DENTINAL ULTRASTRUCTURE IN OSTEOGENESIS IMPERFECTA AND DENTINOGENESIS IMPERFECTA

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DECLARATION OF ORIGINALITY

I hereby declare that the work presented in this thesis was carried out by myself. Information derived from the published and unpublished work of others has been acknowledged in the text and the relevant references are included in this thesis.

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ABSTRACT

Osteogenesis Imperfecta (OI) associated with Dentinogenesis Imperfecta, type I (DI) is a rare genetic condition, where mutations of COL1A1 and COL1A2 genes result in variations in the amino sequence of the collagen α-chains. The collagen fibrils are expected to be abnormally thin due to defective collagen formation. These alterations have been shown to affect the bones, but have not yet been elucidated in the dentinal collagen.

Objectives: Evaluation of demineralisation protocols to expose dentinal collagen for permanent and primary teeth and to characterise the morphology of dentinal collagen ultrastructure in primary DI type I vs. control primary teeth using Atomic Force Microscopy (AFM).

Methods: Primary (6) and permanent (6) control teeth plus one DI type I primary tooth have been used to date in this on-going project. To reveal the dentinal collagen structure, four demineralisation approaches have been evaluated: 1) two blocks of dentine were treated with 10 vol% citric acid for 15second (s) and 6.5 vol% NaOCLaq for 120s; 2) dentine blocks treated with commercial etchant (37% phosphoric acid) and controlled 6.5 vol% NaOCLaq. Raman spectroscopy was used to assess the degree of demineralisation. These demineralised dentine were characterized using AFM imaging; 3) demineralisation protocol in which the teeth were left in 17% Ethylenediaminetetraacetic acid (EDTA) for 12 months before being histology sectioned and stained with Picrosirius red; and 4) dentine block was treated with 17% EDTA for 10 minutes. Both demineralised samples were characterised using scanning electron microscopy (SEM).

Results: All demineralisation protocols achieved partial or complete demineralisation of the dentine. Demineralisation with commercial etchant and NaOCl produced visibly defined collagen fibrils in primary and permanent teeth. A shorter time was sufficient for demineralisation in primary teeth. No signs of degradation of the fibrils suggesting that the demineralisation protocol will be adequate for our DI/OI teeth.
In the ultrastucture findings of OI associated DI primary teeth; AFM images revealed an area of thickened dentinal collagen fibrils that distributed at only one a direction with a wide distribution of D-banding separations distance, suggesting the DI collagen banding was less regular in pattern.

Conclusion: The difference in dentinal collagen fibril arrangement and D-banding distance in the affected primary tooth may lead explain the altered mineralization.

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LIST OF ABBREVIATIONS

α	Alpha
%	Percentage
µm	Micrometer
mm	Milimeter
cm	Centimetre
nm	Nanometer
H	Hydrogen
s	Seconds
NH	Amide
C=O	Carbonyl
3D	three dimensional
AFM	atomic force microcopy
BMP1	bone morphogenetic protein 1
BSP	bone sialoprotein
calcium hydroxyapatite,	$Ca_{10}(PO_4)_6(OH)_2$
CRTAP	cartilage associated protein
COL1A1	collagen type 1, alpha 1
COL1A2	collagen type 1, alpha 2
DEJ	dento-enamel junction
DI	Dentinogenesis Imperfecta
DMP1	dentine matrix protein 1
DPP	dentine phosphoprotein
DSPP	dentine sialophosphoprotein
DSP	dentine sialoprotein
ECM	extracellular matrix
ECF	extracellular fluid
EDH	Eastman Dental Hospital
EDTA	Ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
FKBP10	FK506 binding protein 10, 65 kDa
GAG	glycosaminoglycan
hyald	hydroxylysine- aldehyde

KHN	Knoop Hardness Number
mRNA	messenger ribo-nucleic acid
min	Minutes
NaOCI	Sodium hypochlorite
NHS	National Health Service
OI	Osteogenesis Imperfecta
OPN	Osteopontin
PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2
SEM	scanning electron microscopy
SERPINF1	serpin peptidase inhibitor, clade F, member 1
SMPD3	sphingomyelin phosphodiesterase 3
SP7	Sp7 transcription factor
TEM	transmission electron microscopy
vol	Volume

CHAPTER 1
INTRODUCTION

1.0 INTRODUCTION

1.1 Osteogenesis Imperfecta

1.1.1 History and classification

Osteogenesis Impefecta (OI) is a genetic disorder characterised by abnormal connective tissue development that leads to osseous fragility and bone fracture. These features have led to the trivial name of 'Brittle Bone Disease' (Rauch and Glorieux, 2004) Other secondary features that may present in OI patients include short stature, loose ligaments, hypermobility, hearing deficit, blue sclera, skin hyperlaxity and Dentinogenesis Imperfecta (Byers, 2000)

The study of OI began in 1788, where the Swede Olof Jakob Ekman, described the condition of congenital osteomalacia with hereditary bone fragility, in his doctoral thesis and mentioned cases dating back to 1678. Since then, many other names have been used to describe the condition. Among some of the common alternatives names are Ekman-Lobstein syndromes, Vrolik syndrome and glass bone disease. These names were recognised during the 18th century by Lobstein and Vrolik who lent their names to the syndrome (Byers, 1995).In 1906, further studies proposed by Looser postulated two category of OI; congenital (severe) and tarda (mild). The important milestone in OI research has been the classification of OI into four types which was based on the mode of inheritance, clinical presentation and radiographic findings as characterised by Sillence (Sillence et al., 1979a). With advances in molecular genetic analyses today, attempts have been made to further classify OI, although the Sillence classification is still in used in an adjusted and extended form (Rauch and Glorieux, 2004)

It was reported that the prevalence of OI was approximately 6-7 per 100,000 live births (Steiner et al., 1993). According to Steiner, each of the OI types has different prevalence from each other (Steiner et al., 1993). In 1979, the prevalence of OI type I was reported to be approximately 3-4 per 100,000 live births (Sillence et al., 1979b) The prevalence of type II and type III were about 2-3 per 100, 000 live births (Steiner et al., 1993). Other reports showed that the general prevalence of OI ranged from one per 10 000 to one per 20 000 live birth (Engelbert et al., 1998). Recent accurate prevalence data for OI are currently not available.

The Sillence classification distinguished OI into four types; type I, II, III and IV. Type I is autosomal dominant and the mildest form of the disease. Normally, affected individuals have blue sclera, normal or close to normal stature and infrequently Dentinogenesis Imperfecta. Vertebral fractures are common and can lead to mild scoliosis. The fractures are rarely seen at birth, however begin when the affected child starts to walk and stand. Fractures commonly affect the long bones, ribs and small bones of the feet and hands. The incidence of fracture decreases following puberty. Radiographic bone morphology is generally normal although mild femoral bowing may occur in newborns while in adults the classic 'cod-fish' appearance (vertebral deformity) often develops accompanied with a loss in height (Sillence et al., 1979b).

Type II is the perinatal lethal form of OI that affects newborn infants. The mode of inheritance may be autosomal dominant or autosomal recessive. Affected infants exhibit severe skeletal deformities with short extremities, bowed legs and flexed and abducted hips. Prematurity and low birth weights are common. Radiographic findings showed bowed tibias, short femurs and an absence of mineralisation. The survival rate of infant beyond a year is extremely rare (Sillence et al., 1979b).

Type IIII is the progressive form of OI, which can be autosomal dominant or autosomal recessive. The features are characterised by severe progressive skeletal deformities which often start from conception. This results in in-utero fractures. The incidences of fractures remain high during growth until adulthood. The affected individuals are normally very short with blue sclera at birth and deformities can cause severe scoliosis. Dentinogenesis Imprefecta is common and involves primary and permanent teeth. Radiographs at birth generally demonstrate decreased mineralised calvarium with large fontanelle and features of Wormian bones which showed thin ribs with sign of fractures. Individuals with type III are at risk of respiratory problems and brain stem compression due to bone deformation at associated organs (Sillence et al., 1979b).

Type IV is autosomal dominant with the most clinically diverse group. The features include normal or grayish sclera, mild to moderate deformity, short stature and Dentinogenesis Imperfecta. The incidence of fractures can be variable. Fractures are common during birth and cease during adult life. The life span of individuals with OI type IV is nearly normal (Sillence et al., 1979b).

In 2004, the Sillence classification was expanded and to recognize three more types of OI; type V to VII (Rauch and Glorieux, 2004). Type V is autosomal dominant and a moderately deforming disease. The main features of type V are frequent development of hypertrophic calluses at fracture sites, calcification of the interosseous membranes between the bones of the forearm which may lead to dislocation of the radial head, and patients generally have short stature. Blue sclera and Dentinogenesis Imperfecta are not present in type V (Rauch and Glorieux, 2004)
Type VI is autosomal recessive with moderate to severe skeletal deformities without blue sclera and Dentinogenesis Imperfecta. The distinct features of this type are the

presence of excessive osteoid accumulation on bone-forming surfaces and fishscale-like appearance of the bone lamellae (Rauch and Glorieux, 2004).

Type VII is autosomal recessive with moderate deforming OI. Individuals present with mild short stature, bone fragility and lack blue sclera and dentinogenesis imperfecta. The distinct feature is a shortening of the humerus and the femur. Infants with type VII may develop respiratory insufficiency in the neonatal and postnatal periods and is frequently fatal, as a result of underlying problems such as pulmonary anomalies and infectious disease (Rauch and Glorieux, 2004).

In 2007, Type VIII was proposed as an additional type of OI (Cabral et al., 2007a). This type of OI is described as autosomal recessive and severe deforming lethal type. Patients may present with white sclera, extremely short stature and severe osteoporosis. To date, Type VIII has only been observed in a community of Native Americans in northern Quebec (Glorieux, 2008).

Type	Genetics	Clinical findings
OI I	Autosomal dominant	Blue sclera, normal or close to normal stature, DI (infrequent), fracture with little or no limb deformity
OI II	Autosomal dominant	Lethal perinatal type; affect newborn infants, severe skeletal deformities, short extremities, bowed legs, premature infant Radiograph : absence of bone mineralisation
OI III	Autosomal dominant or autosomal recessive	Progressive skeletal deformities at birth, scleral hue varies, very short stature, DI Radiograph: decreased mineralised calvarium, wormian bone (thin ribs with sign of fracture)
OI IV	Autosomal dominant	Normal sclerae, mild/moderate limb deformity with fracture, variable short stature, DI, hearing loss.
OI V	Autosomal dominant	Frequent development of hypertrophic callus at fracture sites, calcification of intraosseous membrane of bones, dislocation of bone, short stature
OI VI	Autosomal recessive	Moderate to severe skeletal deformities without DI and blue sclera, excessive osteoid accumulation form fishscale-like appearance
OI VII	Autosomal recessive	Moderate deforming OI, bone fragility, congenital fractures, shortening of humerus and femur, respiratory insufficiency
OI VIII	Autosomal recessive	White sclera, extremely short, severe osteoporosis

Table 1. 1: Clinical classification of Osteogenesis Imperfecta

1.1.2 Mutations of gene in Osteogenesis Imperfecta

OI is associated with abnormalities of type I collagen, with the majority of patients having mutations in one of the two genes, COL1A1 and COL1A2 that encode Type 1 collagen chains. The remaining may be due to recessive variants in other genes such as cartilage-associated protein (CRTAP) and leucine proline-enriched proteoglycan (LEPRE) which were found in Type VII and Type VIII OI (Van Dijk et al., 2010).

Recently, molecular and biochemical studies have identified types of OI that are not associated with a defect in collagen gene COL1A1 and COL1A2. These types of OI have an association with deficiency of genes that encode component of collagen. In 2006, partial loss of function cartilage-associated protein (CRTAP) mutations encodes prolyl-3-hydroxylase-1(P3H1) hydroxylation of proline residue in procollagen chain found to cause OI type VII (Morello et al., 2006). The following year, LEPRE1 mutations that encode P3H1 was described in OI type VIII (Cabral et al., 2007b). To date, a few new genes have been determined causing autosomal recessive forms of OI (Byers and Pyott, 2012) ;

- Mineralisation defect- Type VI OI (serpin peptidase inhibitor, clade F, member 1 (SERPINF1),
- Collagen P3H1 defects- Type VII, VIII and IX OI (CRTAP, LEPRE1, Peptidyl-prolyl cis-trans isomerase B (PPIB),
- Collagen chaperone defects – Type X and XI OI (SERPINF1 and FKBP10),
- C-propeptide cleavage enzyme defect – Type XII OI (Bone morphogenetic protein 1 (BMP1),
- Frameshift mutations – unclassified OI types (Sp7 transcription factor (SP7).

1.1.3 Effect of mutation on Type I collagen

Mutation of collagen can be classified into two general categories (Marini et al., 2007).

I. Mutation that cause quantitative defect, with synthesis of structurally normal type I procollagen (affect amount of type I procollagen)

II. Mutation that results in synthesis of collagen with structural abnormalities type I procollagen (affect the structure of type I procollagen)

(Marini et al., 2007)

The first group usually results from one 'null' COL1A1 gene that leads to diminished amounts of type I procollagen. This mutation usually produces premature termination codons in the coding sequence of one COL1A1 allele that cause reduction in stability of the mRNA due to a process known as' nonsense-mediated decay (Byers, 1993). There are several events that can give rise to these premature termination codons. These include points mutation that cause substitution of a triple helical Glycine residue by another amino acid and mutation that alter splice sites (Marini et al., 2007). These mutations have mild clinical consequences as they do not appear to alter the expression of many other genes in bone and other connective tissues. Type I OI almost always results in this type of mutation (Byers, 1993).

The mutations that affect the structure of the procollagen chains have more deleterious phenotypic consequences than those of 'null' mutations (Byers, 1993). Structural mutations of type I collagen results in abnormal proteins chains that can interfere with the collagen cell interaction or the mineralisation mechanism (Marini et al., 2007). The ranges of clinical presentations depend on the genes in which the mutation occurs, the location of abnormal sequence in protein and type of mutations. (Byers, 1993). These comprise the majority of mutations that result in OI type II, III and IV. The earliest model between relationship of mutation and phenotype was

focused on the chain in which mutation occurred. Mutations of COL1A1 gene may have more severe phenotypic consequences and may even be lethal, because they compromise three-quarters of all type I collagen (Prockop et al., 1989). However, if the similar mutation occurs in COL1A2, the effect will be mild as it only causes decreased amounts of collagen production. Figure 1.1 describes the mutation in OI type I which due to decreased synthesis of collagen and in type II,III and IV which were due to mutation that affect the collagen structure (Gajiko-Galicka, 2002). Later, it was proposed that the clinical severity of OI depends on the position of mutation along the chain, with the mutations closest to the carboxyl terminal end resulting in more severe phenotypes than those closer to the amino-terminal end, that result in moderate to mild cases (Starman et al., 1989).

OI type I is mostly characterised by the reduction in the amount of collagen whilst OI type II, III and IV were results from abnormal production of collagen type I (Marini et al., 2007).

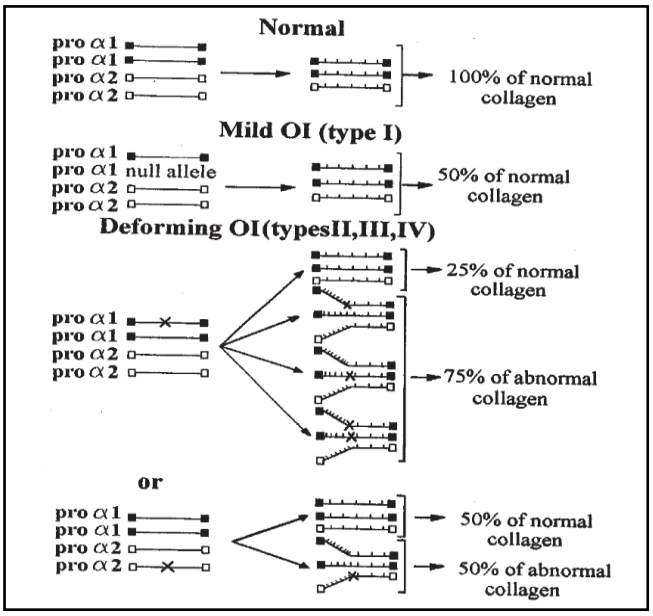


Figure 1.1: Mutations that shows decreased synthesis of normal collagen results in OI type I and mutations that affect the collagen structure that results in OI type II, III and IV (Adapted from Galicka et al, 2002).

1.1.4 Bone collagen in relation to Osteogenesis Imperfecta

Bone is a dynamic tissue, which continuously remodels. Bone has long been known to be responsive to mechanical loading to resist mechanical forces and fractures. Strength is dependent on the bone density and quality of the bone which is characterised by the shape and microarchitecture of the bone and also the turnover of mineral and collagen (Carbonare Dalle L, 2004).

Bone contains complex biominerals and is highly hierarchical in structure. The organic component is primarily composed of type I collagen with minor amounts of type III and type V collagen (Saito and Marumo, 2010). The collagen molecules in bone are typically fibrillar type I collagen which is reinforced with nanoscale hydroxyapatite particles. Collagen type I formed by the osteoblast is typically deposited in parallel or concentric lamellar layers arranged in trabecular and cortical bone (Tzaphlidou, 2008).

The role of type I collagen in the pathogenesis of OI has long been investigated. The strength of the collagen fibrils is derived mainly from intermolecular cross-links. Cross-linking is results of specific three-dimensional arrangements of collagens within the fibril; only molecules that are correctly aligned are able to form cross-links (Bank et al., 2000). These cross links involved the aldehyde side chains which react with amine groups of parallel collagen molecules. Cross links can be divided into two types; enzymatic cross link (lysine hydroxylases and lysyl oxidase) and non-enzymatic cross links (glycation end product) (Bank et al., 2000). A breakdown or reduction in cross-links has been revealed to lead to a loss of strength (Oxlund et al., 1995). The failure of the regulatory mechanism that controls lysyl hydroxylation of the crosslinking in collagen was shown to have close association with bone abnormalities and bone fragility disorder such as OI (Knott and Bailey, 1998)

The vast majority of OI diseases are usually the outcome mutation of COL1A1 and COL1A2 genes which result in the defective production of type I procollagen and abnormal amount of collagen (Byers, 1993). The most abundant mutation that alters the sequence of procollagen is caused by either substitution of the triple helical glycine residue by another amino acid, or splice mutations in genes encoding COL1A1 and COL1A2 (Marini et al., 2007). Mutations are presumably expressed in all type I collagen producing tissue, yet the most affected tissue are skeletal. This may be due to rapid secretion of mutant collagen by bone compared to skin cells. (Gajiko-Galicka, 2002). It was suggested that the patient's osteoblasts expressed type I collagen in a different way than fibroblasts. The selective action towards the osteoblasts compared to the fibroblasts suggests that the incorporation of abnormal sequence is tissue specific (Mundlos et al., 1996). It has been shown that mutant molecules are incorporated into the extracellular matrix of bone efficiently and represent a high percentage of disorganised matrix and had a dramatic effect on the mineral deposition (Gajiko-Galicka, 2002).

Bone contains non collagenous proteins that were believed to be responsible for the initiation and control of the mineralisation process, which includes nucleation and growth of hydroxyapatite crystals, collagen fibril formation and deposition of the matrix (Qin et al., 2004, Gajiko-Galicka, 2002). These mineralisation events and the interactions of the non collagenous proteins in collagen scaffolds were thought to play a significant role in OI bone pathology. The changes in these matrix components were shown to increase the bone brittleness in OI type I, III and IV (Bank et al., 2000).

Information on the ultrastructural abnormalities in OI bone is limited. There have been reports that OI bone collagen diameter are generally smaller, due to a failure of maturation of type I fibrils (Jones et al., 1984). In contrast, another study indicated that the diameter of type I collagen in OI were larger than normal. This is due to the

alteration the primary structure of type I collagen (amino acid sequence) that can reflect changes in the quarternary collagen structure (fibril) which modified the diameter (Cassella et al., 1994).

In the severe forms of OI, abnormal collagen can be seen in the loose fibrous unmineralised regions in lamellar bone of the femoral diaphysis. The abnormal collagen were described as 'pseudo-fibrils' that suggest structural defects featuring thin and fine fibrils (Traub et al., 1994). In OI type III, the collagen fibrils demonstrated abnormal collagen fibrils with flower like, twisted, spiralled and sparsely distributed pattern throughout a very thick osteoid (Sarathchandra and Pope, 2005). However, this was not in the case of OI type I, which demonstrated abnormal thickened type I collagen fibrils, with kinks in the fibrils extending to right angle deformities. These fibrils were closely related to normal collagen molecules with overall D- band periodicity of approximately 67nm (Cassella et al., 1994).

1.1.5 An overview of organic matrix in bone and dentine

Dentine and bone are composite materials that are composed of extracellular matrix (ECM), which primarily comprises of organic matrix (collagen) and an inorganic mineralised phase (hydroxyapatite) (McKee MD et al., 2005). Both are mineralised tissues that are closely related in several aspects. At the ultrastructure scale, the main component of organic matrix in bone and dentine is the collagen type I. This collagen is secreted by well differentiated cells; the osteoblast and odontoblast during osteogenesis and dentinogenesis process respectively (Opsahl Vital et al., 2012). The remaining organic matrix component consists of non-collagenous protein. Some of the proteins include osteopontin (OPN) and bone sialoprotein (BSP) which are believed to be responsible for mineralisation of bone and dentine matrix protein 1

(DMP1) and dentine sialophosphoprotein (DSPP) which are involved in the mineralisation of the dentine (Qin et al., 2004).

Interestingly, the collagen in bone and dentine are interrelated, therefore any bone disease that involves the collagen may disturb the collagen in the dentine too. This can be seen in the condition of Osteogenesis Imperefecta (OI) where one of the important oral finding can be the presence of Dentinogenesis Imperfecta (DI).

1.2 Dentinogenesis Imperfecta

1.2.1 Dentinogenesis Imperfecta in association with Osteogenesis Imperfecta

As mentioned previously, OI results from various gene mutations leading to defects in type I collagen, which is the major component in bone and dentine. The important oral finding in OI is the presence of Dentinogenesis Imperfecta (DI). DI associated with OI is called DI type I (OMIM 166240).

Clinical features: Clinically, affected teeth have an abnormal colour ranging from grey-brown to opalescent blue and have bulbous crowns (Barron et al., 2008). The condition affects both primary and permanent teeth although the primary teeth are often more severely affected (Hart and Hart, 2007). The affected teeth show signs of excessive wear, due to enamel chipping and fracture. The enamel layer is normal but the dentino-enamel junction (DEJ) and the underlying dentine are not. When subjected to occlusal stresses, the dentine is easily exposed as the enamel shears off and cracks away from the dentine (Shields et al., 1973). The dentine also will obliterate the pulp, causing a loss of sensation in the tooth soon after eruption or prior to tooth eruption (Kim and Simmer, 2007).

Radiographic features*:* DI features altered root morphology with short and constricted roots in both dentitions .The degree of pulp of obliteration varies and occurs soon after eruption or sometimes before eruption (Witkop, 1975, Barron et al., 2008).

Pathologic features*:*

The first layer of dentine, the mantle dentine, which forms when dentine forms in the tooth germ, lacks any tubular structures. The mantle dentine is formed by newly differentiated odontoblasts and less mineralized. The mantle dentine has been reported to be normal in patients with DI (Hall et al., 2002) whereas dysplastic manifestations in mantle dentine have been found by others (Malmgren and Lindskog, 2003). The normal appearing mantle dentine suggests an initial normal function of odontoblasts that may be possibly related to different mechanism of 'startup' matrix formation (Hall et al., 2002). The mantle dentine merges with a dysplastic form of dentine which finally becomes hypertrophic and obliterates the pulp chamber and pulp canal (Kim and Simmer, 2007). During initial dentine formation, mesenchymal cells differentiate to form odontoblasts. Clusters of odontoblasts fail to produce regular predentine matrix that may explained changes in further dentine mineralisation process (Wright and Gantt, 1985). The odontoblast differentiation also is controlled by gene expression that may explain the dysplastic manifestations in the dentine.

In an ultrastucture study (using transmission electron microscopy), the collagen observed in the dentine was smaller in diameter than normal (Waltimo et al., 1996). This corresponds with earlier findings on the small diameter of collagen fibril in bone of OI patient (Jones et al., 1984) .

Genetics*:* DI type I is an autosomal dominant trait, associated with OI (type I, III and IV). Individuals with the mutation in type I collagen genes (COL1A1 and COL1A2) and in some other genes which involved in the formation of the collagen and the maturation of collagen can be associated with DI. It is hypothesised that the metabolic pathways involved in dentine and bone matrix formation probably have defects (Bixler, 1976). However, the severity of the dentine and bone defect displayed by the patients varies widely (Marini, 2004). Despite type I collagen being the main organic matrix in bone and dentine, the majority of OI patients have normal teeth (Waltimo et al., 1996). The relationship between how type I collagen mutations effect bone and dentine is poorly understood as it can result in severe bone defects with only mild DI or, DI can be the most penetrant feature in OI (Barron et al., 2008).

The gene mutation can be expressed differently in bone and in dentine which can be assigned partly to specific cell type differences in the transcription of mutated genes (Waltimo, 1996, Waltimo et al., 1996). This was supported by a study showing that tissue specificity exists for mutated collagen, which can be expressed differently in bone and skin (Mundlos et al., 1996). A report of possible mechanisms of dentinal defects due to odontoblast dysfunction was hypothesised to cause the histological changes in DI type I. As the result of the collagen mutation, the dysfunctional odontoblasts may dilate, due to the intracellular accumulation of abnormal procollagen in the rough endoplasmic reticulum. The secreted abnormal matrix mineralizes eventually enveloping the dilated odontoblasts and its process and preventing further collagen secretion (Hall et al., 2002, Majorana et al., 2010). In DI type I, the phenotype presentation of DI can relate to the collagen abnormalities however there is still no strong link between mutations of collagen gene and dental phenotype (Ben Amor et al., 2013).

1.2.2 Dentinogenesis Imperfecta Type II

DI type II (OMIM 125490) can occur as an isolated effect due to mutation of dentine sialophosphoprotein (DSPP).

