Figures 6.9 - 6.15) also showed little visual difference between infiltrations with negatively charged or positively charged (Al_2O_3 coated) nanoparticles. Furthermore, the EDS results on Figure 6.6 also showed no significant difference in infiltration of positively (Al_2O_3 coated) charged and negatively charged nanoparticles.

(b) Effect of glutaraldehyde

It is essential for specimens to be immersed in chemical fixatives before utilising techniques such as SEM, TEM and EDS. It is speculated that fixatives may remove positive charges on amino acid residues, which may prevent adjacent positively charged residues, such as lysine, from being exposed. It was expected that this may affect the adherence ability of the negatively charged silica particles. Additionally, Chapman *et al* discovered that negative stains did not work as well as positive stains when collagen was fixed (Chapman *et al*., 1990). However, there is no scientific evidence to show that glutaraldehyde would affect the amino acids found in collagen type I; glycine, proline and hydroxyprolyl.

The SEM images in 6.4 showed no visual differences between fixed and non-fixed Biogide® specimens, and Table 6.1 showed no change in collagen fibre width between the two. With regards to infiltration ability, Figure 6.5 also showed no visual difference comparing fixed and non-fixed specimens. Statistical analysis of the collagen mass change from the syringe pump experiment, displayed in Figure 6.3, also showed no significant difference when comparing fixed and non-fixed specimens.

The results from this study imply that the use of glutaraldehyde did not affect particle adherence, which allows techniques such as EDS, SEM and TEM to be undertaken without risk of altering the adherence potential of the particles.

(c) Effect of acid exposure

If the infiltration of silica particles were to be applied clinically to a carious tooth, then it is likely that the silica particles would need to be just as effective in adhering to collagen that has been demineralised by a variety of acids. Figures 6.7 and 6.8 show the SEM micrographs of the Biogide® membranes after exposure in various demineralising agents that were used in the previous study in Chapter 5. Visual assessment by SEM showed that the acids did not appear to affect the integrity of the collagen membranes. The widths of the collagen fibres varied within all specimens, as displayed on Table 6.1. Following statistical analysis it was revealed that there was a significant difference when comparing the collagen widths in the fixed non-acid treated specimen and the acetic acid treated specimen. However, there are no other significant differences between any other group combinations. There is no scientific evidence to explain why only acetic acid treated specimens differed from the non-acid specimen. The large standard deviations in Table 6.1 imply that the fibre widths vary considerably within each specimen.

Previous studies have suggested that acid treated dentine is more positively charged than non-acid treated dentine (Stetlerstevenson and Veis, 1986, Zhang *et al*., 2005, Nezu and Winnik, 2000, Besinis *et al*., 2012), which could affect the infiltration and adherence results. A low pH environment can also affect the surface charge of the silica nanoparticles. This is due to hydrogen ions (H^+) causing a reduction of SiO^- groups to Si-OH, resulting in a reduced electrostatic barrier between particles. With regards to the positively charged (Al_2O_3 coated) particles, the increase in H^+ may increase the repulsion forces and help to prevent aggregation. In addition, this increase of positive charge could allow adherence to the collagen.

When visually comparing the SEM images after infiltration of nanoparticles in non-acid treated specimens (Figure 6.5) and after acid treatments (Figures 6.9 – 6.15), there appeared to be more nanoparticles present after the acid treatment, suggesting that acid-treated collagen exposes more binding points on the collagen. However, the results from the syringe pump experiment,

displayed in Figure 6.3, showed no significant difference in silica adherence between specimens treated with an acid or not treated with an acid.

6.5.3 The interaction between silica and collagen

The silica nanoparticles have a large surface area enabling strong bonds to form with materials, and they can undergo ionic and hydrogen interactions, as described in the product specification (Grace, 2007). Although the exact mechanism of their adherence to collagen is uncertain, the interaction is believed to have been quite strong because it withstood the flushing of water.

As described in section 6.5.2 (a), both positively charged (Al_2O_3 coated) and negatively charged silica nanoparticles appeared to infiltrate through the collagen and adhere to collagen fibres. As displayed on Figure 6.5, some of the silica nanoparticles agglomerated to form clumps (approximately 100 nm diameter) and literature suggests that changes in pH can cause silica nanoparticles to stick together (Hall, 2010, Zhang *et al*., 2012a). The negatively charged silica nanoparticles were provided in a solution of pH 11, where they were most stable. At this high pH, they have high levels of repulsion between each other and therefore, agglomerates should not form. In addition, positively charged silica nanoparticles were provided in a solution of pH 4.5, where they were most stable and have high levels of repulsion between each other, preventing agglomeration. One suggestion for the agglomerates displayed in Figure 6.5 is that the water flushing step may have caused the overall net pH to be more neutral, which would have effected the electrostatic repulsion levels of both types of silica nanoparticles, leading to agglomerates of the particles.

Regarding the adherence of silica nanoparticles to collagen fibres, when comparing positively charged silica nanoparticles with negatively charged silica nanoparticles, there was no significant difference in any of the collagen specimens, except for those that were exposed to EDTA (demonstrated on Figure 6.3). Visual assessment of SEM images showed no difference between the two types of silica nanoparticles in any of the collagen specimens. There were also no statistically significant differences in silica adherence when comparing fixed collagen specimens with non-fixed

collagen specimens, as well as acid treated collagen specimens with non-acid treated collagen specimens (also demonstrated on Figure 6.3).

Under normal physiological conditions, dentine collagen has a net charge of zero (Karube *et al*., 1976, Singh *et al*., 1995, Sehgal and Srinivasan, 2009). However, when collagen is exposed to an acid, the hydrogen ions present within the acid are likely to coat the collagen fibres with a positive charge, leaving a positive net charge overall (Zhang *et al*., 2005, Nezu and Winnik, 2000). Therefore, it would be expected that negatively charged nanoparticles would bind better to acid-treated collagen than a) non-acid treated collagen and b) than positively charged (Al_2O_3 coated) nanoparticles. However, there was no significant difference between the two oppositely charged particles when considering the presence of an acid. This suggests that the collagen consists of positive and negative regions, even when it has a positive net charge.

The Biogide® membranes were an appropriate model for testing the ability of silica to adhere to collagen fibres in various conditions but the Biogide® membranes were purified to remove non-collagenous proteins (NCPs) beforehand, which affect the overall charge. However, results from this study have suggested that even when the overall net charge of collagen is polar, both types of silica nanoparticles were still able to bind (either via ionic or hydrogen bonds) to oppositely charged regions of the collagen fibre. This suggests that even though dentine collagen may have an overall negative net charge, it will still have positively charged regions, enabling both positively and negatively charged silica nanoparticles to adhere.

The silica nanoparticles have to be modified in order to have a positive surface charge; the manufacturer states that the surface of the silica particles consists of a single layer of aluminium oxide. The measured thickness of the coating is 5 nm and subsequently, the material behaves as aluminium oxide, not silica.

Therefore, as there was little difference between positively charged (Al_2O_3 coated) and negatively charged particles, it was decided to use negatively charged silica nanoparticles with regards to infiltration and remineralisation

with the future studies because they are pure silica and their behaviour can be monitored more easily than coated silica.

6.5.4 Summary

A novel infiltration method was designed which demonstrated that silica nanoparticles, rather than becoming trapped between collagen fibres, may actually have adhered to them directly. This is an important finding because it suggests that a collagen-silica scaffold can be created which is not separated when in solution. Furthermore, it was revealed that Biogide® specimens could be chemically fixed for analytical purposes without affecting the true results. This is particularly useful because these analytical techniques are essential for all *in vitro* remineralisation studies and the effect of using a fixative has never been investigated previously. Also discovered, was that particles with either a positively charged (Al_2O_3 coated) or negatively charged surface charge were able to adhere to collagen fibres, which broadens the range of nanoparticles that could be investigated in the future. The infiltration method also demonstrated that collagen can be treated with a range of demineralising agents without inhibiting nanoparticle adherence.

6.6 Future work

- Chapter 5 used the Hyp Assay to determine whether the collagen from dentine specimens became denatured following treatment with an acid. The Hyp Assay could be repeated on the collagen membranes to determine whether the collagen membranes become denatured as well. Furthermore, in conjunction with SEM analysis, collagen membrane structure and level of denaturation should be examined after various exposure times in acids with a range of pH values. The syringe pump experiment should also be repeated to assess the relationship between level of silica adherence and level of collagen membrane denaturation.
- In this study, silica was only tested in 1 wt. % solutions. Therefore, a range of silica concentrations and volumes should be investigated to discover the optimum concentration for infiltration with minimal

agglomeration. Furthermore, as the syringe pump is a novel method which has never been tested before, the parameters (such as, infiltration time, flow rate, volume of solutions) should be altered to find the optimum settings.

- Chapter 6 compared the adherence ability between positively charged and negatively charged silica nanoparticles. There was only a statistically significant difference between the two silica groups in the membranes that were treated with EDTA and there is no explanation as to why the EDTA-group was the only group to have these results. Therefore, more repeats are necessary to investigate whether these results are consistent.
- Section 6.5.1 explains the justification for using Biogide® membranes as a model for dentine collagen. In order to further verify this decision, other collagen membranes, from different origins and different preparation methods (e.g. exposure to fixatives) should be tested to a) find the best model for dentine collagen and b) to help explain how silica nanoparticles adhere to collagen fibres.
- As nanoparticles become more prevalent in medical and dental research, the number of studies investigating the cytotoxicity of nanoparticles appears to be increasing. Results regarding the cytotoxicity of silica nanoparticles are still very much inconclusive. Sigma Aldrich confirmed that there was no published literature regarding the biocompatibility of the Ludox Silica CL and HS specifically (email correspondence, July 2016). Fede *et al* have shown that the outcome of toxicity tests on other silica nanoparticles solutions has been dependent on the type of technique and the treatment conditions (Fede *et al*., 2012), suggesting that more research is required before the toxicity of silica nanoparticles can be verified.

7. Remineralisation of Dentine

7.1 Introduction

Nanotechnology is becoming increasingly prevalent in dental remineralisation studies. Notably; nano-hydroxyapatite solutions (Jeong *et al*., 2006, Lv *et al*., 2007), nano-bioactive glasses (Forsback *et al*., 2004, Vollenweider *et al*., 2007), nano-calcium fluorides (Sun and Chow, 2008), nano-amorphous calcium phosphates (Tay and Pashley, 2008, Tay and Pashley, 2009) and nano-silica (Besinis *et al*., 2014b) have recently been under investigation.

As discussed in previous chapters, the study by Besinis *et al* is of particular interest because it involves sub-surface remineralisation of dentine. The *in vitro* studies established that demineralised dentine specimens showed signs of remineralisation after infiltration with silica nanoparticles and subsequent exposure to a remineralising solution. Remineralisation was assessed using transmission electron microscopy (TEM), energy dispersive spectroscopy (EDS) and micro-computed tomography (micro-CT) analysis following a 12 week study. The results showed significantly more remineralisation in the specimens that had been infiltrated with silica nanoparticles compared to specimens that had not been infiltrated. They report that prior infiltration with silica nanoparticles recovered up to 20 % phosphate levels and 16 % mineral volume whilst also decreasing mineral separation to levels similar to sound dentine (Besinis *et al*., 2014b). Their remineralisation strategy depended upon nanoparticles being able to (i) infiltrate through the collagen, (ii) adhere to collagen fibres and (iii) interact with calcium or phosphate ions from a remineralising solution.

However, further research is required to make this strategy more clinically applicable: (1) more clinically relevant demineralised dentine models (produced using erosion/caries acids) are required in addition to the fully demineralised formic acid model that has currently been utilised; (2) a shorter infiltration time and a lower concentration of nanoparticles would make this method more clinically acceptable (Besinis *et al* infiltrated the

dentine blocks for 24 hours with 30 wt. % silica solutions) (3) to understand the remineralisation mechanism in more depth, particularly focusing on the interaction between the silica nanoparticles and the remineralising ions to verify that the nanoparticles directly facilitate remineralisation.

7.1.1 Clinically Relevant Demineralised Dentine Models

It was concluded in Chapter 5 that it is important to consider the type of demineralising agent used for *in vitro* dentine experiments because each agent affects dentine differently. The Besinis *et al* remineralisation strategy only tested dentine that had been fully demineralised using formic acid, which is a laboratory acid often used to produce *in vitro* demineralised dentine models because it has a short demineralisation time and is easy to obtain (Butler *et al*., 1981, Addy *et al*., 1987, Kim *et al*., 2011, Ryou *et al*., 2011, Kato and Fusayama, 1970). However, formic acid is not involved in caries or erosion processes. Furthermore, it is unclear whether silica nanoparticles are able to infiltrate through other demineralised dentine models, for example, through a collapsed structure that may not have the same potential to allow nanoparticle infiltration and subsequent remineralisation. Additionally, the remineralisation study by Besinis *et al* was only carried out on fully demineralised dentine for proof of principle purpose. Therefore, investigating the remineralisation of a range of partially demineralised dentine models would significantly add to the body of knowledge.

7.1.2 Concentration and Exposure Times of Infiltration

Besinis *et al* investigated silica nanoparticles in a 30 wt. % concentration but it is unknown whether this is an optimum concentration for remineralisation. For clinical implications, it would be beneficial to decrease the nanoparticle solutions as much as possible; for example, a 1 wt. % silica solution, as demonstrated in Chapter 6, being sufficient to coat the collagen fibres. Furthermore, Besinis *et al* used a 24 hour infiltration time but in order to develop this remineralisation strategy and to consider clinical implications, it would be beneficial to decrease the infiltration period to more clinically relevant time periods whilst maintaining significant remineralisation.

7.1.3 The Interaction between Nanoparticles and Remineralising Ions

Previous studies have shown that colloidal suspensions of silica nanoparticles can infiltrate into the dentine collagen (Besinis *et al*., 2012). Furthermore, it has been demonstrated in this project (chapter 6) that silica nanoparticles are able to adhere to collagen fibres to create a collagen-nanoparticle scaffold.

However, the interaction between the nanoparticles and the remineralising ions within a remineralising solution has not yet been investigated. There has been some evidence that nanoparticles can facilitate remineralisation because infiltrated specimens showed significantly more calcium and phosphorous than non-infiltrated control specimens (Besinis *et al*., 2014b). Fully demineralised dentine models were used, as opposed to partially demineralised dentine models, which suggest that any presence of calcium or phosphate is a direct result of the nanoparticle treatment. Understanding of the interaction between the nanoparticles and the remineralising ions is essential in order to develop the remineralising strategy further.

It has been established previously that both negatively and positively (Al_2O_3 coated) charged nanoparticles can infiltrate into dentine collagen (Besinis *et al*., 2012). Furthermore, in chapter 6 of this thesis, silica nanoparticles were seen to interact with collagen fibres of the Biogide® membranes. As discussed in section 6.5.3, positively charged silica nanoparticles are coated with 5 nm of aluminium oxide and therefore, cannot be considered to behave as true silica. Henceforth, unmodified negatively charged silica nanoparticles are more appropriate for dentine remineralisation and it is more likely these nanoparticles will interact with calcium ions (Ca^{2+}) rather than phosphate ions (PO_4^-) due to the attraction of opposite charges.

As discussed in the Literature Review (section 2.5.3.2), small changes in calcium concentrations have shown to have a greater effect on remineralisation than similar changes in phosphate concentrations (Lynch, 2004). In fact, some *in vitro* studies have demonstrated calcium to be twenty times more effective than phosphate at deterring demineralisation (Tanaka and Kadoma, 2000, Lynch and ten Cate, 2005). In addition, it has also been

demonstrated that an optimal rate of remineralisation is obtained with a calcium:phosphate ratio of 1.6 (Exterkate *et al*., 1993), whereas plaque fluid has a calcium:phosphate ratio of 0.3 (Margolis, 1990). Therefore, it has been suggested that the calcium ion concentration may be the main rate-limiting mineral constituent, and the delivery of additional calcium could promote remineralisation and prevent further demineralisation (ten Cate, 1994). There have already been investigations that suggest that adding calcium chloride to mouth rinses can increase enamel hardness (Pearce and Nelson, 1988, Pearce, 1982), and other studies showing more remineralisation when calcium ion concentration is increased and fluoride levels remain the same (Lynch *et al*., 2006). Therefore, negatively charged silica nanoparticles, with the potential to attract calcium ions, are more relevant than positively charged (Al_2O_3 coated) silica nanoparticles. However, the interaction between the calcium ions and the negatively charged silica nanoparticles has not yet been investigated. Monitoring the zeta potential of the nanoparticles whilst adding remineralising ions to the solution may help to determine whether they interact with each other.

7.1.3.1 Measuring Zeta Potential

Over the past decade, researchers have increasingly used dynamic light scattering (DLS) to measure the zeta potential of nanoparticles in order to monitor their interaction with other particles or surfaces. For example, the surface interactions of iron oxide nanoparticles (Zhang *et al*., 2008), cerium nanoparticles (Patil *et al*., 2007), titanium oxide nanoparticles (Bouhaik *et al*., 2013) and silica nanoparticles (Wu *et al*., 2015, Puddu and Perry, 2012, Patwardhan *et al*., 2012, Atalay *et al*., 2014).

The magnitude of the zeta potential relates to the degree of electrostatic repulsion between particles in a solution. Any particle with a charge will attract counter ions creating an electrical double layer consisting of an inner region where counter ions are strongly bound (the stern layer), and an outer layer where ions are more loosely attached (the diffuse layer), as highlighted in Figure 7.1. There is a boundary within the diffuse layer at which the ions

form a stable entity with the particle, called the hydrodynamic layer. The potential that exists here is the zeta potential.

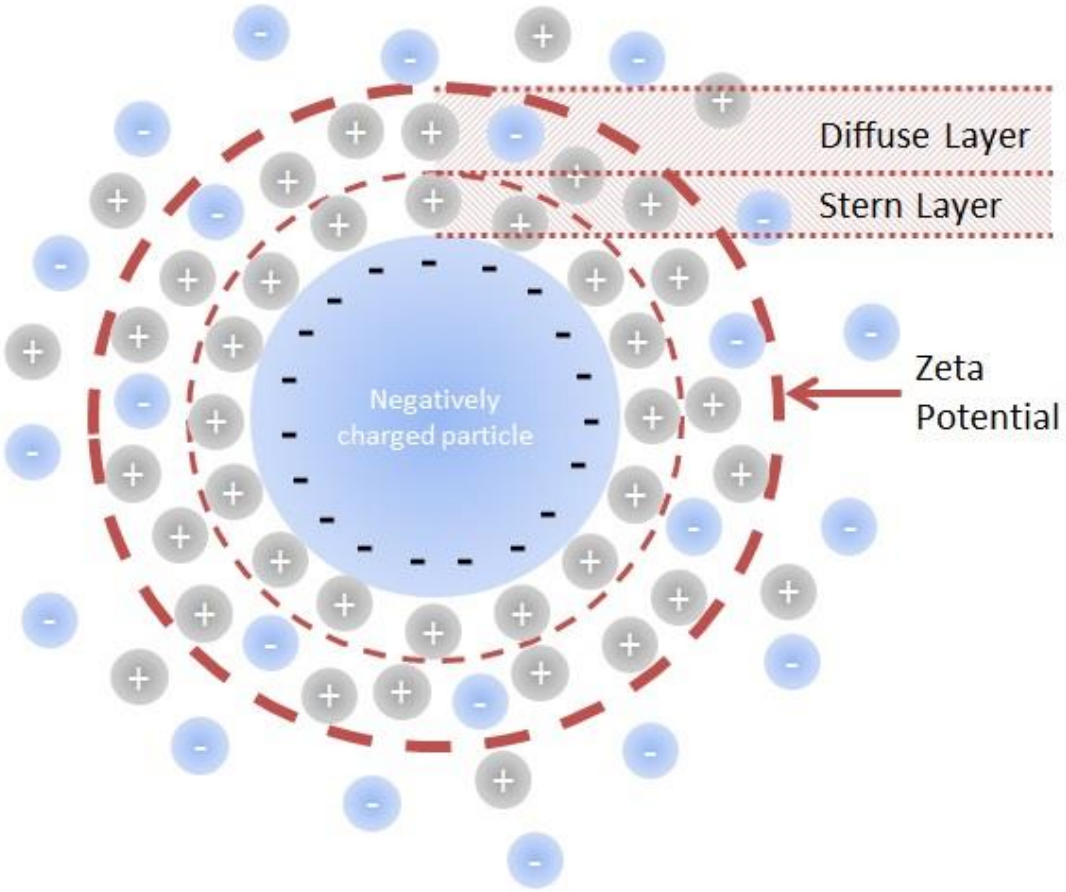

Figure 7.1: A diagram to show the behaviour of a negatively charged particle in a colloidal solution with counter ions (positive ions), illustrating the location of the diffuse layer, stern layer and zeta potential.

The stability of the colloidal system can be predicted by measuring the zeta potential. For example, if all particles have a large zeta potential then they will have high degrees of repulsion and little or no aggregation. pH is the most important factor that affects zeta potential. A particle with a negative zeta potential will acquire a more negative charge in an alkali environment and a more positive charge in an acidic environment. By monitoring the zeta potential of the particles whilst adding ions to the solution, it is possible to assess whether they interact with each other.

7.2 Aims and Objectives

The aim of this study was to gain a greater understanding of the remineralisation mechanism pioneered by Besinis *et al* and to develop the method with more clinical applicability. The specific objectives were:

1) To explore a variety of partially demineralised dentine models to assess the effects on infiltration and remineralisation
2) To compare infiltration of 1 wt. % and 0.1 wt. % nanoparticle solutions using EDS analysis
3) To compare infiltration times of 24 hours and 1 minute using EDS analysis
4) To investigate whether negatively charged silica nanoparticles attract calcium ions, using dynamic light scattering (by measuring zeta potential)
5) To use EDS to measure relative calcium and phosphorous levels in dentine specimens after 4 weeks exposure to a remineralising solution.

7.3 Materials and Methods

7.3.1 Infiltration of Nanoparticles through Dentine Specimens

Dentine blocks were prepared and demineralised for 7 days using EDTA, lactic acid, hydrochloric acid and citric acid; as described in sections 4.2 and 4.2.1. They were then infiltrated for either 24 hours or 1 minute (as explained in section 4.6.2). The infiltration solutions were either 1 wt. % or 0.1 wt. % of negatively charged silica nanoparticle solutions.

Energy dispersive spectroscopy (EDS) analysis was used to examine the presence of the silica (Kα, 1.740 keV) nanoparticles after infiltration.

100 dentine blocks divided into 4 demineralisation groups (EDTA, lactic acid, hydrochloric acid, citric acid), leaving 25 blocks per group. Each demineralisation group was then further divided into 5 subgroups 1) infiltrated for 24 hours 1%, 2) infiltrated for 24 hours 0.1 wt. %, 3) infiltrated 1 minute 1 wt. %, 4) infiltrated 1 minute 0.1 wt. %. or 5) control – no infiltration. Therefore, each subgroup had n = 5.

In this chapter, the inner core of two surfaces of the dentine blocks were analysed; surface A and surface B, as illustrated below in Figure 7.2. Specimens were sliced 1 mm from the outer surface using an ultra-thin steel scalpel blade (0.36 mm thickness) from Sigma Aldrich, Poole, UK. A ruler was used to measure 1 mm from the surface, and calipers were used to confirm that the dentine had been cut 1 mm (+/- 0.2 mm) from the outer surface. The new blocks were then embedded into resin and ground gently using wet silicon carbide papers, without a lubricant, on a grinder-polisher wheel. The blocks were subsequently carbon coated. Further details about EDS sample preparation and method can be found in section 4.7.2.

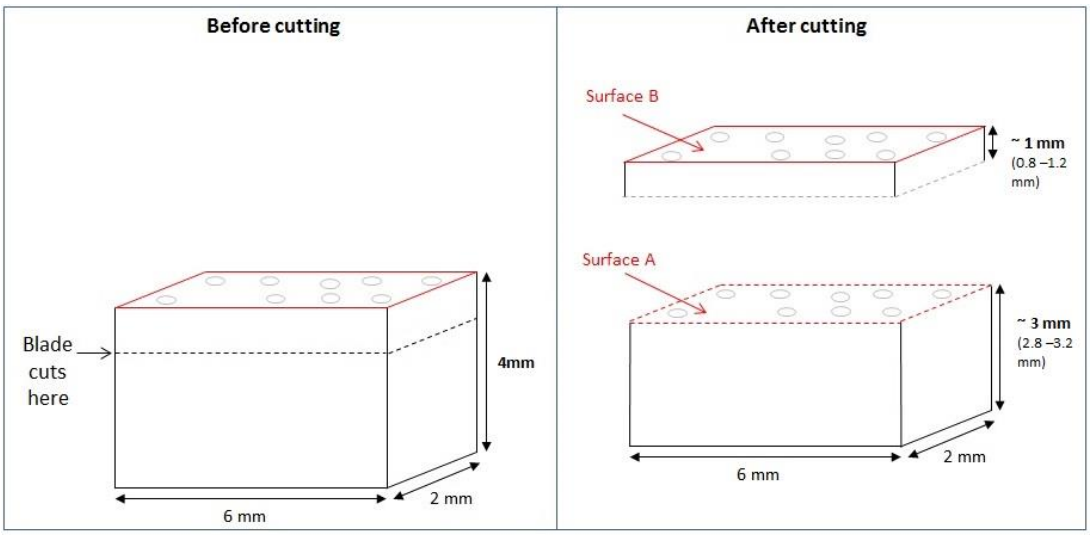

Figure 7.2: A diagram to show surface A and surface B of the dentine specimens after cutting approximately 1 mm beneath the surface

7.3.2 Interaction between Silica and Calcium

DLS was used to measure the zeta potential of the nanoparticles before and after the addition of calcium, to monitor any interaction (n = 3). The protocol can be found in section 4.7.9.

7.3.3 Remineralisation

Thirty six dentine specimens were demineralised and randomly assigned to 1 of 3 groups:

Group i: infiltrated with 1 wt. % Ludox® CL silica for 1 minute

Group ii: Immersed in 20 ml remineralising solution (constituents in section 4.6.1)

Group iii: infiltrated with 1 wt. % Ludox® CL silica for 1 minute and then immersed in 20 ml remineralising solution

Samples were removed from the remineralising solution after 4 weeks and were examined for signs of remineralisation (discussed in section 4.6.3).

Energy dispersive spectroscopy (EDS) analysis was used to examine the presence of calcium (Ca Kα, 3.692 keV) and phosphorous (P Kα, 2.010 keV) after remineralisation.

Thirty six dentine blocks were divided into 4 demineralisation groups (EDTA, lactic acid, hydrochloric acid, citric acid), leaving 9 blocks per group. Each group was then divided into 3 sub-groups as discussed in 4.6.2 (Group i: infiltrated only, Group ii: immersed in a remineralising solution, Group iii: infiltrated and immersed in a remineralising solution), with n = 3 for each sub-group. The specimens were sectioned as outlined in Figure 7.2 and EDS was carried out, as described in section 4.7.2, on surfaces A and B. EDS measurements were taken at 4 different parts of the area of analysis on each dentine block, as illustrated on Figure 4.8.

7.4 Results

7.4.1 Infiltration of Negatively Charged Silica Nanoparticles

7.4.1.1 Energy Dispersive Spectroscopy Results

Energy dispersive spectroscopy (EDS) was used to measure the relative percentage of silica present on the inner core of surface A of dentine specimens after infiltration with negatively charged silica nanoparticles. The control specimens were demineralised, but not infiltrated with silica nanoparticle solutions. All other specimens were infiltrated with either 1 wt. % silica nanoparticle solutions (represented with red bars on Figure 7.3) or 0.1 % silica nanoparticle solutions (represented with blue bars on Figure 7.3) for either 24 hours or 1 minute. As illustrated in Figure 7.3, there was no significant difference comparing the different demineralised dentine models and for this reason, Figure 7.4 displays the results as an average of each demineralised dentine model.

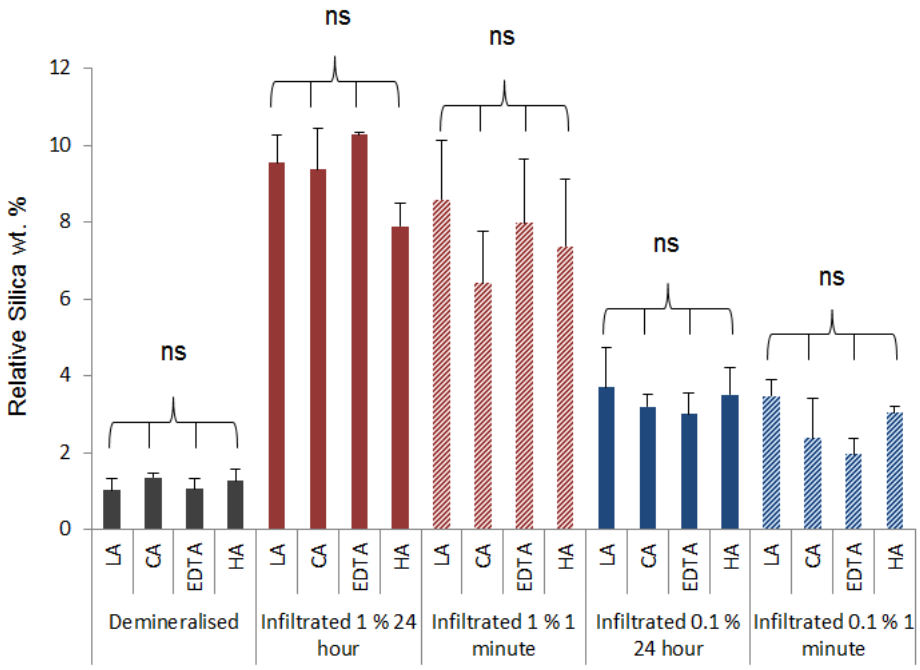

Figure 7.3: Relative wt. % of silica (Kα 1.740 keV) using EDS on dentine specimens (surface A) after demineralisation and subsequent infiltration with silica nanoparticles. Control specimens were not infiltrated. LA = lactic acid, CA = citric acid, HA = hydrochloric ns = no significant difference between variables underneath the brace, using one way ANOVA and Tukey's post hoc analysis.

As highlighted on Figure 7.4, there was no significant difference between the two infiltration times; 24 hours and 1 minute. However, the concentration of the silica nanoparticle solutions did influence the relative % of silica detected by the EDS. For example, specimens that were infiltrated with 1 wt. % silica solutions (red bars) resulted in >50 % more silica on the surfaces than specimens infiltrated with 0.1 wt. % silica solutions (blue bars).

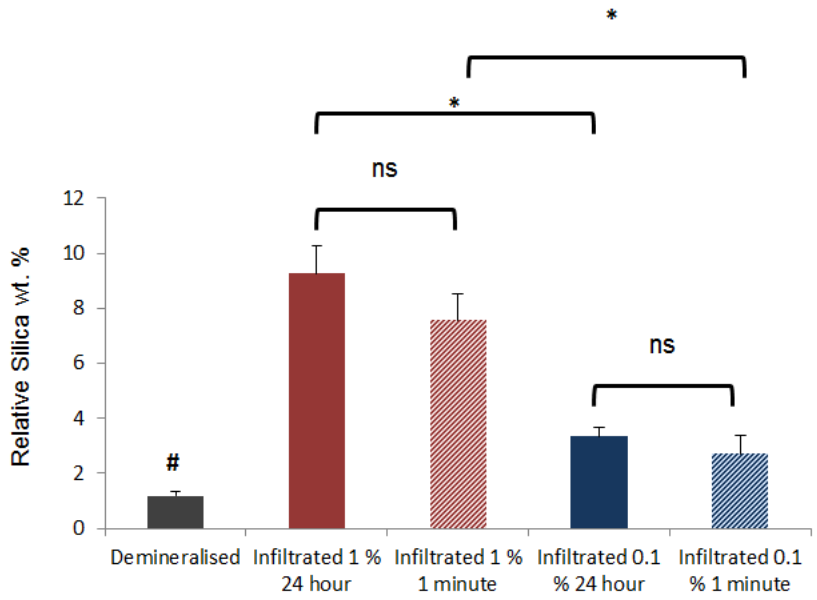

Figure 7.4: Average relative silica wt.% (from EDS) of dentine specimens following demineralisation with the range of demineralising agents, followed by infiltration with silica solutions. Statistical analysis using one way ANOVA with Tukey's post hoc analysis.

= significant difference ($p<0.05$) from all other variables.

***** = significant difference ($p<0.05$) between the two variables under the bracket

ns = no significant difference ($p<0.05$) between the two variables under the bracket

7.4.2 Interaction between Negatively Charged Silica Nanoparticles and Calcium Ions

7.4.2.1 Dynamic Light Scattering Results

Dynamic light scattering (DLS) was used to measure the zeta potential of the silica nanoparticles. Figure 7.5 displays the zeta potential values for the silica nanoparticle solutions at pH 11 (the pH of the solution when unmodified). In these conditions, the zeta potential of the silica nanoparticles was approximately -55 mV. When sodium chloride (NaCl) or calcium chloride ($CaCl_2$) was added to the silica solutions, the zeta potential value of the silica increased. For example, when NaCl was added to the solution, the silica nanoparticles had a zeta potential of approximately -40 mV and when $CaCl_2$ was added to solution instead, they had a zeta potential of approximately -30 mV. $CaCl_2$ increased the zeta potential more than NaCl**.**

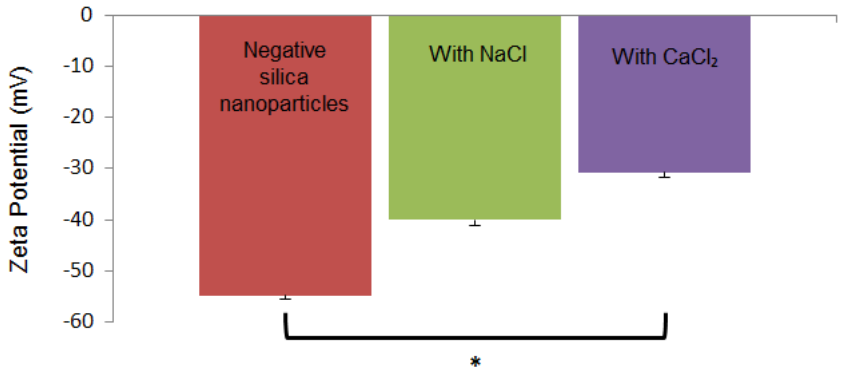

Figure 7.5: Zeta potential (mV) of silica nanoparticles at pH 11: unmodified (red bar), with NaCl (green bar), with $CaCl_2$ (purple bar). Statistical analysis using Kruskal-Wallis H test with Dunn's 1964 post-hoc and Bonferroni's adjustment. No significant differences between groups unless indicated.

***** = significant difference ($p<0.05$) between two variables indicated by the bracket.

Figure 7.6 displays the zeta potential of the silica nanoparticles in a range of pH conditions, with and without the addition of NaCl and $CaCl_2$. For each specimen, the zeta potential of the silica nanoparticles decreased as pH was increased. At all pH conditions, the addition of NaCl or $CaCl_2$ increased the zeta potential of the silica nanoparticles, with $CaCl_2$ increasing the zeta potential more so at each pH value. The isoelectric point of silica is at approximately pH 2.5, (see Figure 7.6) when the silica particles have a zeta potential of 0 mV.

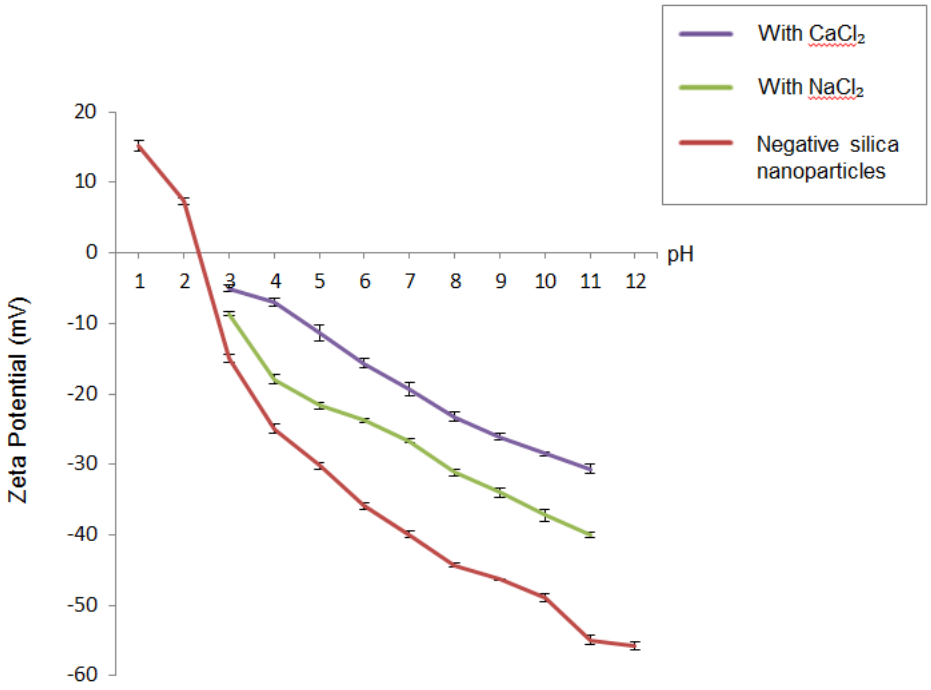

Figure 7.6: Zeta potential of silica nanoparticles in solutions of a range of pH values (red line), with NaCl added (green line) and with $CaCl_2$ added (purple line).

7.4.3 Calcium and Phosphorous Levels

7.4.3.1 Energy Dispersive Spectroscopy Results

Following infiltration with silica nanoparticles and subsequent exposure to an a remineralising solution for 4 weeks, the inner surface (surface A) and outer surface (surface B) of the dentine blocks were analysed using EDS to indicate the relative % of Ca and P (Figure 7.7 and 7.8, respectively).

Group i (demineralised and infiltrated, but not exposed to a remineralising solution) and group ii (demineralised, but not infiltrated, and exposed to a remineralising solution) were the control groups whereas group iii had been demineralised, infiltrated and exposed to a remineralising solution. All specimens had an average relative Ca % of less than 25 wt. % (Figure 7.7) and relative P of below 8 wt. % (Figure 7.8), regardless of type of demineralising agent used. The specimens that were demineralised with citric acid had the highest relative Ca and P %, whereas specimens demineralised with EDTA had the lowest relative Ca % and specimens demineralised with lactic acid had the lowest relative P %. There was no

statistical significant difference when comparing surfaces A and B, when comparing different demineralising agents or when comparing groups i, ii and iii with each other.

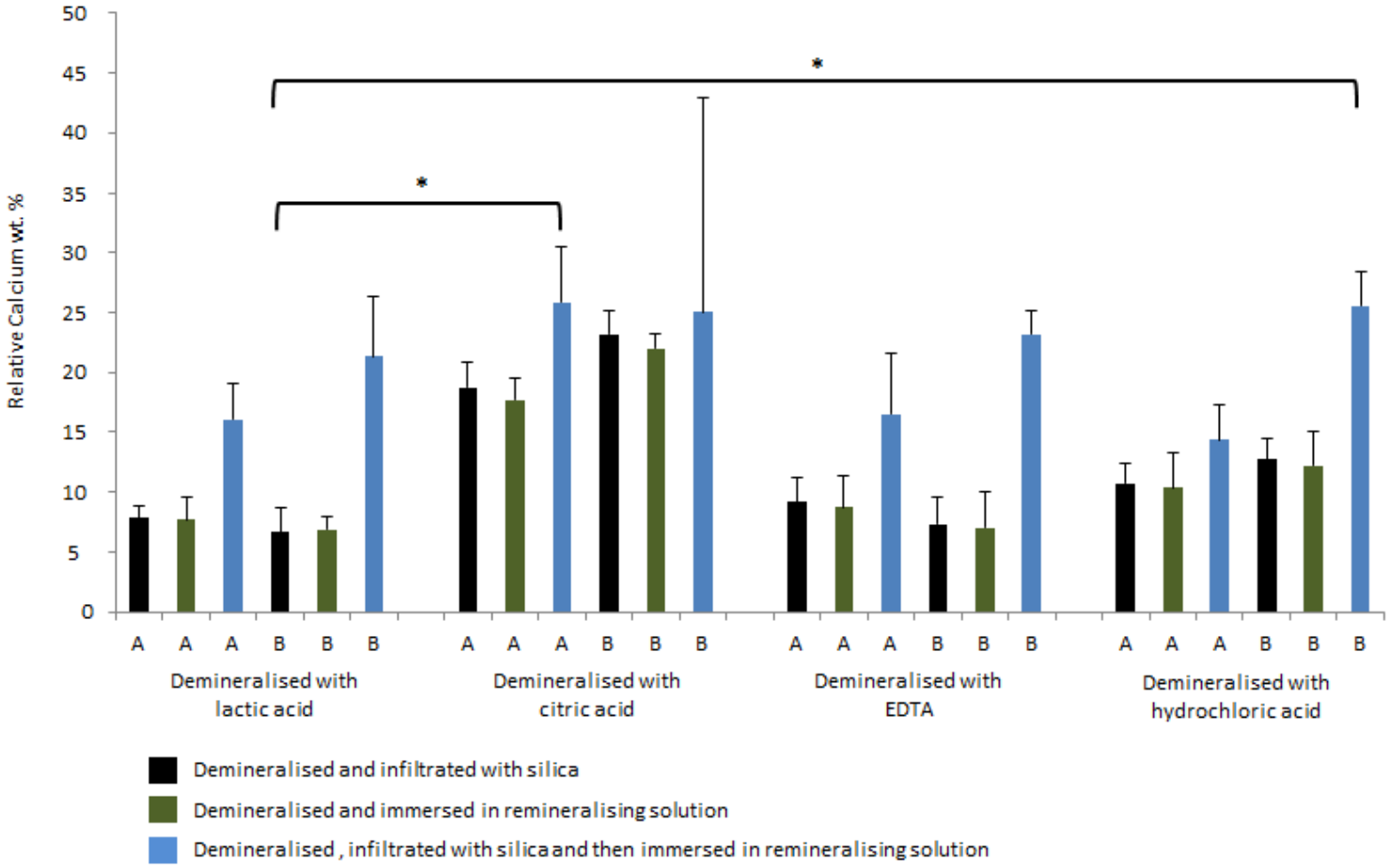

Figure 7.7: Relative calcium wt. % (Kα, 3.692 keV) of dentine specimens (surfaces A and B) using EDS data. Statistical analysis using Kruskal-Wallis H test with Dunn's (1964) post-hoc analysis and Bonferroni's adjustment. No significant difference between variables unless indicated.

* = significant difference ($p<0.05$) between the two variables indicated by the bracket. A = surface A; B = surface B

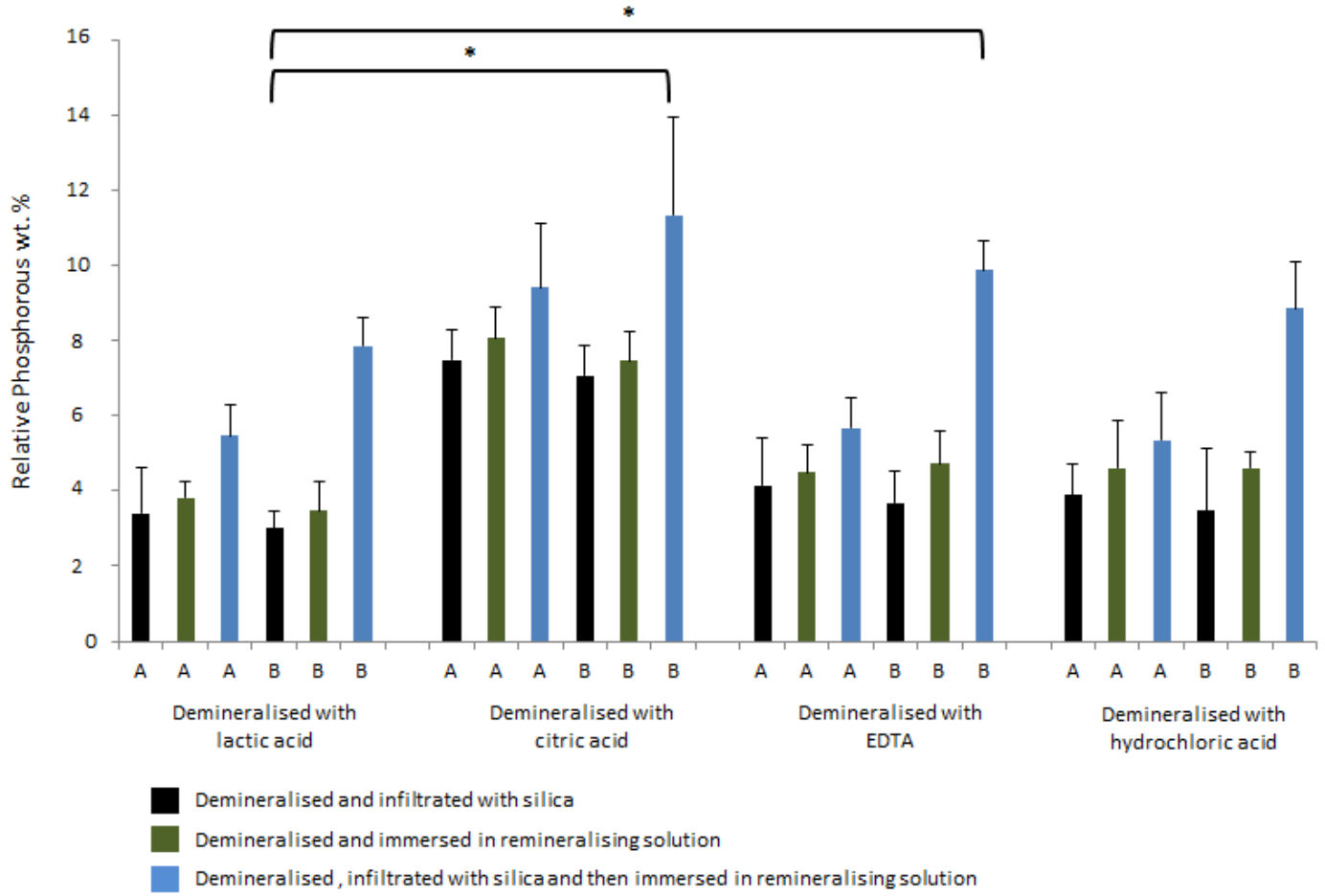

Figure 7.8: Relative phosphorous wt.% (Kα, 2.010 keV) of dentine specimens (surfaces A and B) using EDS data. Statistical analysis using Kruskal-Wallis H test with Dunn's (1964) post-hoc analysis and Bonferroni's adjustment. No significant difference between variables unless indicated.