Clinical features*:* The clinical and radiographic tooth phenotype is indistinguishable from DI type I (Hart and Hart, 2007). Certain characteristics that are recognized in DI type II are:

a) Osteogenesis Imperfecta is not related to DI type II (Bixler, 1976, Witkop, 1975)
b) The severity, colouration and attrition are more profound in DI II (Bixler, 1976) whereas more variable in DI type I

Radiographic features*:* Obliteration of the pulp chamber can begin prior to tooth eruption. Abnormal crown and root morphology is noted with bulbous crowns, a typical feature, with marked cervical constriction. The roots are often narrow with small and short obliterated root canals.

Genetics*:* The non-collagenous part of the organic matrix in the dentine is composed of various proteins with dentine sialophosphoprotein (DSPP) predominating about 50%. DSPP is a human gene that encodes two smaller proteins; dentin sialoprotein (DSP) and dentin phosphoprotein (DPP). These proteins are the main component of the organic matrix which plays an essential role in tooth development. Dentine phosphoprotein helps in the deposition of mineral crystals among the collagen fibrils. Defects in the DSPP gene had been suggested as a causative factor in DI type II (Xiao et al., 2001, Malmgren et al., 2004). Bone defects appear to be absent in individuals with dentine disorder involving mutation of DSPP despite DSPP expression in this tissue. This may due to low expression level

of DSPP in bone and explained the exclusive isolated effect in dentine only in DI type II (Kim and Simmer, 2007).

1.2.3 Dentinogenesis Imperfecta Type III

DI type III (OMIM 125500) is a form of DI found in a triracial isolate from southern Maryland and Washington DC known as the Brandywine isolate (Witkop et al., 1966).

Clinical features*:* Typically severe signs of wear develop on occlusal and incisal surfaces although most of the clinical features are similar to DI type I and DI type II (Levin et al., 1983). The newly erupted crowns have bulbous shape and thickness but are reduced in height as the tooth surface wears over time. Hursey et al reported on a case of DI type III in children where enamel was only present at the gingival margins and the dentine was amber brown in colour without evidence of caries (Hursey et al., 1956). The clinical presentation varies, including a large pulp chamber in the primary teeth, obliteration of the pulp chamber and root canals. The incidence of pulp exposure is high, as the tooth surfaces wear rapidly, especially in the primary teeth (Shields et al., 1973).

Radiographic features*:* Primary teeth in DI type III show considerable variation in appearance, ranging from normal to pulpal obliteration and even to shell teeth (Shields et al., 1973) which manifest as an enlarged pulp chambers surrounded by only a thin layer of dentine. These features were found only in young children (Witkop, 1975).

Genetics*:* The dentine sialophosphoprotein (DSPP) gene has been identified in a family with Dentinogenesis Imperfecta type III (DI type III). Linkage analyses showed that this gene is located on human chromosome 4q21 (MacDougall M et al., 1999). In

DI type III, only DPP level is altered with normal levels of DSP. This alteration affects dentine matrix mineralization and the colour of the teeth (Levin et al., 1983).

It is helpful to consider the formation and structure of normal dentine in order to have a better understanding of the effects of abnormal dentine formation.

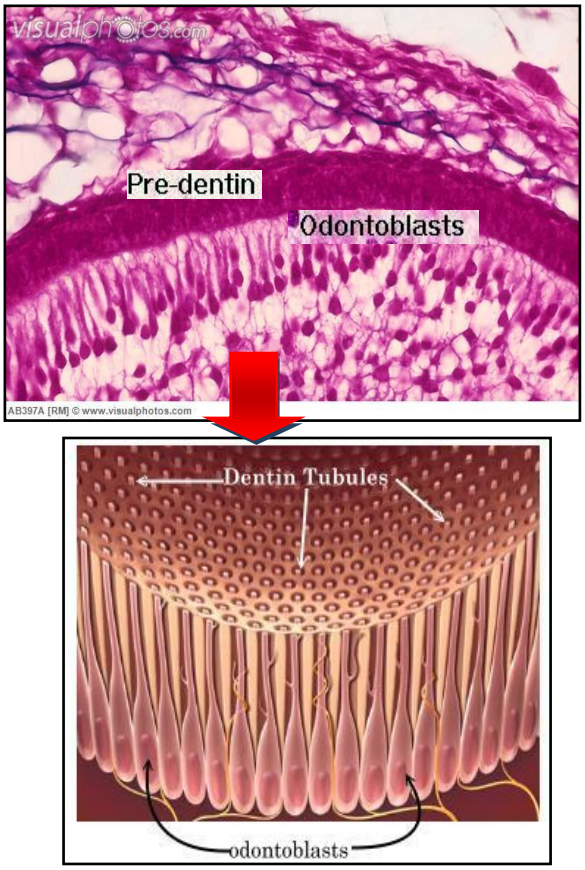
1.3 Dentinogenesis and dentine

1.3.1 Dentinogenesis

Odontoblasts are the cells which are responsible for the formation of the dentine and dentine's constituents. These cells are differentiated from the ectomesenchymal cells of the dental papilla in the inner dental epithelium. The ectomesenchymal cells will enlarge and elongate to become the pre-odontoblasts cells, which in turn change the cytoplasmic volume and mature to become odontoblasts. Odontoblasts are columnar in shape, and form the interface between mineralised dentine and pulp (Steve Weiner et al., 1999).The nucleus of the cell is located at the proximal end of the cells, and the cell is rich with Golgi apparatus and endoplamic reticulum. These organelles are responsible for the protein secretion during the formation of the dentine (Nanci, 2008). The odontoblastic process extends in the tubular as the dentinal tubules extend. The odontoblastic processes are long and straight in the crown region and shorter and more sigmoidal in the root (Byen and Sugaya, 1994). The main functions of odontoblasts are their involvement in synthesis and secretion of the dentine extracellular matrix (Butler and Ritchie, 1995). During formation of dentine, odontoblasts will secrete the organic matrix that is composed of collagen and non-collagenous proteins. The odontoblasts differentiate at the bell stage secrete the organic predentine matrix. The predentine is an unmineralised region containing type I collagen and non collagenous proteins that is thought to act as a nucleator of

hydroxyapatite crystals during mineralisation process. As the dentinogenesis continues, the odontoblasts continue to migrate deeper into the pulp cavity, extending their process as they go, while secreting the new dentine matrix. As the odontoblasts migrate further, the matrix they secrete becomes dominated by type I collagen orientated parallel to the dentine-enamel junction, resulting in a denser mineralised dentine known as primary dentine (Barron et al., 2008).

Mutations in the genes which encode for the organic matrix seem to underlie most of the hereditary dentine defects. The molecular defects in OI involve mutation in type I collagen and genes involved in formation of the collagen can be associated with DI type I. On the other hand, the allelic mutation of DSPP gene that encode dentine sialophosphoprotein ; the most abundant non- collagenous protein in the dentine can be linked to DI type II and DI type III (Barron et al., 2008).



http://doctorspiller.com/Tooth_Anatomy/tooth_anatomy.htm
Figure 1.2: Human odontoblasts in teeth

1.3.2 Composition, types and histology of the dentine

Dentinal matrix composition

Mature dentine is composed of 70% inorganic matrix material, 20% organic matrix and 10 %water on a weight basis and about 45%, 33% and 22% on a volume basis respectively (Nanci, 2008, Rauch and Glorieux, 2004). The inorganic matrix is composed of hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$ which can be described as small flattened plates each of 60 to 70nm in length, 20 to 30nm in width and 3 to 4nm in thickness (Avery, 2002).

On the other hand, the organic matrix is composed of 90% of collagen. The most abundant type of collagen found is type I with minor amounts of type III and V. The basic structure of type I collagen consists of polypeptide chains of two α1 (I) chains and one α 2(I) chain that coil together to form a triple helix (Rest and Garrone, 1991a). These collagens are staggered in an organised manner to form microfibrils.

About 10% of the organic matrix is composed of non-collagenous protein. The non-collagenous protein is mainly made up of dentine phosphoprotein (DPP), dentine sialoprotein (DSP), proteoglycan and other serum proteins.The non-collagenous proteins are produced by odontoblasts and play a role in regulating mineral deposition in dentine (Nanci, 2008). DPP and DSP are the most abundant non collagenous proteins found in dentine. Both proteins are the product of the dentine sialophosphoprotein (DSPP) molecule. DPP is the C- terminal proteolytic cleavage product of DSPP while the DSP is the N-terminal proteolytic cleavage product (M. MacDougall et al., 1997). DPP has been suggested to have a possible role in the dentine mineralisation because its capability to bind to large amounts of calcium (Milan A.M et al., 2006). However, the DSP function is still not clear. It was said that the DSP, which is predominant in the peritubular area have the possibility to promote

the growth of hydroxyapatite crystals when bound to collagen fibrils in vitro as a role in the matrix mineralization reaction (A. Boskey et al., 2000).

Proteoglycan is one of the non-collagenous proteins which containing a polypeptide core that attached to one or more attached glycosaminoglycan (GAG) chain. It has a few functional roles in the dentine matrix. The main involvement is maintaining and stabilizing collagen fibres. Proteoglycan can be found abundantly in the predentine area of the dentine.

Types of dentine

1) Primary dentine

Most part of dentine is composed of the primary dentine. It is the developmental dentine that formed during the formation of teeth. The first layer that forms in the primary dentine is the mantle dentine which can be found at the dentine-enamel junction (DEJ). This region is less mineralised area which consists of the non-collagenous proteins and collagen. The organic component is produced by the young active odontoblasts which will disappear at more mature stage (Linde and Goldberg, 1993).

2) Secondary dentine

Secondary dentine is the subsequent dentine that formed once root formation has occurred and as the teeth start to erupt in the mouth. The deposition of the dentine continues at a slow rate. This occurs as the results of normal physiological factor. This can be contribute by the stimuli from the tooth eruption and age factor (Avery, 2002). The secondary dentine has an uneven deposition of organic matrix and

irregular tubules. The changes of the deposition in secondary dentine is obviously can be seen around the periphery of the pulp chamber (Nanci, 2008).

3) Tertiary dentine

The tertiary dentine is the dentine that deposited as the results of pathological stimuli. Among the factors that can cause the reaction is attrition, caries, or dental procedures. The undifferentiated cells in the pulp will differentiated and become secondary odontoblasts. Tertiary dentin is irregular in structure and the reaction localized to the affected dentinal tubules. The tertiary dentine may have the continuous tubules with the secondary dentine, irregular arrangement of tubules or no tubules at all (Mjör, 2009).

Histology of dentine

1) Dentine tubules

Dentine tubules are the cylindrical channels which extend through the entire thickness from DEJ to the pulp. The diameter of the dentinal tubule is depend on the age (young or old) and their location, approximately 2.5µm near the pulp, 1.2µm in the midportion and 900nm near to the DEJ (Nanci, 2008). The tubules increased in number from about 20,000 in the DEJ to above 50,000 per mm2 towards pulp, respectively (Marshall et al., 1997b). The configuration of the dentinal tubules can be seen in two curvatures which are the primary curvature that is in S shaped and the secondary curvature which is wrinkled in texture. In the crown, the tubules follow the slight curve shape or sometimes take on the S-shaped curvature from the DEJ to dentine-predentine junction (Avery, 2002). The shapes of the tubules are almost straight beneath the incisal edge, the cusps and at the dentine root surface. The curvature shape is made up by the odontoblast during the formation of the dentine. The feature of the tubules in the dentine is depending on the odontoblast process

that embeds into the extracellular matrix which involve during and after mineralisation (Habelitz et al., 2007)The dentinal tubules are divided into a branching system. The major branches more frequently found peripherally, the fine branches were numerous in the root area and the microbranches extended in all part of the dentine (Mjör and Nordahl, 1996).The dentinal tubules are occupied by the nerve fibers in association with the odontoblastic process and the extracellular fluid (ECF). These components are important to provide nutrients and giving the sensation of the tooth (Tidmarsh, 1981).

2) Peritubular dentine

The peritubular dentine which is also known as the intratubular dentine is the term used to describe the area of dentine which surrounds the dentinal tubules. This area extends about 1 to 2 μm. The amount of the peritubular dentin varies in location and it is increased from about 3 vol% at the DEJ to at least 60 vol% close to the pulp (Gotliv et al., 2006). Peritubular dentine is composed of hydroxyapatite integrated with small amount of collagen fibre. It comprises less water and less organic material than the intertubular dentine. At least 60 vol% of hydroxyapatite is present in peritubular dentin (GW Marshall et al., 1997). In the dual secretion model suggested Linde (1989), the peritubular dentine is hypothesised to be formed by non-collagenous and glutamic-acid-rich proteins. This component plays an important role as an inducer for mineralisation where they will induce the apatite nucleation. This results in higher degree of mineralisation at the peritubular area (Linde, 1989). The odontoblasts are dominantly involved in formation of collagen at early stage of dentinogenesis and plays an important role in formation of non-collagenous protein towards the end of crown formation which causing the peritubular area to be less prominent in the root area (Steve et al., 1999).

3) Intertubular dentine

The intertubular dentine is the dentine located between the dentinal tubules. It is composed mainly by interwoven network of type I collagen. The collagen fibrils are arranged in the incremental plane, which is roughly perpendicular to the dentinal tubule direction (Kramer, 1951). The content of the hydroxyapatite in the intertubular dentine is about 45 vol% (GW Marshall et al., 1997). In between these two minerals in the intertubular dentine, the amorphous ground substances can be found in the form of non-collagenous proteins (Nanci, 2008).

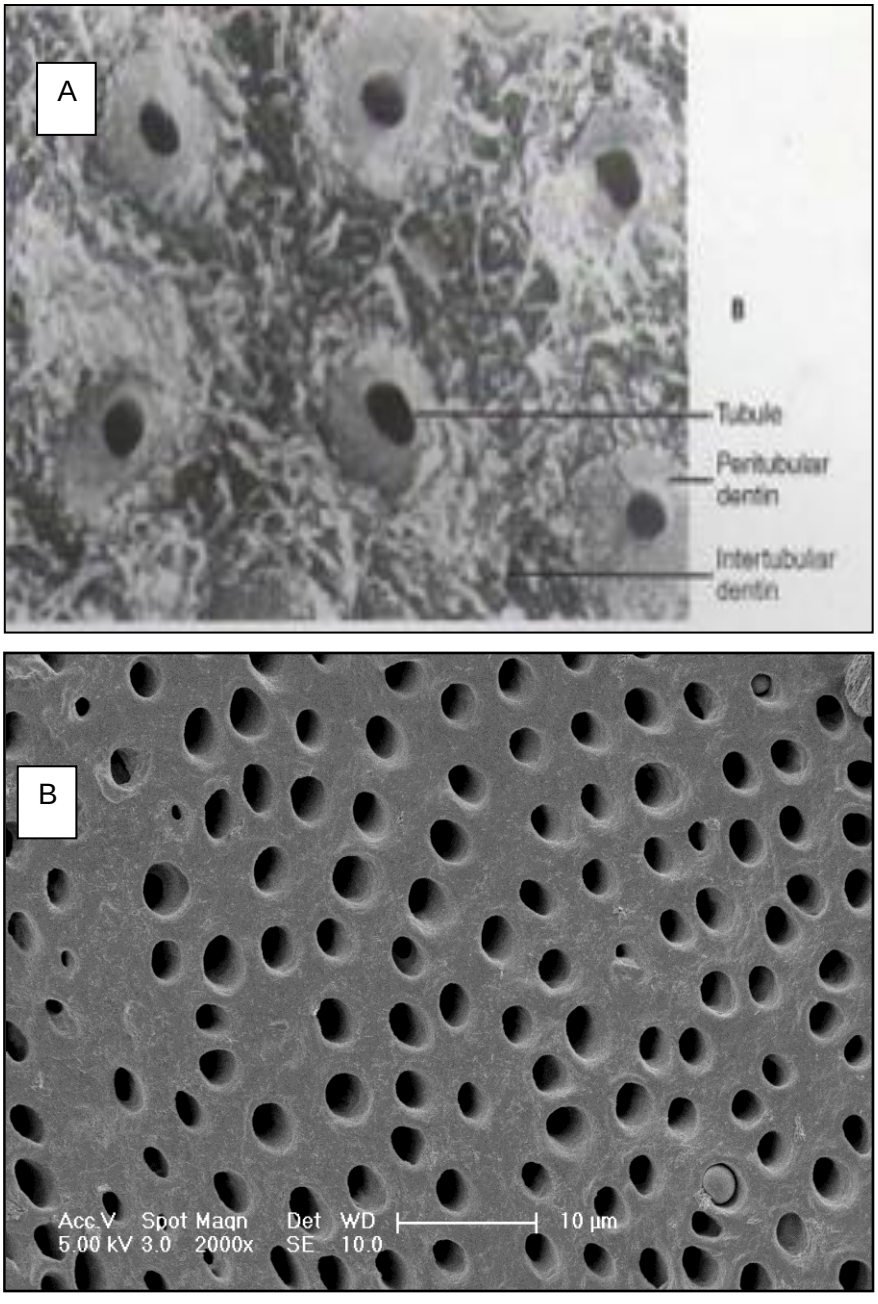


Figure 1.3: (A)Dentinal tubules which are surrounded by highly mineralised peritubular dentine and intertublular dentine (B) Partially demineralised dentine after being treated with 37% phosphoric acid for 15 seconds.

1.3.3 Dentine in primary teeth and permanent teeth

The primary dentine was reported to have substantial microstructural difference to the permanent teeth. Primary dentine has been described to be smaller in size with less thickness of dentinal surface from the dentinoenamel junction to the pulp. The longitudinal ground section of permanent teeth show that the dentinal tubules follows an ‘’S’’-shaped curve whilst most of the primary teeth exhibited straight course dentinal tubules .This suggests that the ‘’S’’ shaped is the results of the crowding of odontoblasts as they moves from the periphery towards the center of the pulp (Nanci, 2008). However, the primary teeth tubules follow a straight path as the result of less crowding of the odontoblasts and significant thin dentine layer towards the pulp (Chowdhary and Reddy, 2010).

The tubules diameter in the primary teeth appears to be greater compare to the permanent teeth (Sumikawa et al., 1999). Analysis in the density of the tubules reported that the number of density appears to be greater in primary teeth than permanent. However, most of the study did not mention the exact location of measurement whether in the middle layer of the dentine, outer layer or inner part of the dentine (Sumikawa et al., 1999). In one of the study, the primary dentine has been described to have lower tubular density at a distance of 0.4 to 0.5mm from the pulpal surface when compared with permanent teeth (Koutsi V et al., 1994).

The dentine hardness in the circumpulpal and peripheral area in both primary and permanent teeth are similar except for the central area of the dentine in the crown and the root area. This is the related to the fact that this area in the permanent teeth is more mineralised (Sumikawa et al., 1999). The concentration of the inorganic mineral in the peritubular and intertubular dentine in primary teeth are lower than the permanent teeth (Hirayama, 1990). A hardness property of the teeth is believed to be related to the degree of mineralisation. The comparison hardness value in the central

dentine area was 60KHN (Knoop Hardness Number) in the primary teeth and 69KHN to 83KHN in the permanent teeth (Johnsen, 1988).

1.4 Dentinal collagen

The organic matrix of the dentine is composed of 90% of collagen. The most abundant type of collagen found is type I with minor amounts of type III and V. Compared to bone, the dentinal collagen is more interwoven with crossing fibrils (Habelitz et al., 2002). The primary function of dentinal collagen is structural; it holds the extracellular protein matrix together as a functional unit for mineralisation (Silver et al., 2003). The extracellular matrix (ECM) is a complex network that occupies the space between cells and is composed of different combinations of collagen and non-collagenous protein like proteoglycans and other glycoproteins. Dentinal collagen also provides nucleation sites that induce the nucleation of hydroxyapatite (Silver and Landis, 2011).

1.4.1 Role of dentinal collagen in clinical dentistry

In dentine adhesive systems, dentinal collagen plays an important role to maintain the bond strength and its durability. The efficiency of this bonding system depends on the micromechanical retention promoted by resin infiltration in partially demineralised dentin, that lead to the formation of the hybrid layer and tags (Pashley et al., 1995). The hybrid layer was described by Nakabayshi as a layer that allow the impregnation of the resin monomer into the intertubular collagen network and dentinal tubules (Nakabayashi et al., 1982). This layer was formed as the results of acid etching that is used to expose the collagen network by removal of the smear layer on the dentine surface.

Several studies have been performed to establish an ideal protocol for adhesive system (Nor et al., 1996, Hosoya et al., 2000, Pioch et al., 2003). In order to expose the dentinal collagen, an ideal etching time is needed (Van Meerbeek et al., 1992). When the tooth was treated in a longer time, the smear layer will be remove completely and subsequently cause unnecessarily excessive demineralisation of the intertubular and peritubular dentine (Perdigao and Lopes, 2001). The impact of this will cause severe collapse of dentinal collagen and formation of thick hybrid layer which increased the susceptibility to failure (Hashimoto et al., 2000). Therefore, the preservation of the dentinal collagen structural integrity is very essential to ensure the success of a restoration.

1.4.2 Ultrastructure studies on dentinal collagen

A number of studies had been reported to observe the dentinal collagen structure by using electron microscopy (Lin et al., 1993, Perdigao et al., 1999). The used of AFM has facilitate imaging and analysis of dentinal collagen. Gwinnett reported that dehydrated collagen may cause dentinal collagen structure to collapse.This condition can be avoided by maintaining the dentine surface under the wet-state (Gwinnett, 1994). In 1998, El, Feninat demonstrated the effect of etching time on dentinal collagen. When there was an increased in etching time, the dentinal collagen structure showed signs of damage; this was reflected by the showing an inability to detect or observe any collagen fibrils (El Feninat et al., 1998). The study of dentinal collagen in permanent teeth was further reported by Habelitz where the images of collagens were achieved by using etching and controlled deproteinisation. A narrow distribution of D-banding between 67 and 68nm were present in hydrated fibrils whilst in dehydrated samples, the D-banding were divided into three groups which were 57nm, 62nm and 67nm (Habelitz et al., 2002). Besides imaging, the AFM also was used for the study of mechanical changes of dentinal collagen on permanent teeth

particularly in D-banding area (Marshall et al., 1993, Balooch et al., 2008, Fawzy, 2010, Bertassoni et al., 2012). Yet, to date, little is known about the detail of ultrastructure study of the dentinal collagen in primary teeth.

1.4.3 Collagen composition and ultrastructure

There are more than 20 genetically distinct type of collagen which can be found in tissues and these can be divided into the fibril forming collagens (including types I, II, III) and non-fibril forming collagens (including types IV, VIII, X). The majority of collagens in vetebrates belong to the fibril forming collagen group, of which the most common type of collagen found is Type I (Henkel and Glanville, 1982). Type I collagen molecules can be found in the dentine, bone, skin, and tendon. This collagen may produce a variety of fibril organisation with different ultrastructure morphology (Kadler, 2007).The basic structure of type I collagen are polypeptide chains of two α1 (I) chains and one α 2(I) chain that coil together to form triple helix (Rest and Garrone, 1991b). Each collagen polypeptide has a large number of repeating amino acid sequences, often follows the pattern Glycine-Proline-X or Glycine-X- Hydroxyproline, where X may be any of various other amino acid residues. For the three polypeptide chains to coil together into a triple helix, they must have the smallest amino acid; glycine (Rest and Garrone, 1991b).

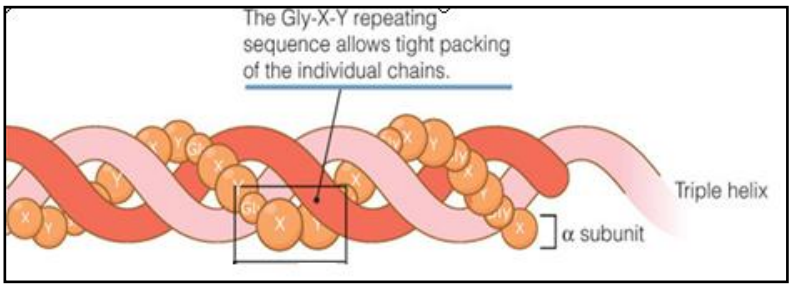


Figure 1.4: Three polypeptide chains that coiled together into a triple helix

Glycine gives flexibility to twist and form the triple helix structure where there is no space for a larger amino acid. The side chain of glycine, an H atom linking the peptide bond NH of a glycine residue with a peptide carboxy (C═O) group in an adjacent polypeptide to form a helix. Its repetition at every third position on the sequence allows close package of chains into helix structure. Any replacement of amino acid at the fixed chain of this C – N peptidyl-proline or peptidyl-hydroxyproline will inhibit triple helical formation (Kadler et al., 1996).

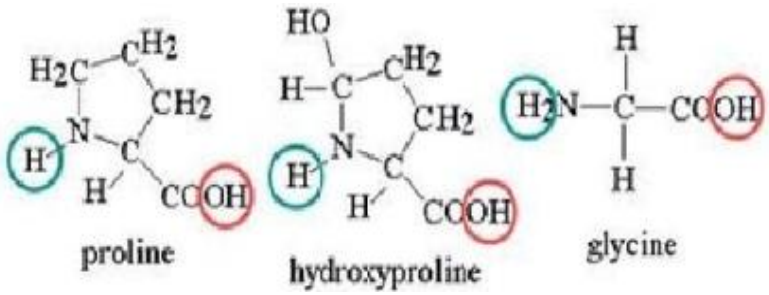


Figure 1.5: Chemical structure of collagen amino acid Glysine-Proline Hydroxyproline

The regular banding pattern in collagen fibril can be observed in transmission electron microscopy (TEM) (Schmitt et al., 1942) and atomic force microscopy (AFM) (Chernoff et al., 1992). The first two dimensional (2D) stacking model adapted by Hodge and Petruska, observed five tropocollagen molecules which are staggered together side by side with the offset distances (D banding) 67nm between two neighbouring collagen molecules (Hodge and Petruska, 1963). The alignment and periodicity shows a dark region known as a ‘gap’ and a light band region known as ‘overlap’. The overlap contains higher collagen molecules density than the gap region.

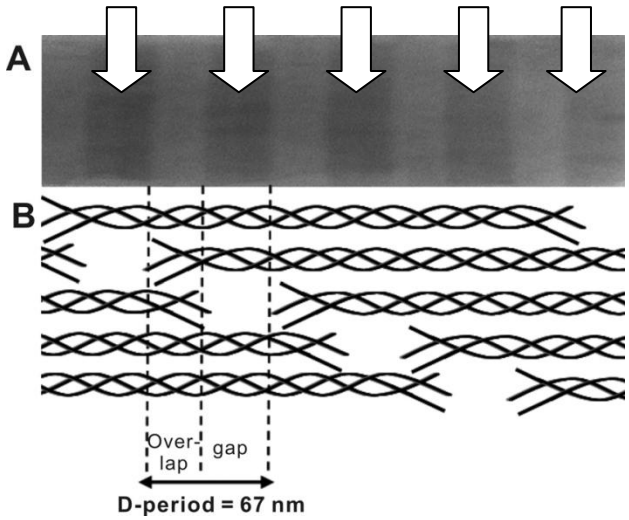


Figure 1.6: A) Transmission electron microscopy (TEM) image of single fibrils with the 67 nm D-period visible. (B) Schematic representation of the two-dimensional axial arrangement of collagen molecules in a microfibril. Arrows show the gap regions. The D-period originates from the staggered aggregation of the collagen molecules in microfibrils (Hodge et al,1963)

In the overlap region, the collagen molecule regions are tightly packed. The model of packing of the molecule microfibrils in three dimensional stacking was first introduced by Smith (Smith, 1968.). In 1998, a model describing compressed microfibrils was explained. The five molecules in a microfibril are tightly packed in the overlap region, forming a crystalline structure while in the gap region; the molecular segments follow an individual path. According to the model, the microfibrils are interconnected by telopeptides that may explain the difficulty in isolating individual microfibrillar structures (Wess et al., 1998).

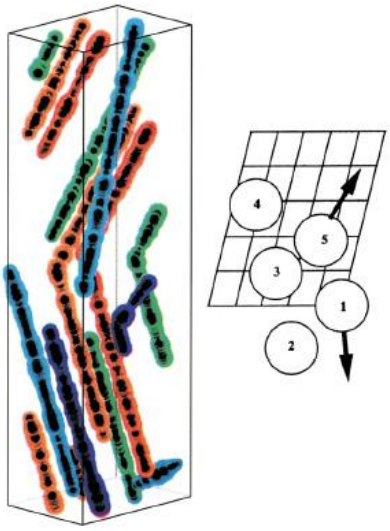


Figure 1.7: Left, a 3D representation of the molecular arrangement of type I collage. Right, the cyclic set of the final model and the telopeptide directions (West et al, 1998)

Bozec et al further proposed a three dimensional stacking model of the collagen fibrils. The modelling shows that the collagen fibrils forming a rope like structure. According to the model, it appears that topological characteristics of the collagen fibril are driven by the inherent and repetitive twist of the collagen molecule as well as the interactions between the strands (Bozec et al., 2005).

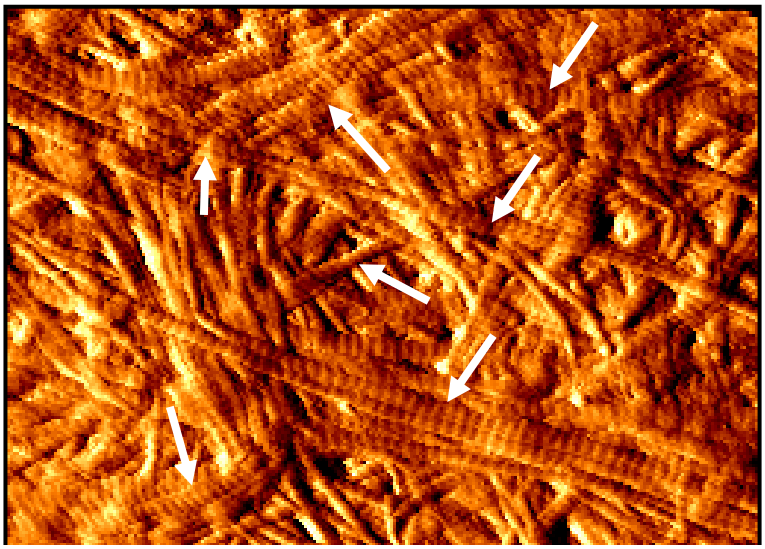


Figure 1.8: Collagen gel from rat tail under atomic force microscopy (AFM) showing presence of clear fibrils arrangement with periodicity of gap and overlap zone, D-banding which indicated by the arrows

In teeth, collagen is synthesized by the odontoblasts and secreted into predentin, where the collagen molecules are arranged into fibres (Leblond, 1989). The collagen molecules are long, rod like structure with a diameter of ~ 1.5 nm and are approximately 300nm in length. The collagenous network is less dense adjacent to the odontoblast cell bodies but reaches its full density closer to the predentin/dentin interface (Avery, 2002)

Dentinal collagen forms from procollagen (proprotein). The biosynthesis of dentinal collagen is initiated within rough endoplasmic reticulum(ER). The dentinal collagen is synthesized and subsequently controlled by the odontoblasts. The procollagen chain consists of a short signal peptide; a central α chain segment and two terminal ends; carboxy (C-terminal) and amino propeptides (N-terminal). The disulphide bond between the C-terminal and N-terminal propeptide sequences align the chain to form a triple helix. As the chains are synthesized, they are translocated into the lumen of the ER, where postranslation modification occurs. The procollagen removes the C-terminal and N-terminal propeptides and the chain is often called tropocollagen. In the extracellular zone, the tropocollagens will assemble in a staggered to form making a microfibril (Minor, 1980).

Three important post translational modifications of collagen seem to occur extracellularly; removal of the N- and C- propeptides, mediated by specific endopeptidases, and the oxidation of lysine residues by the enzyme lysyl oxidase. This enzyme acts on lysines and hydroxylysines producing aldehyde groups, which will eventually undergo covalent bonding between tropocollagen molecules and staggering to form collagen fibrils (Minor, 1980). The dentinal collagens are stabilised in fibrils by inter-covalent molecular cross-linking. The collagen crosslinking is initiated with the conversion of specific lysine and hydroxylysine residues. The major collagen cross-linking in dentine is derived from hydroxylysine-aldehyde (Hylald). The

newly deposited fibrils are small in diameter and length. As the tissue mature, the diameter and length increases.

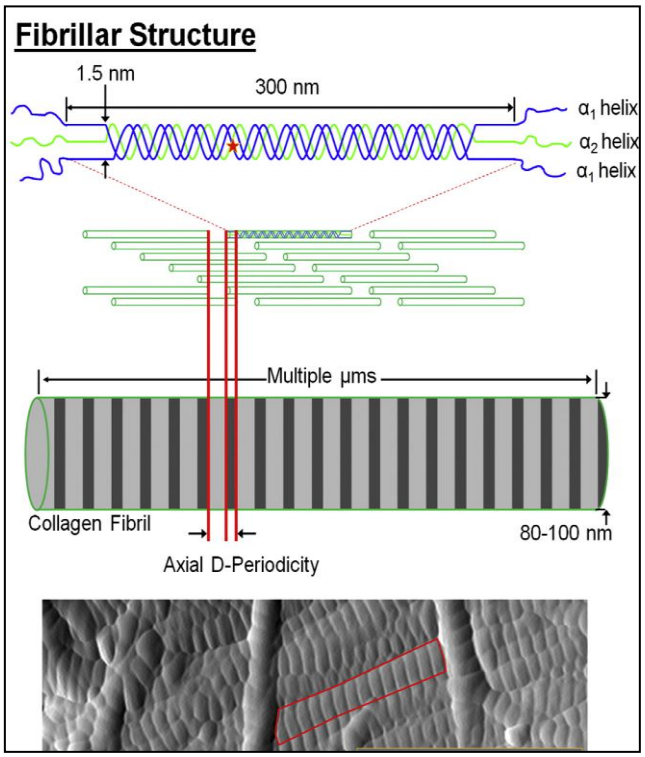


Figure 1.9: Biosynthesis of dentinal collagen
http://bhgroup.lsa.umich.edu/research/collagen

1.4.3 Dentinal collagen in relation to OI and DI

DI type I can be associated with OI mostly in Type III and Type IV. From the previous studies, the prevalence of DI in patients with OI was ranging from 21% to 73% (Lund et al., 1998, Malmgren and Norgren, 2002).

A study carried out by Lund et al. in 88 patients with OI demonstrated that only 28 patients presented with DI showed that the majority of these patients with DI had a qualitative collagen defect and only a minority of patients had a quantitative collagen defect (Lund et al., 1998). The ultrastructure findings on dentinal collagen defect of primary teeth in OI patients were varied; in the normal areas, the dentinal collagen fibers encircled the dentinal tubules demonstrating normal cross sectioned collagen orientation. However, in the abnormal area, the collagen orientations were not

uniform; some collagen fibers were densely packed and some may appeared loose. Most of the fibres in the loosly textured area can be divided in two groups with normal diameter of 80-100nm or those with reduced diameter about 40nm (Waltimo et al., 1996).

Dentinal collagen defect due to odontoblast dysfunction has also been theorized to be the reason of histological changes in DI type I (Hall et al., 2002, Majorana et al., 2010). Majorana carried out a study in sixteen children who were diagnosed with OI. The findings showed that the orientation of the collagen was parallel to the long axis when near to the dentinal tubules and randomly oriented when away from the tubules (Majorana et al., 2010).

However, to date, the topological and characterisation of affected dentinal collagen on DI associated with OI in both primary and permanent teeth has not been reported. Further studies regarding the dentinal collagen topological and ultrastucture findings in the dentinal collagen in DI type I are required. Therefore, with high resolution electron microscopy, the dentine collagen structure and topography can be imaged to obtain the information about the collagen fibrils in the affected dentine (DI type I) and the reaction of the dentinal collagen towards chemical treatment. With better understanding of the collagen structure in DI and OI, it is relevant to help in providing the importance of structure- properties relationship of collagen fibrils in aspect of selective disease and restorative treatment such as etching and bonding.

1.4.4 Overview of dentinal collagen and bone collagen in OI and DI

Reviews of a few studies in the previous literature were summarized as below:

Fibril diameter

Bone collagen in OI: Generally, in the normal human bone, the fibrils show uniform diameter ranging from 100-300nm (Traub et al., 1994). The results of diameter of bone collagen in OI were varied. There have been reports that generally the bone collagen diameters were smaller due to failure of maturation of type I fibrils (Jones et al., 1984). This was in agreement with Sarathchandra, who also demonstrated significantly reduced diameters of bone collagen in OI (Sarathchandra et al., 1999). However, there were also reports that showed that the diameter of type I collagen were larger due to alteration of the primary structure of type I collagen (Cassella et al., 1994).

Dentinal collagen in DI type I/ DI associated with OI: In 2002, Habelitz demonstrated that the hydrated fibril diameters in human dentinal collagen were spread between 75nm to 105nm (Habelitz et al., 2002). For the dehydrated sample, the diameters were ranged between 50nm and 100nm (Pashley, 1991, Perdigao et al., 1996). Waltimo observed slightly reduced diameters of dentinal collagen in patient with mild DI associated with OI (Waltimo et al., 1996). The normal fibril diameters were ranging between 80-100nm whilst the reduced dentinal collagen fibrils diameter were about 40nm.

Fibril architectures

Bone collagen in OI : The lamellar structure in the bone can be divide into two different architectures known as 'plywood' like structure ; ' classical plywood' ; collagen fibrils are parallel in each lamella and 'twisted plywood'; fibrils are helical in nature and continuously change their direction (Giraud-Guille, 1988). In OI bones,

Traub reported that there was a good normal lamellar bone structure even in severe OI. However, in OI type III, there was an abnormal thin collagen in loose fibrous regions described as ‘pseudo-fibrils’ that suggest structural defective features of fibrils (Traub et al., 1994). The abnormal findings in OI type III were also reported by Sarathchandra in which the collagen fibrils appeared unravelled in longitudinally sectioned bundles with no periodicity and in transversely sectioned bundles they were flower-like with some fused, composite fibrils. In some areas, the collagen fibre bundles appeared twisted and loosely packed throughout a very thick osteoid (Sarathchandra and Pope, 2005). In OI type I, the collagen fibres were organised within thin osteoid lining (Sarathchandra et al., 2000).

Dentinal collagen in DI type I / DI associated with OI: The dentinal collagens were arranged in interwoven arrangements with numerous crossing of fibrils (Kramer, 1951). The minerals in the dentine (hydroxyapatite) located in the gaps between collagen molecles (intrafibrillar) or attached to the collagen fibrils (extrafibrillar). In hydrated fibrils, the D-banding distance between 67and 68 nm was recorded (Chernoff et al., 1992, Habelitz et al., 2002). In the DI type I patient, the dentinal collagen defects were varied. In the TEM findings by Waltimo, the affected DI teeth showed in loose texture area, there were variations of density and thickness of collagen fibres; some were densely packed and some appeared loose. In the normal area, the dentinal collagen fibers encircled the dentinal tubules, demonstrating the normal cross section (Waltimo et al., 1996). In another ultrastructure findings of DI type I, the collagen fibrils orientation were described to appear in random orientation when away from the tubules (Majorana et al., 2010). Overall, the ultrastructure findings on the dentinal collagen are still limited and further study will be required.

1.5 Experimental design in dentinal collagen studies

1.5.1 Atomic Force Microscopy (AFM)

AFM produces high-resolution, three dimensional images by scanning a sharp tip over the sample surface with feedback mechanism that enables the tip to track the sample surface in a controlled manner. The images are obtained by measuring changes in magnitude of the tip position when dragged over the sample surface. A tip is attached to the underside of a reflective cantilever which is mounted on a cylindrical piezoelectric tube. A diode laser is focused onto the back of a reflective cantilever. As the tip scans the sample surface, the deflection of the tip is measured by the laser, reflected onto a photodiode. The photo detector measures the difference in light intensities between the upper and lower photo detectors, and then converts to voltage. Voltages applied to the X and Y electrodes on the piezoelectric tube deflect the tube horizontally to produce a precise raster scan over the sample surface. A voltage applied to the Z electrode on the piezo tube controls the vertical height of tip. The information feedback through a software control from the computer, enables the tip to maintain either a constant force or constant height above the sample.

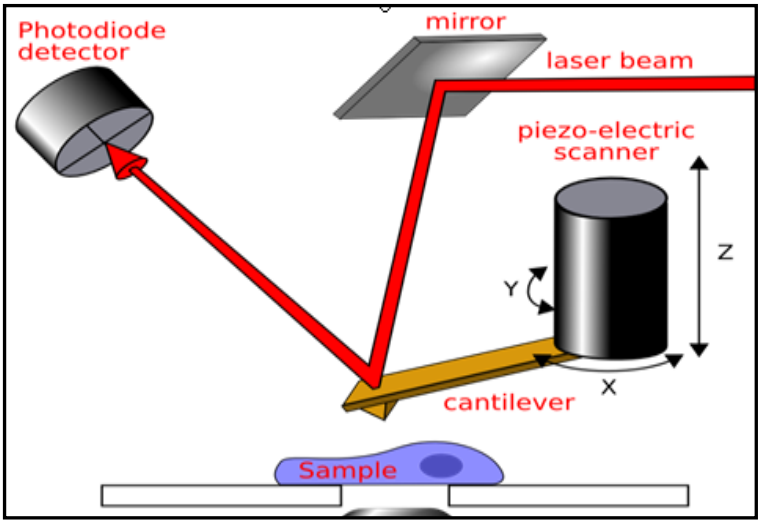


Figure 1.10: AFM detection method showing tip of cantilever approaching sample surfaces (http://www.freesbi.ch/en/illustration/figures)

AFM, which uses a sharp tip to probe the surface features by raster scanning, can image the surface topography with extremely high magnifications, up to 1,000,000 times comparable or even better than electronic microscopes. The measurement of an AFM is made in three dimensions, the horizontal X-Y plane and the vertical Z dimension. AFM produces precise topographic images of a sample by scanning the surface with a nanometer-scale probe (lateral resolution ~ 1 nm, vertical ~ 0.1 nm). Examples of sample that can be imaged under AFM are polymers, biomolecules, fibres, cells, minerals and ceramics. A unique advantage of AFM is that it enables imaging with minimal sample preparation, in air or liquid environment.

In comparison, with other forms of microscopy, the AFM is better or comparable:

- AFM versus Scanning Electron Microscopy (SEM): AFM provides extraordinary topographic contrast, direct height measurements, without complicated sample preparation being required (no coating is necessary).
- AFM versus Transmission Electron Microscopy (TEM): Three dimensional AFM images are obtained without expensive sample preparation and yield more information than two dimensional profiles from TEM.

AFM can be operated in a few modes such as contact mode and tapping mode depends on the application(Veeco, 2000);

1. The contact mode is common mode used on solid and static surfaces. This mode was used extensively in this study. Basically, the cantilever is constantly in contact with the sample during imaging whether at the constant height mode where the height of the tip is fixed whereas the cantilever deflection will generate data or at the constant force mode where the deflection of the cantilever is fixed whereas the scanner movement is recorded. When the tip of cantilever scans through uneven surface, the deflection of the cantilever increased and the feedback

system will move the whole cantilever and adjusted back to original position. This can minimized the scratching on the sample surface which may happened when the cantilever deflects more as the tip scans over bumpy surface areas. Advantages of the contact mode; the skills of operating are simple and direct, can scan quite fast and easily. However, there will be possibility to damage sample surface because the tip of cantilever is constantly contact with the samples.

2. In the non contact mode, the probe does not touch the sample surface, but oscillates above the surface during scanning. The vibrating tip is brought near to the sample surface and induces a frequency shift in resonant frequency. Surface topography can be measured by using the feedback loop to monitor the amplitude of the cantilever oscillation. The total force between the tip and the sample is very low which is suitable for the soft dry samples. However, due to the oscillation measurement, contamination layer on samples can have a great effect on the reading.

3. In the tapping mode (intermittent contact mode, AC mode, vibrating), the cantilever does make contact with the sample surface but the tip oscillates up and down (bounces up and down) very fast in short period during slow lateral scanning. Data obtained by measuring the change in oscillation amplitude detected by laser beam to photodiode. Advantages are; this method is suitable for soft sample surface and the tip does no scratch the samples. The operating mode is more intensive and the tip is more expensive and scanning will need more time.

1.5.2 Raman spectroscopy

Raman spectroscopy is a light scattering technique that is based on the interaction between photons of light which interact with a sample to produce scattered radiation of different wavelengths. During this process, the laser will interact with a sample; energy is exchanged between the photon and molecules such that the scattered photon will have the same frequency / energy as the incident radiation or there is energy exchanged between the photon during the collision. The difference in energy is made up by a change in the rotational and vibrational energy of the molecule. The scattering process without any change of frequency is called the Rayleigh scattering whilst when there is a shift of frequency; is known as Raman scattering. Some advantages of the Raman technique include little or no special sample preparation needed, non-destructive sample, linear response to mineral/chemical concentrations (Tsuda and Arends, 1997) and capable to identify distributions of organic and inorganic compunds (Tsuda et al., 1993, Karan et al., 2009).

Various Raman spectra studies on mineral relevant to dental research were reported (Tsuda et al., 1996, Wang et al., 2006). In dentin spectrum, the bands associated with collagen occur at 1667 (amide I), 1273 (amide III), and 1240cm^{-1} (amide III) whilst the bands associated with mineral occur at 1070 (carbonate) and 961cm^{-1} (P—O) (Tsuda and Arends, 1997, Wang et al., 2006).

Peak	Wavenumber cm^{-1}
Amide I	1667
Amide III C-N	1273
Amide III N-H	1240
Carbonate	1070
Phosphate	960

Table 1. 2: Raman spectra peak that relevant to dental research

These analyses are useful for chemical identification and quantification to investigate chemical changes of mineral and organic content in the dentine

1.5.3 Scanning Electron Microscopy (SEM)

SEM uses a beam of highly energetic electrons to form an image at a micron scale. The principle of field emission SEM instrument is based on the interaction of electron beam and the atoms on the sample surfaces. The electrons that produce by the electron gun (electron probe) will travel through an electromagnetic field and secondary electrons will be emitted from the sample surface. The output signals from the secondary electron detector are amplified and transferred to display unit. The topography of the surface can be observed in two-dimensional scanning that will be generated on screen.

In terms of sample preparation, the samples must have conductivity in principle. The most typical method in preparing the sample for SEM imaging is coating the non-conductive specimen with highly conductive thin metal film such as palladium or gold. The reason for using a noble metal is that it is highly stable and it can produce a quality images without any distortion.

1.6 Effect of demineralising agents

1.6.1 Effect of dentine etching

Compared to the highly complex hierarchical structure of bone, tooth dentine has a rather simple microstructure, the mineralised collagen fibrils being oriented in a plane perpendicular to the direction of dentine formation. It is believed that approximately 70 to 75% of the mineral may be extra-fibrillar. The apatite minerals have been found to be needle-like near the pulp, but plate-like near the enamel (Lin et al., 1993, Kinney et al., 2001).

It is important to realise that the action of acid on the dentine occurs mostly on the mineralised component. Initial stages of dissolution involved a process of mineral softening where the diffusion of acid solution into the structure results in loss of calcium and phosphate ions (Eisenburger et al., 2004). The action of the acid also reacts with the organic components of the dentine, contributing to changes in the morphology of peritubular and intertubular dentine. It was known that after demineralisation, the dentinal tubules opened and increased in diameter. The collagen network resulting from the demineralisation protocol was left intact (Nakabayashi and Pashley, 1998). A linear etching time with the depth of demineralisation was reported by Marshall. When the time was increased, rapid dissolution of the peritubular zone leaves widened dentinal tubules and collapse of collagen was shown at intertubular area (Marshall et al., 1993). In 2004, Nakabayshi reported that phosphoric acid partially solublizes the non collagenous phosphoproteins, glycosaminoglucans and low molecular weight peptides including collagen (Nakabayashi et al., 2004).This was supported by Spencer, i.e; suggested that phosphoric acid could modify the organic components of the collagen molecule (Sencer et al., 2001).

1.6.2 Effect of sodium hypochlorite

Sodium hypochlorite solutions are widely used in dental procedures especially in endodontic treatment. Sodium hypochlorite (NaOCl) exhibits a dynamic balance as shown below:

$$NaOCl + H_2O \leftrightarrow NaOH + HOCl \leftrightarrow Na^+ + OH^- + H^+ + OCl^-$$

It have been believed that hypochlorous acid (HOCl) in sodium hypochlorite solution acts as a solvent by forming chloramines (ion chlorine that combined with protein amino group) which then lead to amino acid degradation (Estrela et al., 2002). Due to their non- specific deproteinising action, the effects of sodium hypochlorite may affect the mechanical properties of the dentine. Several studies have been reported on the detrimental effect of NaOCl, which alter the modulus and hardness of the dentine (Marshall et al., 2001, Sim et al., 2001, Correr et al., 2004). It also could affect the resin penetration into the dentine structure and subsequently influence the quality of restoration (Sim et al., 2001). The effect of NaOCl treatment has also been shown to alter the organic substrate without changing the molecular content in the inorganic material (O'Driscoll et al., 2002, Ramirez-Bommer et al., 2007, Morgan A et al., 2010).

1.6.3 Effect of Ethylenediaminetetraacetic acid (EDTA)

EDTA is generally accepted as the most effective chelating agent at the neutral pH. It is used to demineralise the smear layers, open the dentinal tubules, softening the dentine and increased the dentine permeability (Calt and Serper, 2002, Hulsmann et al., 2003). It was first introduced clinically in 1957 (Ostby, 1957).

The major factors that contribute to the properties of EDTA are the chelating mechanism and protonation mechanisms. At neutral pH, the binding of calcium ions to EDTA solution will increase the dissociation of hydroxyapatite. This will cause the

pH decrease as the results of EDTA's proton (H^+) exchange with the calcium ions. As the reaction proceeds, increased hydrogen ion as the results of the dissociation will cause the protonation mechanism to prevail. This is because the chelating mechanism may be limited by the availability of calcium ions (Cury et al., 1981). Once the process reached the equilibrium between chelating mechanism and protonation mechanism, EDTA was said to limit the amount of dissolves dentine (Carvalho et al., 1996).

1.7 Aims of the study

The aim of this project was to characterise the morphology of the dentinal collagen ultrastructure in affected OI and DI teeth using Atomic Force Microscopy (AFM).

Specific objectives were to:

1. establish the demineralisation techniques to reveal collagen ultrastructure in permanent teeth
2. establish the demineralisation techniques to reveal collagen ultrastructure in primary teeth
3. compare the topological of dentinal collagen ultrastructure of control teeth and affected OI teeth under AFM

CHAPTER 2
MATERIAL AND METHODS

2.0 Materials and methods

This chapter outlines the materials and methods that were used throughout this project.

2.1 Development of the database

2.1.1 Study registration and ethical approval

This study was designed and conducted in the Department of Paediatric Dentistry, Eastman Dental Hospital's (EDH), University College London Hospital NHS Foundation Trust. Ethical approval for this study was obtained from the National Health Services Research Ethics Committee (NHS REC) registration reference number 11/LO/0777. The lists of documents approved by the committee were;

1. participant consent form; Parent Consent Form
2. participant consent form; Patient Consent Form
3. participant consent form for the Phenodent database,
4. participant information sheet ; Main study ; Parent
5. participant information sheet ; Main study ; Patient
6. participant information sheets ; Colour spectroscopy ; Parent
7. participant information sheets ; Colour spectroscopy ; Patient

2.1.2 Sample selection

All patients referred to the Department with dental anomalies were allocated to the dedicated dental anomalies clinics. Patients attending for dental appointments were also invited to participate in this study. Inclusion criteria included:

- Patients with DI and /or OI
- Patient and parents with sufficient English to give informed consent to participate in the study

2.1.3 Anomalies clinic and data collection

The dental anomalies clinics were held once a month. All clinicians involved in this study underwent training and calibration sessions on how to use the forms and how to obtain consent and the procedures required for data collection.

Any patients and parents who attended the clinic were informed about the study at the beginning of the appointment. Information leaflets were provided (Appendix 1) and the voluntary nature of their participation was reinforced. Once they agreed, written consents were obtained (Appendix 2), one copy was given to the patient/parent, another copy was filed in the patient's notes and one copy was stored by supervisor in a filing cabinet in a locked office.