***** = significant difference ($p<0.05$) between the two variables indicated by the bracket. A = surface A; B = surface B

7.5 Discussion

This study used DLS to investigate whether silica nanoparticles had an affinity for calcium ions and used EDS analysis in an attempt to reveal the levels of silica, calcium and phosphorous on the dentine specimen surfaces. The results allowed comparisons to be made between specimens and enabled variables such as infiltration times and solution concentrations to be compared.

7.5.1 Negatively charged silica nanoparticles seemed to infiltrate and adhere beneath the surface of partially demineralised dentine specimens

(a) Silica nanoparticles infiltrated all demineralised dentine models

Results from Chapter 6 revealed that silica nanoparticles appeared to bind to collagen type I and III fibres from porcine membranes, suggesting that silica nanoparticles may also bind to human dentine collagen, which is also composed of type I and III collagen fibres. However, the organic dentine network contains many non-collagenous proteins (NCPs) as well as some residual mineral and it was unclear whether these additional components in the dentine would affect the silica nanoparticle adherence. Previous to this work, silica nanoparticle infiltration had only been tested on fully demineralised dentine and did not consider the effect of any remaining mineral (Besinis *et al*., 2012). Furthermore, as results from Chapter 5 showed that each demineralising agent had a different effect on the dentine collagen structure, it was speculated that certain demineralising agents, such as citric acid, might damage the collagen network to a degree that would prevent successful infiltration.

Therefore, this study used a range of partially demineralised dentine specimens (produced as described in section 4.2.1 and as characterised in Chapter 5), which were infiltrated with silica nanoparticle solutions and then flushed with water in an attempt to dislodge any non-adhered nanoparticles. The results (displayed in Figures 7.3 and 7.4) demonstrated that infiltration occurred in partially demineralised dentine regardless of the type of

demineralising agent used, suggesting that silica nanoparticles may be able to infiltrate demineralised dentine *in vivo*.

(b) Silica nanoparticles infiltrated beneath the dentine surface and appeared to adhere to collagen fibres

Previous studies outside of this thesis have suggested that particle size is the significant factor when infiltrating solutions through dentine and the colloidal behaviour of the particles can further facilitate the infiltration ability (Forsback *et al*., 2004, Jeong *et al*., 2006, Lee *et al*., 2012, Eglin *et al*., 2006). In particular, Besinis *et al* demonstrated that nanoparticles with a diameter of 10-15 nm were small enough to pass through the collagen matrix (intra-tubular and inter-tubular areas) facilitated by their colloidal properties (Besinis *et al*., 2012).

In order to assess whether the silica nanoparticles had indeed infiltrated beneath the dentine surface, the outer surface of the infiltrated specimen was removed so that surface A could be analysed (as demonstrated in Figure 7.2). It was decided that at least 1 mm of the top surface would need to be removed to reflect recent studies which have indicated that leaving behind at least 1 mm of dentine during cavity preparation is enough to reduce the likelihood of bacterial toxins reaching the pulp by 90 % and therefore, increasing the likelihood survival of the tooth (Hernandez and Marshall, 2014, About *et al*., 2001, Murray *et al*., 2003).

EDS results (Figures 7.3 and 7.4) showed that silica nanoparticles were present on surface A, after infiltrating beneath the dentine surface, and had remained there after the subsequent flushing with water, suggesting that they had adhered to the dentine in some way. This also indicated that the nanoparticles would not become dislodged when the dentine specimens were exposed to the remineralising solution in the next stage of the remineralisation experiment. As described in Chapter 6, it was unclear exactly how the nanoparticles adhered to the collagen but it is possible that it was *via* ionic and/or hydrogen bonding.

(c) Limitations

Along with the limitations of using EDS on dentine specimens (described in section 5.5.1), the consequences of cutting the infiltrated specimens needs to be considered. As shown on Figure 7.2, although the blade was positioned 1 mm beneath the surface, due to the thickness of the blade and human error in measuring using a ruler, calipers were used to check that between 0.8 and 1.2 mm had been removed from the surface. Surface A was then polished, removing more of the surface. Therefore, although the exact distance between surface A and B is unknown, it is approximately 1 mm, but at least 0.8 mm minimum.

Creating surface A may have created a smear layer, as discussed in section 5.5.1; the smear layer is believed to be composed of inorganic and organic material. The presence of a smear layer may have concealed some of the silica nanoparticles and therefore it could have affected the EDS results. The surface was polished in an attempt to flatten the surface and remove some of the smear layer and there was continuous water flowing during the polishing process to prevent any contamination from the silicon carbide paper.

Figure 7.4 shows that silica was found on the non-infiltrated specimens at surface A. The source of this silica may have been contamination from the sample preparation (silicon carbide paper) although this is unlikely due to the water flushing. Furthermore, it may be that some traces of silica were present in the teeth from silica-containing toothpastes. It must also be considered that EDS data is less accurate when results are less than 2 % and therefore, more data is needed to investigate the presence of silica on the control specimens. Nevertheless, there was a still a significant difference in silica when comparing non-infiltrated and infiltrated specimens.

Another limitation of this study was that the protocol has never been used before meaning that the optimum parameters (e.g. silica concentration or infiltration time) have not yet been defined. SEM analysis from Chapter 6 showed that 1 wt. % silica solutions were able to infiltrate and adhere to collagen fibres evenly and therefore, were also used for the dentine infiltration/remineralisation experiments in Chapter 7. Since the protocols in

this study are novel and have not been undertaken before, it was decided that 0.1 wt. % silica solutions should also be used to investigate whether the two silica concentrations would produce different results and thereby not only help to find the optimum parameters for future studies but could also help to decipher the mechanism involved in silica adherence and infiltration. As expected, when dentine was infiltrated with the 1 wt. % silica solution, there was statistically significant increase in silica on the surface compared to the 0.1 wt. % solution, however, this increase was not 10 fold, as expected. This suggested that although increasing the silica solution concentration led to more silica on the collagen surface, silica could only bind when there were available binding sites.

In terms of infiltration time, two different infiltration periods were explored; 24 hours (used by Besinis *et al*) and 1 minute (more clinically appropriate). There was little difference in the amount of silica measured by EDS regardless of the infiltration times. This suggested that the silica nanoparticles had strong colloidal properties allowing immediate infiltration through the pores in dentine and rapid adherence to the collagen fibres.

7.5.2. Negatively charged silica nanoparticles interacted with calcium ions

Due to the law of electromagnetic attraction, it is understood that particles of the same charge are repulsed by one another, and particles of opposite charges are attracted to one another. Therefore, it can be assumed that the negatively charged silica nanoparticles may attract positively charged calcium ions (Ca^{2+}) and the positively charged (Al_2O_3 coated) silica nanoparticles may attract negatively charged phosphate ions (PO_4^-). There is evidence that suggests that calcium ions are much more important than phosphate ions for successful remineralisation (Lynch, 2004, Lynch and ten Cate, 2005, Tanaka and Kadoma, 2000). Therefore, this study focused on the use of negatively charged silica in the hope that they could attract calcium ions from a remineralising solution into the demineralised dentine network.

To explore this, DLS was employed to monitor the zeta potential of the silica nanoparticles. The magnitude of the zeta potential indicates the degree of repulsion between particles in dispersion. When the zeta potential is very positive or very negative (± 40 to 60 mV) this confers stability (no aggregation). When the potential is low (± 0 to 30 mV), the colloids may coagulate or flocculate (Greenwood, 2003). By observing Figure 7.6 it is clear that the negatively charged silica nanoparticles were in a colloidal suspension with a good level of repulsion when pH was above 5. When the pH was lower than 5, the zeta potential was very small and the particles were less stable. The particles were most stable at a high pH of 11-12, the pH of unmodified silica solutions. The results of the zeta potential measurements presented in Figure 7.6 illustrate that the isoelectric point was pH 2.5, when the silica solutions no longer possess colloidal properties. These findings are significant for this study because it indicated that silica solutions would need to be administrated at a pH above 5 in order to be stable, although they are most stable at pH 11, these conditions would be too damaging to the oral mucosa.

Sodium chloride dissociates into mono-valent sodium ions (Na+) when in solution. When sodium chloride was added to the silica solution, the silica particles had a smaller zeta potential. This indicated that the sodium ions were being adsorbed onto the silica surface and thereby making the surface more positive (or less negative). However, when calcium chloride was added to the silica nanoparticles, their surface zeta potential was even more affected. Calcium chloride dissociates into divalent calcium ions (Ca^{2+}) in solution and the valency appears to have had a direct effect on the double layer of the silica particles. Divalent ions compress the double layer to a greater extent than monovalent ions and therefore, a small addition of divalent calcium ions had a much greater effect on the surface charge of the silica particles than the same addition of monovalent sodium ions. The calcium ions, therefore, produced a smaller double layer and smaller zeta potential. This information about the changes in zeta potential demonstrated that the negatively charged silica nanoparticles had a high affinity for the

calcium ions. This may explain the mechanism behind the remineralisation witnessed by Besinis *et al* (Besinis *et al*., 2014b).

7.5.3. Remineralisation

In this current study, EDS was used to measure the calcium and phosphorous in dentine specimens after exposure to a remineralising solution for 4 weeks following a 1 minute infiltration with 1 wt. % nanoparticle solutions. EDS analysis has been used many times to monitor mineral levels for indicating surface mineralisation (Arnold *et al*., 2001, Earl *et al*., 2011, Besinis *et al*., 2012, Besinis *et al*., 2014b). A remineralisation period of 4 weeks was employed because this seems to be a minimum duration for remineralisation of partially demineralised dentine according to literature (Vollenweider *et al*., 2007, Shibata *et al*., 2008).

Figure 7.7 (showing calcium results) and Figure 7.8 (showing phosphorous results) revealed no significant difference between surfaces A and B (refer to Figure 7.2 for positioning of surfaces A and B). For the demineralised dentine group (black bars) this was surprising because it would be expected that the outer surface (B) would have had lower levels of mineral than the inner surface (A). To investigate this further, a cross-section analysis of the specimen could be performed to ascertain if there was a gradient from the outer surface to the inner core. It was also surprising that there was no significant difference in levels of calcium and phosphorous between surfaces A and B that had been immersed in a remineralising solution (green bars). It would have been expected that the outer surface (B) would have had higher levels of calcium and phosphorous than the inner surface (A) because the outer surface (B) was exposed to the remineralising solution.

Figures 7.7 and 7.8 also revealed no significant difference between the specimens not treated with a remineralising solution (black bars) and specimens immersed in a remineralising solution (green bars). This suggested that no additional calcium or phosphorous from the remineralising solution was deposited on the surface. This may have been due to the constant agitation that the specimens were exposed to, which may have dislodged the ions.

However, Figures 7.7 and 7.8 indicated an increase in calcium and phosphorous when specimens were pre-treated with silica infiltration before immersion in the remineralising solution (blue bars) but this was not verified statistically. A non-parametric statistical analysis test was performed because not all of the variables had normally distributed data. The non-parametric test is less powerful that parametric tests, however, assessing the normality of data when N is <5 is also less effective. More data is required to increase the N number so that normality can be assessed more effectively, which may allow more powerful statistics to be used. The results also indicated that there was no significant difference between surfaces A and B that had been pre-infiltrated with silica and immersed in a remineralising solution. This suggests that the silica nanoparticles had infiltrated beneath the surface and were able to facilitate the movement of calcium and phosphrous beneath the surface too. More powerful statistical analysis would be needed to verify this claim.

By visual assessment, the bar charts suggest that the citric acid group had a lower increase of calcium and phosphorous when exposed to infiltration and remineralisation solution. Although this was not confirmed statistically, it may suggest a difference with this specimen group, which could be connected to the difference seen with the citric acid group in Chapter 5. Results from chapter 5 suggested that citric acid caused the most denatured collagen, which may have prevented the calcium and phosphorus from attaching in some way. However, these are just speculations and more data would be required in order to draw firm conclusions.

Previous studies by Besinis *et al* have used Micro-CT, TEM, SEM and EDS to demonstrate that infiltration with silica nanoparticles can lead to remineralisation levels (Besinis *et al*., 2014b).

7.5.4. Summary

This study has shown that the concentration of the nanoparticle solutions and exposure time in the infiltration solution can be reduced and still show significantly increased levels of silica approximately 1 mm beneath the surfaces of partially demineralised dentine models. Furthermore, this study

has shown, by dynamic light scattering, that there is an interaction between the negatively charged silica nanoparticles and calcium ions. More data is required to test the reproducibility of the results.

Although more data is required to verify any claims, this study has indicated that pre-infitlration with a silica nanoparticle solution may lead to an increase in calcium and phosphorous beneath the surface.

7.6 Future work

- This study attempted to measure silica, calcium and phosphorous 1mm beneath the surface but as described in section 7.5.1 (d), it is likely that the measurement was actually taken from the range of 0.8 to 1.2 mm under the surface. Future work should focus on measuring the infiltration at various depths more accurately; this may involve producing cross sections of dentine.
- The limitations of EDS are explained in section 5.5.1, concluding that although EDS may indicate general trends in levels of silica, calcium and phosphorous, it cannot be used alone to quantify the amounts. Therefore, future work should focus on employing other techniques (for example, phosphate assay, transmission electron microscopy and micro or nano-CT) to complement EDS analysis.
- More experimental repeats for the remineralisation study are necessary in order to increase the quality of the statistical analysis described in section 7.5.3.
- A range of silica solution concentrations and infiltration times should be investigated in order to explore the optimum parameters for the remineralisation process. It would be beneficial to assess the levels of remineralisation at other time points (rather than just at 4 weeks) to investigate when the first signs of remineralisation are observed.
- To compare levels of calcium and phosphorous in specimens that are agitated in remineralisation solution with specimens that are not agitated, to explore whether agitation prevents calcium and phosphorous from adhering to the non-infiltrated specimens. This would help to refine and optimise the remineralisation protocol.

- Finally, a range of other nanoparticles solutions could be tested (for example, zinc, silver, copper which have all proven to have antibacterial properties), as well as a range of remineralisation solutions (for example, those containing fluoride or increased calcium which may encourage more remineralisation) as well as testing remineralistion on natural caries and erosion specimens.

8. General Discussion

Nanotechnology is increasingly considered as a potential way of enhancing remineralisation beneath the dentine surface. This study investigated the use of colloidal silica nanoparticle solutions to infiltrate partially demineralised dentine and act as a scaffold to encourage remineralisation; this was developed from the proof of concept study undertaken by Besinis *et al* (Besinis *et al*., 2014b).

All *in vitro* remineralisation studies require a demineralised dentine model but there are many acids from different origins (bacteria, gastric and diet) that are involved in caries and/or erosion. There is an assumption that all types of acids affect dentine in the same way and consequently, most studies are not very discerning of the type of acid used. This has led to a standard practice of using laboratory acids (e.g. formic acid) or chelating agents (e.g. EDTA) to create artificial demineralised dentine models with the presumption that they have the same effect as acids involved in caries or erosion (Eggert and Germain, 1979, Besinis *et al*., 2014a, Besinis *et al*., 2014b, Kim *et al*., 2011, Habelitz *et al*., 2002, Wang and Spencer, 2002).

This current study used a range of demineralising agents: bacterial (lactic acid), dietary (citric acid, phosphoric acid, acetic acid), gastric (hydrochloric acid) and laboratory (EDTA and formic acid) acids (properties displayed in Table 5.1) to create a variety of partially demineralised dentine models. A number of characterisation techniques were employed to compare the dentine structure and composition of these models to investigate whether the demineralising agents affected dentine in the same way, previously never considered.

Energy dispersive spectroscopy (EDS), a phosphate assay and x-ray fluorescence (XRF) were used to indicate the levels of calcium and phosphorous following demineralisation; whilst x-ray diffraction (XRD) was utilized to characterise the remnant mineral and micro-hardness test to compare the levels of mineralisation. There is increasing evidence that remnant mineral is essential for further remineralisation to occur (Tay and

Pashley, 2008, Tay and Pashley, 2009), therefore it is important to characterise and assess the remnant mineral in a variety of artificially demineralised dentine models; this has not been explored before.

The results indicated that each demineralising agent had a unique effect on the mineral levels, which was a significant finding because it demonstrated that the choice of demineralising agent is important when considering an artificial caries or erosion model. The XRD data also demonstrated that there were no other calcium phosphate phases, apart from hydroxyapatite, present during demineralisation with any of the acids; this is the first time that the phase composition of the remnant mineral has been monitored and it has provided a better understanding of the demineralisation process.

This study also considered the mechanism by which the demineralising agents work, which has been overlooked until now. Chelating agents, strong acids and weak acids are often used without considering their different demineralising mechanisms. Although this study did not identify significant differences regarding the demineralising mechanism or acid type, it did highlight that not all acids affect dentine in the same way. This has highlighted the need to characterise demineralised dentine models in terms of the collagen structure as well as the mineral content. The results of this study have further explained the process of dentine demineralisation during caries or erosion *in vivo*, where a range of these acids attack the tooth surface at once.

It is essential that demineralised dentine models have an intact collagen structure to replicate the outer zones of carious lesions which often contain undamaged collagen (Deyhle *et al*., 2011), as well as increasing evidence showing remineralisation to require an intact collagen matrix in order to occur (Gower, 2008, Olszta *et al*., 2003b, Forsback *et al*., 2004, Vollenweider *et al*., 2007). Despite this, the level of collagen denaturation in artificial demineralised dentine models has never been investigated before. In this study, scanning electron microscopy (SEM) was employed to observe the dentine collagen network following demineralisation and the hydroxyproline (Hyp) assay to quantify any collagen that had denatured. The results demonstrated that all of the demineralising agents caused some degree of

collagen denaturation, citric acid causing the most damage. These major findings suggest the presence of a denaturation mechanism in the absence of bacteria. As discussed in section 2.4.3.1, there are various possible ways in which dentine becomes damaged during caries; but there has been no evidence to date to indicate the dentine collagen becomes denatured by a demineralising agent *in vitro.* Therefore, this study implies there is another method of denaturation, which may replicate the findings by Zhang *et al* (Zhang *et al*., 1998) that suggest a low pH can alter the tertiary structure of collagen. Additionally, the assumption that higher degrees of demineralisation result in higher levels of collagen denaturation was dismissed; each acid affected the collagen structure and the mineral composition independently.

For the first time, the hypothesis that all acids used for demineralising dentine have the same effect and this is comparable to caries or erosion acids, was proven to be untrue. Previous proof of concept studies by Besinis *et al* used fully demineralised dentine models using formic acid. However, results from this current study suggest that formic acid does not behave in the same way as acids involved in caries or erosion, and therefore, rationalises the theory that a range of more clinically similar acids should be used in remineralisation studies. Furthermore, many studies use fully demineralised dentine models, but it is speculated that partially demineralised dentine models are more clinically similar due to the importance of remnant mineral found in outer carious zones (Tay and Pashley, 2009, Tay and Pashley, 2008).

Therefore, the results from the dentine characterisation study suggest that careful consideration must be taken when choosing a demineralising agent for the production of an artificial caries or erosion model. Demineralised dentine models should be well characterised, in terms of organic and inorganic components, before being used in remineralisation studies. For this study, demineralised dentine models did not include components such as buffers or proteins. This was to reduce the number of variables and allow comparisons to be made between the models, which leaves opportunities for further research.

The study proceeded to characterise the Ludox® silica nanoparticles using dynamic light scattering (DLS) and transmission electron microscopy (TEM), results indicating silica nanoparticles had a 15 nm diameter and were in a colloidal suspension when pH was above 5. Although the initial proof of concept study by Besinis *et al* (Besinis *et al*., 2014b) used positively charged silica nanoparticles (Al_2O_3 coated), this current study took a new approach. It focused on negatively charged silica because they are available without any surface modification, making analysis more reliable; and, due to the law of electrostatic attraction, they are more likely to attract calcium ions. It was important to entice calcium ions from the remineralising solution, rather than phosphate ions, because there is growing evidence that increasing calcium concentrations is much more effective than increasing phosphate concentrations (Lynch, 2004, Lynch and ten Cate, 2005, Tanaka and Kadoma, 2000).

It was also important that the nanoparticles adhered to the dentine collagen so that they are not dislodged during exposure to the remineralising solution. Although Besinis *et al* speculate that the silica nanoparticles do attach to the dentine collagen (Besinis *et al*., 2012), this assumption was based on TEM observation and is inconclusive. In this study however, Biogide® collagen membranes (Geistlich, Manchester, UK) were utilised instead of human dentine collagen because it enabled the interaction between the two surfaces to be monitored without the presence of other components. Furthermore, dentine specimens have huge variability between both the different teeth and the different parts of the tooth; the variability between specimens reduced by using Biogide® membranes. A novel infiltration method was designed using a syringe pump, involving flushing the pre-infiltrated membranes with water in an attempt to dislodge any non-adhered nanoparticles. Nanoparticle adherence was determined by weighing the membranes before and after infiltration. The infiltration method pioneered in this study was successful for monitoring the infiltration of negatively charged silica nanoparticles and may have potential use for other studies in the future.

A concern with undertaking this type of study in an *in vitro* scenario is the need to treat and prepare the specimens in a particular manner to enable

effective characterisation; a process that does not occur *in vivo*. Principally, it was considered that chemically fixing the specimens for EDS, SEM and TEM may affect particle infiltration and remineralisation. Using the novel infiltration method, pioneered in this study, the effect of chemical fixatives and demineralising agents on the nanoparticle adherence could also be tested. Fixed and non-fixed specimens were compared and it was verified that chemical fixation did not affect the adherence of the nanoparticles, enabling characterisation techniques to be used without concern for unintentionally altering the true results. This is the first study to not only consider the effect of the fixatives during remineralisation experiments, but to also demonstrate that chemical fixation with glutaraldehyde did not affect the true results, opening further research opportunities to test other chemical fixatives. This is a significant addition to the field because most remineralisation studies rely upon chemical fixation.

Although previous studies have demonstrated that infiltrated silica nanoparticles may encourage remineralisation (Besinis *et al*., 2014b), the chemistry of the nanoparticle-remineralising ion interaction has never been investigated. This study used DLS to monitor the zeta potential of the negatively charged silica nanoparticles at various pH conditions. This is a method used numerous times previously to monitor interactions with other nanoparticles (Zhang *et al*., 2008, Patil *et al*., 2007, Wu *et al*., 2015, Puddu and Perry, 2012). It was demonstrated that when calcium ions or sodium ions were added to the solution, the surface of the silica nanoparticles became much less negative, indicating the ions were bound to the silica surfaces. It was also evident that calcium ions affected the silica surface more than sodium ions because of their bivalency, which further demonstrates the surface-surface interaction was occurring. Therefore, these findings add knowledge to the field by providing a potential mechanism behind some of the results found by Besinis *et al.*

This remineralisation method has many potential clinical applications. As well as providing sub-surface remineralisation for deep dentine caries, severe erosion/hypersensitivity and dentine root caries, this method may particularly help with the management of caries in developing communities

that do not have access to sophisticated dental treatment. Infiltration with silica nanoparticles could be a quick and cheap way to facilitate remineralisation to these communities.

9. Conclusions

Tooth tissue demineralisation is a major challenge in dentistry, and nanoscale materials have considerable potential as a basis for a therapeutic intervention. Current knowledge at the time of starting this study was insufficient to support real clinical progress, and the aims of this research was therefore to investigate remineralisation with silica nanoparticles as described in full in Chapter 3. The conclusion of this research are summarised below.

9.1 Characterisation of Dentine

- **It was concluded that dentine tissue models vary depending on the type of agent selected to induce demineralisation.** There are two broad mechanisms by which demineralisation can occur: (i) chelation of calcium ions from the hydroxyapatite crystal, and (ii) removal of phosphate ions from hydroxyapatite *via* dissociated hydrogen ions. This study did not show a direct link between type of mechanism and degree of demineralisation but it did show significant differences in mineral content and collagen denaturation of dentine when exposed to different demineralising agents. This research has established that laboratory acids do not necessarily affect dentine in an identical way to the acids involved in caries or erosion and consideration must be taken when choosing a demineralising agent for dentine research studies.
- **It was also concluded that, regardless of the type of demineralising agent used, all demineralised dentine models contained some denatured collagen in the absence of bacterial collagenases. It is likely that the low pH environment activates MMPs and cathepsins or alters the tertiary structure using the mechanism suggested by Zhang *et al* (Zhang *et al*., 1998).** There was no correlation between types of demineralising agent i.e. weak acid, strong acid or chelating agent, and the levels of denatured collagen. Also, there was no correlation between the degrees of

demineralisation and the levels of collagen denaturation. It is recommended that great care be taken in choosing a specific acid to create a demineralised model.

9.2 Interaction between Nanoparticles and Collagen

- **It was discovered that silica nanoparticles infiltrated and were able to adhere to fixed and non-fixed collagen networks, demonstrating that chemical fixation does not alter the remineralisation potential of silica nanoparticles.** Silica nanoparticles appeared to adhere to the collagen fibres, creating a nanoparticle-collagen complex or scaffold that remained in place during exposure to the remineralising solution. Although glutaraldehyde removed positive charges from collagen lysine and hydroxylysine residues, this did not affect the infiltration or adherence of negatively charged silica nanoparticles.
- **It was also demonstrated that both positively charged and negatively charged silica nanoparticles were able to infiltrate and adhere to both acid treated and non-acid treated collagen.** There was no significant difference between the two types of silica nanoparticles (positive surface charge versus negative surface charge) suggesting that collagen had negative and positive binding sites. The exposure of collagen to an acid did not affect the adhering or infiltrating ability of the silica nanoparticles, suggesting that this proof of concept process could be transferred to natural demineralised dentine.

9.3 Remineralisation of Dentine

- **It was concluded that negatively charged silica nanoparticles may facilitate the remineralisation of partially demineralised dentine.** Negatively charged silica nanoparticles were able to infiltrate approximately 1 mm beneath the partially demineralised dentine surface, regardless of the type of demineralising agent used. In addition, the infiltration time was reduced from 24 hours to 1 minute

without significantly affecting the levels of silica, and this substantial reduction in time delay makes the process clinically viable. Dynamic light scattering demonstrated that negatively charged silica nanoparticles had a high affinity for calcium ions, suggesting that infiltrated dentine specimens may become remineralised beneath the surface after exposure to a remineralising solution.

To summarise, this research has made a significant contribution to the knowledge of remineralisation of dentine by nanoscale silica. The thesis has also provided a far greater understanding of models of dentine demineralisation. The new knowledge reported here will contribute to the clinical and commercial search for new therapies for this dental challenge.

10. Future Work

The work carried out in this study demonstrated that a colloidal suspension of silica nanoparticles can be used to create nucleation sites in the collagen matrix of partially demineralised dentine and in this way encourage sub-surface dentine remineralisation. Further work is required in the following areas:

10.1 Characterisation of Demineralised Dentine

A detailed list of future work ideas are listed in section 5.6. The future work mainly focuses on addressing the limitations of the study described in section 5.5.1. This involves increasing the variety of techniques to overcome limitation of sample preparation such as sample surface polishing or sample dehydration. One of the other main limitations of the study was the variability of the dentine specimens. The future work section in 5.6 describes increasing the number of repeats in order to improve statistical analysis and to assess the regional differences of dentine (for example between dentine near the enamel and dentine near the pulp) as well as characterising the microstructure of dentine in more detail (for example, considering the number and orientation of tubules).

Future work also involes using a wider range of variables, for example, different fixatives, combinations of demineralising agents, buffers and remineralisation solutions (for example, including fluoride).

10.2 Infiltration of Nanoparticles through Collagen

A detailed list of future work ideas are listed in section 6.6. Future work focuses on testing a range of protocol parameters (such as infiltration flow rate and silica solution concentrations) in order to optimise the syringe pump experiment. It was also noted that the Hyp Assay could be used to identify whether the collagen membranes were denatured by the demineralising agent. Furthermore, the use of other collagen membranes was suggested in order to find the most appropriate dentine collagen model.

10.3 Remineralisation of Dentine

A detailed list of future work ideas are listed in section 7.6. As with Chapter 5, future work mainly focuses on addressing the limitations of the study. For example; using other techniques as well as EDS to measure infiltration and remineralisation, increasing the number of repeats to enable more powerful statistical analysis and measuring levels of infiltration and remineralisation on a cross section of the specimen in order to accurately measure the depth. Other future work ideas include testing a range of parameters (for example, remineralisation time points), other nanoparticles, other remineralisation solutions and testing on natural caries and erosion.

11. References

AAS, J. A., GRIFFEN, A. L., DARDIS, S. R., LEE, A. M., OLSEN, I., DEWHIRST, F. E., LEYS, E. J. & PASTER, B. J. 2008. Bacteria of dental caries in primary and permanent teeth in children and young adults. *Journal of Clinical Microbiology,* 46**,** 1407-1417.

ABOUT, I., MURRAY, P. E., FRANQUIN, J. C., REMUSAT, M. & SMITH, A. J. 2001. The effect of cavity restoration variables on odontoblast cell numbers and dental repair. *Journal of Dentistry,* 29**,** 109-117.

ADDY, M. 2005. Tooth brushing, tooth wear and dentine hypersensitivity - are they associated? *International Dental Journal,* 55**,** 261-267.

ADDY, M., ABSI, E. G. & ADAMS, D. 1987. Dentin hypersensitivity - the effects invitro of acids and dietary substances on root-planed and burred dentin. *Journal of Clinical Periodontology,* 14**,** 274-279.

AGGELI, A., BELL, M., BODEN, N., KEEN, J. N., KNOWLES, P. F., MCLEISH, T. C. B., PITKEATHLY, M. & RADFORD, S. E. 1997a. Responsive gels formed by the spontaneous self-assembly of peptides into polymeric beta-sheet tapes. *Nature,* 386**,** 259-262.

AGGELI, A., BELL, M., BODEN, N., KEEN, J. N., MCLEISH, T. C. B., NYRKOVA, I., RADFORD, S. E. & SEMENOV, A. 1997b. Engineering of peptide beta-sheet nanotapes. *Journal of Materials Chemistry,* 7**,** 1135-1145.

AGGELI, A., FYTAS, G., VLASSOPOULOS, D., MCLEISH, T. C. B., MAWER, P. J. & BODEN, N. 2001. Structure and dynamics of self-assembling beta-sheet peptide tapes by dynamic light scattering. *Biomacromolecules,* 2**,** 378-388.

AGRAWAL, N., SHASHIKIRAN, N. D., SINGLA, S., RAVI, K. S. & KULKARNI, V. K. 2015. Effect of remineralizing agents on surface microhardness of primary and permanent teeth after erosion. *Journal of dentistry for children (Chicago, Ill.),* 81**,** 117-21.

AIMUTIS, W. R. 2004. Bioactive properties of milk proteins with particular focus on anticariogenesis. *Journal of Nutrition,* 134.

ALVARES, K., KANWAR, Y. S. & VEIS, A. 2006. Expression and potential role of dentin phosphophoryn (DPP) in mouse embryonic tissues involved in epithelial-mesenchymal interactions and branching morphogenesis. *Developmental Dynamics,* 235.

AMAECHI, B. T. & HIGHAM, S. M. 2005. Dental erosion: possible approaches to prevention and control. *Journal of Dentistry,* 33**,** 243-252.

ANDERSSON, O. H. & KANGASNIEMI, I. 1991. Calcium-phosphate formation at the surface of bioactive glass invitro. *Journal of Biomedical Materials Research,* 25.

ARNOLD, W. H., KONOPKA, S. & GAENGLER, P. 2001. Qualitative and quantitative assessment of intratubular dentin formation in human natural carious lesions. *Calcified Tissue International,* 69**,** 268-273.

ASSEV, S. & ROLLA, G. 1986. FURTHER-STUDIES ON THE GROWTH-INHIBITION OF STREPTOCOCCUS-MUTANS OMZ-176 BY XYLITOL. *Acta Pathologica Microbiologica Et Immunologica Scandinavica Section B-Microbiology,* 94.

ATALAY, S., MA, Y. & QIAN, S. 2014. Analytical model for charge properties of silica particles. *Journal of Colloid and Interface Science,* 425**,** 128-130.

AYAD, F., AYAD, N., ZHANG, Y. P., DEVIZIO, W., CUMMINS, D. & MATEO, L. R. 2009. Comparing the efficacy in reducing dentin hypersensitivity of a new toothpaste containing 8.0% arginine, calcium carbonate, and 1450 ppm fluoride to a commercial sensitive toothpaste containing 2% potassium ion: an eight-week clinical study on Canadian adults. *The Journal of clinical dentistry,* 20.

BADER, J. D., VOLLMER, W. M., SHUGARS, D. A., GILBERT, G. H., AMAECHI, B. T., BROWN, J. P., LAWS, R. L., FUNKHOUSER, K. A., MAKHIJA, S. K., RITTER, A. V., LEO, M. C. & GRP, X. A. C. R. 2013. Results from the Xylitol for Adult Caries Trial (X-ACT). *Journal of the American Dental Association,* 144**,** 21-30.

BAIRATI, A., TARELLI, L. T. & PETRUCCI.MG 1972. Birefringence of collagen fibres after glutaraldehyde treatment. *Journal of Submicroscopic Cytology,* 4**,** 89-&.

BANERJEE, A., KIDD, E. A. M. & WATSON, T. F. 2000a. Scanning electron microscopic observations of human dentine after mechanical caries excavation. *Journal of Dentistry,* 28**,** 179-186.

BANERJEE, A., PAOLINELIS, G., SOCKER, M., MCDONALD, F. & WATSON, T. F. 2008. An in vitro investigation of the effectiveness of bioactive glass air-abrasion in the 'selective' removal of orthodontic resin adhesive. *European Journal of Oral Sciences,* 116**,** 488-492.

BANERJEE, A., WATSON, T. F. & KIDD, E. A. M. 2000b. Dentine caries excavation: a review of current clinical techniques. *British Dental Journal,* 188**,** 476-482.

BARBOUR, M. E. & REES, G. D. 2006. The role of erosion, abrasion and attrition in tooth wear. *The Journal of clinical dentistry,* 17**,** 88-93.

BARTLETT, D. 2006. Intrinsic causes of erosion. *Monographs in Oral Science,* 20**,** 119-139.

BECKER, J., SCHUPPAN, D., BENZIAN, H., BALS, T., HAHN, E. G., CANTALUPPI, C. & REICHART, P. 1986. Immunohistochemical distribution of collagens types iv, v, and vi and of pro-collagens types i and iii in human alveolar bone and dentin. *Journal of Histochemistry & Cytochemistry,* 34**,** 1417-1429.

BECKER, M. R., PASTER, B. J., LEYS, E. J., MOESCHBERGER, M. L., KENYON, S. G., GALVIN, J. L., BOCHES, S. K., DEWHIRST, F. E. &

GRIFFEN, A. L. 2002. Molecular analysis of bacterial species associated with childhood caries. *Journal of Clinical Microbiology,* 40**,** 1001-1009.

BEELEY, J. A., YIP, H. K. & STEVENSON, A. G. 2000. Chemochemical caries removal: a review of the techniques and latest developments. *British Dental Journal,* 188**,** 427-430.

BEERENS, M. W., VAN DER VEEN, M. H., VAN BEEK, H. & TEN CATE, J. M. 2010. Effects of casein phosphopeptide amorphous calcium fluoride phosphate paste on white spot lesions and dental plaque after orthodontic treatment: a 3-month follow-up. *European Journal of Oral Sciences,* 118**,** 610-617.

BEIER, G. & ENGEL, J. 1966. Renaturation of soluble collagen . Products formed at different temperatures. *Biochemistry,* 5**,** 2744-&.

BELLA, J., EATON, M., BRODSKY, B. & BERMAN, H. M. 1994. Crystal-structure and molecular-structure of a collagen-like peptide at 1.9-angstrom resolution. *Science,* 266**,** 75-81.

BESINIS, A. 2010. *The use of Nanoparticles as a Strategy for Dentine Remineralisation.* PhD, University of Sheffield.

BESINIS, A., DE PERALTA, T. & HANDY, R. D. 2014a. The antibacterial effects of silver, titanium dioxide and silica dioxide nanoparticles compared to the dental disinfectant chlorhexidine on Streptococcus mutans using a suite of bioassays. *Nanotoxicology,* 8**,** 1-16.

BESINIS, A., DE PERALTA, T., TREDWIN, C. J. & HANDY, R. D. 2015. Review of nanomaterials in dentistry: interactions with the oral microenvironment, clinical applications, hazards, and benefits. *ACS nano,* 9**,** 2255-89.

BESINIS, A., VAN NOORT, R. & MARTIN, N. 2012. Infiltration of demineralized dentin with silica and hydroxyapatite nanoparticles. *Dental Materials,* 28**,** 1012-1023.

BESINIS, A., VAN NOORT, R. & MARTIN, N. 2014b. Remineralization potential of fully demineralized dentin infiltrated with silica and hydroxyapatite nanoparticles. *Dental Materials,* 30**,** 249-262.

BLEICHER, F., COUBLE, M. L., FARGES, J. C., COUBLE, P. & MAGLOIRE, H. 1999. Sequential expression of matrix protein genes in developing rat teeth. *Matrix Biology,* 18**,** 133-143.

BONETA, A. R. E., RAMIREZ, K., NABOA, J., MATEO, L. R., STEWART, B., PANAGOKOS, F. & DE VIZIO, W. 2013. Efficacy in reducing dentine hypersensitivity of a regimen using a toothpaste containing 8% arginine and calcium carbonate, a mouthwash containing 0.8% arginine, pyrophosphate and PVM/MA copolymer and a toothbrush compared to potassium and negative control regimens: An eight-week randomized clinical trial. *Journal of Dentistry,* 41**,** S42-S49.

BONUCCI, E. 1992. *Calcification in Biological Systems*.

BORNSTEIN, P. & SAGE, H. 1980. STRUCTURALLY DISTINCT COLLAGEN TYPES. *Annual Review of Biochemistry,* 49**,** 957-1003.

BOSKEY, A. L., MARESCA, M., DOTY, S., SABSAY, B. & VEIS, A. 1990. Concentration-dependent effects of dentin phosphoryn in the regulation of invitro hydroxyapatite formation and growth. *Bone and Mineral,* 11**,** 55-65.

BOSKEY, A. L., MARESCA, M., ULLRICH, W., DOTY, S. B., BUTLER, W. T. & PRINCE, C. W. 1993. Osteopontin-hydroxyapatite interactions in-vitro - inhibition of hydroxyapatite formation and growth in a gelatin-gel. *Bone and Mineral,* 22**,** 147-159.

BOUHAIK, I. S., LEROY, P., OLLIVIER, P., AZAROUAL, M. & MERCURY, L. 2013. Influence of surface conductivity on the apparent zeta potential of TiO2 nanoparticles: Application to the modeling of their aggregation kinetics. *Journal of Colloid and Interface Science,* 406**,** 75-85.

BOUKPESSI, T., MENASHI, S., CAMOIN, L., TENCATE, J. M., GOLDBERG, M. & CHAUSSAIN-MILLER, C. 2008. The effect of stromelysin-1 (MMP-3) on non-collagenous extracellular matrix proteins of demineralized dentin and the adhesive properties of restorative resins. *Biomaterials,* 29**,** 4367-4373.

BOUSHELL, L. W., NAGAOKA, H. & YAMAUCHI, M. 2011. Increased Matrix Metalloproteinase-2 and Bone Sialoprotein Response to Human Coronal Caries. *Caries Research,* 45**,** 453-459.

BOWES, J. H. & CATER, C. W. 1968. Interaction of aldehydes with collagen. *Biochimica Et Biophysica Acta,* 168**,** 341-&.

BRACKETT, M. G., TAY, F. R., BRACKETT, W. W., DIB, A., DIPP, F. A., MAI, S. & PASHLEY, D. H. 2009. In Vivo Chlorhexidine Stabilization of Hybrid Layers of an Acetone-based Dentin Adhesive. *Operative Dentistry,* 34**,** 379-383.

BRESCHI, L., CAMMELLI, F., VISINTINI, E., MAZZONI, A., VITA, F., CARRILHO, M., CADENARO, M., FOULGER, S., MAZZOTI, G., TAY, F. R., DI LENARDA, R. & PASHLEY, D. 2009. Influence of Chlorhexidine Concentration on the Durability of Etch-and-Rinse Dentin Bonds: A 12-month In Vitro Study. *Journal of Adhesive Dentistry,* 11**,** 191-198.

BRESCHI, L., LOPES, M., GOBBI, P., PRATI, C., FALCONI, M. & PERDIGAO, J. 2002. Immunocytochemical analysis of the hybrid layer: a high resolution SEM analysis. *Journal of Dental Research,* 81.

BRESCHI, L., MAZZONI, A., NATO, F., CARRILHO, M., VISINTINI, E., TJADERHANE, L., RUGGERI, A., JR., TAY, F. R., DORIGO, E. D. S. & PASHLEY, D. H. 2010. Chlorhexidine stabilizes the adhesive interface: A 2-year in vitro study. *Dental Materials,* 26**,** 320-325.

BRUNS, R. & GROSS, J. 1974. High-resolution analysis of modified quarter-stagger model of collagen fibril. *Biopolymers,* 13**,** 931-&.

BUCHWALOW, I. B. & BÖCKER, W. 2010. Immunohistochemistry: Basics and Methods. Springer Science and Business Media.

BULLOCK, G. R. 1984. The current status of fixation for electron-microscopy - a review. *Journal of Microscopy-Oxford,* 133**,** 1-15.

BURNS, J. A., LI, Y., CHENEY, C. A., OU, Y., FRANLIN-PFEIFER, L. L., KUKLIN, N. & ZHANG, Z.-Q. 2009. Choice of Fixative Is Crucial to Successful Immunohistochemical Detection of Phosphoproteins in Paraffin-embedded Tumor Tissues. *Journal of Histochemistry & Cytochemistry,* 57**,** 257-264.

BURWELL, A. K., LITKOWSKI, L. J. & GREENSPAN, D. C. 2009. Calcium sodium phosphosilicate (NovaMin): remineralization potential. *Advances in dental research,* 21**,** 35-9.

BUTLER, W. T. 1987. MINERALIZED TISSUES - an overview. *Methods in Enzymology,* 145**,** 255-261.

BUTLER, W. T. 1998. Dentin matrix proteins. *European Journal of Oral Sciences,* 106**,** 204-210.

BUTLER, W. T., BHOWN, M., DIMUZIO, M. T. & LINDE, A. 1981. Nonocollagenous proteins of dentin - isolation and partial characterization of rat dentin proteins and proteoglycans using a 3-step preparative method. *Collagen and Related Research,* 1.

BUTLER, W. T., MUNKSGAARD, E. C. & RICHARDSON, W. S. 1979. Dentin proteins - chemistry, structure and biosynthesis. *Journal of Dental Research,* 58**,** 817-824.

CAMERON, J. A. 1983. The use of ultrasonics in the removal of the smear layer - a scanning electron-microscope study. *Journal of Endodontics,* 9**,** 289-292.

CAO, Y., MEI, M. L., LI, Q.-L., LO, E. C. M. & CHU, C. H. 2014. Agarose Hydrogel Biomimetic Mineralization Model for the Regeneration of Enamel Prismlike Tissue. *Acs Applied Materials & Interfaces,* 6**,** 410-420.

CAO, Y., MEI, M. L., XU, J., LO, E. C. M., LI, Q. & CHU, C. H. 2013. Biomimetic mineralisation of phosphorylated dentine by CPP-ACP. *Journal of Dentistry,* 41**,** 818-825.

CARAFOLI, E. 1987. Intracellular calcium homeostasis. *Annual Review of Biochemistry,* 56**,** 395-433.

CARRIGAN, P. J., MORSE, D. R., FURST, M. L. & SINAI, I. H. 1984. A scanning electron-microscopic evaluation of human dentinal tubules according to age and location. *Journal of Endodontics,* 10**,** 359-363.

CARRILHO, M. R., TAY, F. R., DONNELLY, A. M., AGEE, K. A., TJAEDERHANE, L., MAZZONI, A., BRESCHI, L., FOULGER, S. & PASHLEY, D. H. 2009. Host-Derived Loss of Dentin Matrix Stiffness Associated With Solubilization of Collagen. *Journal of Biomedical Materials Research Part B-Applied Biomaterials,* 90B**,** 373-380.

CARVALHO, R. M., YOSHIYAMA, M., PASHLEY, E. L. & PASHLEY, D. H. 1996. In vitro study on the dimensional changes of human dentine after demineralization. *Archives of Oral Biology,* 41**,** 369-377.

CDH SURVEY 2013. Child Dental Health Survey. Health and Social Care Information Centre.

CHAO, W., LIU, L.-L., AI-TIBNG, Z., XIE, P., LU, J.-J. & ZOU, X.-T. 2012. Antibacterial effects of zinc oxide nanoparticles on Escherichia coli K88. African Journal of Biotechnology 11.

CHAPMAN, J. A., TZAPHLIDOU, M., MEEK, K. M. & KADLER, K. E. 1990. The collagen fibril - a model system for studying the staining and fixation of a protein. *Electron Microscopy Reviews,* 3**,** 143-182.

CHAUSSAIN, C., BOUKPESSI, T., KHADDAM, M., TJADERHANE, L., GEORGE, A. & MENASHI, S. 2013. Dentin matrix degradation by host matrix metalloproteinases: inhibition and clinical perspectives toward regeneration. *Frontiers in Physiology,* 4.