All data information obtained from the patient was collected using a modified version of the Phenodent form (Appendix 3). This form contained the demographic details of the patient, extra oral examination findings, intra- oral examination including specific information regarding the enamel and dentine defects, radiographic appearance, diagnosis of the patient and treatment plan. The extra oral (profile view) and intra-oral photograph (upper and lower arch) were taken after the completion of the dental examination. All of the data obtained from the patient were transferred to an electronic database under the control of the second supervisor. Patient's details remained anonymous using a coding number. At the end of the appointment, patients were given a plastic container as a storage medium for any exfoliated teeth and saliva was also collected as part of the study protocol.

2.2 Teeth samples

2.2.1 Teeth selection

Any primary or permanent teeth that were extracted as part of the patient's treatment, or had exfoliated and for whom consent had been obtained were included in this study. Healthy teeth extracted for orthodontic purposes were included for control samples.

2.2.2 Sample storage

Teeth were stored according to the protocols approved by the Human Tissue Act 2004 in Eastman Dental Institute, 256 Gray's Inn Road, London. All samples were thoroughly cleaned before storage. Remnants of the soft tissues that were attached to the root surface of the sample were removed with the scalpel blade. Teeth were then stored in 70% of ethanol at 4°C in locked refrigerator on the premises for 7 days. After this stage, the teeth were stored in Thymol 0.01% until prepared.

2.2.3 Preparation of teeth in epoxy resin

Teeth were placed in a vertical position and were embedded in an acrylic block using an ultra-low shrinkage, dry-curing epoxy resin (Epofix, Electron Microscopy Sciences, Fort Washington, PA). The blocks were left set for 24 hours at room temperature. Bisecting the embedded samples using a rotary saw with twin-mounted, diamond-tipped copper blades (Testbourne, UK) exposed the coronal dentine surface as well as levelling the surface on the samples. The samples were sectioned again to produce of 10mm thickness.

Polishing removed any scratches present on the dentine surfaces following cutting and any superficial debris. The samples were polished using a polishing machine under flowing water coolant (MD Fuga, MD Largo) according to a series of silicone-carbide grit paper (500, 1200grade and 2400 grade).

The outer surfaces of the samples then were cut to produce 5x5x5mm size of dentine blocks, leaving only the flat surface of the exposed dentine block. Finally, the samples were cleaned in the ultrasonic bath for 30 seconds. Once ultrasonication completed, the samples were left in plastic container filled with ultra high quality (UHQ) water until imaging.

SAMPLE PREPARATION IN EPOXY RESIN

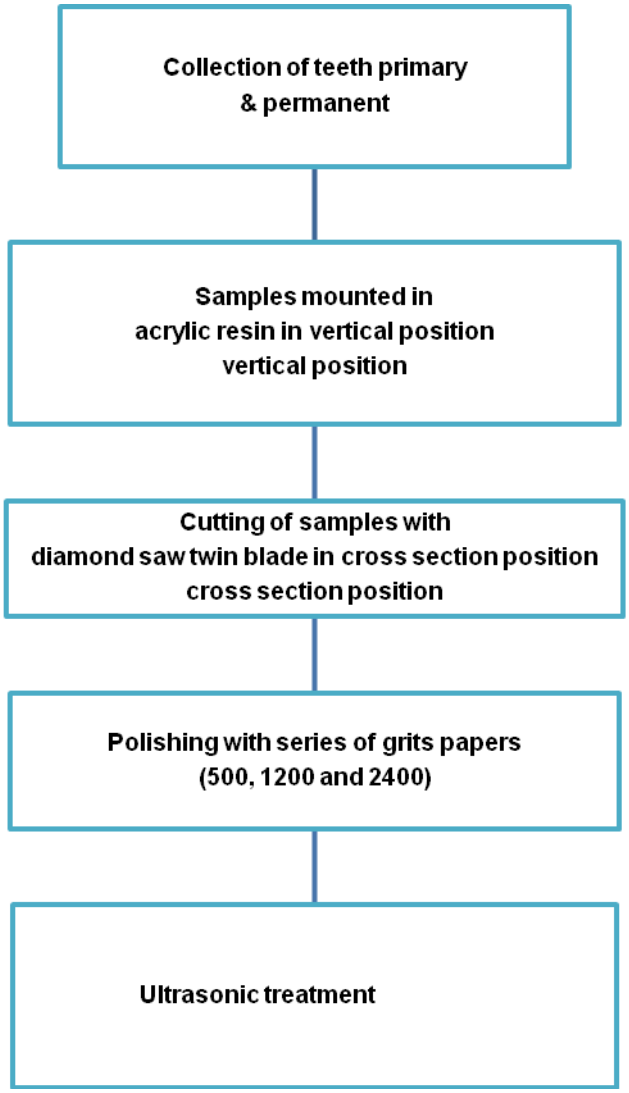


Figure 2.1: Sample preparation in epoxy resin

2.2.4 Preparation of teeth for histopathology sections

From the sample, two primary and two permanent control teeth were immersed in 17% Ethylenediaminetetraacetic acid (EDTA) solution and were kept at room temperature for 12 months. The EDTA was changed on weekly basis and the samples were placed on the rocking plate in order to allow the maximum effect of the EDTA solution.

After the storage period, the teeth were sent to the Pathology department, Queens Mary Hospital, Barts and the Royal London NHS trust for histopathology sections. The teeth were prepared into sections with the thickness of 5µm mounted on the glass slide, and stained with Picrosirius red.

Picrosirius red staining was used because of the Sirius red, an elongated molecule that contains strong cationic dye reacts with basic amino acid in the collagen molecules. The reaction promotes an enhancement of normal birefringence due to the fact that many dye molecules are attached to the collagen fibres in such way that their long axes are parallel (Junqueira et al., 1979).

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2.3 Experimental design for demineralisation study

This protocol aimed to assess the most suitable demineralisation technique to image the collagen in the dentine of primary and permanent teeth.

2.3.1 List of solutions used in the demineralisation protocols:

1) 10 vol% citric acid
2) 6.5 vol% sodium hypochlorite
3) 17% EDTA
4) Commercial etchant (37% phosphoric acid)

2.3.2 Demineralisation using published protocol

The initial demineralisation protocol was set out to replicate a method previously published (Habelitz et al., 2002).This protocol appears to be a valid method to expose collagen in partially demineralised human dentine. The dentine blocks were etched with 10%vol citric acid for 15 seconds. The purpose of etching was to dissolve the mineral content in the dentine. Then, the blocks were cleaned under steady flow of ultra high quality (UHQ) water before being treated with 6.5%vol sodium hypochlorite (NaOCL) for 120 seconds. The sodium hypochlorite was used as a deproteinising agent which removes the non-collagenous matrix proteins covering the collagen fibrils (Marshall et al., 2001). Finally, ultrasonic treatments in UHQ water for 10 seconds were used to clean the surface area.

2.3.3 Demineralisation of experimental protocol using commercial etchant (37% phosphoric acid)

Dentine etching is a common technique used in dental treatment for providing a better substrate for bonding to dental adhesives (Marshall et al., 1997a). In this group, a commercial etchant was used as demineralisation agent. The main component of the etchant was 37% phosphoric acid. Dentine blocks were treated

with the commercial etchant according to an assay of etching times. The assay was developed using the following sequence; 1 minute, 3 minutes, 5 minutes and onwards with the interval of 2 minutes of each exposure until the calcium phosphate inorganic mineral were totally dissolved. For each defined times point of exposure, Raman microspectroscopy measurements were carried out to measure the degree of mineralisation of the dentine.

- ✓ Assessment of etching time with Raman spectroscopy

In this study, the degree of the demineralisation of the dentine was determined using Raman spectroscopy (HR 800, Jobin Yvon, Horiba, Japan) calibrated with known lines of silicone. The spectrometer was operated with monochromatic radiation by He-Ne laser with 633nm wave length at 20mW (Balooch et al., 2008). Spectra were obtained under a focusing laser beam of 50x optical lens (Nikon) and all spectra were offset from 500 and 1600 cm^{-1}. The sample dentine block was set on the glass slide and immobilized with Blu-Tack® (Bostik S. A., Paris, France). Once all of the spectra had been recorded, they were analysed using LabSpec5® Raman software (Jobin Yvon, Horiba, France). All spectra were adjusted using polynomial baseline correction and were arranged for direct visual comparison. All spectra were normalised on the carbonate (CO_2^{-3}) peaks (1072 cm^{-1}.) and at 8-point of spectral smoothing. The peak maxima absorbance for the phosphate (1667 cm^{-1}) and amide III (1243 cm^{-1}) were recorded. The data were then exported to Microsoft Excel®(Microsoft Inc., Redmond, WA, USA) software. Data also have been analysed using OriginLab® (OriginLab Corporation, Northampton, MA, USA) software.

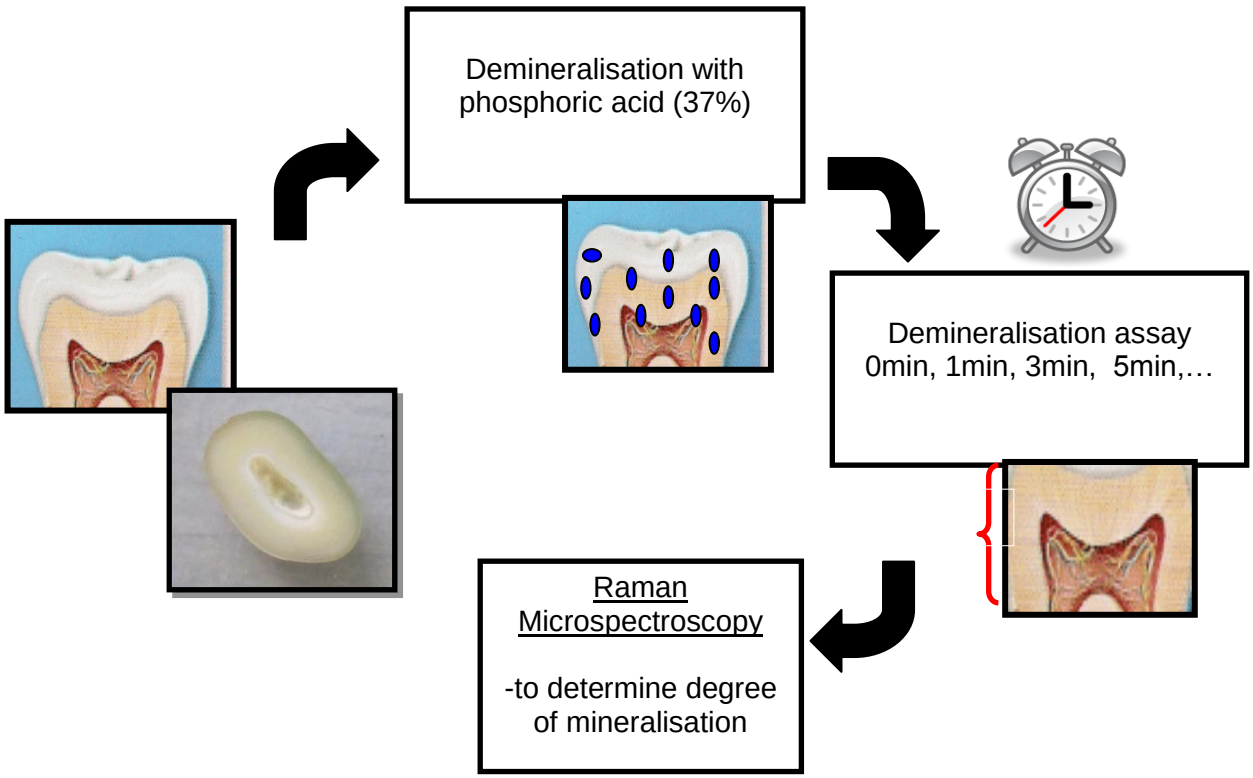


Figure 2.2: Demineralisation technique sequence using commercial etching

2.3.4 Further steps in the experimental protocol with 6.5vol% of NaoCl

Once the whole demineralisation assay was completed, the dentine blocks were rinsed with UHQ water and dried in air for 1 min. According to Marshall, the dissolution of most of the mineral phase followed by gradual removal of the non-collagenous matrix proteins, should expose the collagen. Sodium hypochlorite was suggested, based on their deproteinising action (Marshall et al., 2001). Therefore, the dentine blocks were then treated with 6.5%vol sodium hypochlorite. The samples were immersed at time intervals of 20 seconds for up to 120 seconds total time. Finally, the blocks were ultrasonicated in UHQ water for 10 seconds and were air dried for 1 second. The prepared blocks were imaged using AFM.

2.3.5 Comparing the demineralisation protocols with 17% EDTA

EDTA is a chelating agent with a neutral pH that removes the calcium ions of the collagen dentin matrix more selectively and can be used to remove the smear layer (Calt and Serper, 2002). In this study, two groups of samples were allocated to assess the effects of demineralisation using 17% of EDTA at pH 7:

a) One permanent and one primary tooth were stored in 17% EDTA for 12 month at room temperature. Samples were prepared as described in section 2.2.3.

b) One permanent and one primary tooth were prepared in epoxy resin (section 2.2.2) and the prepared samples were immersed in 17% EDTA for 10 minutes. Ultrasonic treatment in UHQ water were used to clean the surfaces (Serper and Calt, 2002).

2.4 Topological assessment of demineralised dentine

2.4.1 Atomic Force Microscopy (AFM)

Collagen imaging was performed by using two type of AFM which were Dimension 3100 AFM (Bruker, Santa Barbara, CA) and XE-100 AFM (Park Instrument Korea) shown in figure 2.3 and 2.4 respectively, as used in a previous study (Bozec et al., 2005). Initially, excess water from the ultrasonic treatments was removed by gentle blotting with absorbent paper, leaving the dentine surface visibly moist. Then, the partially demineralised dentine blocks were mounted on a glass slide for AFM imaging. Thin double sided tapes were placed on the bottom of the dentine blocks to hold the sample securely on the glass slide. For imaging purpose, silicone nitride (Si3N4) tips with a spring constant of 0.6N.m-1 were used (μMasch, Talinn, Estonia). Typically scan rates up to 2.5Hz were employed. The integral and proportional gains were adjusted accordingly to keep the height and the deflection of the cantilever

feedback nearly constant. The deflection set point used was optimised to minimise the contact force and typical set up at 2nN. Images were scanned up to area of 50 μm^2 at a resolution of 512 x 512 pixels with a typical Z-servo gain of 1.5 (XE-100 AFM (Park Instrument Korea). Topography images were recorded and analysed using Nanoscope Analysis Software (Version v140r1). All images were subjected to a standard image processing technique. This process involved plane flattening and optimization of the images contrast and brightness (Habelitz et al., 2002).

Measuring D-banding distance

The collagen fibril D-banding distances were measured from the profile of the fibrils in modified images. Analysis was made by determining the step height of individual fibrils. The length of the sections were recorded and divided by the number of bands present as shown in section 3.2.4 (b)

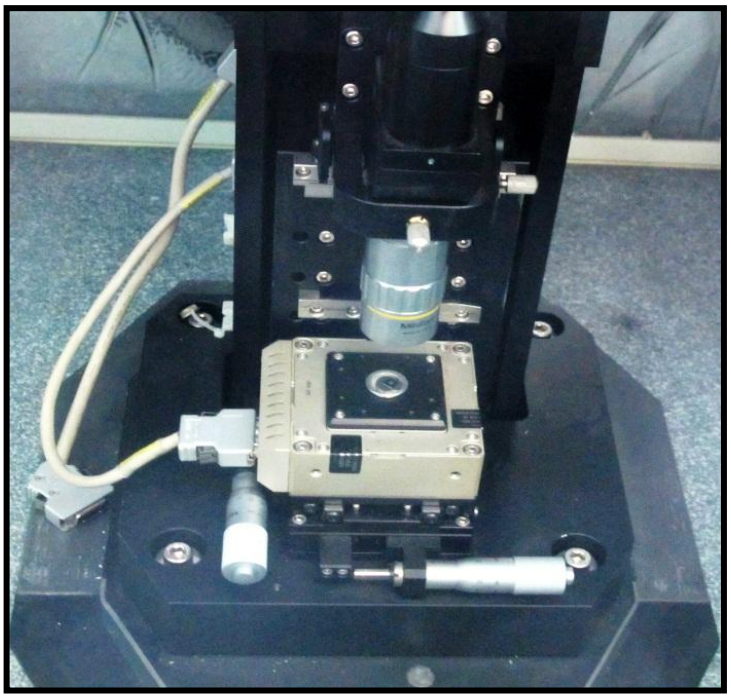


Figure 2.3: XE-100 AFM (Park Instrument Korea)

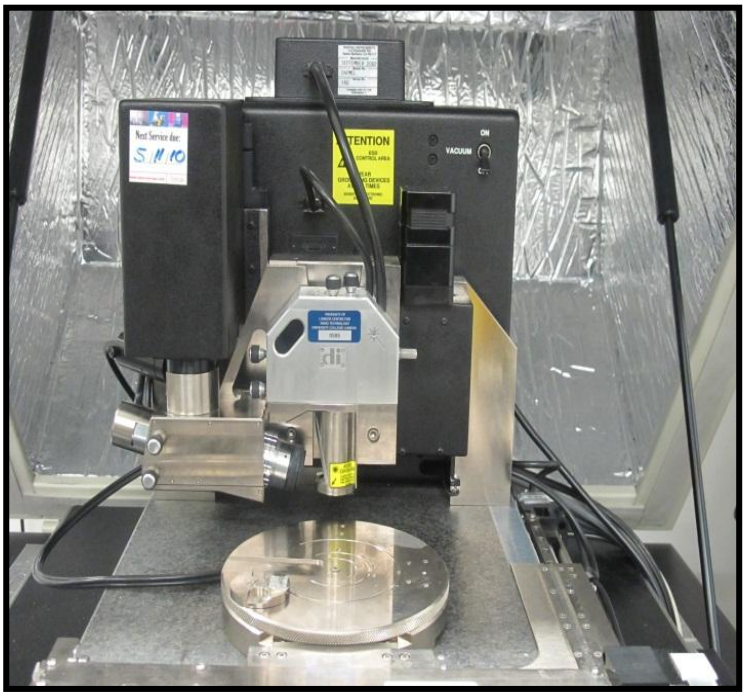


Figure 2.4: Dimension 3100 AFM (Veeco, Santa Barbara, CA)

2.4.2 Scanning Electron Microscopy (SEM)

High resolution SEM images were employed for the histopathology sections. The Philips XL30 Field Emission SEM was used for imaging at 20 kV. The histopathology slides were cut into small square sections. The sectioned samples were sputter-coated with gold-pallidium (Au(95%) / Pd (5%) prior to visualisation. The prepared samples should be mounted on the stubs. The SEM employed 5 kV for imaging and the magnification used were between 2000x – 15000x.

The sequence for collection, preparation and imaging of four experimental groups is shown in the following figure 2.5.

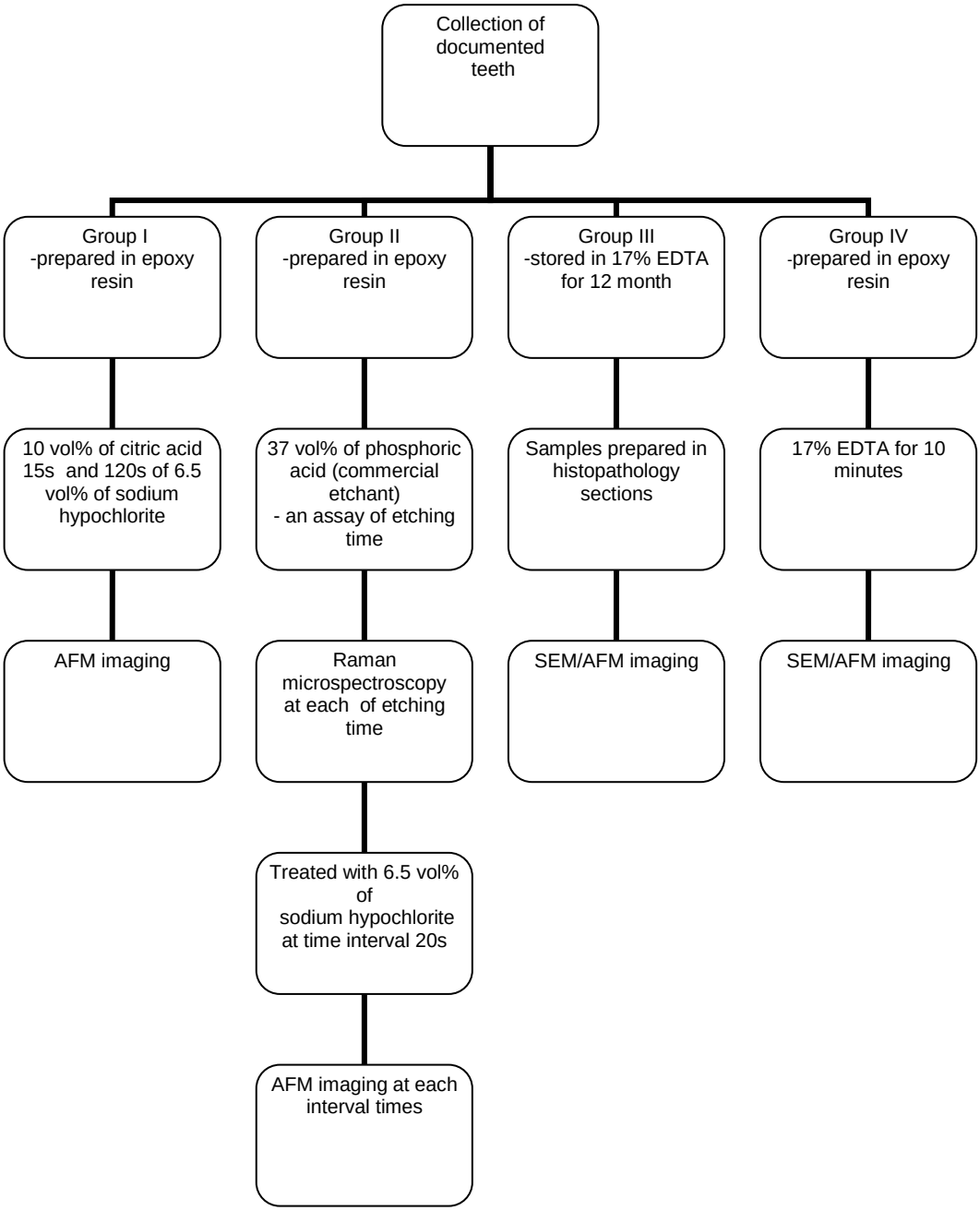


Figure 2.5: Experimental design for demineralisation studies

CHAPTER 3

EVALUATION OF DEMINERALISATION PROTOCOLS FOR PERMANENT TEETH

3.0 Evaluation of demineralisation protocols for permanent teeth

3.1 Introduction

Dentine has been characterised as a biological composite consisting of tubules, a highly mineralised zone of peritubular dentine and the intertubular dentine (Ten Cate, 1994). The mineral component in the dentine is exclusively hydroxyapatite. The mineral in the dentine occupies in two sites within the collagen scaffold; intrabrillar (inside the gap between the collagen fibril) and extrafibrillar (attached to collagen fibrils) (Lees et al., 1997, Wassen et al., 2000). The shapes of the mineral crystallites were reported to look like a needle when near to the pulp; and changes to a plate-like appearance nearer to the enamel (LeGeros, 1991, Kinney et al., 2001). The dense matrix of collagen fibrils lies in the intertubular dentine located in between the dentinal tubules. The collagen is associated with non collagenous proteins such as DPP, DSP, glysaminoglycans and proteoglycan that cover the collagen fibril and are reinforced by the incorporation of hydroxyapatite.

The concept of dentine demineralisation has been introduced in bonding systems (Pashley, 1992, Van Meerbeek et al., 1999). The purpose of the procedure were to ; 1) eliminate the smear layer that contains large quantity of organic and inorganic substances and create micropores on the enamel and dentine surface ,2) uncover the collagen and expose the dentinal tubules so that resin can infiltrate and form a hybrid layer (Nakabayashi et al., 1991).During dentine etching, the demineralisation process dissolved the inorganic content at certain depths and caused morphological changes on the dentine surface (Marshall et al., 1993). After the removal of the inorganic content, the dentinal collagen becomes destabilized and exposed to enzymatic degradation. Some studies have reported that dentine demineralisation results in shrinkage of the dentine structure due to collapse of the collagen matrix

(Garberoglio and Brannstrom, 1976a). Another study reported that the intrafibrillar minerals act to stabilize the dentinal collagen and protect it from the extracellular fluids (Balooch et al., 2008).

Published studies that have suggested dentine demineralisation protocols have been mainly in collagen network research (Marshall et al., 1993, El Feninat et al., 2001, Habelitz et al., 2001, Ma et al., 2009, Fawzy, 2010). These protocols induce important changes including exposing the collagen matrix and even alteration of the collagen structure and properties (Nakabayashi et al., 1982, Balooch et al., 2008).The quality of demineralisation depends on the demineralising agents used, demineralisation time, the composition of the dentine surface and sometimes depends on the acid concentrations (Garberoglio and Brannstrom, 1976a, Van Meerbeek et al., 1999). Common demineralisation agents used include citric acid, maleic acid, phosphoric acid, nitric acid and EDTA.

In this chapter, assessment for the demineralisation protocols on the control permanent teeth were carried out; to determine which protocol exposed the dentinal collagen without inducing any changes or minimal changes in the morphology of the collagen fibrils. Images of collagen fibrils of a rat tails were used as a form of baseline image. Selective demineralisation protocols were used in order to reveal the collagen networks that are shielded by inorganic and non collagenous proteins in the dentine.

3.2 Materials and methods

The sequence for collection, preparation and imaging of four experimental groups in control permanent teeth are shown in the figure 3.1. Total of six control permanent teeth were involved in the chapter.

3.2.1 Demineralisation using published protocols

In this demineralisation study, two control permanent teeth were embedded in epoxy resin as described in section 2.2.3. Then, the prepared teeth were treated based on replicated protocols from the published study described in section 2.3.2 (Habelitz et al., 2002).