CHENG, L., WEIR, M. D., XU, H. H. K., ANTONUCCI, J. M., KRAIGSLEY, A. M., LIN, N. J., LIN-GIBSON, S. & ZHOU, X. 2012. Antibacterial amorphous calcium phosphate nanocomposites with a quaternary ammonium dimethacrylate and silver nanoparticles. *Dental Materials,* 28.

CHERNG, A. M., CHOW, L. C. & TAKAGI, S. 2004. Reduction in dentin permeability using mildly supersaturated calcium phosphate solutions. *Archives of Oral Biology,* 49.

CHHOUR, K. L., NADKARNI, M. A., BYUN, R., MARTIN, F. E., JACQUES, N. A. & HUNTER, N. 2005. Molecular analysis of microbial diversity in advanced caries. *Journal of Clinical Microbiology,* 43**,** 843-849.

COUSINS, B. G., ALLISON, H. E., DOHERTY, P. J., EDWARDS, C., GARVEY, M. J., MARTIN, D. S. & WILLIAMS, R. L. 2007. Effects of a nanoparticulate silica substrate on cell attachment of Candida albicans. *Journal of Applied Microbiology,* 102**,** 757-765.

CRAIG, R. G., GEHRING, P. E. & PEYTON, F. A. 1959. Relation of structure to the micro-hardness of human dentin. *Jour Dental Res,* 38**,** 624-630.

CRAIG, R. G. & PEYTON, F. A. 1958. The microhardness of enamel and dentin. *Jour Dental Res,* 37**,** 661-668.

CROSS, K. J., HUQ, N. L. & REYNOLDS, E. C. 2007. Casein phosphopeptides in oral health - Chemistry and clinical applications. *Current Pharmaceutical Design,* 13.

CUI, F.-Z., LI, Y. & GE, J. 2007. Self-assembly of mineralized collagen composites. *Materials Science & Engineering R-Reports,* 57**,** 1-27.

CURTIS, A. R., WEST, N. X. & SU, B. 2010. Synthesis of nanobioglass and formation of apatite rods to occlude exposed dentine tubules and eliminate hypersensitivity. *Acta Biomaterialia,* 6**,** 3740-3746.

DAI, L., DOUGLAS, E. P. & GOWER, L. B. 2008. Compositional analysis of a polymer-induced liquid-precursor (PILP) amorphous CaCO3 phase. *Journal of Non-Crystalline Solids,* 354**,** 1845-1854.

DASANAYAKE, A. P., WIENER, H. W., LI, Y., VERMUND, S. V. & CAUFIELD, P. W. 2002. Lack of effect of chlorhexidine varnish on

Streptococcus mutans transmission and caries in mothers and children. *Caries Research,* 36**,** 288-293.

DAWES, C. 2003. What is the critical pH and why does a tooth dissolve in acid? *Journal (Canadian Dental Association),* 69**,** 722-4.

DELANY, A. M. & HANKENSON, K. D. 2009. Thrombospondin-2 and SPARC/osteonectin are critical regulators of bone remodeling. *Journal of cell communication and signaling,* 3**,** 227-38.

DEYHLE, H., BUNK, O. & MUELLER, B. 2011. Nanostructure of healthy and caries-affected human teeth. *Nanomedicine-Nanotechnology Biology and Medicine,* 7**,** 694-701.

DICKSON, I. R., DIMUZIO, M. T., VOLPIN, D., ananthanarayanan, s. & veis, a. 1975. Extraction of phosphoproteins from bovine dentin. *Calcified Tissue Research,* 19.

DIMUZIO, M. T. & VEIS, A. 1978. PHOSPHOPHORYNS - MAJOR NON-COLLAGENOUS PROTEINS OF RAT INCISOR DENTIN. *Calcified Tissue Research,* 25**,** 169-178.

DOCIMO, R., MONTESANI, L., MATURO, P., COSTACURTA, M., BARTOLINO, M., DEVIZIO, W., ZHANG, Y. P., CUMMINS, D., DIBART, S. & MATEO, L. R. 2009. Comparing the efficacy in reducing dentin hypersensitivity of a new toothpaste containing 8.0% arginine, calcium carbonate, and 1450 ppm fluoride to a commercial sensitive toothpaste containing 2% potassium ion: an eight-week clinical study in Rome, Italy. *The Journal of clinical dentistry,* 20.

DOI, Y., HORIGUCHI, T., KIM, S. H., MORIWAKI, Y., WAKAMATSU, N., ADACHI, M., IBARAKI, K., MORIYAMA, K., SASAKI, S. & SHIMOKAWA, H. 1992. Effects of noncollagenous proteins on the formation of apatite in calcium beta-glycerophosphate solutions. *Archives of Oral Biology,* 37**,** 15-21.

DOI, Y., HORIGUCHI, T., MORIWAKI, Y., KITAGO, H., KAJIMOTO, T. & IWAYAMA, Y. 1996. Formation of apatite-collagen complexes. *Journal of Biomedical Materials Research,* 31**,** 43-49.

DONOGHUE, P. C. J., SANSOM, I. J. & DOWNS, J. P. 2006. Early evolution of vertebrate skeletal tissues and cellular interactions, and the canalization of skeletal development. *Journal of Experimental Zoology Part B-Molecular and Developmental Evolution,* 306B**,** 278-294.

DOWNER, M. C. 1995. The 1993 national survey of childrens dental-health. *British Dental Journal,* 178**,** 407-412.

DUCKWORTH, R. M. & HUNTINGTON, E. 2006. On the relationship between calculus and caries. *Teeth and Their Environment: Physical, Chemical and Biochemical Influences,* 19**,** 1-28.

DUKE, S. A., REES, D. A. & FORWARD, G. C. 1979. Increased plaque calcium and phosphorus concentrations after using a calcium-carbonate toothpaste containing calcium glycerophosphate and sodium monofluorophosphate - pilot-study. *Caries Research,* 13**,** 57-59.

DUNG, S. Z., LI, Y., DUNIPACE, A. J. & STOOKEY, G. K. 1994. Degradation of insoluble bovine collagen and human dentin collagen pretreated in-vitro with lactic-acid, PH-4.0 AND PH-5.5. *Archives of Oral Biology,* 39.

DUNG, T. S. Z. & LIU, A. H. H. 1999. Molecular pathogenesis of root dentin caries. *Oral Diseases,* 5**,** 92-99.

EARL, J. S., LEARY, R. K., MULLER, K. H., LANGFORD, R. M. & GREENSPAN, D. C. 2011. Physical and chemical characterization of dentin surface following treatment with NovaMin technology. *The Journal of clinical dentistry,* 22**,** 62-7.

EDGAR, W. M., HIGHAM, S. M. & MANNING, R. H. 1994. Saliva stimulation and caries prevention. *Advances in dental research,* 8**,** 239-45.

EDWARDSSON, S., BIRKHED, D. & MEJARE, B. 1977. Acid production from lycasin, maltitol, sorbitol and xylitol by oral streptococci and lactobacilli. *Acta Odontologica Scandinavica,* 35.

EGGERT, F. M. & GERMAIN, J. P. 1979. Rapid demineralization in acidic buffers. *Histochemistry,* 59**,** 215-224.

EGLIN, D., MAALHEEM, S., LIVAGE, J. & CORADIN, T. 2006. In vitro apatite forming ability of type I collagen hydrogels containing bioactive glass and silica sol-gel particles. *Journal of Materials Science-Materials in Medicine,* 17**,** 161-167.

EICK, J. D., ROBINSON, S. J., CHAPPELL, R. P., COBB, C. M. & SPENCER, P. 1993. The dentinal surface: its influence on dentinal adhesion. Part III. *Quintessence international (Berlin, Germany : 1985),* 24.

EICK, J. D., WILKO, R. A., ANDERSON, C. H. & SORENSEN, S. E. 1970. Scanning electron microscopy of cut tooth surfaces and identification of debris by use of the electron microprobe. *Journal of dental research,* 49.

EKSTRAND, K. R., POULSEN, J. E., HEDE, B., TWETMAN, S., QVIST, V. & ELLWOOD, R. P. 2013. A Randomized Clinical Trial of the Anti-Caries Efficacy of 5,000 Compared to 1,450 ppm Fluoridated Toothpaste on Root Caries Lesions in Elderly Disabled Nursing Home Residents. *Caries Research,* 47**,** 391-398.

EL FENINAT, F., ELLIS, T. H., SACHER, E. & STANGEL, I. 2001. A tapping mode AFM study of collapse and denaturation in dentinal collagen. *Dental Materials,* 17**,** 284-288.

ELIADES, G., PALAGHIAS, G. & VOUGIOUKLAKIS, G. 1997. Effect of acidic conditioners on dentin morphology, molecular composition and collagen conformation in situ. *Dental Materials,* 13.

EMBERY, G., HALL, R., WADDINGTON, R., SEPTIER, D. & GOLDBERG, M. 2001. Proteoglycans in dentinogenesis. *Critical Reviews in Oral Biology & Medicine,* 12**,** 331-349.

ERICKSON, R. L. 1992. Surface interactions of dentin adhesive materials. *Operative dentistry,* Suppl 5**,** 81-94.

ERICSON, D., ZIMMERMAN, M., RABER, H., GOTRICK, B., BORNSTEIN, R. & THORELL, J. 1999. Clinical evaluation of efficacy and safety of a new method for chemo-mechanical removal of caries - A multi-centre study. *Caries Research,* 33.

ESSIG, M. E., BODDEN, W. R., BRADLEY, E. L., KOULOURIDES, T. & HOUSCH, T. 1985. Enamel microhardness change and plaque ph measurements in an intra-oral model in humans. *Journal of Dental Research,* 64.

EXTERKATE, R. A. M., DAMEN, J. J. M. & TENCATE, J. M. 1993. A single-section model for enamel demineralization and remineralization studies .1. The effects of different ca/p ratios in remineralization solutions. *Journal of Dental Research,* 72**,** 1599-1603.

FEATHERSTONE, J. D. B. & LUSSI, A. 2006. Understanding the chemistry of dental erosion. *Dental Erosion: from Diagnosis to Therapy,* 20**,** 66-76.

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**,** 377-385.

FEDE, C., SELVESTREL, F., COMPAGNIN, C., MOGNATO, M., MANCIN, F., REDDI, E. & CELOTTI, L. 2012. The toxicity outcome of silica nanoparticles (Ludox (R)) is influenced by testing techniques and treatment modalities. *Analytical and Bioanalytical Chemistry,* 404**,** 1789-1802.

FEJERSKOV, O. & KIDD, E. 2008a. *Dental Caries. The Disease and its Clinical Management*, Blackwell Munksgaard Ltd.

FEJERSKOV, O. & KIDD, E. 2008b. *Dental Caries: The Disease and Its Clinical Management*.

FEJERSKOV, O. & KIDD, E. 2008c. *Dental Caries: The Disease and its Clinical Management*.

FENG, J. Q., ZHANG, J., TAN, X., LU, Y., GUO, D. & HARRIS, S. E. 2002. Identification of Cis-DNA regions controlling Bmp4 expression during tooth morphogenesis in vivo. *Journal of Dental Research,* 81**,** 6-10.

FEYNMAN, R. 1960. There's Plenty of Room at the Bottom.

FLEISCHMANNOVA, J., MATALOVA, E., TUCKER, A. S. & SHARPE, P. T. 2008. Mouse models of tooth abnormalities. *European Journal of Oral Sciences,* 116**,** 1-10.

FONSECA, R. B., HAITER-NETO, F., CARLO, H. L., SOARES, C. J., SINHORETI, M. A. C., PUPPIN-RONTANI, R. M. & CORRER-SOBRINHO, L. 2008. Radiodensity and hardness of enamel and dentin of human and bovine teeth, varying bovine teeth age. *Archives of Oral Biology,* 53**,** 1023-1029.

FORSBACK, A. P., AREVA, S. & SALONEN, J. I. 2004. Mineralization of dentin induced by treatment with bioactive glass S53P4 in vitro. *Acta Odontologica Scandinavica,* 62**,** 14-20.

FOWLER, C., WILLSON, R. & REES, G. D. 2006. In vitro microhardness studies on a new anti-erosion desensitizing toothpaste. *The Journal of clinical dentistry,* 17**,** 100-5.

FRANCISCONI, L. F., HONORIO, H. M., RIOS, D., MAGALHAES, A. C., MACHADO, M. A. A. M. & BUZALAF, M. A. R. 2008. Effect of erosive pH cycling on different restorative materials and on enamel restored with these materials. *Operative Dentistry,* 33**,** 203-208.

FROST, H. M. & JEE, W. S. S. 1992. On the rat model of human osteopenias and osteoporosis. *Bone and Mineral,* 18**,** 227-236.

FUSAYAMA, T. 1979. 2 layers of carious dentin - diagnosis and treatment. *Operative Dentistry,* 4**,** 63-70.

FUSAYAMA, T. & KUROSAKI, N. 1972. Structure and removal of carious dentine. *International Dental Journal,* 22**,** 401-&.

GAGE, J. P. 1984. Electrophoretic characterization of peptides from normal mature human-dentin. *Archives of Oral Biology,* 29**,** 575-580.

GAIKWAD, R. M. & SOKOLOV, I. 2008. Silica nanoparticles to polish tooth surfaces for caries prevention. *Journal of Dental Research,* 87**,** 980-983.

GAISHUN, V., TULENKOVA, O., MELNICHENKO, I., BARYSHNIN, S., POTAPENOK, Y. & AP, X. 2002. Preparation and properties of colloidal nanosize silica dioxide for polishing of monocrystalline silicon wafers. Materials Science - Poland.

GANSS, C. 2006. Definition of erosion and links to tooth wear. *Monographs in Oral Science,* 20**,** 9-16.

GANSS, C. 2008. How valid are current diagnostic criteria for dental erosion? *Clinical Oral Investigations,* 12**,** S41-S49.

GARBEROGLIO, R. & BRANNSTROM, M. 1976. Scanning electron-microscopic investigation of human dentinal tubules. *Archives of Oral Biology,* 21**,** 355-362.

GARNERO, P., FERRERAS, M., KARSDAL, M. A., NICAMHLAOIBH, R., RISTELI, J., BOREL, O., QVIST, P., DELMAS, P. D., FOGED, N. T. & DELAISSE, J. M. 2003. The type I collagen fragments ICTP and CTX reveal distinct enzymatic pathways of bone collagen degradation. *Journal of Bone and Mineral Research,* 18**,** 859-867.

GEISTLICH, P. & ECKMAYER, Z. 1995. *Membrane comprising collagen for use in guided tissue regeneration*. 05072022.0.

GEORGE, A., BANNON, L., SABSAY, B., DILLON, J. W., MALONE, J., VEIS, A., JENKINS, N. A., GILBERT, D. J. & COPELAND, N. G. 1996. The carboxyl-terminal domain of phosphophoryn contains unique extended triplet amino acid repeat sequences forming ordered carboxyl-phosphate interaction ridges that may be essential in the

biomineralization process. *Journal of Biological Chemistry,* 271**,** 32869-32873.

GEORGE, A., SABSAY, B., SIMONIAN, P. A. L. & VEIS, A. 1993. Characterization of a novel dentin matrix acidic phosphoprotein - implications for induction of biomineralization. *Journal of Biological Chemistry,* 268**,** 12624-12630.

GIBBONS, R. J. & MACDONALD, J. B. 1961. Degradation of collagenous substrates by bacteroides melaninogenicus. *Journal of Bacteriology,* 81**,** 614-&.

GILLAM, D. G., TANG, J. Y., MORDAN, N. J. & NEWMAN, H. N. 2002. The effects of a novel Bioglass (R) dentifrice on dentine sensitivity: a scanning electron microscopy investigation. *Journal of Oral Rehabilitation,* 29**,** 305-313.

GJORGIEVSKA, E. S. & NICHOLSON, J. W. 2010. A preliminary study of enamel remineralization by dentifrices based on Recalden (CPP-ACP) and Novamin (calcium-sodium-phosphosilicate). *Acta odontologica latinoamericana : AOL,* 23**,** 234-9.

GOLDBERG, H. A. & HUNTER, G. K. 1995. The inhibitory activity of osteopontin on hydroxyapatite formation in vitro. *Annals of the New York Academy of Sciences; Osteopontin: Role in cell signalling and adhesion,* 760**,** 305-308.

GOLDBERG, M. & BOSKEY, A. L. 1996. Lipids and biomineralizations - Introduction. *Lipids and Biomineralizations,* 31**,** 1-39.

GOLDBERG, M., SEPTIER, D., RAPOPORT, O., IOZZO, R. V., YOUNG, M. F. & AMEYE, L. G. 2005. Targeted disruption of two small leucine-rich proteoglycans, biglycan and decorin, excerpts divergent effects on enamel and dentin formation. *Calcified Tissue International,* 77**,** 297-310.

GOLDBERG, M., SEPTIER, D., RAPOPORT, O., YOUNG, M. & AMEYE, L. 2002. Biglycan is a repressor of amelogenin expression and enamel formation: An emerging hypothesis. *Journal of Dental Research,* 81**,** 520-524.

GOLDMAN, L. B., GOLDMAN, M., KRONMAN, J. H. & LIN, P. S. 1981. The efficacy of several irrigating solutions for endodontics - a scanning electron-microscopic study. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics,* 52**,** 197-204.

GOLUB, E. E. 2009. Role of matrix vesicles in biomineralization. *Biochimica Et Biophysica Acta-General Subjects,* 1790**,** 1592-1598.

GOTLIV, B.-A., ROBACH, J. S. & VEIS, A. 2006. The composition and structure of bovine peritubular dentin: Mapping by time of flight secondary ion mass spectroscopy. *Journal of Structural Biology,* 156**,** 320-333.

GOTLIV, B.-A. & VEIS, A. 2007. Peritubular dentin, a vertebrate apatitic mineralized tissue without collagen: Role of a phospholipid-proteolipid complex. *Calcified Tissue International,* 81**,** 191-205.

GOWER, L. B. 2008. Biomimetic Model Systems for Investigating the Amorphous Precursor Pathway and Its Role in Biomineralization. *Chemical Reviews,* 108**,** 4551-4627.

GRACE, W. R. 2007. *LUDOX® Colloidal Silica in Coatings Lithium Polysilicate in Coatings* [Online]. Available: https://grace.com/coatings-and-inks/en-us/Documents/LUDOX%20Coatings%20TI.pdf [Accessed].

GRANSTROM, G. & LINDE, A. 1981. Atp-dependent uptake of ca-2+ by a microsomal fraction from rat incisor odontoblasts. *Calcified Tissue International,* 33**,** 125-128.

GRANT, R. A., COX, R. W. & HORNE, R. W. 1967. The structure and assembly of collagen fibres ii an inst electron microscope study of cross linked collagen rat. *Journal of the Royal Microscopical Society,* 87**,** 143-155.

GREENWOOD, R. 2003. Review of the measurement of zeta potentials in concentrated aqueous suspensions using electroacoustics. *Advances in Colloid and Interface Science,* 106**,** 55-81.

GROSS, E. L., BEALL, C. J., KUTSCH, S. R., FIRESTONE, N. D., LEYS, E. J. & GRIFFEN, A. L. 2012. Beyond Streptococcus mutans: Dental Caries Onset Linked to Multiple Species by 16S rRNA Community Analysis. *Plos One,* 7.

GROSS, E. L., LEYS, E. J., GASPAROVICH, S. R., FIRESTONE, N. D., SCHWARTZBAUM, J. A., JANIES, D. A., ASNANI, K. & GRIFFEN, A. L. 2010. Bacterial 16S Sequence Analysis of Severe Caries in Young Permanent Teeth. *Journal of Clinical Microbiology,* 48**,** 4121-4128.

GU, K., CHANG, S. R., RITCHIE, H. H., CLARKSON, B. H. & RUTHERFORD, R. B. 2000. Molecular cloning of a human dentin sialophosphoprotein gene. *European Journal of Oral Sciences,* 108**,** 35-42.

GU, L.-S., KIM, J., KIM, Y. K., LIU, Y., DICKENS, S. H., PASHLEY, D. H., LING, J.-Q. & TAY, F. R. 2010. A chemical phosphorylation-inspired design for Type I collagen biomimetic remineralization. *Dental Materials,* 26**,** 1077-1089.

GU, L.-S., KIM, Y. K., LIU, Y., TAKAHASHI, K., ARUN, S., WIMMER, C. E., OSORIO, R., LING, J.-Q., LOONEY, S. W., PASHLEY, D. H. & TAY, F. R. 2011. Immobilization of a phosphonated analog of matrix phosphoproteins within cross-linked collagen as a templating mechanism for biomimetic mineralization. *Acta Biomaterialia,* 7**,** 268-277.

GUNDBERG, C. M., HAUSCHKA, P. V., LIAN, J. B. & GALLOP, P. M. 1984. Osteocalcin - isolation, characterization, and detection. *Methods in Enzymology,* 107**,** 516-544.

GUSTAVSON, K. H. 1956. The chemistry and reactivity of collagen. *The chemistry and reactivity of collagen***,** ix+342p.-ix+342p.

GUTIÉRREZ-SALAZAR, M. D. P. & REYES-GASGA, J. 2003. Microhardness and chemical composition of human tooth. *Materials Research,* 6**,** 367-373.

HABELITZ, S., BALOOCH, M., MARSHALL, S. J., BALOOCH, G. & MARSHALL, G. W. 2002. In situ atomic force microscopy of partially demineralized human dentin collagen fibres. *Journal of Structural Biology,* 138**,** 227-236.

HAHN, C. L., FALKLER, W. A. & MINAH, G. E. 1991. Microbiological studies of carious dentin from human teeth with irreversible pulpitis. *Archives of Oral Biology,* 36**,** 147-153.

HALL, A. F., BUCHANAN, C. A., MILLETT, D. T., CREANOR, S. L., STRANG, R. & FOYE, R. H. 1999. The effect of saliva on enamel and dentine erosion. *Journal of Dentistry,* 27**,** 333-339.

HALL, C. 2010. *Deposition of Aluminium Oxide Modified Core-shell*

Silica Particles onto Silica Surfaces. Master of Philosophy, University of Manchester.

HANNAS, A. R., PEREIRA, J. C., GRANJEIRO, J. M. & TJADERHANE, L. 2007. The role of matrix metalloproteinases in the oral environment. *Acta Odontologica Scandinavica,* 65**,** 1-13.

HANNIG, M. & HANNIG, C. 2010. Nanomaterials in preventive dentistry. *Nature Nanotechnology,* 5**,** 565-569.

HARRINGTON, D. J. & RUSSELL, R. R. B. 1994. Identification and characterization of 2 extracellular proteases of streptococcus-mutans. *Fems Microbiology Letters,* 121**,** 237-241.

HAYAT, M. A. 1981. Fixation for electron microscopy. *Fixation for electron microscopy.***,** i-xix, 1-501.

HE, G., GAJJERAMAN, S., SCHULTZ, D., COOKSON, D., QIN, C. L., BUTLER, W. T., HAO, J. J. & GEORGE, A. 2005. Spatially and temporally controlled biomineralization is facilitated by interaction between self-assembled dentin matrix protein 1 and calcium phosphate nuclei in solution. *Biochemistry,* 44**,** 16140-16148.

HE, G. & GEORGE, A. 2004. Dentin matrix protein 1 immobilized on type I collagen fibres facilitates apatite deposition in vitro. *Journal of Biological Chemistry,* 279**,** 11649-11656.

HENCH, L. L. 2006. The story of Bioglass (R). *Journal of Materials Science-Materials in Medicine,* 17**,** 967-978.

HENCH, L. L. & WILSON, J. 1984. Surface-active biomaterials. *Science,* 226**,** 630-636.

HERNANDEZ, M. & MARSHALL, T. A. 2014. Reduced odds of pulpal exposure when using incomplete caries removal in the treatment of dentinal cavitated lesions. *Journal of the American Dental Association,* 145**,** 574-576.

HILLEMANN, H. H. & LEE, C. H. 1953. Organic chelating agents for decalcification of bones and teeth. *Stain Technology,* 28**,** 285-287.

HO, S. P., YU, B., YUN, W., MARSHALL, G. W., RYDER, M. I. & MARSHALL, S. J. 2009. Structure, chemical composition and mechanical properties of human and rat cementum and its interface with root dentin. *Acta Biomaterialia,* 5**,** 707-718.

HOPPENBROUWERS, P. M. M., DRIESSENS, F. C. M. & BORGGREVEN, J. 1987. The mineral solubility of human tooth roots. *Archives of Oral Biology,* 32**,** 319-322.

HORNBY, K., EVANS, M., LONG, M., JOINER, A., LAUCELLO, M. & SALVADERI, A. 2009. Enamel benefits of a new hydroxyapatite containing fluoride toothpaste. *International Dental Journal,* 59**,** 325-331.

HUNTER, G. K., KYLE, C. L. & GOLDBERG, H. A. 1994. Modulation of crystal-formation by bone phosphoproteins - structural specificity of the osteopontin-mediated inhibition of hydroxyapatite formation. *Biochemical Journal,* 300**,** 723-728.

IMAZATO, S., TORII, M., TSUCHITANI, Y., MCCABE, J. F. & RUSSELL, R. R. B. 1994. Incorporation of bacterial inhibitor into resin composite. *Journal of Dental Research,* 73**,** 1437-1443.

IOZZO, R. V. 1998. Matrix proteoglycans: From molecular design to cellular function. *Annual Review of Biochemistry,* 67**,** 609-652.

IOZZO, R. V. 1999. The biology of the small leucine-rich proteoglycans - Functional network of interactive proteins. *Journal of Biological Chemistry,* 274**,** 18843-18846.

ITOTA, T., NAKABO, S., TORII, Y., NARUKAMI, T., DOI, J. & YOSHIYAMA, M. 2006. Effect of fluoride-releasing liner on demineralized dentin. *Quintessence International,* 37**,** 297-303.

IZUTANI, N., IMAZATO, S., NAKAJO, K., TAKAHASHI, N., TAKAHASHI, Y., EBISU, S. & RUSSELL, R. R. B. 2011. Effects of the antibacterial monomer 12-methacryloyloxydodecylpyridinium bromide (MDPB) on bacterial viability and metabolism. *European Journal of Oral Sciences,* 119**,** 175-181.

JAMES, P., PARNELL, C. & WHELTON, H. 2010. The Caries-Preventive Effect of Chlorhexidine Varnish in Children and Adolescents: A Systematic Review. *Caries Research,* 44**,** 333-340.

JEE, S.-S., THULA, T. T. & GOWER, L. B. 2010a. Development of bone-like composites via the polymer-induced liquid-precursor (PILP) process. Part 1: Influence of polymer molecular weight. *Acta Biomaterialia,* 6**,** 3676-3686.

JEE, S. S., CULVER, L., LI, Y., DOUGLAS, E. P. & GOWER, L. B. 2010b. Biomimetic mineralization of collagen via an enzyme-aided PILP process. *Journal of Crystal Growth,* 312**,** 1249-1256.

JENTSCH, H. F. R., ECKERT, F. R., ESCHRICH, K., STRATUL, S. I. & KNEIST, S. 2014. Antibacterial action of Chlorhexidine/thymol containing varnishes in vitro and in vivo. *International Journal of Dental Hygiene,* 12**,** 168-173.

JEONG, S. H., HONG, S. J., CHOI, C. H. & KIM, B. I. 2007. *Effect of new dentifrice containing nano-sized carbonated apatite on enamel remineralization*.

JEONG, S. H., JANG, S. O., KIM, K. N., KWON, H. K., PARK, Y. D. & KIM, B. I. 2006. *Remineralization potential of new toothpaste containing nano-hydroxyapatite*.

JOHANSEN, E. & PARKS, H. F. 1961. Electron-microscopic observations on soft carious human dentin. *Journal of Dental Research,* 40**,** 235-&.

JOINER, A., SCHAFER, F., HORNBY, K., LONG, M., EVANS, M., BEASLEY, T. & ABRAHAM, P. 2009. Enhanced enamel benefits from a novel fluoride toothpaste. *International Dental Journal,* 59**,** 244-253.

KANDELMAN, D. 1997. Sugar, alternative sweeteners and meal frequency in relation to caries prevention: New perspectives. *British Journal of Nutrition,* 77**,** S121-S128.

KARLINSEY, R. L. & MACKEY, A. C. 2009. Solid-state preparation and dental application of an organically modified calcium phosphate. *Journal of Materials Science,* 44**,** 346-349.

KARLINSEY, R. L., MACKEY, A. C., WALKER, E. R. & FREDERICK, K. E. 2009. Spectroscopic evaluation of native, milled, and functionalized beta-TCP seeding into dental enamel lesions. *Journal of Materials Science,* 44**,** 5013-5016.

KARLINSEY, R. L., MACKEY, A. C., WALKER, E. R. & FREDERICK, K. E. 2010a. Preparation, characterization and in vitro efficacy of an acid-modified beta-TCP material for dental hard-tissue remineralization. *Acta Biomaterialia,* 6**,** 969-978.

KARLINSEY, R. L., MACKEY, A. C., WALKER, E. R. & FREDERICK, K. E. 2010b. Surfactant-modified beta-TCP: structure, properties, and in vitro remineralization of subsurface enamel lesions. *Journal of Materials Science-Materials in Medicine,* 21**,** 2009-2020.

KARUBE, I., NAKAMOTO, Y. & SUZUKI, S. 1976. Photocontrol of urease activity in spiropyran collagen membrane. *Biochimica Et Biophysica Acta,* 445**,** 774-779.

KATO, S. & FUSAYAMA, T. 1970. Recalcification of artificially decalcified dentin in-vivo. *Journal of Dental Research,* 49**,** 1060-&.

KAWASAKI, K. & WEISS, K. M. 2008. SCPP gene evolution and the dental mineralization continuum. *Journal of Dental Research,* 87**,** 520-531.

KELLER, U. & HIBST, R. 1997. Effects of Er:YAG laser in caries treatment: A clinical pilot study. *Lasers in Surgery and Medicine,* 20**,** 32-38.

KIDD, E. 2004. *Essentials of dental caries: the disease and its management*, Oxford University Press.

KIELTY, C. M., HOPKINSON, I. & GRANT, M. E. 1993. Collagen: The collagen family: Structure, assembly, and organization in the extracellular matrix. *Connective tissue and its heritable disorders: Molecular, genetic, and medical aspects*, 103-147.

KIM, D.-S., KIM, J., CHOI, K.-K. & KIM, S.-Y. 2011. The influence of chlorhexidine on the remineralization of demineralized dentine. *Journal of Dentistry,* 39, 855-862.

KINNEY, J. H., BALOOCH, M., MARSHALL, S. J., MARSHALL, G. W. & WEIHS, T. P. 1996. Hardness and Young's modulus of human peritubular and intertubular dentine. *Archives of Oral Biology,* 41, 9-13.

KINNEY, J. H., HABELITZ, S., MARSHALL, S. J. & MARSHALL, G. W. 2003. The importance of intrafibrillar mineralization of collagen on the mechanical properties of dentin. *Journal of Dental Research,* 82.

KIRKHAM, J., FIRTH, A., VERNALS, D., BODEN, N., ROBINSON, C., SHORE, R. C., BROOKES, S. J. & AGGELI, A. 2007. Self-assembling peptide scaffolds promote enamel remineralization. *Journal of Dental Research,* 86, 426-430.

KITASAKO, Y., SHIBATA, S., COX, C. F. & TAGAMI, J. 2002. Location, arrangement and possible function of interodontoblastic collagen fibres in association with calcium hydroxide-induced hard tissue bridges. *International Endodontic Journal,* 35, 996-1004.

KITASAKO, Y., TANAKA, M., SADR, A., HAMBA, H., IKEDA, M. & TAGAMI, J. 2011. Effects of a chewing gum containing phosphoryl oligosaccharides of calcium (POs-Ca) and fluoride on remineralization and crystallization of enamel subsurface lesions in situ. *Journal of Dentistry,* 39, 771-779.

KLEINBERG, I. 2002. SensiStat. A new saliva-based composition for simple and effective treatment of dentinal sensitivity pain. *Dentistry today,* 21.

KLETER, G. A., DAMEN, J. J. M., BUIJS, M. J. & TEN CATE, J. M. 1998. Modification of amino acid residues in carious dentin matrix. *Journal of Dental Research,* 77, 488-495.

KORN, A. H., FEAIRHEL.SH & FILACHIO.EM 1972. Glutaraldehyde - nature of reagent. *Journal of Molecular Biology,* 65, 525-&.

KUBOKI, Y., OHGUSHI, K. & FUSAYAMA, T. 1977. Collagen biochemistry of 2 layers of carious dentin. *Journal of Dental Research,* 56, 1233-1237.

KURAMOTO, A., IMAZATO, S., WALLS, A. W. G. & EBISU, S. 2005. Inhibition of root caries progression by an antibacterial adhesive. *Journal of Dental Research,* 84, 89-93.

LAGERLOF, F. & OLIVEBY, A. 1994. Caries-protective factors in saliva. *Advances in dental research,* 8, 229-38.

LANDIS, W. J. 1995. The strength of a calcified tissue depends in part on the molecular-structure and organization of its constituent mineral crystals in their organic matrix. *Bone,* 16**,** 533-544.

LEE, E.-J., JUN, S.-H., KIM, H.-E. & KOH, Y.-H. 2012. Collagen-silica xerogel nanohybrid membrane for guided bone regeneration. *Journal of Biomedical Materials Research Part A,* 100A**,** 841-847.

LENANDER-LUMIKARI, M. & LOIMARANTA, V. 2000. Saliva and dental caries. *Advances in dental research,* 14**,** 40-7.

LESOT, H., BEGUEKIRN, C., KUBLER, M. D., MEYER, J. M., SMITH, A. J., CASSIDY, N., RUCH, J. V., AUBIN, J. E., GOLDBERG, M. & MAGLOIRE, H. 1993. Experimental induction of odontoblast differentiation and stimulation during reparative processes. *Cells and Materials,* 3**,** 201-217.

LI, X. & CHANG, J. 2008. Preparation of bone-like apatite-collagen nanocomposites by a biomimetic process with phosphorylated collagen. *Journal of Biomedical Materials Research Part A,* 85A**,** 293-300.

LINDE, A. 1973. Glycosaminoglycans of odontoblast-predentine layer in dentinogenically active porcine teeth. *Calcified Tissue Research,* 12**,** 281-294.

LINDE, A. 1984. Dentin and Dentinogensis. CRC Press.

LINDE, A. 1989. Dentin matrix proteins - composition and possible functions in calcification. *Anatomical Record,* 224.

LINDE, A. & GOLDBERG, M. 1993. Dentinogenesis. *Critical Reviews in Oral Biology and Medicine,* 4**,** 679-728.

LINDE, A. & LUSSI, A. 1989. Mineral induction by polyanionic dentin and bone proteins at physiological ionic conditions. *Connective tissue research,* 21**,** 197-203.

LITKOWSKI, L. J., HACK, G. D., SHEAFFER, H. B. & GREENSPAN, D. C. 1997. Occlusion of dentin tubules by 45S5 Bioglass (R). *Bioceramics, Vol 10*.

LITONJUA, L. A., ANDREANA, S., BUSH, P. J. & COHEN, R. E. 2003. Tooth wear: Attrition, erosion, and abrasion. *Quintessence International,* 34**,** 435-446.

LIU, D. M., YANG, Q. Z., TROCZYNSKI, T. & TSENG, W. J. J. 2002. Structural evolution of sol-gel-derived hydroxyapatite. *Biomaterials,* 23.

LIU, Y., MAI, S., LI, N., YIU, C. K. Y., MAO, J., PASHLEY, D. H. & TAY, F. R. 2011. Differences between top-down and bottom-up approaches in mineralizing thick, partially demineralized collagen scaffolds. *Acta Biomaterialia,* 7**,** 1742-1751.

LOGUERCIO, A. D., STANISLAWCZUK, R., POLLI, L. G., COSTA, J. A., MICHEL, M. D. & REIS, A. 2009. Influence of chlorhexidine

digluconate concentration and application time on resin-dentin bond strength durability. *European Journal of Oral Sciences,* 117**,** 587-596.

LORENZ, K., BRUHN, G., HEUMANN, C., NETUSCHIL, L., BRECX, M. & HOFFMANN, T. 2006. Effect of two new chlorhexidine mouthrinses on the development of dental plaque, gingivitis, and discolouration. A randomized, investigator-blind, placebo-controlled, 3-week experimental gingivitis study. *Journal of Clinical Periodontology,* 33**,** 561-567.

LOWER, S. K. 2005. Directed natural forces of affinity between a bacterium and mineral. *American Journal of Science,* 305**,** 752-765.

LU, Y., YE, L., YU, S., ZHANG, S., XIE, Y., MCKEE, M. D., LI, Y. C., KONG, J., EICK, J. D., DALLAS, S. L. & FENG, J. Q. 2007. Rescue of odontogenesis in Dmp1-deficient mice by targeted re-expression of DMP1 reveals roles for DMP1 in early odontogenesis and dentin apposition in vivo. *Developmental Biology,* 303**,** 191-201.

LUNDGREN, T. & LINDE, A. 1988. Na+/ca-2+ antiports in membranes of rat incisor odontoblasts. *Journal of Oral Pathology & Medicine,* 17**,** 560-563.

LUSSI, A. & CARVALHO, T. S. 2014. Erosive tooth wear: a multifactorial condition of growing concern and increasing knowledge. *Monographs in oral science,* 25**,** 1-15.

LUSSI, A., JAGGI, T. & SCHARER, S. 1993. The influence of different factors on in-vitro enamel erosion. *Caries Research,* 27**,** 387-393.

LUSSI, A. & LINDE, A. 1993. Mineral induction in-vivo by dentin proteins. *Caries Research,* 27**,** 241-248.

LV, K., ZHANG, J., MENG, X. & LI, X. 2007. *Remineralization effect of the nano-HA toothpaste on artificial caries*.

LY, K. A., MILGRORN, P. & ROTHEN, M. 2006. Xylitol, sweeteners, and dental caries. *Pediatric Dentistry,* 28.

LYNCH, R. J. M. 2004. Calcium glycerophosphate and caries: a review of the literature. *International Dental Journal,* 54**,** 310-314.

LYNCH, R. J. M., MONY, U. & TEN CATE, J. M. 2006. The effect of fluoride at plaque fluid concentrations on enamel de- and remineralisation at low pH. *Caries Research,* 40**,** 522-529.

LYNCH, R. J. M. & TEN CATE, J. M. 2005. The anti-caries efficacy of calcium carbonate-based fluoride toothpastes. *International Dental Journal,* 55**,** 175-178.

LYNCH, R. J. M. & TEN CATE, J. M. 2006. Effect of calcium glycerophosphate on demineralization in an in vitro biofilm model. *Caries Research,* 40**,** 142-147.

MACDOUGALL, M., SIMMONS, D., LUAN, X., GU, T. T. & DUPONT, B. R. 1997. Assignment of dentin sialophosphoprotein (DSPP) to the critical DGI2 locus on human chromosome 4 band q21.3 by in situ hybridization. *Cytogenetics and Cell Genetics,* 79**,** 121-122.

MADER, C. L., BAUMGARTNER, J. C. & PETERS, D. D. 1984. Scanning electron-microscopic investigation of the smeared layer on root-canal walls. *Journal of Endodontics,* 10**,** 477-483.

MAGALHAES, A. C., DE ALMEIDA FURLANI, T., DE MORAES ITALIANI, F., IANO, F. G., DELBEM, A. C. B. & BUZALAF, M. A. R. 2007. Effect of calcium pre-rinse and fluoride dentifrice on remineralisation of artificially demineralised enamel and on the composition of the dental biofilm formed in situ. *Archives of Oral Biology,* 52**,** 1155-1160.

MAGLOIRE, H., BOUVIER, M. & JOFFRE, A. 1992. Odontoblast response under carious lesions. *Proceedings of the Finnish Dental Society. Suomen Hammaslaakariseuran toimituksia,* 88 Suppl 1**,** 257-74.

MAGLOIRE, H., JOFFRE, A., AZERAD, J. & LAWSON, D. E. M. 1988. Localization of 28 kda calbindin in human odontoblasts. *Cell and Tissue Research,* 254**,** 341-346.

MANNAA, A., CAMPUS, G., CARLEN, A. & LINGSTROM, P. 2014. Caries-risk profile variations after short-term use of 5000 ppm fluoride toothpaste. *Acta Odontologica Scandinavica,* 72**,** 228-234.

MARGOLIS, H. C. 1990. An assessment of recent advances in the study of the chemistry and biochemistry of dental plaque fluid. *Journal of Dental Research,* 69**,** 1337-1342.

MARINHO, V. C. C., WORTHINGTON, H. V., WALSH, T. & CLARKSON, J. E. 2013. Fluoride varnishes for preventing dental caries in children and adolescents. *The Cochrane database of systematic reviews,* 7**,** CD002279-CD002279.

MARSHALL, G. W., BALOOCH, M., TENCH, R. J., KINNEY, J. H. & MARSHALL, S. J. 1993. Atomic-force microscopy of acid effects on dentin. *Dental Materials,* 9.

MARSHALL, G. W., HABELITZ, S., GALLAGHER, R., BALOOCH, M., BALOOCH, G. & MARSHALL, S. J. 2001. Nanomechanical properties of hydrated carious human dentin. *Journal of Dental Research,* 80.

MARSHALL, G. W., MARSHALL, S. J., KINNEY, J. H. & BALOOCH, M. 1997. The dentin substrate: structure and properties related to bonding. *Journal of Dentistry,* 25**,** 441-458.

MARTIN-DE LAS HERAS, S., VALENZUELA, A. & OVERALL, C. M. 2000. Gelatinase A in human dentin as a new biochemical marker for age estimation. *Journal of Forensic Sciences,* 45**,** 807-811.

MARTINEK, N., SHAHAB, J., SODEK, J. & RINGUETTE, M. 2007. Is SPARC an evolutionarily conserved collagen chaperone? *Journal of Dental Research,* 86**,** 296-305.

MASAMURA, K., INABA, R. & IWATA, H. 1995. Salivary calcium and total protein in relation to dental caries. *Nihon eiseigaku zasshi. Japanese journal of hygiene,* 50**,** 886-92.

MATHEWS, M. S., AMAECHI, B. T., RAMALINGAM, K., CCAHUANA-VASQUEZ, R. A., CHEDJIEU, I. P., MACKEY, A. C. & KARLINSEY,

R. L. 2012. In situ remineralisation of eroded enamel lesions by NaF rinses. *Archives of Oral Biology,* 57**,** 525-530.

MAZZONI, A., PAPA, V., NATO, F., CARRILHO, M., TJADERHANE, L., RUGGERI, A., JR., GOBBI, P., MAZZOTTI, G., TAY, F. R., PASHLEY, D. H. & BRESCHI, L. 2011. Immunohistochemical and biochemical assay of MMP-3 in human dentine. *Journal of Dentistry,* 39**,** 231-237.

MEHTA, R., NANDLAL, B. & PRASHANTH, S. 2013. Comparative evaluation of remineralization potential of casein phosphopeptide-amorphous calcium phosphate and casein phosphopeptide-amorphous calcium phosphate fluoride on artificial enamel white spot lesion: an in vitro light fluorescence study. *Indian journal of dental research : official publication of Indian Society for Dental Research,* 24**,** 681-9.

MERGENHAGEN, S. E. & SCHERP, H. W. 1960. LYSIS OF RECONSTITUTED COLLAGEN AND CATABOLISM OF PRODUCTS OF COLLAGENOLYSIS BY THE ORAL MICROBIOTA. *Archives of Oral Biology,* 1**,** 333-&.

MEYER-LUECKEL, H., WIERICHS, R. J., SCHELLWIEN, T. & PARIS, S. 2015. Remineralizing Efficacy of a CPP-ACP Cream on Enamel Caries Lesions in situ. *Caries Research,* 49**,** 56-62.

MILAN, A. M., SUGARS, R. V., EMBERY, G. & WADDINGTON, R. J. 2004. Dentinal proteoglycans demonstrate an increasing order of affinity for hydroxyapatite crystals during the transition of predentine to dentine. *Calcified Tissue International,* 75**,** 197-204.

MILAN, A. M., SUGARS, R. V., EMBERY, G. & WADDINGTON, R. J. 2005. Modulation of collagen fibrillogenesis by dentinal proteoglycans. *Calcified Tissue International,* 76**,** 127-135.

MILGROM, P., LY, K. A., TUT, O. K., MANCL, L., ROBERTS, M. C., BRIAND, K. & GANCIO, M. J. 2009. Xylitol Pediatric Topical Oral Syrup to Prevent Dental Caries A Double-blind Randomized Clinical Trial of Efficacy. *Archives of Pediatrics & Adolescent Medicine,* 163.

MITCHELL, J. C., MUSANJE, L. & FERRACANE, J. L. 2011. Biomimetic dentin desensitizer based on nano-structured bioactive glass. *Dental Materials,* 27**,** 386-393.

MIURA, H., ISOGAI, E., HIROSE, K., WAKIZAKA, H., UEDA, I. & ITO, N. 1991. Application of a sucrose indicator strip to evaluate salivary sucrose clearance. *Journal of Dentistry,* 19**,** 189-191.

MIYAUCHI, H., IWAKU, M. & FUSAYAMA, T. 1978. Physiological re calcification of carious dentin. *Bulletin of Tokyo Medical and Dental University,* 25**,** 169-180.

MJOR, I. A. & NORDAHL, I. 1996. The density and branching of dentinal tubules in human teeth. *Archives of Oral Biology,* 41**,** 401-412.

MOODNIK, R. M., DORN, S. O., FELDMAN, M. J., LEVEY, M. & BORDEN, B. G. 1976. Efficacy of biomechanical instrumentation: a scanning electron microscopic study. *Journal of endodontics,* 2**,** 261-6.

MOORE, B. W., CARTER, W. J., DUNN, J. K. & FOSDICK, L. S. 1956. The formation of lactic acid in dental plaques. I. Caries-active individuals. *Jour Dental Res,* 35**,** 778-785.

MOREAU, J. L., SUN, L., CHOW, L. C. & XU, H. H. K. 2011. Mechanical and acid neutralizing properties and bacteria inhibition of amorphous calcium phosphate dental nanocomposite. *Journal of Biomedical Materials Research Part B-Applied Biomaterials,* 98B.