3.2.2 Demineralisation of experimental group using commercial etchant

The purpose of this protocol was to investigate the reaction of teeth using commercial etchant that have been used in our clinical setting. Two control permanent teeth were embedded in epoxy resin as described in section 2.2.3. The prepared samples were etched using the method in 2.3.3 and images were recorded using AFM. To relate our findings to the chemical composition of the dentine, Raman spectroscopy was recorded at every etching time. Then, the samples were treated using 6.5 vol% NaOCI as mentioned in section 2.3.4 and images were recorded by using AFM.

3.2.3 Demineralisation using 17% EDTA for 12 months

One control permanent tooth was stored in 17% EDTA followed the protocol in section 2.3.5 (a), after which the tooth were prepared for histopathology section as described in section 2.2.4. Images were recorded by using light microscope and SEM.

3.2.4 Demineralisation using 17% EDTA for 10 minutes

One control permanent tooth was embedded in epoxy resin as described in section 2.2.3. The prepared sample was treated with 17% EDTA for 10 minutes and images were recorded using SEM.

The sequence for collection, preparation and imaging of four experimental groups for control permanent teeth is shown in the following figure 3.1.

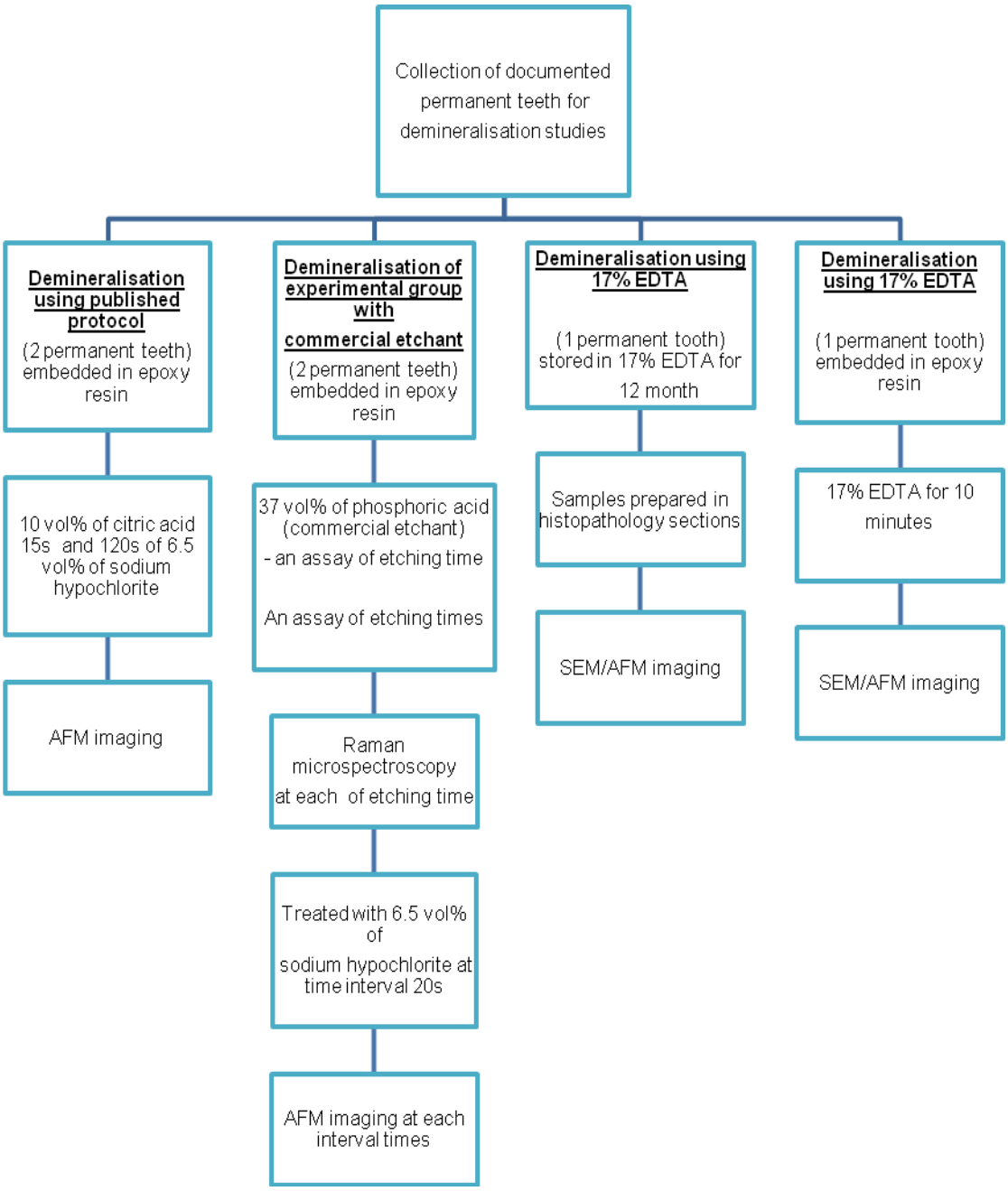


Figure 3.1: Experimental groups for control permanent teeth

3.3 Results

3.3.1 Demineralisation using published protocols

The results of replicated protocols from the published study as mentioned in 3.2.1 were demonstrated in Figure 3.2. The purpose of this protocol was to evaluate the published methods that proved to enable in situ imaging of dentinal collagen.

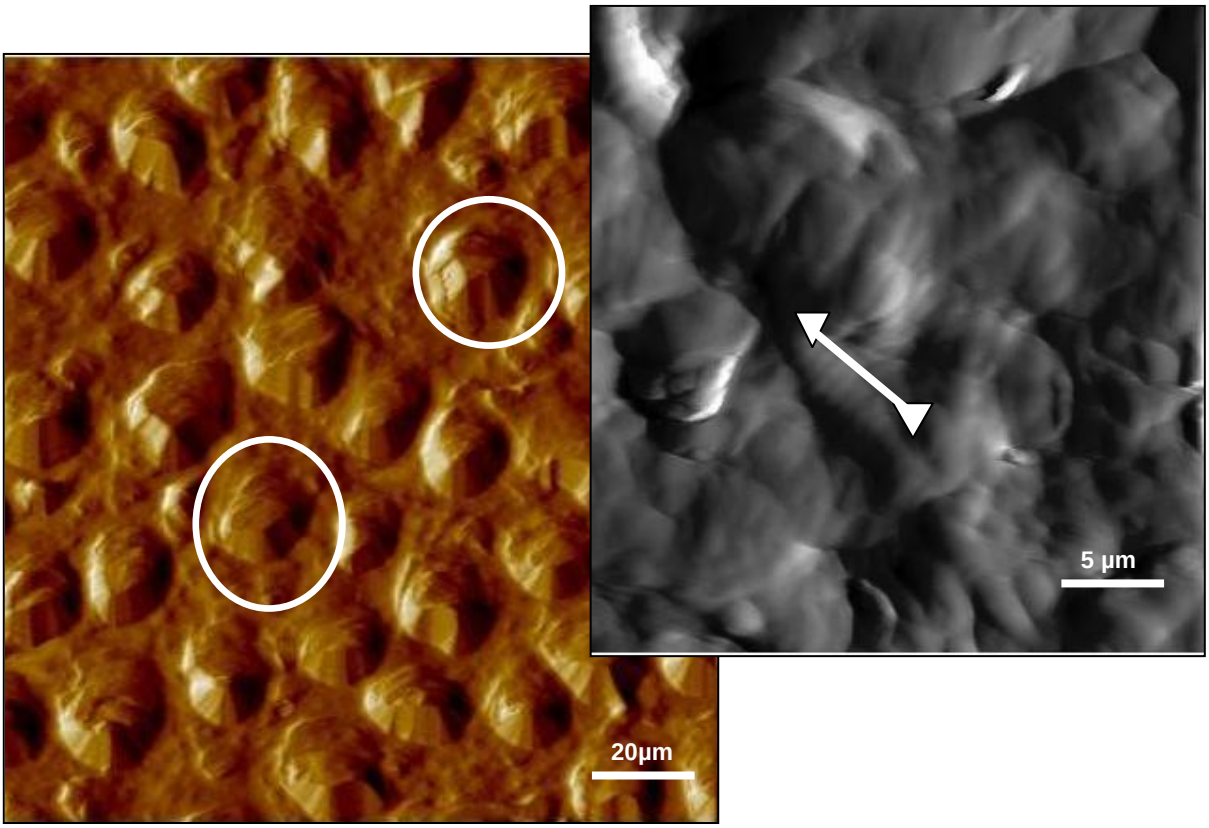


Figure 3.2: Contact mode AFM images of occlusal section of permanent human dentine. Pictures showing polished samples after partially demineralised by 10vol% of citric acid for 15 s, and subsequently treated with 6.5 vol% of sodium hypochlorite for 120 s. (a) polished samples at 20µm, increased diameter of dentinal tubules(indicated in circle) (b) at 5µm Presence of D-banding (arrows) at lower scan size. Images were obtained using Dimension 3100 AFM (Veeco, Santa Barbara, CA).

In this protocol, contact mode images at different scan size were obtained. At 20 µm, dissolved peritubular dentine after demineralisation and deproteinisation treatments can be seen. The diameters of dentinal tubules were increased due to recession of the peritubular dentine (indicated in circle). At lower scan size, we suggested that the fibrils can only be seen in certain areas (indicated by arrows). The images contrasts were not strong enough to reveal the clear collagen fibrils. The aim to expose the collagen network as mentioned in the published data was not fully achieved.

3.3.2 Demineralisation of experimental group using commercial etchant

As been mentioned in section 3.2.2, there were a few steps involved in this demineralisation protocol. The protocol began with the samples preparation for two control permanent teeth in epoxy resin. When preparing the dentine blocks, we cannot readily obtain clean surface as shown in Figure 3.3

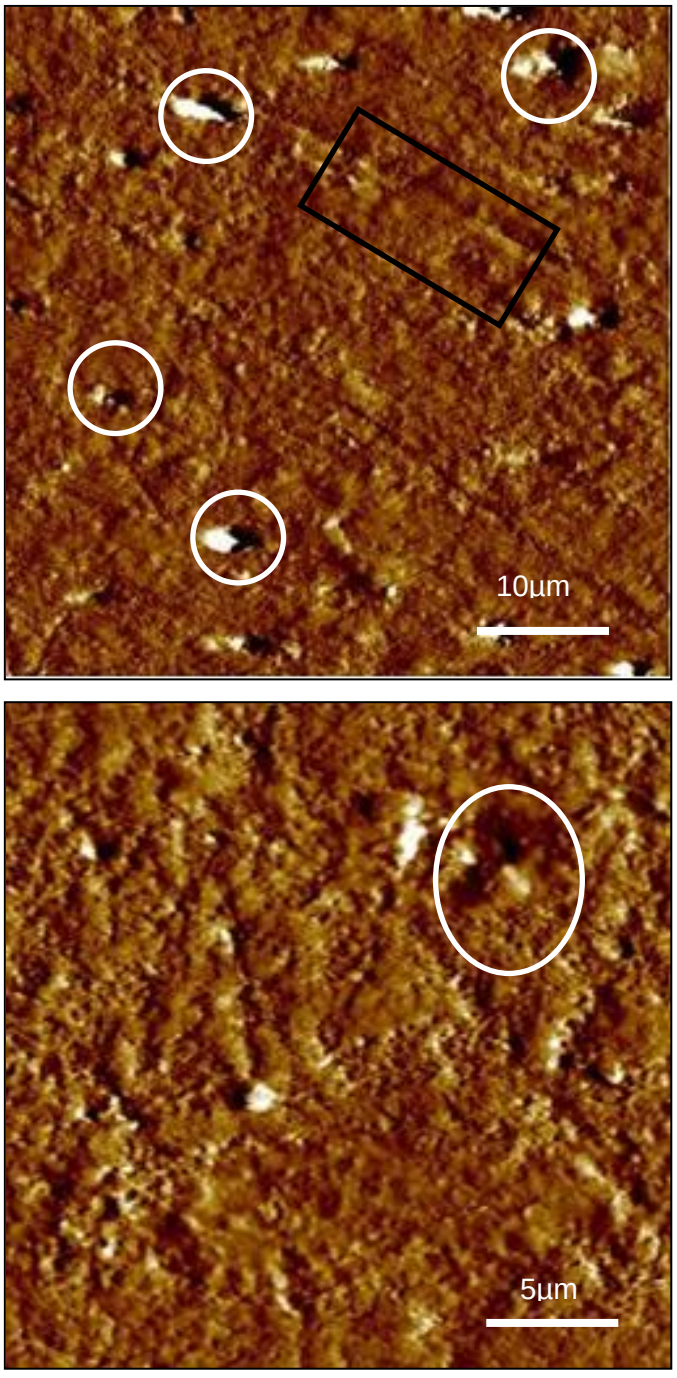


Figure 3.3: Contact mode AFM images of permanent teeth after sample preparation. Presence of small opening of dentinal tubules (indicated in circle) and polishing marks as the results of sample preparation (indicated in box).

During sample preparation, smear layers that contain inorganic and organic substances were produced and as a result it was not possible to identify the collagen fibrils. To relate our structural findings to the chemical composition of the dentine, baseline Raman spectra of the permanent dentine was recorded as shown in figure 3.4.

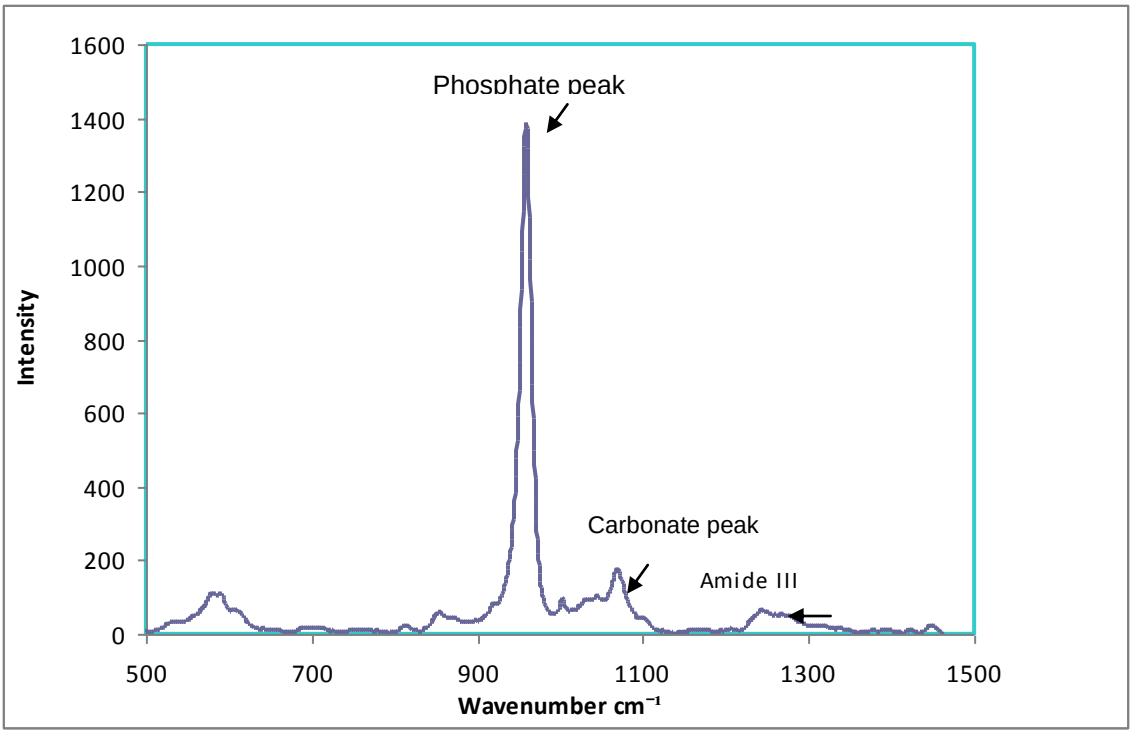


Figure 3.4: Raman spectra of permanent teeth with phosphate, carbonate and amide III peak

The spectra were normalized against the carbonate peak (CO_2^{3-}) at 1072 cm^{-1} which is considered a stable hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) spectrum. For clarity and ease of interpretation, the x-axis has been rescaled to show only wavenumbers from 500 cm^{-1} to 1500 cm^{-1} and two main characteristic peaks; phosphate (PO_4^{3-}) at 960 cm^{-1} and amide III at 1240 cm^{-1} were identified.

Based on the protocol in section 3.2.2, once samples were embedded in epoxy resin, the clinical application of commercial etching that contains 37% phosphoric acid was undertaken. The effects of different etching time were obtained using Raman spectroscopy. At a different etching time, the effects of the Raman spectral would be expected to show a significant different at both peaks; phosphate band (PO_4^3) at 960 cm^{-1} and amide III band at 1240 cm^{-1}

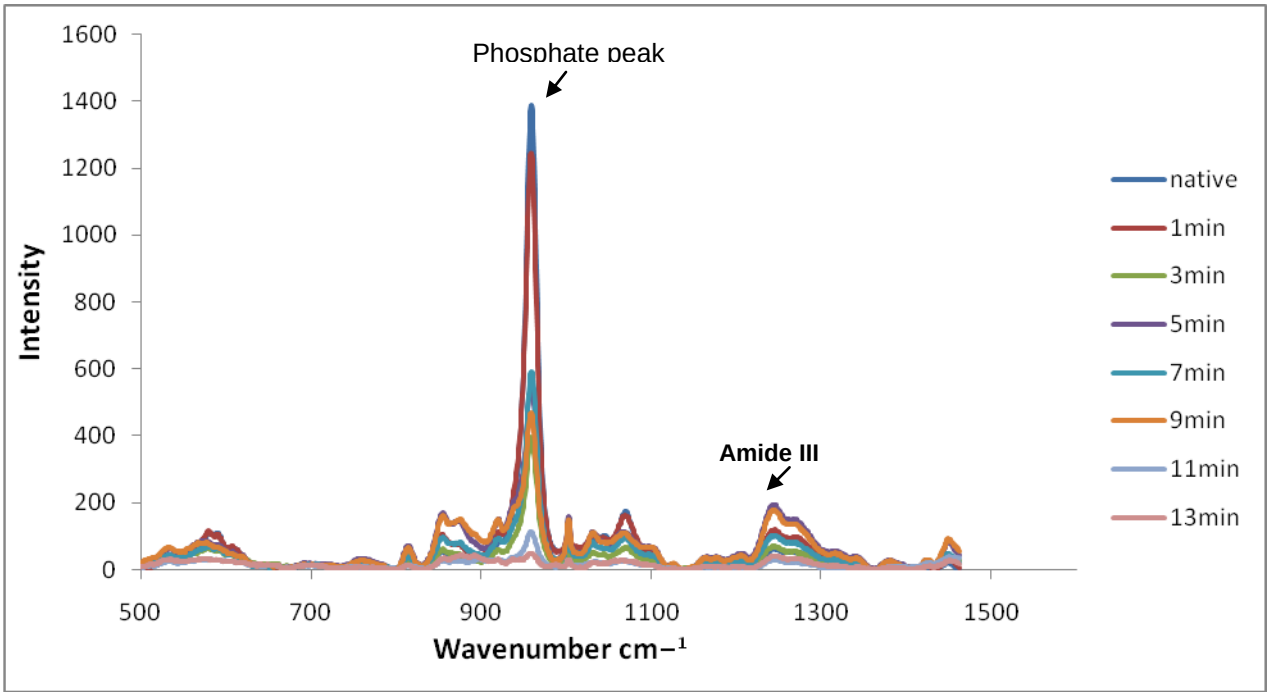


Figure 3.5: Schematic raman spectra at different times of demineralisation with acid phosphoric 37% on permanent teeth

The ratio of phosphate band to amide III was shown in Figure 3.6 to determine significant changes of minerals during demineralisation. As stated by Kinney et al, during demineralisation about 70-75% of minerals were removed rapidly while the remaining minerals were removed at lower rate (Kinney et al., 1995) This observation potentially explained that at certain particular time, we can observe significant changes of minerals in the dentine. Although etching can be a method to reveal the dentinal collagen, it also can interfere with the original features of the collagen (El Feninat et al., 2001) In a condition where the sample surface was over etched, the

collagen fibrils were totally lost or altered their shape and distribution. Therefore, an appropriate time was needed to avoid the denaturating of collagen fibrils.

Ratio of Phosphate band / Amide III band

As mentioned in Figure 3.5, a band can be observed in the Raman spectra at 1240cm^{-1}, which relates to amide III (N-H). The band was weak in Raman spectra and was not been observed previously because of the overlapping of the spectra. In order to assess the efficiency of the demineralisation protocol, it was decided to take the ratio of the Phosphate band (which should decrease in intensity as a function of the demineralisation time) to the Amide III band (which is related to the presence of collagen in the dentine and should not vary unless the demineralisation agent start denaturing the collagen). The ratio between intensity of phosphate peak at 960 cm^{-1} and the amide III peak at 1240cm^{-1} were compared and result was shown in figure 3.6.

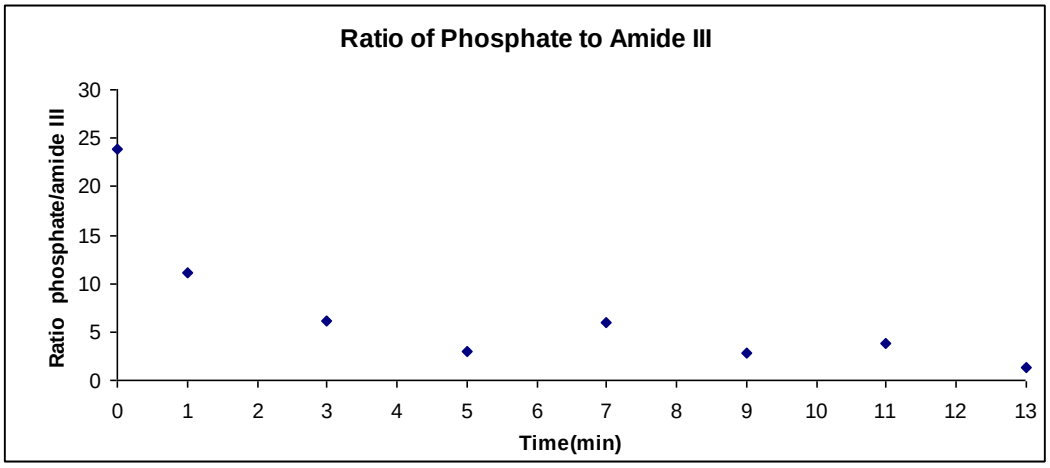


Figure 3.6 : Graph showing the ratio data of phosphate peak and amide III

Figure 3.6 shows the ratio of phosphate band intensity to amide III intensity. From the graph, it shows that the intensity of the phosphate band decreased with time. After 5 minutes, the intensity of phosphate band to amide III was not consistent. It can be hypothesised that after 5 minutes, changes in dissolution of phosphate minerals in hydroxyapatite in the dentine became unchanged.

For each demineralisation treatment with commercial etchant on control permanent teeth, Raman spectra were obtained and were followed by observation of topological surfaces by using AFM. The purpose of imaging at each demineralisation time was to observe the morphology changes on the dentinal surface of control permanent teeth.

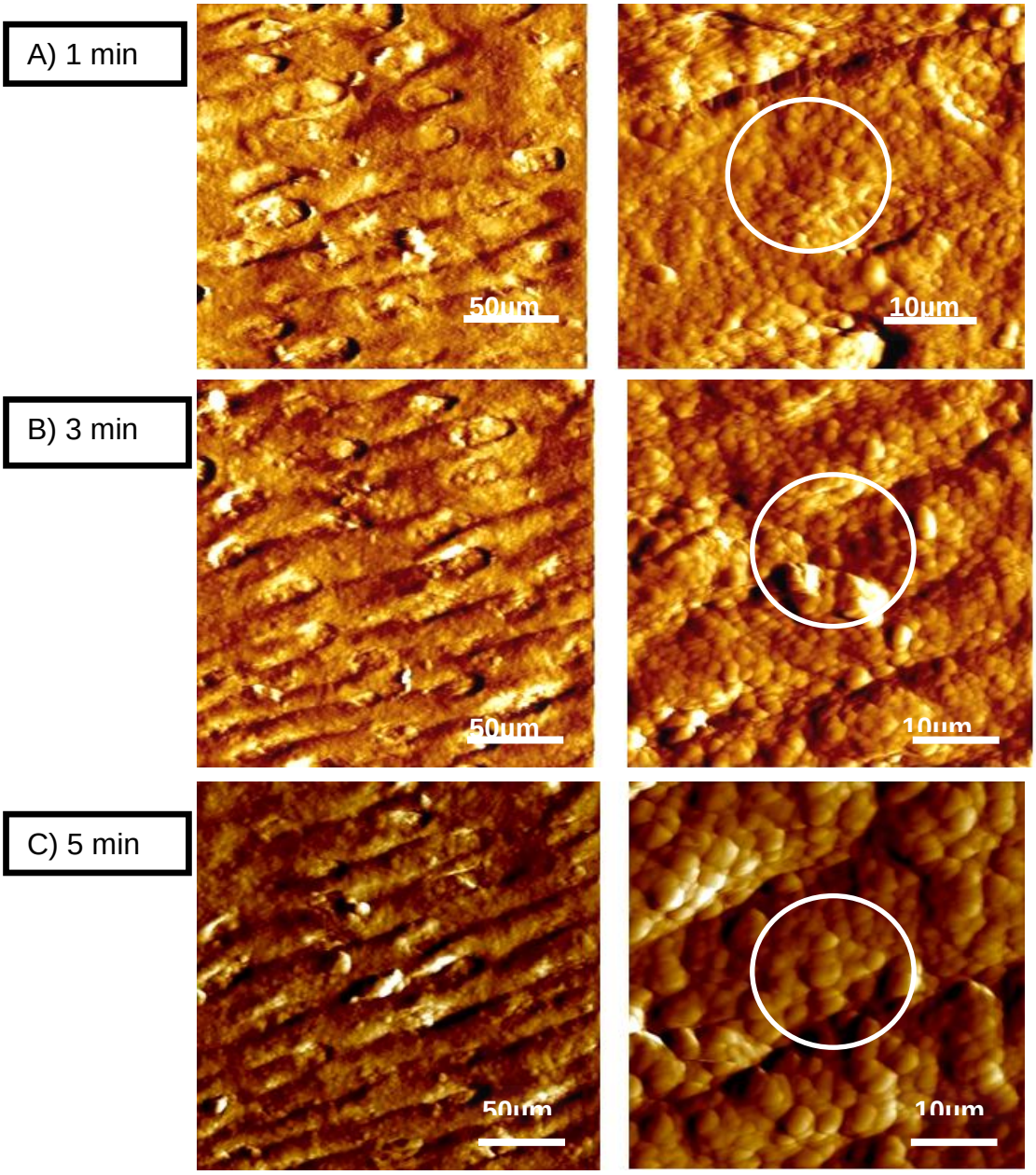


Figure 3.7: Contact mode AFM images of demineralisation sequences in commercial etchant (37% phosphoric acid) of control permanent teeth. An assay of etching times starting from Figure (A) demineralisation at 1 min, (B) 3min and (C) 5 min. Changes of dentinal surface where the granulated particle (smear plugs) became more obvious and rounded (indicated by circle)

The images shows a series of two different scan sizes which were 50x50 µm and 10x10 µm of control permanent teeth after treated by commercial etchant (37% phosphoric acid). The teeth were exposed according to an assay of etching time: Figure (A) shows the microstructure of the dentine after acid treatment for 1 minute. The granulated particles (smear plugs) became more obvious. Some opening of the dentinal tubules can be seen which suggest partial removal of smear layer and smear plugs. Figure (B), demonstrates the dentinal tubules opening that became clearer with removal of smear layer and in Figure (C), massive particles of smear layer that covered the intertubular dentine can be clearly distinguished. The particle became rounded. These features suggested that the dentine surface reacted towards the commercial etchant which caused topological changes on the surface. However, no presence of collagen can be seen at this stage.

Further experimental protocol with 6.5 vol% NaOCl

Further step in the experimental group with commercial etchant was continued with deproteinisation protocol as described in section 2.3.4. The partially demineralised control permanent teeth were treated with 6.5vol% NaOCl at different time with intervals of 20seconds in each sequence. For each interval, topological surfaces of control permanent teeth were imaged by using AFM.

Initial image of the treatment was compared to the image when collagen network became visible was shown in figure 3.8. At 20 seconds exposure to 6.5 vol% NaOCl, the surface was covered with dispersive particles. When the time was increased to 100 seconds, the topology of the dentine surface showed significant changes whereby the interwoven collagen network became visible.

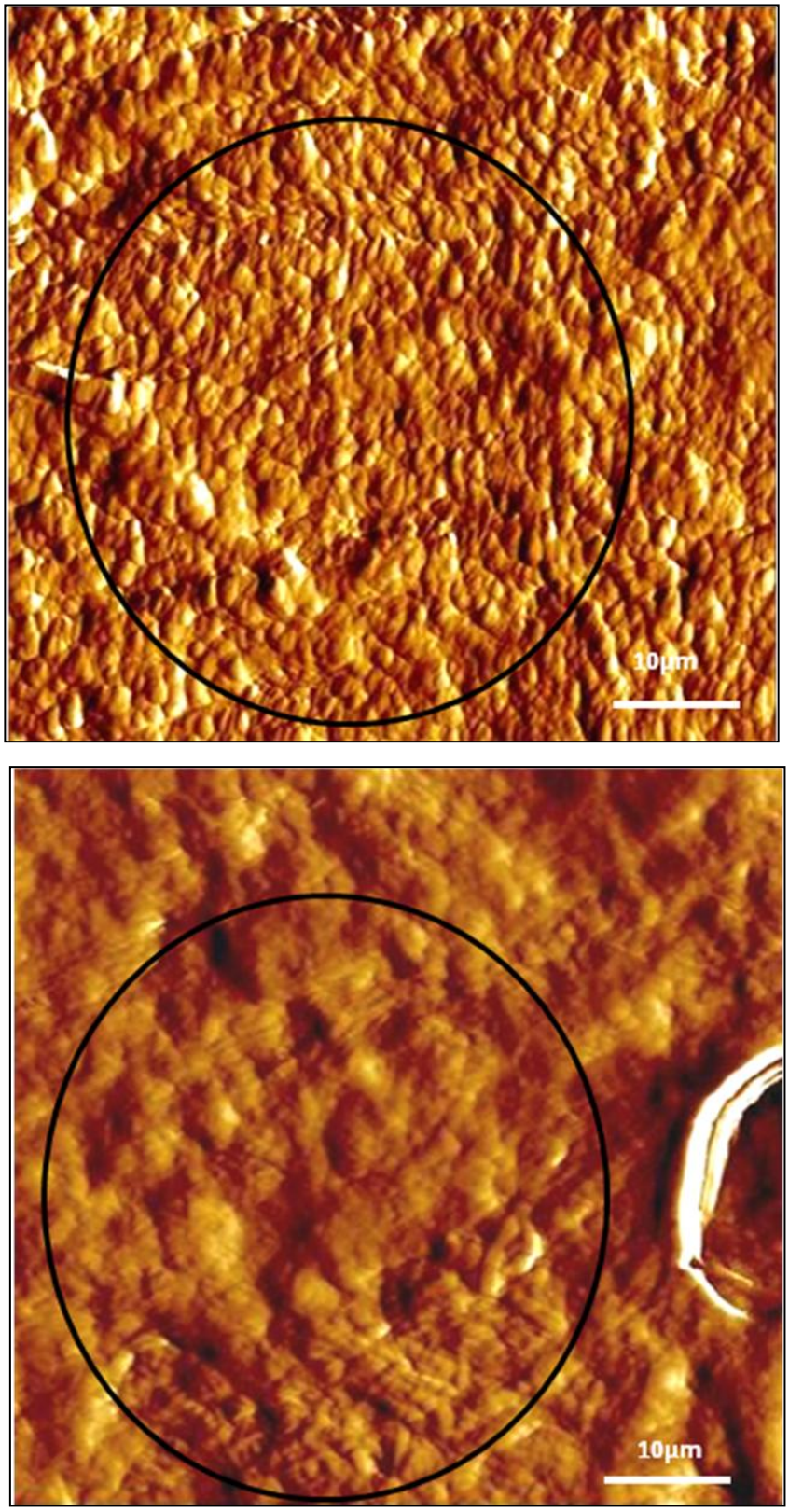


Figure 3.8: AFM images of demineralised dentine surface in 37% phosphoric acid at 5 minutes with deproteinisation treatment in 6.5 vol% sodium hypochlorite. Topological surface at intervals of 20 seconds (above picture) and 100 seconds (below picture)

Samples treated with NaOCI for 100 seconds were observed at lower scan size. The generalized appearance of interwoven area of collagen fibrils at 10µm (indicated in circle in figure 3.7) was hypothesised to allow clear pattern of D-banding distance at lower scan size.

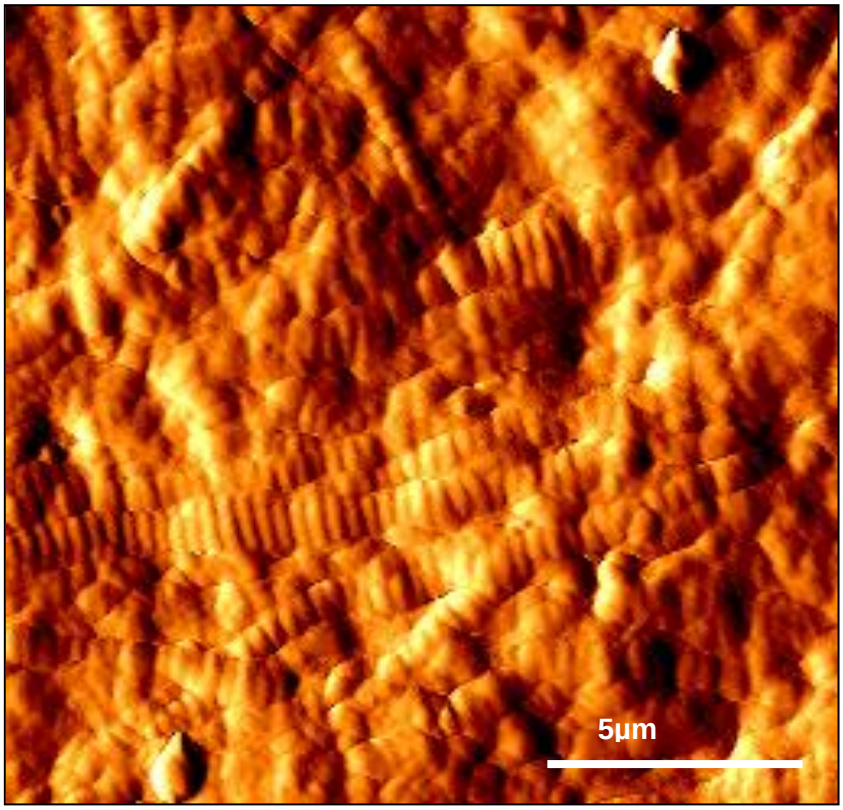


Figure 3.9: Contact mode XE-100 Park AFM images of permanent human dentine after partial demineralisation by 37%phosphoric acid for 5 minutes and subsequently treated with 6.5 vol% NaOCI for 100 seconds. Collagen fibrils were revealed.

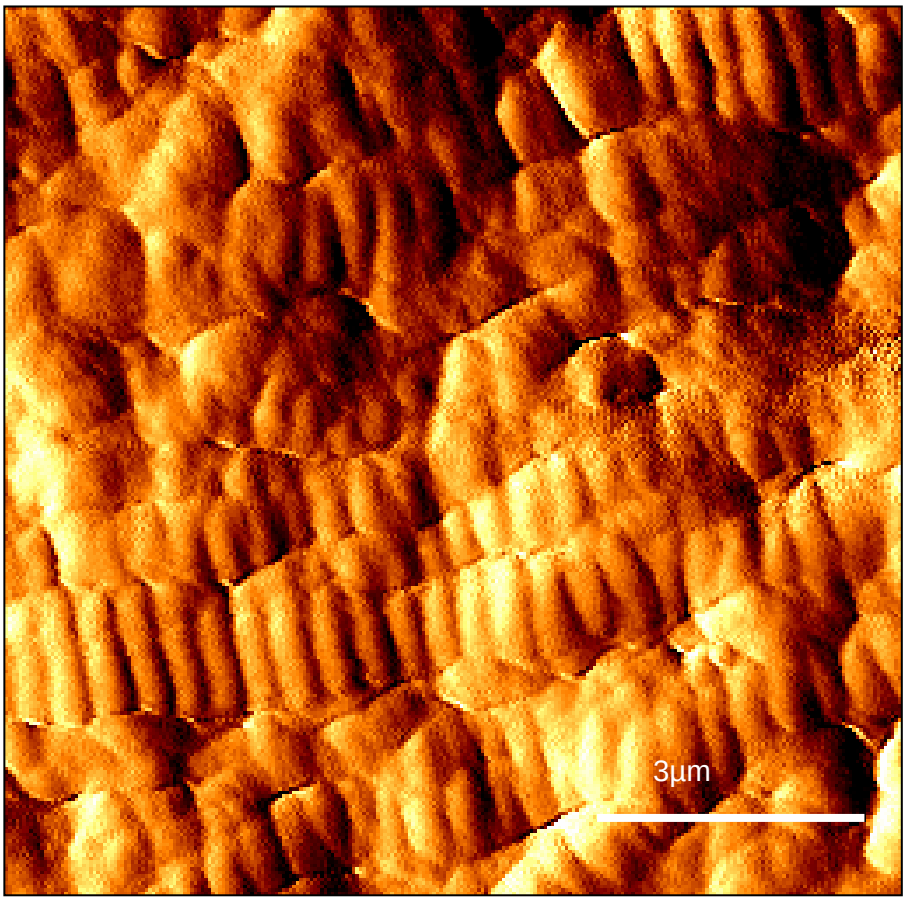


Figure 3.10: Collagen fibrils can be observed showing the repeat pattern and random distribution of fibrils in the intertubular dentine.

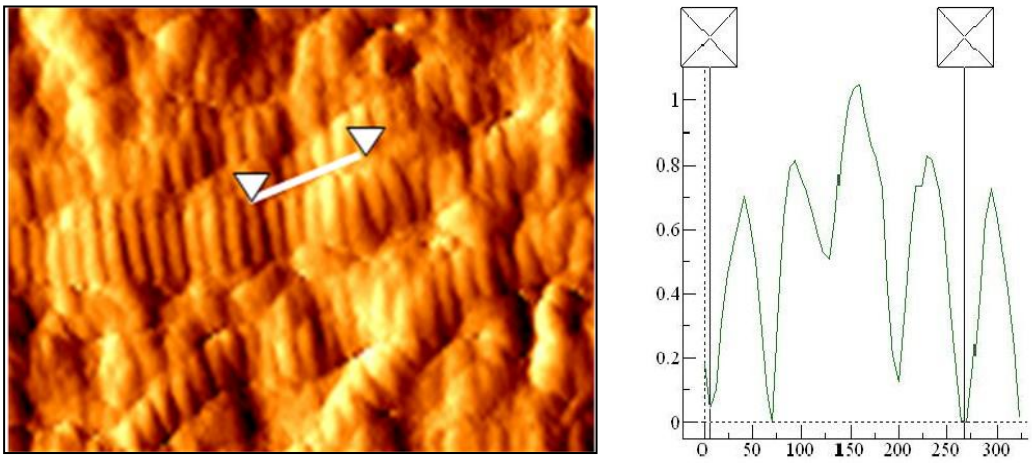


Figure 3.11 :Sectional analysis along the fibrils shows D-banding distance along collagen fibril by WSxM analysis

Figure 3.9 and 3.10 shows dentinal collagen topography at lower scan size. The images showed the repeat pattern and random distribution of collagen fibrils. This is a novel result that shows the topographic profile of collagen fibrils were able to be viewed under AFM by using commercial etchant (37% phosphoric acid) at 5 minutes and controlled deproteinisation agent, 6.5vol% NaOCI for 100 seconds. With clear and descent collagen images, the D-banding distance can be measured.

Figure 3.11, shows the measurement of D-banding distance of control permanent teeth at one collagen fibril (indicated by arrow) that can be determined at 65.03nm. Further analysis about the distribution of D-banding distance in control permanent teeth will be needed for comparison purpose to permanent teeth of DI type I.

3.3.3 Demineralisation using 17% EDTA for 12 months

In this group, the effect of 17% EDTA on one control permanent tooth was evaluated. The tooth was stored in 17%EDTA for 12 months and was prepared into histopathology section, followed the protocol described in section 3.2.3. The section was viewed under light microscope before SEM images were taken.

Light microscopy for control histopathology section

The image obtained using light microscope revealed the demineralised layer, stained red. The results demonstrated a uniform and organised pattern of dentinal tubules with network of extracellular matrix in between the tubules as shown in figure 3.11.

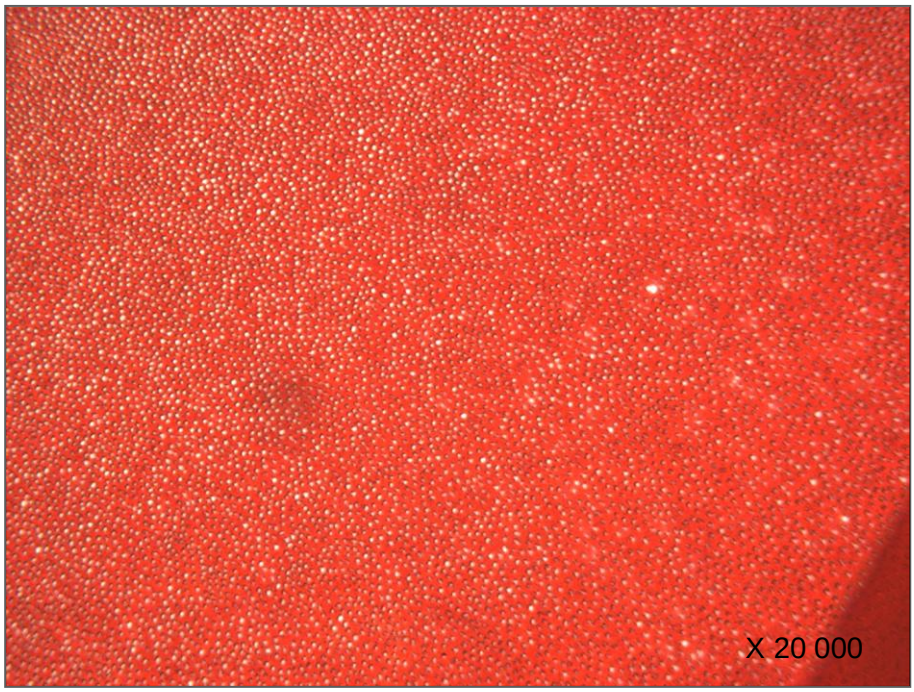
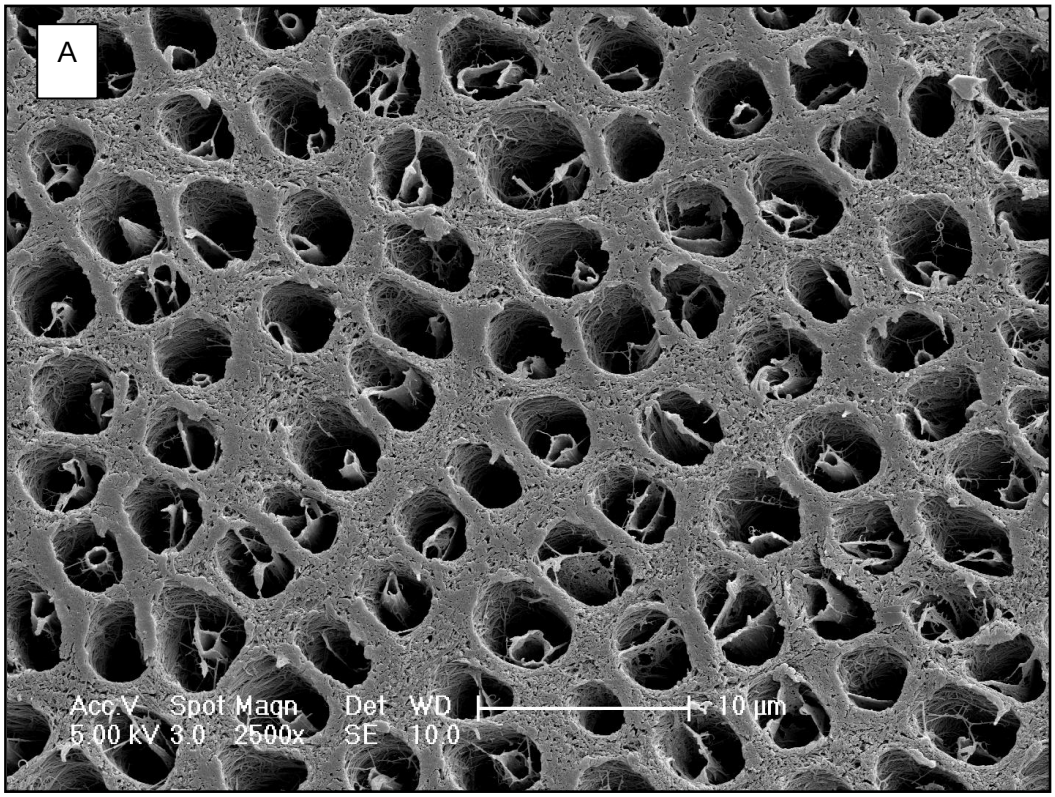


Figure 3.12: Picrosirius red stained histopathology cross- section of permanent teeth after been stored in 17% EDTA for 12 months. The bright red field manifested in between dentinal tubules (white rounded area).

Imaging control histopathology section using SEM

In order to obtained images from the SEM, histopathology section was prepared using protocol in section 2.42. Images were recorded in two scan sizes; 10µm at 2500x magnification and 2µm at 15000x magnification



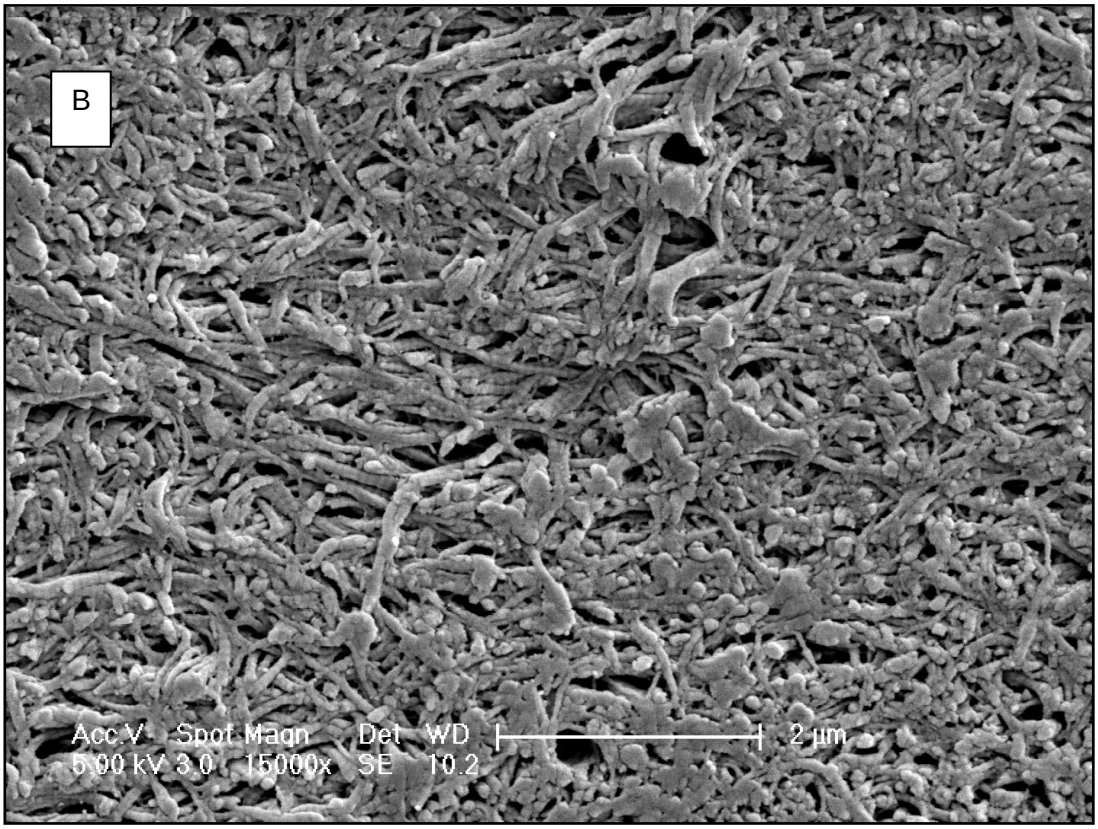
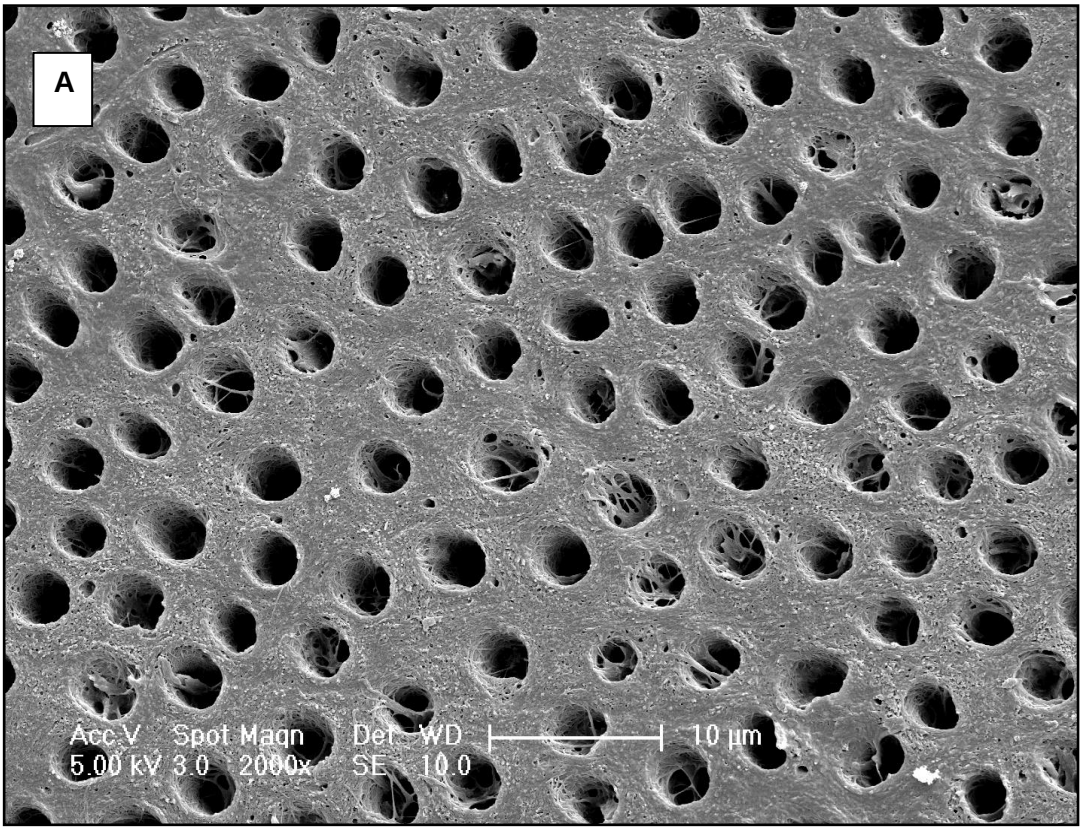


Figure 3.13: Experimental procedure of permanent teeth stored in 17% EDTA for 12 month (A) Enlargement of dentinel tubule openings with sign of erosion of peritubular and intertubular dentine. Projection of odontoblastic process can be seen in certain dentinal tubules openings. (B*)* Higher magnification of this specimen at intertubular dentine showing localised collagen scaffold that surrounded by irregular variation structure which scattered almost at all areas.