MORGAN, M. V., ADAMS, G. G., BAILEY, D. L., TSAO, C. E., FISCHMAN, S. L. & REYNOLDS, E. C. 2008. The anticariogenic effect of sugar-free gum containing CPP-ACP nanocomplexes on approximal caries determined using digital bitewing radiography. *Caries Research,* 42.

MORITZ, A., GUTKNECHT, N., SCHOOP, U., GOHARKHAY, K., WERNISCH, J. & SPERR, W. 1996. Alternatives in enamel conditioning: a comparison of conventional and innovative methods. *Journal of clinical laser medicine & surgery,* 14**,** 133-6.

MORONES, J. R., ELECHIGUERRA, J. L., CAMACHO, A., HOLT, K., KOURI, J. B., RAMIREZ, J. T. & YACAMAN, M. J. 2005. The bactericidal effect of silver nanoparticles. *Nanotechnology,* 16**,** 2346-2353.

MOUNT, G., WALSH, L. & BROSTEK, A. 2005. *Preservation and restoration of tooth structure*.

MOWBRAY, D. 2016. Private communication with Professor David Mowbray, Professor of Physics, The University of Sheffield.

MUNSON, M. A., BANERJEE, A., WATSON, T. F. & WADE, W. G. 2004. Molecular analysis of the microflora associated with dental caries. *Journal of Clinical Microbiology,* 42**,** 3023-3029.

MURPHY, G. & KNAUPER, V. 1997. Relating matrix metalloproteinase structure to function: Why the "hemopexin" domain? *Matrix Biology,* 15**,** 511-518.

MURRAY, P. E., SMITH, A. J., WINDSOR, L. J. & MJOR, I. A. 2003. Remaining dentine thickness and human pulp responses. *International Endodontic Journal,* 36**,** 33-43.

NAGATA, K., HUANG, Y. H., OHSAKI, Y., KUKITA, T., NAKATA, M. & KURISU, K. 1992. Demonstration of type-iii collagen in the dentin of mice. *Matrix,* 12**,** 448-455.

NAKAMURA, O., GOHDA, E., OZAWA, M., SENBA, I., MIYAZAKI, H., MURAKAMI, T. & DAIKUHARA, Y. 1985. Immunohistochemical studies with a monoclonal-antibody on the distribution of phosphophoryn in predentin and dentin. *Calcified Tissue International,* 37**,** 491-500.

NAKASHIMA, S., YOSHIE, M., SANO, H. & BAHAR, A. 2009. Effect of a test dentifrice containing nano-sized calcium carbonate on remineralization of enamel lesions in vitro. *Journal of oral science,* 51.

NANCI, A. 2008. *Ten Cate's Oral Histology*, Mosby Elsevier.

NARAYANAN, K., RAMACHANDRAN, A., HAO, J. J., HE, G., PARK, K. W., CHO, M. & GEORGE, A. 2003. Dual functional roles of dentin matrix protein 1 - Implications in biomineralization and gene transcription by activation of intracellular Ca2+ store. *Journal of Biological Chemistry,* 278**,** 17500-17508.

NASCIMENTO, F. D., MINCIOTTI, C. L., GERALDELI, S., CARRILHO, M. R., PASHLEY, D. H., TAY, F. R., NADER, H. B., SALO, T., TJADERHANE, L. & TERSARIOL, I. L. S. 2011. Cysteine Cathepsins in Human Carious Dentin. *Journal of Dental Research,* 90**,** 506-511.

NEZU, T. & WINNIK, F. M. 2000. Interaction of water-soluble collagen with poly(acrylic acid). *Biomaterials,* 21**,** 415-419.

NIMNI, B., PAULE, W. & BERNICK, S. 1991. Changes in the ratio of non-calcified collagen to calcified collagen in human vertebrae with advancing age. *Clinical Research,* 39.

NISSIM, K., WITT, R. & SHIP, J. 2006. *Salivary gland diseases: Surgical and medical management*.

NIU, L. N., ZHANG, L., JIAO, K., LI, F., DING, Y. X., WANG, D. Y., WANG, M. Q., TAY, F. R. & CHEN, J. H. 2011. Localization of MMP-2, MMP-9, TIMP-1, and TIMP-2 in human coronal dentine. *Journal of Dentistry,* 39**,** 536-542.

NORDBO, H., LEIRSKAR, J., NGO, H., MOUNT, G. J. & WAHLGREN, J. 2003. The influence of a matrix metalloproteinase on the remineralization of artificially demineralized dentin. *Oral health & preventive dentistry,* 1**,** 267-72.

NYRKOVA, I. A., SEMENOV, A. N., AGGELI, A. & BODEN, N. 2000. Fibril stability in solutions of twisted beta-sheet peptides: a new kind of micellization in chiral systems. *European Physical Journal B,* 17**,** 481-497.

OGAWA, K., YAMASHITA, Y., ICHIJO, T. & FUSAYAMA, T. 1983. The ultrastructure and hardness of the transparent layer of human carious dentin. *Journal of Dental Research,* 62**,** 7-10.

OHSAKI, Y. & NAGATA, K. 1994. Type-iii collagen is a major component of interodontoblastic fibers of the developing mouse molar root. *Anatomical Record,* 240**,** 308-313.

OLSZTA, M. J., CHENG, X., JEE, S. S., KUMAR, R., KIM, Y.-Y., KAUFMAN, M. J., DOUGLAS, E. P. & GOWER, L. B. 2007. Bone structure and formation: A new perspective. *Materials Science & Engineering R-Reports,* 58**,** 77-116.

OLSZTA, M. J., DOUGLAS, E. P. & GOWER, L. B. 2003a. Scanning electron microscopic analysis of the mineralization of type I collagen via a

polymer-induced liquid-precursor (PILP) process. *Calcified Tissue International,* 72**,** 583-591.

OLSZTA, M. J., ODOM, D. J., DOUGLAS, E. P. & GOWER, L. B. 2003b. A new paradigm for biomineral formation: Mineralization via an amorphous liquid-phase precursor. *Connective Tissue Research,* 44**,** 326-334.

ORSINI, G., RUGGERI, A., JR., MAZZONI, A., PAPA, V., MAZZOTTI, G., DI LENARDA, R. & BRESCHI, L. 2007. Immunohistochemical identification of decorin and biglycan in human dentin: A correlative field emission scanning electron Microscopy/Transmission electron microscopy study. *Calcified Tissue International,* 81**,** 39-45.

ORSINI, G., RUGGERI, A., MAZZONI, A., NATO, F., MANZOLI, L., PUTIGNANA, A., DI LENARDA, R., TJADERHANE, L. & BRESCHI, L. 2012. A review of the nature, role, and function of dnetin non-collagenous proteins. Part 1: proteoglycans and glycoproteins. *Endodontic Topics,* 21**,** 1-18.

OZDEMIR, B., URAZ, A., ISCAN, D., BOZKURT, S., TUNCER, B. B., ENGIN, D., PEHLIVAN, S. & ISCAN, H. 2014. Influence of Cervitec gel on periodontal health of patients wearing fixed orthodontic appliances. *Journal of Dental Sciences,* 9**,** 265-271.

PANDA, R. N., HSIEH, M. F., CHUNG, R. J. & CHIN, T. S. 2003. FTIR, XRD, SEM and solid state NMR investigations of carbonate-containing hydroxyapatite nano-particles synthesized by hydroxide-gel technique. *Journal of Physics and Chemistry of Solids,* 64**,** 193-199.

PAPAS, A., RUSSELL, D., SINGH, M., KENT, R., TRIOL, C. & WINSTON, A. 2008. Caries clinical trial of a remineralising toothpaste in radiation patients. *Gerodontology,* 25.

PAPAS, A., RUSSELL, D., SINGH, M., STACK, K., KENT, R., TRIOL, C. & WINSTON, A. 1999. Double blind clinical trial of a remineralizing dentifrice in the prevention of caries in a radiation therapy population. *Gerodontology,* 16.

PAPAS, A. S., VOLLMER, W. M., GULLION, C. M., BADER, J., LAWS, R., FELLOWS, J., HOLLIS, J. F., MAUPOME, G., SINGH, M. L., SNYDER, J., BLANCHARD, P. & GRP, P. C. 2012. Efficacy of Chlorhexidine Varnish for the Prevention of Adult Caries: A Randomized Trial. *Journal of Dental Research,* 91**,** 150-155.

PASHLEY, D., OKABE, A. & PARHAM, P. 1985. The relationship between dentin microhardness and tubule density. *Endodontics & dental traumatology,* 1**,** 176-9.

PASHLEY, D. H., ANDRINGA, H. J., DERKSON, G. D., DERKSON, M. E. & KALATHOOR, S. R. 1987. Regional variability in the permeability of human-dentin. *Archives of Oral Biology,* 32**,** 519-523.

PASHLEY, D. H., MICHELICH, V. & KEHL, T. 1981. Dentin permeability - effects of smear layer removal. *Journal of Prosthetic Dentistry,* 46**,** 531-537.

PATIL, S., SANDBERG, A., HECKERT, E., SELF, W. & SEAL, S. 2007. Protein adsorption and cellular uptake of cerium oxide nanoparticles as a function of zeta potential. *Biomaterials,* 28**,** 4600-4607.

PATWARDHAN, S. V., EMAMI, F. S., BERRY, R. J., JONES, S. E., NAIK, R. R., DESCHAUME, O., HEINZ, H. & PERRY, C. C. 2012. Chemistry of Aqueous Silica Nanoparticle Surfaces and the Mechanism of Selective Peptide Adsorption. *Journal of the American Chemical Society,* 134**,** 6244-6256.

PEARCE, E. I. F. 1982. Effect of plaque mineralization on experimental dental-caries. *Caries Research,* 16**,** 460-471.

PEARCE, E. I. F., DONG, Y. M., YUE, L., GAO, X. J., PURDIE, G. L. & WANG, J. D. 2002. Plaque minerals in the prediction of caries activity. *Community Dentistry and Oral Epidemiology,* 30**,** 61-69.

PEARCE, E. I. F. & NELSON, D. G. A. 1988. Invivo comparison of caries inhibition by a plaque mineral enriching mouthrinse and a fluoride dentifrice. *Caries Research,* 22**,** 362-370.

PETERSEN, P. E., BOURGEOIS, D., OGAWA, H., ESTUPINAN-DAY, S. & NDIAYE, C. 2005. The global burden of oral diseases and risks to oral health. *Bulletin of the World Health Organization,* 83**,** 661-669.

PETROU, I., HEU, R., STRANICK, M., LAVENDER, S., ZAIDEL, L., CUMMINS, D., SULLIVAN, R. J., HSUEH, C. & GIMZEWSKI, J. K. 2009. A breakthrough therapy for dentin hypersensitivity: how dental products containing 8% arginine and calcium carbonate work to deliver effective relief of sensitive teeth. *The Journal of clinical dentistry,* 20.

PHILLIPS, M. J., DARR, J. A., LUKLINSKA, Z. B. & REHMAN, I. 2003. Synthesis and characterization of nano-biomaterials with potential osteological applications. *Journal of Materials Science-Materials in Medicine,* 14**,** 875-882.

POGGIO, C., CECI, M., BELTRAMI, R., LOMBARDINI, M. & COLOMBO, M. 2014. Atomic force microscopy study of enamel remineralization. *Annali di stomatologia,* 5**,** 98-102.

POLLARD, M. A., DUGGAL, M. S., FAYLE, S. A., TOUMBA, K. J. & CURZON, M. E. J. 2000. *Caries Preventive Strategies,* Washington, International Life Sciences Institute.

PRADEEP, A. R. & SHARMA, A. 2010. Comparison of Clinical Efficacy of a Dentifrice Containing Calcium Sodium Phosphosilicate to a Dentifrice Containing Potassium Nitrate and to a Placebo on Dentinal Hypersensitivity: A Randomized Clinical Trial. *Journal of Periodontology,* 81**,** 1167-1173.

PRASAD, M., BUTLER, W. T. & QIN, C. 2010. Dentin sialophosphoprotein in biomineralization. *Connective Tissue Research,* 51**,** 404-417.

PRESTES, L., SOUZA, B. M., COMAR, L. P., SALOMAO, P. A., RIOS, D. & MAGALHAES, A. C. 2013. In situ effect of chewing gum containing

CPP-ACP on the mineral precipitation of eroded bovine enamel-A surface hardness analysis. *Journal of Dentistry,* 41**,** 747-751.

PROSKIN, H. M. & VOLPE, A. R. 1995. Comparison of the anticaries efficacy or dentifrices containing fluoride as sodium-fluoride or sodium monofluorophosphate. *American Journal of Dentistry,* 8.

PUDDU, V. & PERRY, C. C. 2012. Peptide Adsorption on Silica Nanoparticles: Evidence of Hydrophobic Interactions. *Acs Nano,* 6**,** 6356-6363.

PUGACH, M. K., STROTHER, J., DARLING, C. L., FRIED, D., GANSKY, S. A., MARSHALL, S. J. & MARSHALL, G. W. 2009. Dentin Caries Zones: Mineral, Structure, and Properties. *Journal of Dental Research,* 88**,** 71-76.

QIN, C., BABA, O. & BUTLER, W. T. 2004. Post-translational modifications of sibling proteins and their roles in osteogenesis and dentinogenesis. *Critical Reviews in Oral Biology & Medicine,* 15**,** 126-136.

QIN, C., D'SOUZA, R. & FENG, J. Q. 2007. Dentin matrix protein 1 (DMP1): New and important roles for biomineralization and phosphate homeostasis. *Journal of Dental Research,* 86**,** 1134-1141.

RAMACHAN.G & SASISEKHARAN, V. 1961. Structure of collagen. *Nature,* 190**,** 1004-&.

REN, G., HU, D., CHENG, E. W. C., VARGAS-REUS, M. A., REIP, P. & ALLAKER, R. P. 2009. Characterisation of copper oxide nanoparticles for antimicrobial applications. *International Journal of Antimicrobial Agents,* 33**,** 587-590.

REYNOLDS, E. C. 2008. Calcium phosphate-based remineralization systems: scientific evidence? *Australian dental journal,* 53**,** 268-73.

REYNOLDS, E. C., CAIN, C. J., WEBBER, F. L., BLACK, C. L., RILEY, P. F., JOHNSON, I. H. & PERICH, J. W. 1995. Anticariogenicity of calcium-phosphate complexes of tryptic casein phosphopeptides in the rat. *Journal of Dental Research,* 74.

REZVANI, M. B., KARIMI, M., AKHAVAN RASOOLZADE, R. & HAGHGOO, R. 2015. Comparing the Effects of Whey Extract and Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP) on Enamel Microhardness. *Journal of dentistry (Shiraz, Iran),* 16**,** 49-53.

RICH, A. & CRICK, F. H. C. 1961. Molecular structure of collagen. *Journal of Molecular Biology,* 3**,** 483-&.

RICKETTS, D., LAMONT, T., INNES, N. P. T., KIDD, E. & CLARKSON, J. E. 2013. Operative caries management in adults and children. *Cochrane Database of Systematic Reviews*.

RIMONDINI, L., PALAZZO, B., IAFISCO, M., CANEGALLO, L., DEMAROSI, F., MERLO, M. & ROVERI, N. 2007. *The remineralizing effect of carbonate-hydroxyapatite nanocrystals on dentine*.

RIOS, D., HONORIO, H. M., MAGALHAES, A. C., BUZALAF, M. A. R., PALMA-DIBB, R. G., MACHADO, M. A. D. A. M. & DA SILVA, S. M.

B. 2006a. Influence of toothbrushing on enamel softening and abrasive wear of eroded bovine enamel: an in situ study. *Brazilian oral research,* 20**,** 148-54.

RIOS, D., HONORIO, H. M., MAGALHAES, A. C., DELBEM, A. C. B., MACHADO, M., SILVA, S. M. B. & BUZALAF, M. A. R. 2006b. Effect of salivary stimulation on erosion of human and bovine enamel subjected or not to subsequent abrasion: An in situ/ex vivo study. *Caries Research,* 40**,** 218-223.

RITCHIE, H. H. & LI, X. R. 2001. A novel rat dentin mRNA coding on ly for dentin sialoprotein. *European Journal of Oral Sciences,* 109**,** 342-347.

ROLLAND, S. L., MCCABE, J. F., IMAZATO, S. & WALLS, A. W. G. 2011. A Randomised Trial Comparing the Antibacterial Effects of Dentine Primers against Bacteria in Natural Root Caries. *Caries Research,* 45**,** 574-580.

ROMBERG, R. W., WERNESS, P. G., RIGGS, B. L. & MANN, K. G. 1986. Inhibition of hydroxyapatite crystal-growth by bone-specific and other calcium-binding proteins. *Biochemistry,* 25**,** 1176-1180.

ROVERI, N., BATTISTELLA, E., FOLTRAN, I., FORESTI, E., IAFISCO, M., LELLI, M., PALAZZO, B. & RIMONDINI, L. 2008. *Synthetic biomimetic carbonate-hydroxyapatite nanocrystals for enamel remineralization*.

RYOU, H., NIU, L. N., DAI, L., PUCCI, C. R., AROLA, D. D., PASHLEY, D. H. & TAY, F. R. 2011. Effect of Biomimetic Remineralization on the Dynamic Nanomechanical Properties of Dentin Hybrid Layers. *Journal of Dental Research,* 90**,** 1122-1128.

SABATINI, D. D., BENSCH, K. & BARRNETT, R. J. 1963. Cytochemistry and electron microscopy - preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. *Journal of Cell Biology,* 17**,** 19-&.

SAITO, T., ARSENAULT, A. L., YAMAUCHI, M., KUBOKI, Y. & CRENSHAW, M. A. 1997. Mineral induction by immobilized phosphoproteins. *Bone,* 21**,** 305-311.

SCHAEFER, F., BEASLEY, T. & ABRAHAM, P. 2009. In vivo delivery of fluoride and calcium from toothpaste containing 2% hydroxyapatite. *International Dental Journal,* 59**,** 321-324.

SCHEININ, A. & BANOCZY, J. 1985. Collaborative who xylitol field studies in hungary - an overview. *Acta Odontologica Scandinavica,* 43.

SCHELLENBERG, U., KREY, G., BOSSHARDT, D. & NAIR, P. N. R. 1992. Numerical density of dentinal tubules at the pulpal wall of human permanent premolars and 3rd molars. *Journal of Endodontics,* 18**,** 104-113.

SCHILKE, R., LISSON, J. A., BAUSS, O. & GEURTSEN, W. 2000. Comparison of the number and diameter of dentinal tubules in human and bovine dentine by scanning electron microscopic investigation. *Archives of Oral Biology,* 45**,** 355-361.

SCHMIT, J. L., STALEY, R. N., WEFEL, J. S., KANELLIS, M., JAKOBSEN, J. R. & KEENAN, P. J. 2002. Effect of fluoride varnish on demineralization adjacent to brackets bonded with RMGI cement. *American Journal of Orthodontics and Dentofacial Orthopedics,* 122**,** 125-134.

SCHUPBACH, P., NEESER, J. R., GOLLIARD, M., ROUVET, M. & GUGGENHEIM, B. 1996. Incorporation of caseinoglycomacropeptide and caseinophosphopeptide into the salivary pellicle inhibits adherence of mutans streptococci. *Journal of Dental Research,* 75.

SCOTT, P. G. & LEAVER, A. G. 1974. Degradation of human dentin collagen by trypsin. *Connective Tissue Research,* 2.

SEHGAL, P. K. & SRINIVASAN, A. 2009. Collagen-coated microparticles in drug delivery. *Expert Opinion on Drug Delivery,* 6**,** 687-695.

SEVINC, B. A. & HANLEY, L. 2010. Antibacterial activity of dental composites containing zinc oxide nanoparticles. *Journal of Biomedical Materials Research Part B-Applied Biomaterials,* 94B.

SHARIF, M., AHMED, F. & WORTHINGTON, H. 2015. Xylitol-containing products for preventing dental caries in children and adolescents. *Cochrane Database of Systematic Reviews*.

SHAW, L., MURRAY, J. J., BURCHELL, C. K. & BEST, J. S. 1983. Calcium and phosphorus-content of plaque and saliva in relation to dental-caries. *Caries Research,* 17**,** 543-548.

SHI, S. R., COTE, R. J. & TAYLOR, C. R. 2001. Antigen retrieval techniques: Current perspectives. *Journal of Histochemistry & Cytochemistry,* 49**,** 931-937.

SHI, S. R., KEY, M. E. & KALRA, K. L. 1991. Antigen retrieval in formalin-fixed, paraffin-embedded tissues - an enhancement method for immunohistochemical staining based on microwave-oven heating of tissue-sections. *Journal of Histochemistry & Cytochemistry,* 39**,** 741-748.

SHIBATA, Y., HE, L. H., KATAOKA, Y., MIYAZAKI, T. & SWAIN, M. V. 2008. Micromechanical property recovery of human carious dentin achieved with colloidal nano-beta-tricalcium phosphate. *Journal of Dental Research,* 87**,** 233-237.

SHIMIZU, C., YAMASHITA, Y., ICHIJO, T. & FUSAYAMA, T. 1981. CARIOUS CHANGE OF DENTIN OBSERVED ON LONGSPAN ULTRATHIN SECTIONS. *Journal of Dental Research,* 60**,** 1826-1831.

SHUTTLEWORTH, C. A., WARD, J. L. & HIRSCHMANN, P. N. 1978. Presence of type-iii collagen in developing tooth. *Biochimica Et Biophysica Acta,* 535**,** 348-355.

SIDI, A. D. & WILSON, R. F. 1991. Fluoride, calcium and inorganic phosphorus concentrations in approximal plaque collected from young-adults 1 and 24 h after toothbrushing with fluoride toothpastes. *Caries Research,* 25**,** 330-334.

SILVERSTONE, L. M. 1973. Structure of carious enamel, including the early lesion. *Oral sciences reviews,* 3**,** 100-60.

SINGH, M. P., STEFKO, J., LUMPKIN, J. A. & ROSENBLATT, J. 1995. The effect of electrostatic charge interactions on release rates of gentamicin from collagen matrices. *Pharmaceutical Research,* 12**,** 1205-1210.

SMALES, R. & YIP, K. 2006. *Tooth erosion: Prevention and treatment,* New Delhi, Jaypee Brothers Medical Publishers.

SMITH, A. J. & LESOT, H. 2001. Induction and regulation of crown dentinogenesis: Embryonic events as a template for dental tissue repair? *Critical Reviews in Oral Biology & Medicine,* 12**,** 425-437.

SMITH, B. G. N. & ROBB, N. D. 1996. The prevalence of toothwear in 1007 dental patients. *Journal of Oral Rehabilitation,* 23**,** 232-239.

SORSA, T., TJADERHANE, L., KONTTINEN, Y. T., LAUHIO, A., SALO, T., LEE, H.-M., GOLUB, L. M., BROWN, D. L. & MANTYLA, P. 2006. Matrix metalloproteinases: Contribution to pathogenesis, diagnosis and treatment of periodontal inflammation. *Annals of Medicine,* 38**,** 306-321.