In order to investigate the effects of prolonged application of EDTA, the control permanent tooth was stored in 17% EDTA (pH 7) for 12 months. The histopathology section of the control permanent tooth demonstrated excessive erosive effect of intertubular and peritubular dentine that leads to the enlargement of dentinal tubule openings. The intertubular dentine almost completely disappears between the tubules as the result of longer demineralisation effect. At lower scan size, the sporadic pattern of collagen scaffold at the intertubular areas can be observed. However, most of the region was covered with irregular surfaces which may be due to the results of denaturation.

3.3.4 Demineralisation using 17% EDTA for 10 minutes

In this group, one of the control permanent tooth was prepared in epoxy resin as described in 2.2.3. Then, the prepared sample was treated with 17% EDTA for 10 minutes and imaging were obtained using SEM



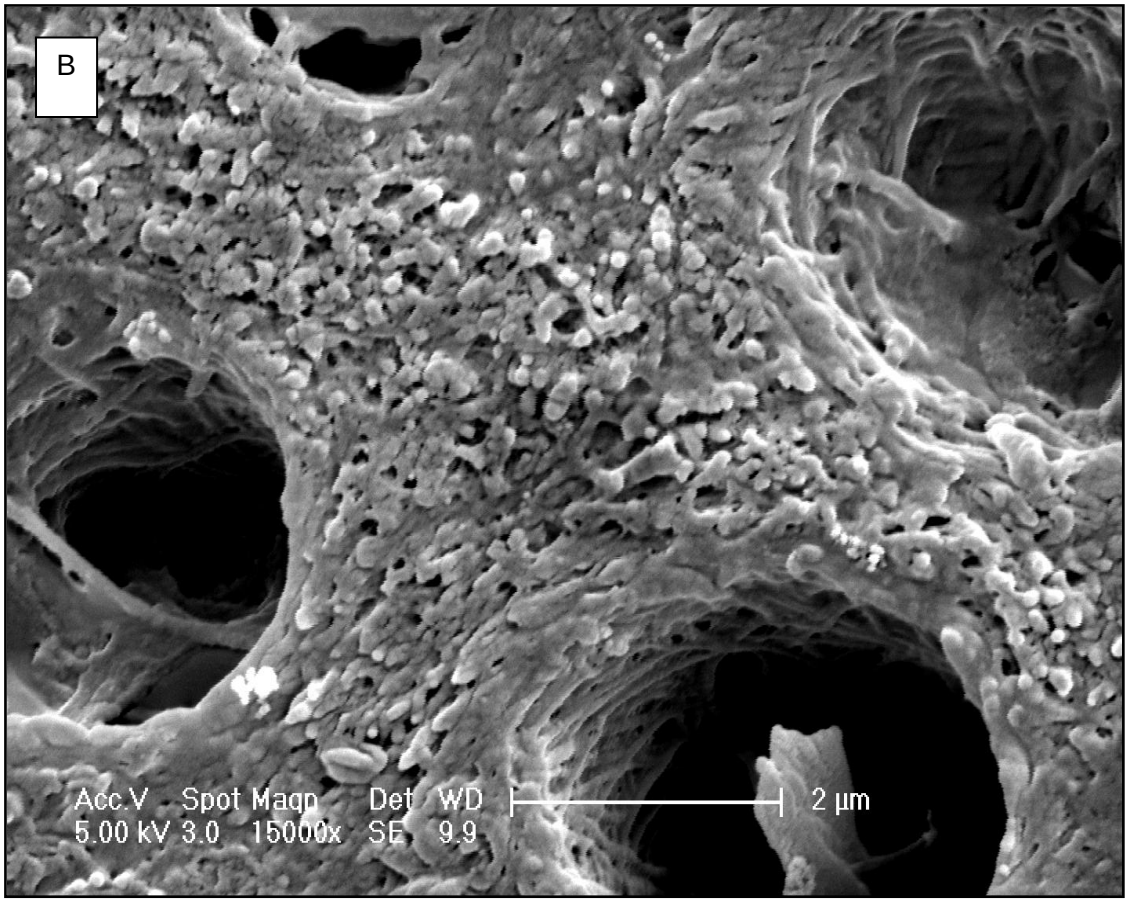


Figure 3.14: Effect of 17% EDTA for 10 minutes on the dentinal surface of control permanent tooth (A)The smear layer was almost completely removed and overall the tubule openings were clearly visible (B) At higher magnification, no presence of collagen can be noted in the intertubular area, however the collagen fibrils arragement begin to appear at the peritubular area.

SEM observation shows that the smear layer was almost completely removed and the dentinal tubule openings were clearly visible. There were slight increased in the dentinal tubules opening observed surrounding the tubules. At 2µm, collagen scaffold begins to appear at the peritubular areas however at the intertubular areas, no defined collagen fibrils can be detected. In this area, it was hypothesised that the area may not be demineralised yet.

3.4 Discussion

3.4.1 Demineralisation using published protocols

The combined techniques of demineralisation (10 vol% citric acid) and controlled deproteinisation (6.5 vol% sodium hypochlorite) of dentine has been reported to enable imaging of dentinal collagen under AFM. The suggestion that dissolution of most mineral with demineralisation protocol followed by gradually removal of non-collagenous proteins with deproteinisation procedure allowed in situ study of dentinal collagen (Habelitz et al., 2002). The same approach was repeated in this chapter and results proved the presence of collagen scaffold under contact mode AFM. The image qualities shown in section 3.2 were of poor contrast. These findings showed that the collagen fibrils only can be viewed at certain areas with some of the fibrils demonstrated irregular appearance. It was assumed that the collagen may go through changes as the results of the non specific effect of the sodium hypochlorite agent.

3.4.2 Demineralisation of experimental group using commercial etchant

Smear layers were created as the results of cutting during sample preparation steps. This layer contains tenacious debris with organic and inorganic substances (Pashley, 1992). During the sample preparation in epoxy resin, I cannot readily obtain a clean surface because the exposed mineralised dentine surfaces were covered by the smear layer. This was shown in the findings in section 3.3. Therefore, in this study, the smear layer must be removed.

As part of restorative procedures, chemical treatment by acid etching was introduced to remove and dissolved the smear layer allowing exposure of partially demineralised dentine (Nakabayashi et al., 1991, Pashley, 1992). To date, the etching methods have been extensively used in restorative dental treatment. Etching with 30-40%

orthophosphoric acid has commonly been used in the modern adhesive restoration (Meerbeek et al, 2000). The use of phosphoric acid in clinics was well documented (Nakabayashi and Pashley, 1998, Sasaki et al., 2008).

In this study, demineralisation protocol using a commercial etchant gel (37% phosphoric acid) that commonly used in our clinical setting was investigated. The conventional method reported that 15 seconds application of 37% phosphoric acid to etch the enamel was required in order to provide a better substrate for bonding to dental adhesives (Buonocore, 1955). In routine studies of etched dentine, it was necessary to remove the smear layers completely so that the collagen fibrils network can be exposed. The first step of this experiment mirrored the published protocol in which the ultrastructure collagen network on demineralised dentine can be observed at 60 seconds of etching time in phosphoric acid (Ma et al, 2009). The results were inconclusive as dispersive particles (smear layer and smear plugs) still can be observed on the dentine surface at similar etching time. This may be affected by the viscosity of the phosphoric acid, in which gel solution provide less etching effects than liquid solution and did require longer application time (Pashley et al, 1992)

The ratio of phosphate band to amide III was shown in Figure 3.6 to determine significant changes of minerals during demineralisation. As stated by Kinney et al, during demineralisation about 70-75% of minerals were removed rapidly while the remaining minerals were removed at lower rate (Kinney et al., 1995). This observation potentially explained that at certain particular time, we can observe significant changes of minerals in the dentine. Although etching can be a method to reveal the dentinal collagen, it also can interfere with the original features of the collagen (El Feninat et al., 2001). In a condition where the sample surface was over etched, the collagen fibrils were totally lost or altered their shape and distribution.

Therefore, an appropriate time was needed to avoid the denaturating of collagen fibrils.

Further experimental protocol with 6.5 vol% NaOCl

The body of this chapter has shown that etching alone would not completely expose the dentinal collagen. This was supported by the results in Figure 3.7 that demonstrated the presence of smear layers and smear plugs after treatment using commercial etchant (37% phosphoric acid) for 5 minutes. No collagen fibrils can be observed. Attempted to track the collagen fibrils in varies of location on the samples were carried out but no defined collagen images can be obtained throughout the experiment. This study confirms the previous speculation that the application of acid treatment would only dissolves the minerals in the dentine while non-collagenous proteins appear remain associated with the collagen network which hindered the imaging of collagen fibrils by AFM (Habelitz et al , 2003). As there have been difficulties of imaging the collagen fibrils, deprotienisation technique was suggested to facilitate immediate AFM imaging. In contrast, Bertasssoni *et al* suggested no further enzymatic treatments of the dentine substrate were required in order to reveal the collagen fibrils. Yet, they failed to reproduce the physiological conditions of the dentine as the results of dry specimens in the study (Bertassoni et al., 2012).

The method of deproteinisation provides guidance for the further experiment on demineralised samples in 37% phosphoric acid for 5 minutes. The concept of using NaOCl after etching leads to removal of non collagenous proteins in extracellular matrix. The initial etching treatment showed enlarged dentinal tubules as the results of recession of peritubular dentine. This can be observed in Figure 3.7. However, acid treatment alone would not completely open the tubules since they remained filed with the proteins. On the other hand, deprotenisation alone does not open the

dentinal tubules since minerals closely integrated with the proteins. Having to said, the etching serves a precondition area to the NaOCI treatment which allows exposure of the collagen after the removal of non collagenous proteins (Toledano et al., 1999, Marshall et al., 2001, Fawzy, 2010). My observation showed that at time interval between 80 seconds and 100 seconds in 6.5 vol% NaOCI, the fibrous collagen network can be revealed using AFM. The organisations of the collagen scaffold were randomly distributed and interlocking with each other. At lower scan size, the fibrils D-banding distance can be observed clearly. This observation may allow further study in characterising the topographic and subsequently assessing the mechanical properties of the collagen fibrils. However, uncontrolled deproteinisation led to total removal of collagen fibrils, leaving a 'moth eaten' appearance of dentine. This was supported by Habelitz et al when similar images occured during bleaching as the time increased to 240 seconds (Habelitz et al., 2002). In addition, previous studies reported that mechanical properties such as hardness (Oliveira et al., 2007) strength and elasticity of the dentine (Sim et al., 2001, Marending et al., 2007) were significantly reduced with the used of the NaOCI. In view of this, further follow up studies may take into account to measure the mechanical properties of the collagen fibrils revealed by these protocols.

3.4.3 Demineralisation using 17% EDTA for 12 months

Another demineralising agent that has been used in the clinic is Ethylenediaminetetraacetic acid (EDTA). It was first introduced clinically in 1957. (Ostby, 1957). The purpose of EDTA demineralisation protocols was to compare the effect of 17% EDTA (pH7) with respect to the duration of application. In this experimental study, the control permanent tooth was stored in EDTA for 12 months. It was hypothesised that with the changing of EDTA in weekly basis, the binding of calcium will continue till all the calcium ions completely attached to the EDTA. The chelating action of EDTA will bound the non collagneous proteins together as these

proteins intimately integrated with the hydroxyapatite. Based on this experimental study, the tooth was prepared for histopathology sections as the results of softening effect on the dentinal surface by EDTA activity. The findings proved the presence of sporadic collagen scaffold on the dentine surface. These collagen fibrils presented in normal cross banding pattern however the distributions of collagens were hindered by random precipitations on and within the demineralised dentine surface. These precipitations may due to the calcium ion precipitation as the results of binding to EDTA. One could argue that it maybe the sign of collagen denaturation as the erosive effect of EDTA can directly be observed in within 10 minutes of EDTA application. Some would assume that it may due to the histopathology protocol that could cut the collagen network and leave the irregular random structure of the dentine.

3.4.4 Demineralisation using 17% EDTA for 10 minutes

The major factors that contribute to the properties of EDTA are the chelating mechanism and protonation mechanism as been explained in previous literature. Study conducted by Serper and Calt showed that the phosphorous releasing effect of EDTA rapidly rises in within 1 minute, and further exposure to EDTA only doubled this effect at 15 minutes exposure (Serper and Calt, 2002). This was probably due to the rapid removal action of smear layer in the first one minute and then followed by slower released of phosphorus as the results of chelating mechanism (Serper and Calt, 2002).In 2004, the same study was repeated to see the liberation of phosphorus at longer time and the results showed that further exposure till 25 minutes did not cause any significant increase in dentine demineralisation (Parmar and Chhatariya, 2004) . Another study of time dependent effects of EDTA on dentine structure proved that a 10 minutes application of 17% EDTA, pH 7.4 caused excessive peritubular and intertubular dentinal erosion under SEM imaging (Calt and Serper, 2002).This study

was in agreement with the previous authors about the effect of erosion at the dentinal tubules when the control permanent tooth was treated using 17% EDTA (pH) for 10 minutes. However the findings did not achieved to demineralise the dentine completely up to the extent where the collagen scaffold can be exposed. As the peritubular dentine is more mineralised than the intertubular dentine, collagen arrangement can only be observed at the peritubular area at this point. Demineralised groups with EDTA were observed under SEM imaging. Significant amount of work were put into this protocol to enable imaging under AFM however up to this extent, no defined images can be detected. A better result perhaps can be achieved if the effect on the dentine structure according to an assay of time exposure to 17% EDTA can be obtained.

3.4.5 Potential issues

In this section, several potential concerns that may be relevant to the findings were discussed.

1) Sample preparation

According to the Centers for Disease Control and Prevention (CDC) guideline, teeth that were used in research need to be stored in solution for infection control purposes (Lee et al., 2007). At the same time, the choice of solutions also needs to have the ability to conserve the integrity of the tooth's surfaces. In 1993, Goodis reported that among five common storage solutions (ethanol, formalin, distilled water, distilled water with thymol, phosphate-buffered saline with thymol), saline storage resulted in large significant decreased in permeability (Goodis et al., 1993).Various type of common solutions were also discussed in a previous studies include ethanol, gamma irradiation, distilled water, formalin and saline (Addy and Mostafa, 1988, Cooley and Dodge, 1989, White et al., 1994). In this study, 70% of ethanol was used as a short term disinfectant medium for 7 days (Strawn et al., 1996). Ethanol is also known as a fixation solution. It can affect the natural composition of the dentine by breaking the hydrogen bonds on proteins molecules (Kiernan, 1981). As for this reason, the teeth were placed in 0.01% Thymol after remained in the ethanol for 1 week until prepared.

Hydrated samples

Moist bonding techniques have been reported to be effective in maintaining collagen fibril structure during dentin bonding procedures (Kanca, 1992, Gwinnett and Kanca, 1992). It also was reported that the technique can prevent the collapse of demineralised dentin matrix that occurs as a result of drying during sample preparation (Garberoglio and Brannstrom, 1976a, Pashley et al., 1993). In situ observation of dentinal collagen for hydrated and dehydrated collagen fibrils were reported by Habelitz showed that dehydration induced significant changes to the collagen fibrils (Habelitz et al., 2002). In this study, the prepared samples were stored

in plastic container that filled with purified water and dried in air for 1 second before imaging. It was hypothesised that without air-blowing and air drying for more than 1 second, the samples can still sustain its hydration.

2) Imaging

AFM imaging

The advent of AFM has opened new perspective in investigating the collagen at high resolution. The quality of images taken by AFM is important in data analysing. Good images are those with sharp and clear characteristics of the sample surfaces. Recording the topography may carry the possibility of the surface deformation. Essentially, one should recognise any sources of artefacts which can appear in the images. Selection of the tip is critical and important to ensure that the images can resemble closely to the sample topography (Keller and Cung, 1991). One of the troubleshooting that we encountered was contamination of the probe tip. Probe tip should be clean and sharp as any debris that contaminated by the tip will generated unusual images. This may continue along the scanning which can mislead the data analysis.

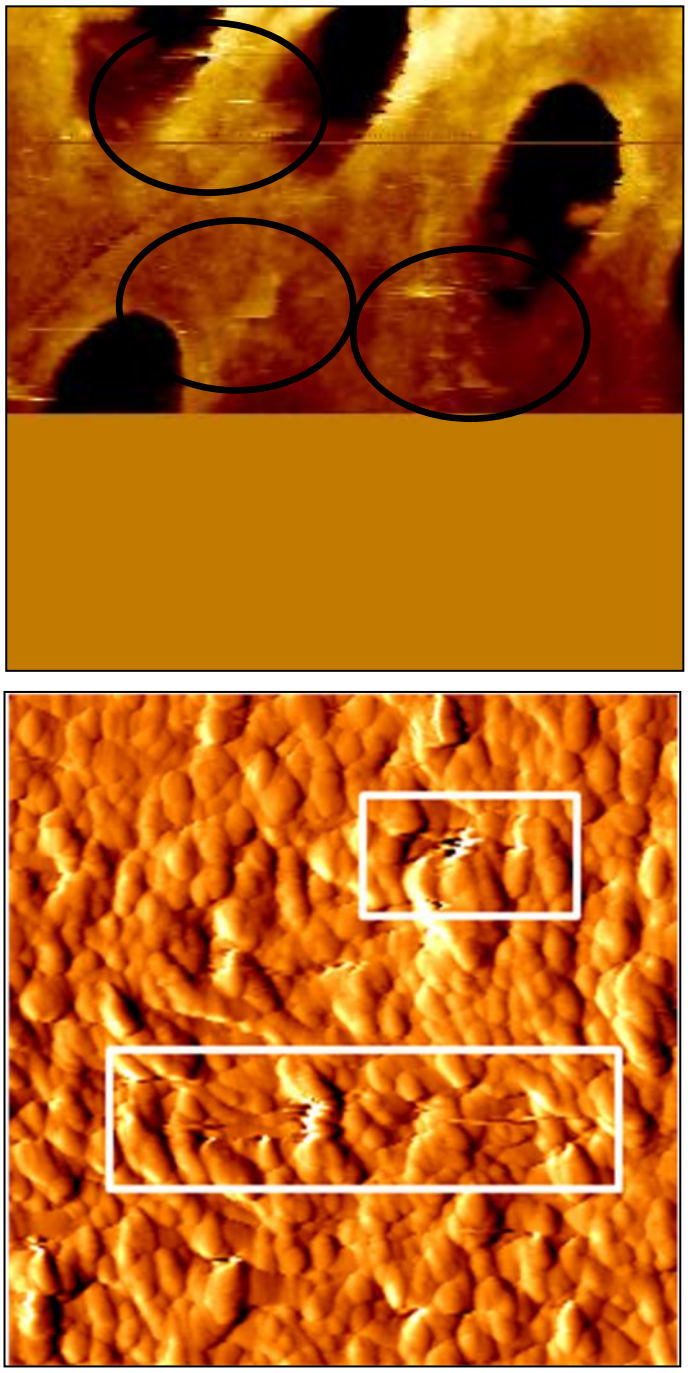


Figure 3.15: The samples did not move simultenously with the scanner, the image became not clear. Scratch marks on the sample surface can be observed.

SEM imaging

The observation on the SEM images showed that there were sign of unusual findings that indicated by circle in Figure 3.15. It was hypothesised that these artefacts may due to the effect of cutting steps during histopathology section preparation.

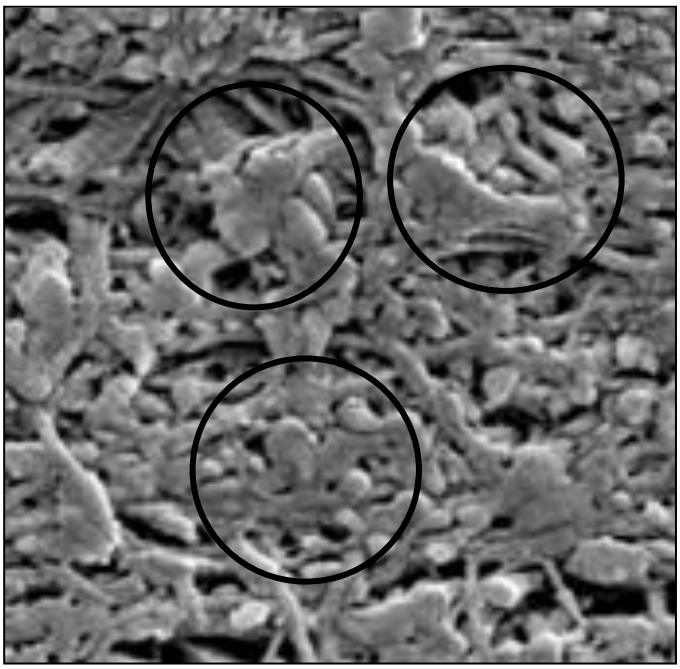


Figure 3.16: SEM image showing irregular pattern or disarrangement on the sample surface. This may be the effect of cutting during sample preparation.

3.5 Conclusion

Finalised new protocol for permanent demineralisation to expose collagen

This chapter has shown the comparison of demineralisation protocols to investigate the feasibility of topological assessment of collagen fibrils in the dentine of permanent teeth. This work has been set out to verify different chemical treatments to expose collagen fibrils in the dentine. The initial assessment of demineralised dentine with commercial etchant suggested that further deproteinisation were required to reveal the D-banding distances of the collagen fibrils. By combining Raman spectroscopy, a comprehensive representation of topological changes of the dentine substrate can be obtained at high resolution electron microsocpy. Etching protocol with 10% citric acid and controlled deproteinisation also enabled the imaging of collagen structure under AFM however the contrast were poor. The comparison results with EDTA demineralisation protocols were restricted as images using AFM could not be obtained, however this study proved the presence of the interlocking collagen scaffold under SEM for 17% EDTA in 12 months. I assumed that the difficulty in imaging the topography may attribute to certain circumstances which include the methodology of samples preparation.

All of the images in this study were generated using contact mode AFM. The skills operating in contact mode were more direct and simple. These findings provide additional insight tools to discover the topological assessment just using the contact mode. The limitation that needs to be highlighted was that this study did not discuss about the analysis of the collagen fibrils. The work just focused on characterising the morphology of dentinal collagen in permanent teeth. In conclusion, the most effective demineralising protocol for control permanent teeth in this study was the demineralisation using commercial etchant (37% phosphoric acid) followed by 6.5 vol% of NaOCI. With the established protocols described, studies on the primary teeth will be further explored in the next chapter.

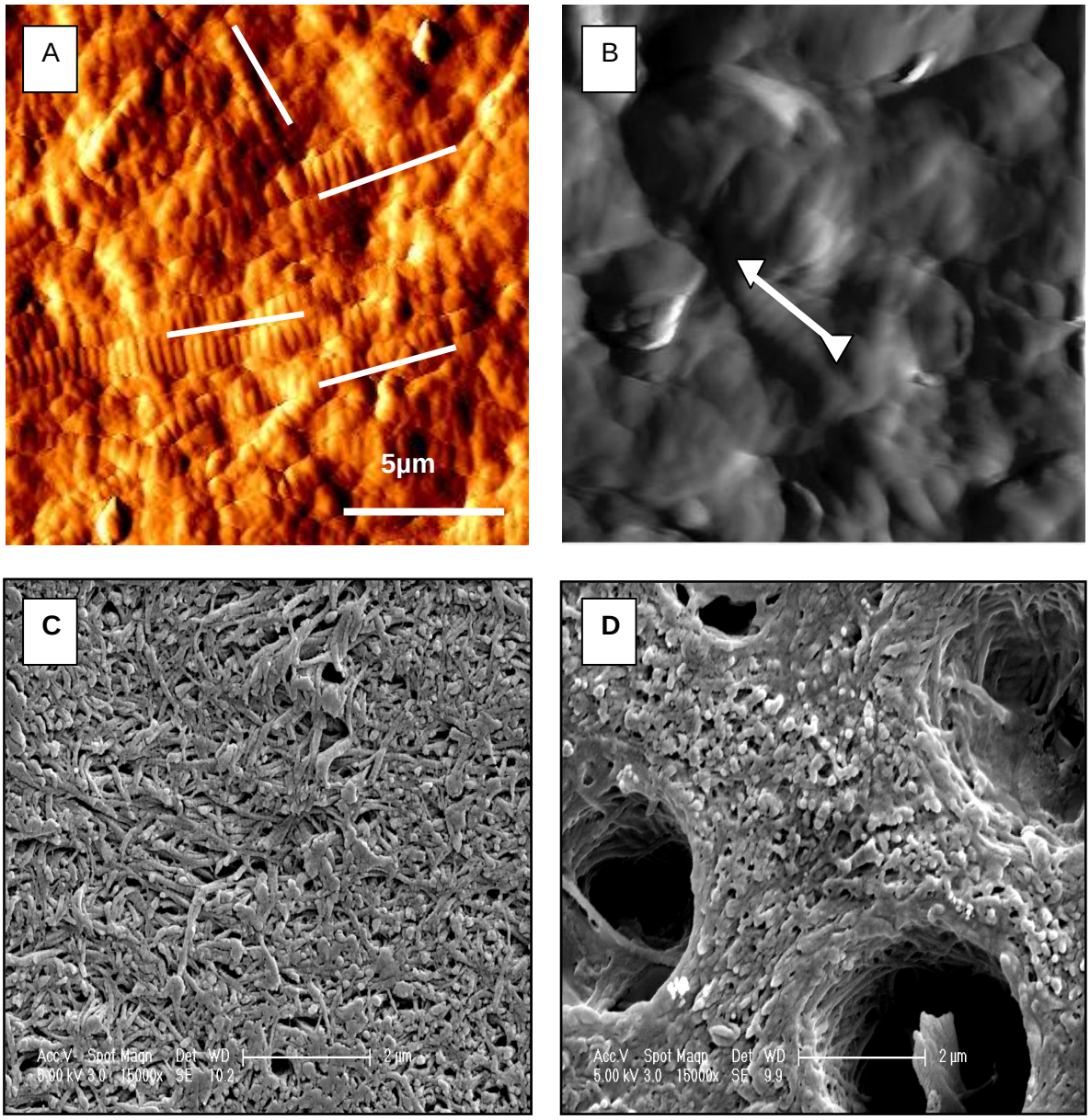


Figure 3. 17: A) Partially demineralised with phosphoric acid and NaOCl, B) partially demineralised with citric acid and NaOCl C) EDTA demineralisation for 12 month D) EDTA demineralisation for 10 minutes. The arrows in (A) and (B) showing the collagen fibrils

CHAPTER 4

EVALUATION OF DEMINERALISATION PROTOCOLS FOR PRIMARY TEETH

4.0 Evaluation of demineralisation protocols for primary teeth

4.1 Introduction

Chapter 3 detailed the application of demineralisation protocols which enabled the imaging of fibrous collagen network of control permanent teeth by using AFM. Demineralisation was carried out in order to remove the smear plugs and smear layers and at the same time to demineralise the dentine surface. This will lead to the enlargement of dentinal tubules and produce zones consisting of organic minerals that rich with collagen network. Gradual removals of non collagenous matrix proteins in the collagen rich zone have been proven to expose the organisation of collagen framework as suggested in the previous literature.