SORSA, T., TJADERHANE, L. & SALO, T. 2004. Matrix metalloproteinases (MMPs) in oral diseases. *Oral Diseases,* 10**,** 311-318.

SPAK, C. J., JOHNSON, G. & EKSTRAND, J. 1994. Caries incidence, salivary flow-rate and efficacy of fluoride gel treatment in irradiated patients. *Caries Research,* 28**,** 388-393.

SREENATH, T., THYAGARAJAN, T., HALL, B., LONGENECKER, G., D'SOUZA, R., HONG, S., WRIGHT, J. T., MACDOUGALL, M., SAUK, J. & KULKARNI, A. B. 2003. Dentin sialophosphoprotein knockout mouse teeth display widened predentin zone and develop defective dentin mineralization similar to human dentinogenesis imperfecta type III. *Journal of Biological Chemistry,* 278**,** 24874-24880.

STANLEY, H. R., PEREIRA, J. C., SPIEGEL, E., BROOM, C. & SCHULTZ, M. 1983. The detection and prevalence of reactive and physiologic sclerotic dentin, reparative dentin and dead tracts beneath various types of dental lesions according to tooth surface and age. *Journal of Oral Pathology & Medicine,* 12**,** 257-289.

STETLERSTEVENSON, W. G. & VEIS, A. 1986. Type-i collagen shows a specific binding-affinity for bovine dentin phosphophoryn. *Calcified Tissue International,* 38**,** 135-141.

STOOKEY, G. K. 2008. The effect of saliva on dental caries. *Journal of the American Dental Association,* 139**,** 11S-17S.

SULKALA, M., TERVAHARTIALA, T., SORSA, T., LARMAS, M., SALO, T. & TJADERHANE, L. 2007. Matrix metalloproteinase-8 (MMP-8) is the major collagenase in human dentin. *Archives of Oral Biology,* 52.

SUN, L. & CHOW, L. C. 2008. Preparation and properties of nano-sized calcium fluoride for dental applications. *Dental Materials,* 24.

TAKAGI, S., CHOW, L. C. & ISHIKAWA, K. 1998. Formation of hydroxyapatite in new calcium phosphate cements. *Biomaterials,* 19.

TAKAGI, Y., FUJISAWA, R. & SASAKI, S. 1986. Identification of dentin phosphophoryn localization by histochemical stainings. *Connective Tissue Research,* 14**,** 279-292.

TANAKA, M. & KADOMA, Y. 2000. Comparative reduction of enamel demineralization by calcium and phosphate in vitro. *Caries Research,* 34**,** 241-245.

TAY, F. R., GWINNETT, A. J., PANG, K. M. & WEI, S. H. Y. 1996. Resin permeation into acid conditioned, moist, and dry dentin: A paradigm using water-free adhesive primers. *Journal of Dental Research,* 75**,** 1034-1044.

TAY, F. R. & PASHLEY, D. H. 2008. Guided tissue remineralisation of partially demineralised human dentine. *Biomaterials,* 29.

TAY, F. R. & PASHLEY, D. H. 2009. Biomimetic Remineralization of Resin-bonded Acid-etched Dentin. *Journal of Dental Research,* 88.

TEN CATE, A. 1994. *Oral histology: Development, Structure and Function*.

TEN CATE, J. M. 1994. In situ models, physico-chemical aspects. *Advances in dental research,* 8**,** 125-33.

TENCATE, J. M. & DUIJSTERS, P. P. E. 1982. Alternating demineralization and remineralization of artificial enamel lesions. *Caries Research,* 16**,** 201-210.

TENCATE, 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**,** 283-296.

TENUTA, L. M. A., CEREZETTI, R. V., DEL BEL CURY, A. A., TABCHOURY, C. P. M. & CURY, J. A. 2008. Fluoride Release from CaF2 and Enamel Demineralization. *Journal of Dental Research,* 87.

TENUTA, L. M. A. & CURY, J. A. 2010. Fluoride: its role in dentistry. *Brazilian oral research,* 24 Suppl 1.

TEZVERGIL-MUTLUAY, A., AGEE, K. A., HOSHIKA, T., CARRILHO, M., BRESCHI, L., TJADERHANE, L., NISHITANI, Y., CARVALHO, R. M., LOONEY, S., TAY, F. R. & PASHLEY, D. H. 2010. The requirement of zinc and calcium ions for functional MMP activity in demineralized dentin matrices. *Dental Materials,* 26**,** 1059-1067.

TEZVERGIL-MUTLUAY, A., MUTLUAY, M., SESEOGULLARI-DIRIHAN, R., AGEE, K. A., KEY, W. O., SCHEFFEL, D. L. S., BRESCHI, L., MAZZONI, A., TJAEDERHANE, L., NISHITANI, Y., TAY, F. R. & PASHLEY, D. H. 2013. Effect of Phosphoric Acid on the Degradation of Human Dentin Matrix. *Journal of Dental Research,* 92**,** 87-91.

THULA, T. T., RODRIGUEZ, D. E., LEE, M. H., PENDI, L., PODSCHUN, J. & GOWER, L. B. 2011. In vitro mineralization of dense collagen substrates: A biomimetic approach toward the development of bone-graft materials. *Acta Biomaterialia,* 7**,** 3158-3169.

TJADERHANE, L., LARJAVA, H., SORSA, T., UITTO, V. J., LARMAS, M. & SALO, T. 1998. The activation and function of host matrix metalloproteinases in dentin matrix breakdown in caries lesions. *Journal of Dental Research,* 77**,** 1622-1629.

TJADERHANE, L., NASCIMENTO, F. D., BRESCHI, L., MAZZONI, A., TERSARIOL, I. L. S., GERALDELI, S., TEZVERGIL-MUTLUAY, A., CARRILHO, M., CARVALHO, R. M., TAY, F. R. & PASHLEY, D. H. 2013. Strategies to prevent hydrolytic degradation of the hybrid layer-A review. *Dental Materials,* 29**,** 999-1011.

TSCHOPPE, P., ZANDIM, D. L., MARTUS, P. & KIELBASSA, A. M. 2011. Enamel and dentine remineralization by nano-hydroxyapatite toothpastes. *Journal of Dentistry,* 39**,** 430-437.

TUBERT-JEANNIN, S., AUCLAIR, C., AMSALLEM, E., TRAMINI, P., GERBAUD, L., RUFFIEUX, C., SCHULTE, A. G., KOCH, M. J., REGE-WALTHER, M. & ISMAIL, A. 2011. Fluoride supplements (tablets, drops, lozenges or chewing gums) for preventing dental caries in children. *Cochrane Database of Systematic Reviews*.

TZIAFAS, D., SMITH, A. J. & LESOT, H. 2000. Designing new treatment strategies in vital pulp therapy. *Journal of Dentistry,* 28**,** 77-92.

UHLEN, M.-M., TVEIT, A. B., STENHAGEN, K. R. & MULIC, A. 2014. Self-induced vomiting and dental erosion - a clinical study. *Bmc Oral Health,* 14.

VAN LOVEREN, C. 2004. Sugar alcohols: What is the evidence for caries-preventive and caries-therapeutic effects? *Caries Research,* 38.

VANAMERONGEN, J. P., LEMMENS, I. G. & TONINO, G. J. M. 1983. The concentration, extractability and characterization of collagen in human dental-pulp. *Archives of Oral Biology,* 28**,** 339-345.

VANDIVER, J., DEAN, D., PATEL, N., BONFIELD, W. & ORTIZ, C. 2005. Nanoscale variation in surface charge of synthetic hydroxyapatite detected by chemically and spatially specific high-resolution force spectroscopy. *Biomaterials,* 26.

VANHOUTE, J. 1994. ROLE OF MICROORGANISMS IN CARIES ETIOLOGY. *Journal of Dental Research,* 73**,** 672-681.

VANMEERBEEK, B., DHEM, A., GORETNICAISE, M., BRAEM, M., LAMBRECHTS, P. & VANHERLE, G. 1993. Comparative sem and tem examination of the ultrastructure of the resin-dentin interdiffusion zone. *Journal of Dental Research,* 72.

VANSTRIJP, A. J. P., VANSTEENBERGEN, T. J. M., DEGRAAFF, J. & TENCATE, J. M. 1994. Bacterial-colonization and degradation of demineralized dentin matrix in-situ. *Caries Research,* 28**,** 21-27.

VANSTRIJP, A. J. P., VANSTEENBERGEN, T. J. M. & TENCATE, J. M. 1997. Bacterial colonization of mineralized and completely demineralized dentine in situ. *Caries Research,* 31**,** 349-355.

VEIS, A. & PERRY, A. 1967. Phosphoprotein of dentin matrix. *Biochemistry,* 6.

VERRAN, J., SANDOVAL, G., ALLEN, N. S., EDGE, M. & STRATTON, J. 2007. Variables affecting, the antibacterial properties of nano and pigmentary titania particles in suspension. *Dyes and Pigments,* 73**,** 298-304.

VIDAL, C. M. P., TJADERHANE, L., SCAFFA, P. M., TERSARIOL, I. L., PASHLEY, D., NADER, H. B., NASCIMENTO, F. D. & CARRILHO, M. R. 2014. Abundance of MMPs and Cysteine Cathepsins in Caries-affected Dentin. *Journal of Dental Research,* 93**,** 269-274.

VIEIRA, A., LUGTENBORG, M., RUBEN, J. L. & HUYSMANS, M. 2006. Brushing abrasion of eroded bovine enamel pretreated with topical fluorides. *Caries Research,* 40**,** 224-230.

VOET, D. & VOET, J. 2010. *Biochemistry*.

VOGEL, G. L., CHOW, L. C., CAREY, C. M., SCHUMACHER, G. E. & TAKAGI, S. 2006. Effect of a calcium prerinse on salivary fluoride after a 228-ppm fluoride rinse. *Caries Research,* 40**,** 178-180.

VOGEL, G. L., SCHUMACHER, G. E., CHOW, L. C., TAKAGI, S. & CAREY, C. M. 2008. Ca pre-rinse greatly increases plaque and plaque fluid F. *Journal of Dental Research,* 87**,** 466-469.

VOLLENWEIDER, M., BRUNNER, T. J., KNECHT, S., GRASS, R. N., ZEHNDER, M., IMFELD, T. & STARK, W. J. 2007. Remineralization of human dentin using ultrafine bioactive glass particles. *Acta Biomaterialia,* 3**,** 936-943.

VYAVHARE, S., SHARMA, D. S. & KULKARNI, V. K. 2015. Effect of three different pastes on remineralization of initial enamel lesion: an in vitro study. *The Journal of clinical pediatric dentistry,* 39**,** 149-60.

WADA, T., MCKEE, M. D., STEITZ, S. & GIACHELLI, C. M. 1999. Calcification of vascular smooth muscle cell cultures inhibition by osteopontin. *Circulation Research,* 84**,** 166-178.

WADDINGTON, R. J., HALL, R. C., EMBERY, G. & LLOYD, D. M. 2003. Changing profiles of proteoglycans in the transition of predentine to dentine. *Matrix Biology,* 22**,** 153-161.

WADDINGTON, R. J., MOSELEY, R., SMITH, A. J., SLOAN, A. J. & EMBERY, G. 2004. Fluoride-induced changes to proteoglycan structure synthesised within the dentine-pulp complex in vitro. *Biochimica Et Biophysica Acta-Molecular Basis of Disease,* 1689**,** 142-151.

WALLS, A. W. G. & MEURMAN, J. H. 2012. Approaches to caries prevention and therapy in the elderly. *Advances in dental research,* 24**,** 36-40.

WALSH, T., OLIVEIRA-NETO, J. M. & MOORE, D. 2015. Chlorhexidine treatment for the prevention of dental caries in children and adolescents. *Cochrane Oral Health Group*.

WALTIMO, J., RISTELI, L., RISTELI, J. & LUKINMAA, P. L. 1994. Altered collagen expression in human dentin - increased reactivity of type-iii and presence of type-vi in dentinogenesis imperfecta, as revealed by immunoelectron microscopy. *Journal of Histochemistry & Cytochemistry,* 42**,** 1593-1601.

WANG, R. Z. & WEINER, S. 1998. Human root dentin: Structural anisotropy and Vickers microhardness isotropy. *Connective Tissue Research,* 39**,** 269-279.

WANG, Y. & SPENCER, P. 2002. Analysis of acid-treated dentin smear debris and smear layers using confocal Raman microspectroscopy. *Journal of Biomedical Materials Research,* 60.

WEGEHAUPT, F., GRIES, D., WIEGAND, A. & ATTIN, T. 2008. Is bovine dentine an appropriate substitute for human dentine in erosion/abrasion tests? *Journal of Oral Rehabilitation,* 35**,** 390-394.

WEINER, S., VEIS, A., BENIASH, E., ARAD, T., DILLON, J. W., SABSAY, B. & SIDDIQUI, F. 1999. Peritubular dentin formation: Crystal organization and the macromolecular constituents in human teeth. *Journal of Structural Biology,* 126**,** 27-41.

WU, C., CHANG, J. & FAN, W. 2012. Bioactive mesoporous calcium-silicate nanoparticles with excellent mineralization ability, osteostimulation, drug-delivery and antibacterial properties for filling apex roots of teeth. *Journal of Materials Chemistry,* 22**,** 16801-16809.

WU, C., WANG, L., HARBOTTLE, D., MASLIYAH, J. & XU, Z. 2015. Studying bubble-particle interactions by zeta potential distribution analysis. *Journal of colloid and interface science,* 449**,** 399-408.

XIAO, S. X., YU, C., CHOU, X. M., YUAN, W. J., WANG, Y., BU, L., FU, G., QIAN, M. Q., YANG, J., SHI, Y. Z., HU, L. D., HAN, B., WANG, Z. M., HUANG, W., LIU, J., CHEN, Z., ZHAO, G. P. & KONG, X. Y. 2001. Dentinogenesis imperfecta 1 with or without progressive hearing loss is associated with distinct mutations in DSPP. *Nature Genetics,* 27.

XU, H. H. K., WEIR, M. D. & SUN, L. 2007. Nanocomposites with Ca and PO4 release: Effects of reinforcement, dicalcium phosphate particle size and silanization. *Dental Materials,* 23.

YAMAKOSHI, Y., HU, J. C. C., IWATA, T., KOBAYASHI, K., FUKAE, M. & SIMMER, J. P. 2006. Dentin sialophosphoprotein is processed by MMP-2 and MMP-20 in vitro and in vivo. *Journal of Biological Chemistry,* 281**,** 38235-38243.

YAMAKOSHI, Y., HU, J. C. C., LIU, S., ZHANG, C., OIDA, S., FUKAE, M. & SIMMER, J. P. 2003. Characterization of porcine dentin sialoprotein (DSP) and dentin sialophosphoprotein (DSPP) cDNA clones. *European Journal of Oral Sciences,* 111**,** 60-67.

YAMAMURA, T. 1985. Differentiation of pulpal cells and inductive influences of various matrices with reference to pulpal wound-healing. *Journal of Dental Research,* 64**,** 530-540.

YEUNG, C. A., CHONG, L. Y. & GLENNY, A.-M. 2015. Fluoridated milk for preventing dental caries. *Cochrane Database of Systematic Reviews*.

YIP, H. K. & SAMARANAYAKE, L. P. 1998. Caries removal techniques and instrumentation: a review. *Clinical oral investigations,* 2**,** 148-54.

YUAN, G., WANG, Y., GLUHAK-HEINRICH, J., YANG, G., CHEN, L., LI, T., WU, L.-A., CHEN, Z., MACDOUGALL, M. & CHEN, S. 2009. Tissue-specific expression of dentin sialophosphoprotein (DSPP) and its polymorphisms in mouse tissues. *Cell Biology International,* 33**,** 816-829.

ZAMATARO, C. B., TENUTA, L. M. A. & CURY, J. A. 2008. Low-fluoride dentifrice and the effect of postbrushing rinsing on fluoride availability in saliva. *European archives of paediatric dentistry : official journal of the European Academy of Paediatric Dentistry,* 9.

ZAURA, E. & TEN CATE, J. M. 2004. Dental plaque as a biofilm: A pilot study of the effects of nutrients on plaque pH and dentin demineralization. *Caries Research,* 38**,** 9-15.

ZERO, D. T. 2009. Recaldent--evidence for clinical activity. *Advances in dental research,* 21**,** 30-4.

ZHANG, J., SENGER, B., VAUTIER, D., PICART, C., SCHAAF, P., VOEGEL, J. C. & LAVALLE, P. 2005. Natural polyelectrolyte films based on layer-by layer deposition of collagen and hyaluronic acid. *Biomaterials,* 26**,** 3353-3361.

ZHANG, K., LI, F., IMAZATO, S., CHENG, L., LIU, H., AROLA, D. D., BAI, Y. & XU, H. H. K. 2013. Dual antibacterial agents of nano-silver and 12-methacryloyloxydodecylpyridinium bromide in dental adhesive to inhibit caries. *Journal of Biomedical Materials Research Part B-Applied Biomaterials,* 101B**,** 929-938.

ZHANG, W., CRITTENDEN, J., LI, K. & CHEN, Y. 2012a. Attachment Efficiency of Nanoparticle Aggregation in Aqueous Dispersions: Modeling and Experimental Validation. *Environmental Science & Technology,* 46**,** 7054-7062.

ZHANG, X., NEOH, K. G., LIN, C. C. & KISHEN, A. 2012b. Remineralization of partially demineralized dentine substrate based on a biomimetic strategy. *Journal of Materials Science-Materials in Medicine,* 23**,** 733-742.

ZHANG, X. H., ZHAO, J., LI, C. F., GAO, S., QIU, C. C., LIU, P., WU, G. Y., QIANG, B. Q., LO, W. H. Y. & SHEN, Y. 2001. DSPP mutation in dentinogenesis imperfecta Shields type II. *Nature Genetics,* 27.

ZHANG, Y., AGEE, K., NOR, J., CARVALHO, R., SACHAR, B., RUSSELL, C. & PASHLEY, D. 1998. Effects of acid-etching on the tensile properties of demineralized dentin matrix. *Dental Materials,* 14.

ZHANG, Y., YANG, M., PORTNEY, N. G., CUI, D., BUDAK, G., OZBAY, E., OZKAN, M. & OZKAN, C. S. 2008. Zeta potential: a surface electrical characteristic to probe the interaction of nanoparticles with normal and

cancer human breast epithelial cells. *Biomedical Microdevices,* 10**,** 321-328.

ZHAO, W., CHANG, J. & ZHAI, W. 2008. Self-setting properties and in vitro bioactivity of Ca3SiO5/CaSO4 center dot 1/2H(2)O composite cement. *Journal of Biomedical Materials Research Part A,* 85A**,** 336-344.

ZHAO, W. Y., WANG, J. Y., ZHAI, W. Y., WANG, Z. & CHANG, J. 2005. The self-setting properties and in vitro bioactivity of tricalcium silicate. *Biomaterials,* 26**,** 6113-6121.

ZHU, Q., GIBSON, M. P., LIU, Q., LIU, Y., LU, Y., WANG, X., FENG, J. Q. & QIN, C. 2012. Proteolytic Processing of Dentin Sialophosphoprotein (DSPP) Is Essential to Dentinogenesis. *Journal of Biological Chemistry,* 287**,** 30426-30435.

12. Appendices

12.1 Appendix 1: Production of Biogide Membranes

As discussed in section 4.3.1, resorbable collagen membranes were used to analyse the interaction between collagen and silica nanoparticles. The following details regarding the origin and purification of these membranes have been taken from patent EP1676592B1 (Geistlich and Eckmayer, 1995).

The membranes were derived directly from naturally occurring membranes in the peritoneum of piglets, ensuring that the natural collagen structure is retained. This collagen in the membranes is pure, native (not denatured) and insoluble.

As part of the preparation of the membranes, they are chemically purified to remove any substances accompanying the collagen, whilst ensuring that the collagen properties are unmodified.

Purification involves immersion in an alkali (0.2 – 4 % sodium hydroxide) to remove fats and alkali-sensitive proteins such as glycosaminoglycans (GAGs) and proteoglycans (PGs), followed by immersion in an acid (hydrochloric acid) to eliminate acid sensitive substances and cause the fibres to swell and the structure to loosen. The membranes are then washed with distilled water to increase the pH before being levelled out to achieve a uniform thickness (approximately 1mm). For this, the material is treated with a salt solution and the fibres de-swell. The membranes are then washed with an alkali to achieve pH7 and rinsed with water before being dried in acetone.

The level of amide nitrogen in the membrane is measured to ensure that every membrane is made up of at least 95 % native (not denatured) collagen. The degree of purification of the collagen is then determined by amino acid analysis. This ensures that collagen is pure and native, whilst eliminating non-collagenous proteins (NCPs). The membranes are not treated with a fixative because it may affect resorption rates.

Products for clinical use would then be subjected to gamma irradiation, however, the membranes used in this project were not irradiated.

12.2 Appendix 2: Publications

12.2.1 Conference Presentations

Ashworth, E M., Miller, C A., Deery C & Martin, N. (March 2015). Choice of Demineralising Agent is Critical for Partially-demineralised Dentine Models. *93rd General Session and Exhibition of the International Association for Dental Research,* Boston, USA. Oral Presentation.

Ashworth, E M., Betamar, N., Deery C., Miller, C A., & Martin, N. (September 2014). Adherence of Silica Nanoparticles to Acid-Affected Fixed and Non-Fixed Collagen. *7th International Association for Dental Research/Pan European Regional Congress,* Dubrovnik, Croatia. Oral Presentation.

Ashworth, E M., Miller, C A., Deery C & Martin, N. (August 2014). Characterisation of Partially-Demineralised Dentine is a Prerequisite for Remineralisation Studies. *26th Annual Conference of the European Society of Biomaterials,* Liverpool, UK. Oral presentation.

Ashworth, E M., Miller, C A., Deery C & Martin, N. (May 2014). Using Remineralisation of Dentine using Nanoparticles. *GlaxoSmithKline Science Symposium*, Weybridge, UK. Oral Presentation.

Ashworth, E M., Miller, C A., Deery C & Martin, N. (September 2013). Effect of Dietary, Gastric, Bacterial and Experimental Acids on Dentine. *60th Annual Scientific Meeting and Exhibition of the British Society for Oral and Dental Research*, Bath, UK. Poster presentation.

Ashworth, E M., Miller, C A., Deery C & Martin, N. (May 2012). Using Nanoparticles as part of a Remineralising Strategy for Dentine affected by Caries or Acid Erosion. *GlaxoSmithKline Science Symposium*, Weybridge, UK. Poster Presentation.

12.2.2 Awards for Presentations

GlaxoSmithKline - Mineralised Tissue Research Group (GSK-MINTIG) Award (September 2014). Ashworth, E M., Betamar, N., Deery C., Miller, C A., & Martin, N. Adherence of Silica Nanoparticles to Acid-Affected Fixed and Non-Fixed Collagen. *7th International Association for Dental Research/Pan European Regional Congress,* Dubrovnik, Croatia. Oral Presentation.

Third Year Presentation Competition (March 2014). Remineralisation of Dentine Using Nanoparticles. *Annual Research Day,* School of Clinical Dentistry, University of Sheffield, UK. Oral Presentation.