There were established substantial microstructural differences between permanent and primary teeth as discussed in Section 1.3.3. Primary dentine has been described to be slightly less mineralised than the permanent human dentine (Hosoya et al., 2000, Angker et al., 2003). The concentration of calcium and phosphorus in the peritubular dentine and intertublular dentine were showed to be reduced in primary teeth (Hirayama et al, 1990). When the primary teeth were exposed to acid etching, the smear layers were removed more quickly and the teeth demineralised more easily, causing deeper demineralisation in the dentine (Koutsi et al., 1994, Nor et al., 1996, Osorio et al., 2010, Shashikiran et al., 2002). With the variation in micro morphology and chemical reaction of primary teeth, the protocols for imaging the collagen network on the dentine surface of primary teeth may differ from the permanent teeth. For this reason, this chapter set out to assess the demineralisation protocols to image the collagen network in primary teeth. It will aim to repeat the basic protocols in chapter 3 and latter should provide a comparison imaging of fibrous network of partially demineralised dentinal collagen by AFM between primary and permanent teeth.

4.2 Materials and methods

Six control primary teeth were involved in this study. The material and methods for each experimental group in control primary teeth were discussed as below:

4.2.1 Demineralisation using published protocols

Two control primary teeth were embedded in epoxy resin as described in section 2.2.3. The same replicated protocol in control permanent teeth in section 3.2.1 was repeated (Habelitz et al., 2002).

4.2.2 Demineralisation of experimental group using commercial etchant

In this demineralisation group, several steps were involved. Two control primary teeth were embedded in epoxy resin and the same steps in section 3.2.2 were followed. The sequences of steps that involved in this group were shown in figure 4.1.

4.2.3 Demineralisation using 17% EDTA for 12 months

One control primary tooth was stored in 17% EDTA followed the protocol in section 2.3.5 (a), after which the tooth were prepared for histopathology section as described in section 2.2.4. Images were recorded by using light microscope and SEM.

4.2.4 Demineralisation using 17% EDTA for 10 minutes

One control primary tooth was prepared as described in section 2.2.3 and demineralisation protocol explained in section 3.2.4 was repeated.

The sequence for collection, preparation and imaging of four experimental groups for control primary teeth is shown in the following figure 4.1.

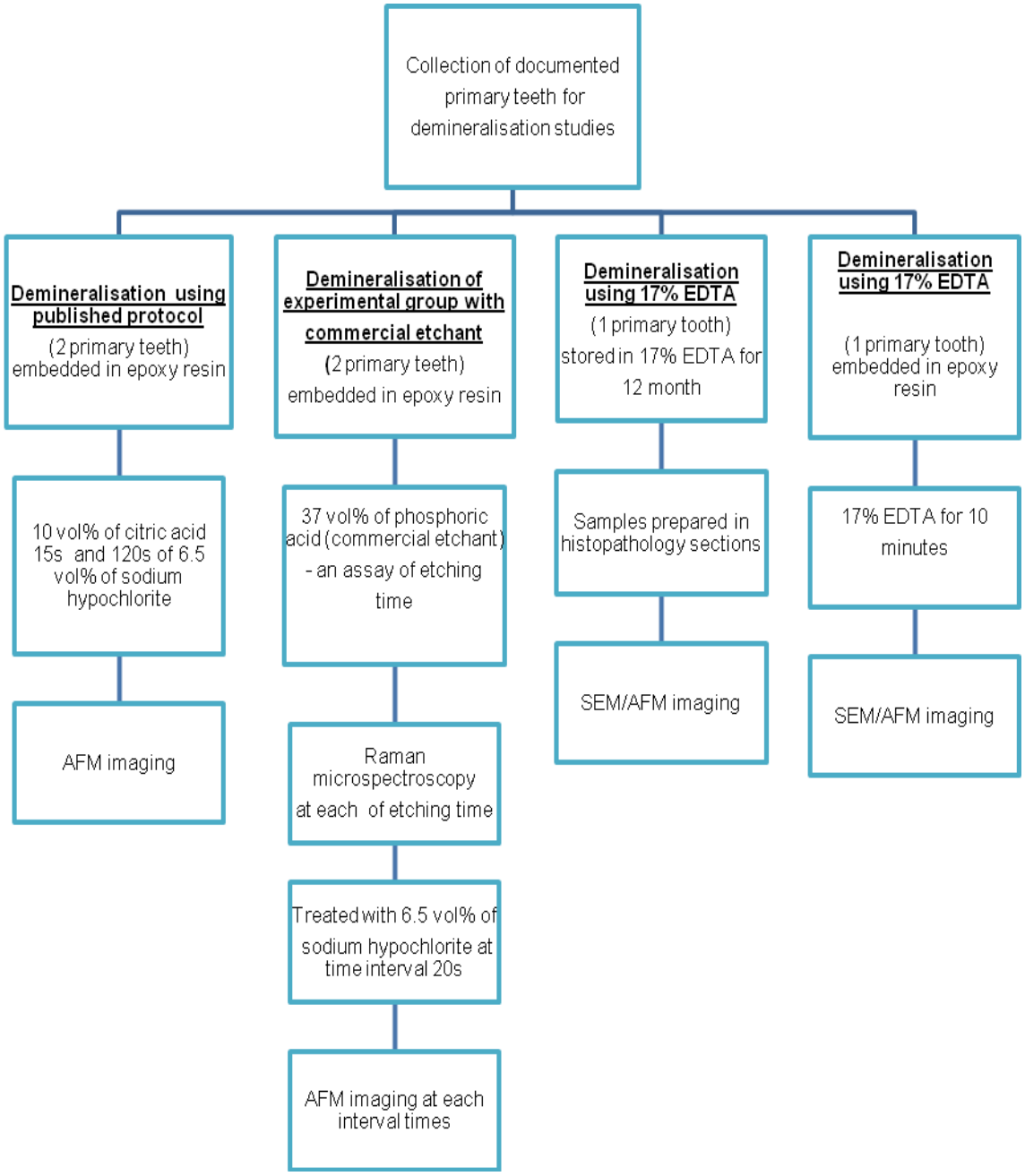


Figure 4.1: Experimental groups for control primary teeth

4. 3. Results

4.3.1 Demineralisation using published protocols

The results of replicated protocols from the published study as mentioned in 4.2.1 were demonstrated in Figure 4.2.

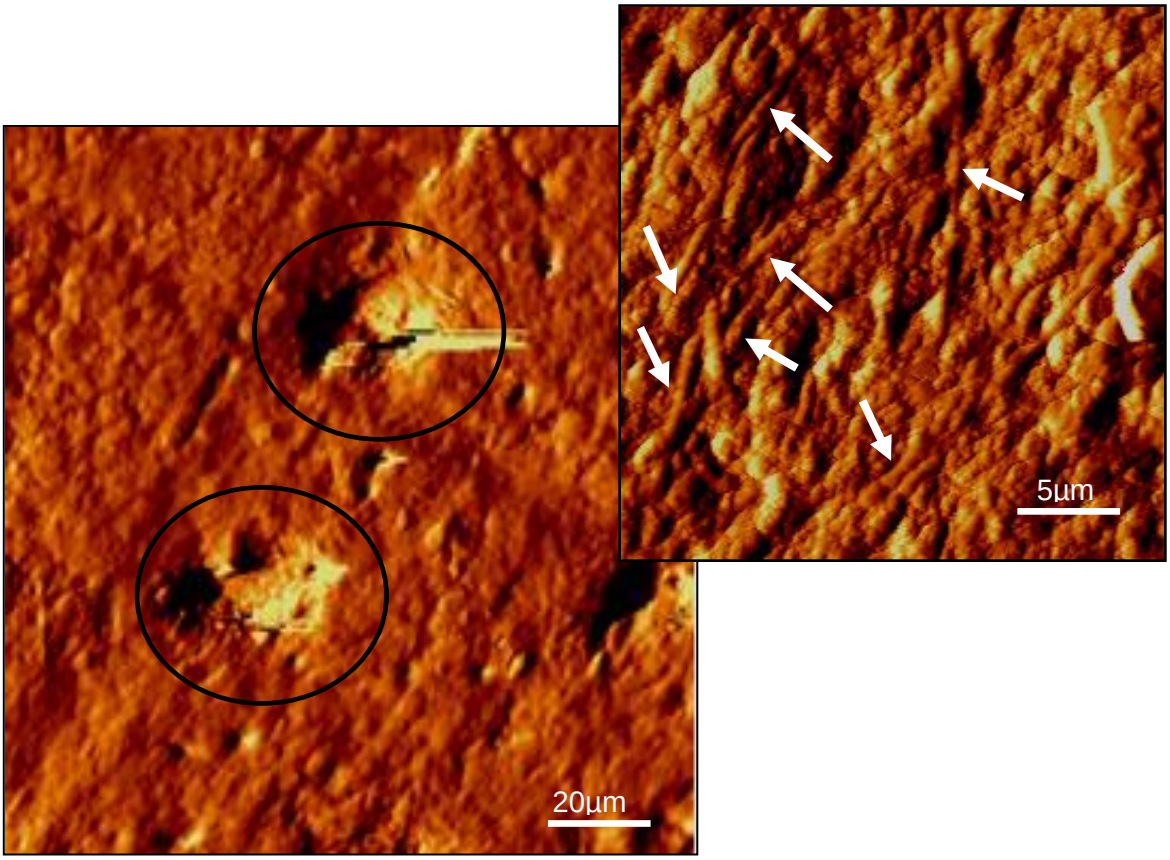


Figure 4.2: Contact mode AFM images showing the occlusal section of primary teeth after demineralisation by acid etching with citric acid and deproteinisation of 6.5 vol% NaOCl. Right: showing partially demineralised primary dentine that treated with 10vol% of citric acid at 15s followed by 6.5vol% of sodium hypochlorite at 80s. Left: revealing the collagen fibrils as shown by the arrows at lower scan size.

Figure 4.2 shows AFM images that revealed a demineralised collagen fibrils network structure after treated with 10vol% of citric acid for 15s, and subsequently treated with 6.5 vol% of sodium hypochlorite at time interval of 80s. At 20µm, the diameter of dentinal tubules were increased due to recession of peritubular dentine (indicated in circle) .At 5 µm, the collagen fibrils network can be revealed (indicated in arrows). However at this point, the D-banding distances cannot be determined due to difficulty in obtaining a defined image at lower scan size.

4.3.2 Demineralisation of experimental group using commercial etchant

Two of control primary teeth were embedded in epoxy resin as described in section 2.2.3. When preparing the dentine blocks, clean surface cannot be obtained as shown in Figure 4.3. These results were similar to the control permanent teeth in previous chapter that have been discussed (Figure 3.3)

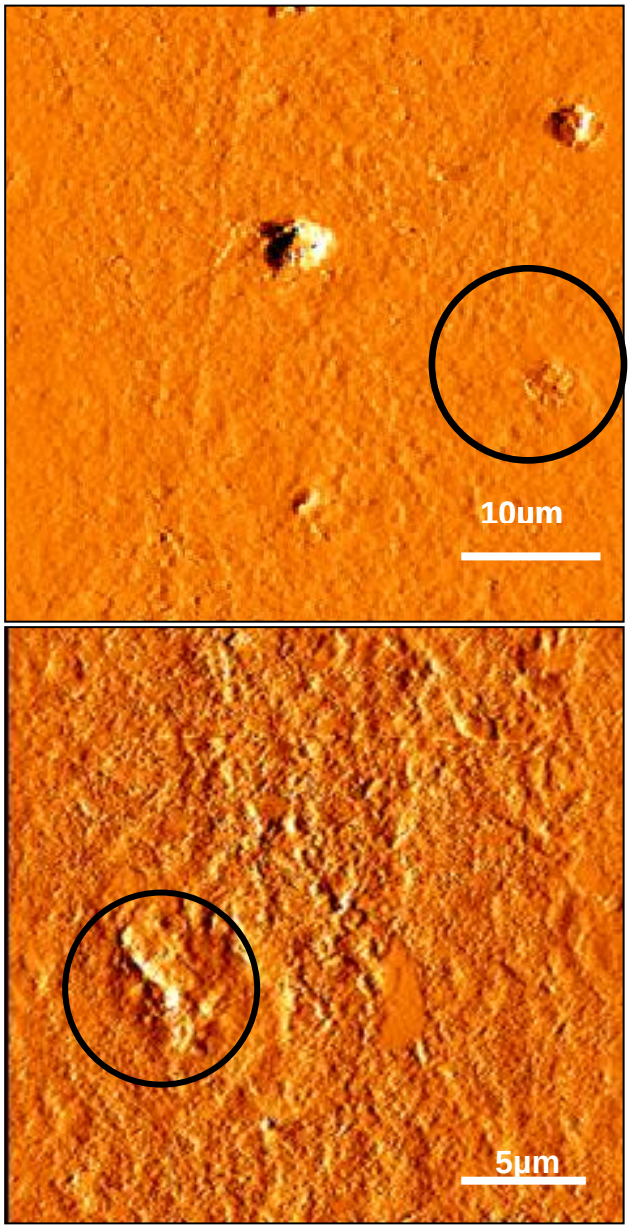


Figure 4.3: Contact mode AFM images of primary teeth after sample preparation. Obstructed dentinal tubules openings (indicated in circle) that may due smear plugs

To relate these topological findings of polished samples to the chemical composition of the dentine, baseline Raman spectra of the control primary dentine was recorded as shown in figure 4.4.

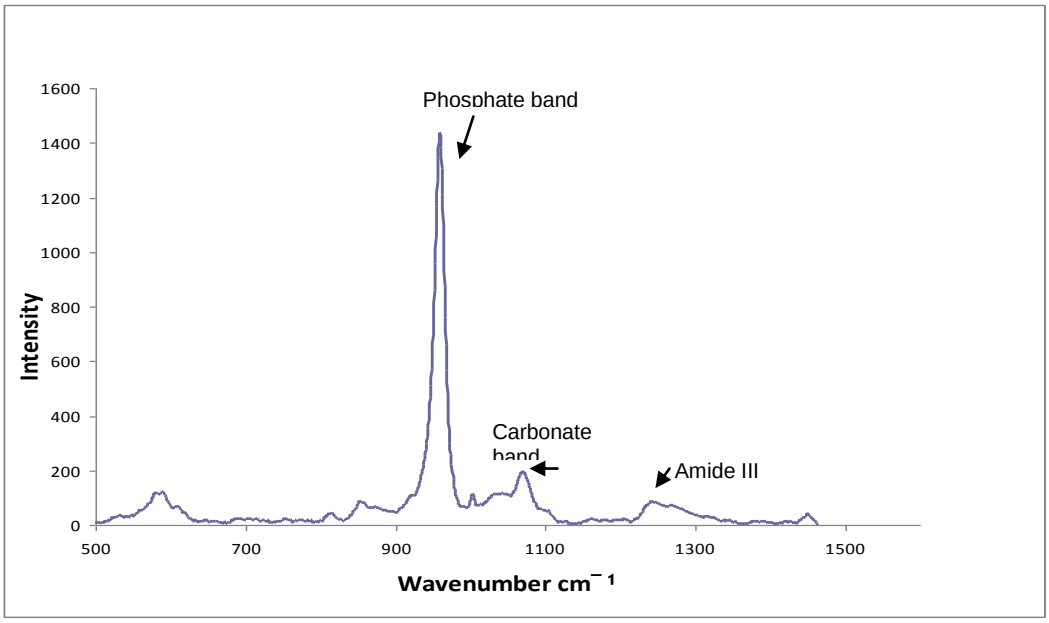


Figure 4.4: Raman spectra of control primary teeth with phosphate, carbonate and amide III peak

In this study, spectra from the control primary dentine were analysed using phosphate stretching band at ~960 cm^{-1}. The data was normalised in order to make sure that the intensity of the spectra will be on the similar scale. The spectra were normalized against the carbonate peak (CO_2^{3}-) at 1072 cm^{-1} which is considered a stable hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) spectrum. The x-axis has been rescaled to show only wavenumber from 500 cm^{-1} to 1500 cm^{-1} and two main characteristic peaks; phosphate (PO_4^{3-}) at 960 cm^{-1} and amide III at 1240 cm^{-1} were identified.

In this study, the prepared samples were treated with commercial etchant as described in section 2.3.3. The effects of different etching time were obtained using Raman spectroscopy.

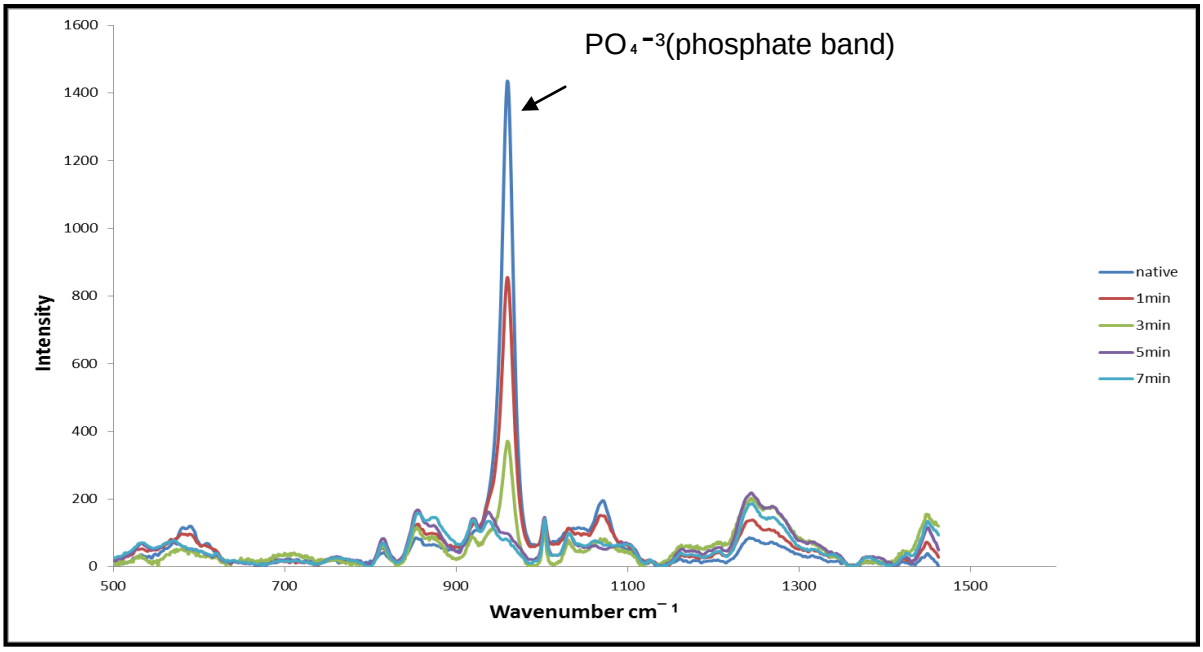


Figure 4.5: Representative raman spectra obtained at different times of demineralisation with commercial etchant (37% acid phosphoric) on control primary teeth

Raman spectra were obtained from control primary teeth at different times of demineralisation using commercial etchant (37% phosphoric acid). A strong defined phosphate band was identified. The intensity of the peak decreased with the increased of exposure time to commercial etchant. After 3 minutes of acid exposure, the peak intensity reduced to nearly 70% and at 7 minutes, the peak of ~960 cm^{-1} was not distinguishable from the background. This suggests that at this point, the inorganic material in the control primary teeth were almost completely removed.

Ratio of Phosphate band / Amide III band

The ratio between phosphate band and amide III was obtained to observe the changes of minerals in the dentine. As described earlier, amide III band can be observed at 1240cm^{-1}, which relates the collagen fingerprint. This ratio can provide significant changes of minerals in relation to time.

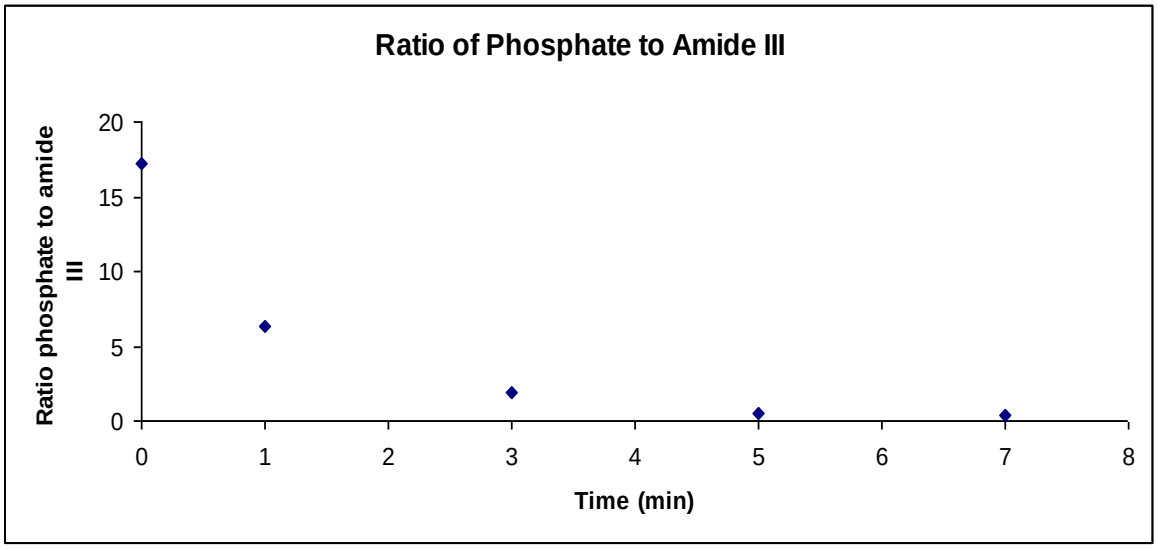


Figure 4.6: Graph showing the ratio data of phosphate peak and amide III

Figure 4.6 shows the ratio of phosphate band intensity to amide III intensity. From the graph, it shows that the intensity of the phosphate band decreased with time. This was in agreement with the results achieved for the control permanent teeth in Figure 3.6. It was hypothesised that the significant time to observe mineral changes would be at 2-3 minutes. After 3 minutes, the intensity of the phosphate band to amide III started to show a massive decline, which was nearly 90% decreased from the initial intensity value.

Images for the demineralisation protocol using commercial etchant were observed under AFM. The images were observed at 2 minutes which was hypothesised to produce significant changes based on the previous result.

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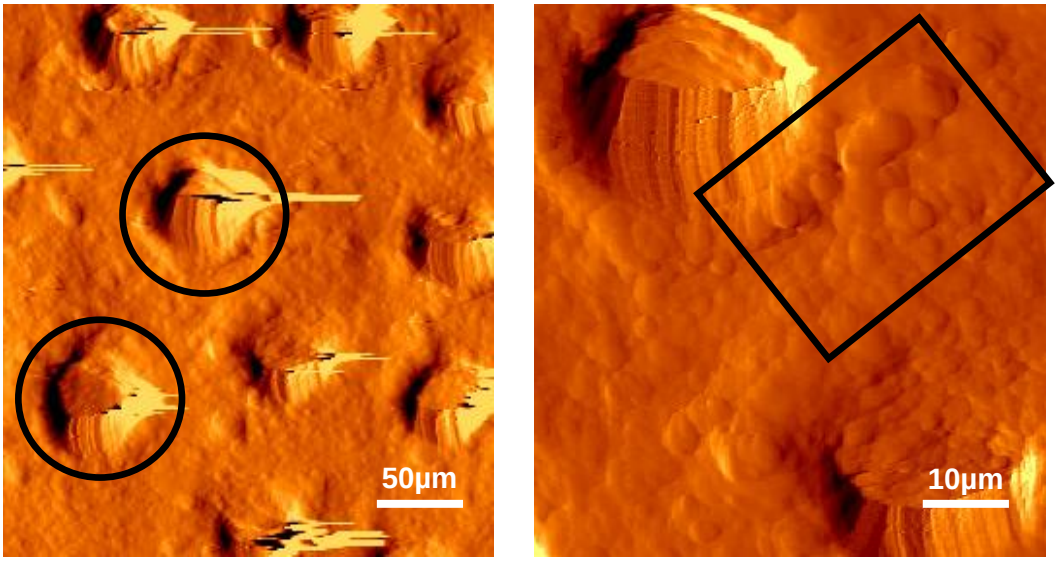


Figure 4.7: Contact mode AFM images of demineralisation using commercial etchant (37% phosphoric acid) of control primary teeth at 2 minutes. Images were recorded and shows sign of peritubular erosion (indicated by circle) and noted the presence of granulated particles on the dentine surface (indicated in box).

Polished control primary tooth shows the microstructure of dentine surface after acid treatment with commercial etchant for 2 minutes. The images show the dentinal tubules diameter increased with sign of peritubular recession (indicated in circle) and granulated particles can be observed on the dentine surface. It was hypothesised that the changes were due to the reaction of the acid towards dentine surface. Yet, at this point no collagen fibrils can be observed.

Further experimental protocol with 6.5 vol% NaOCl

The following step in this group was continued with deproteinisation protocol as described in 2.3.4. The effect of 6.5vol% NaOCl has been proved to enable imaging of fibrous network of partially demineralised dentine by AFM (Habelitz et al., 2002). This was in agreement with the results discussed in chapter 3. The results of deproteinisation on control primary teeth were shown in Figure 4.8.

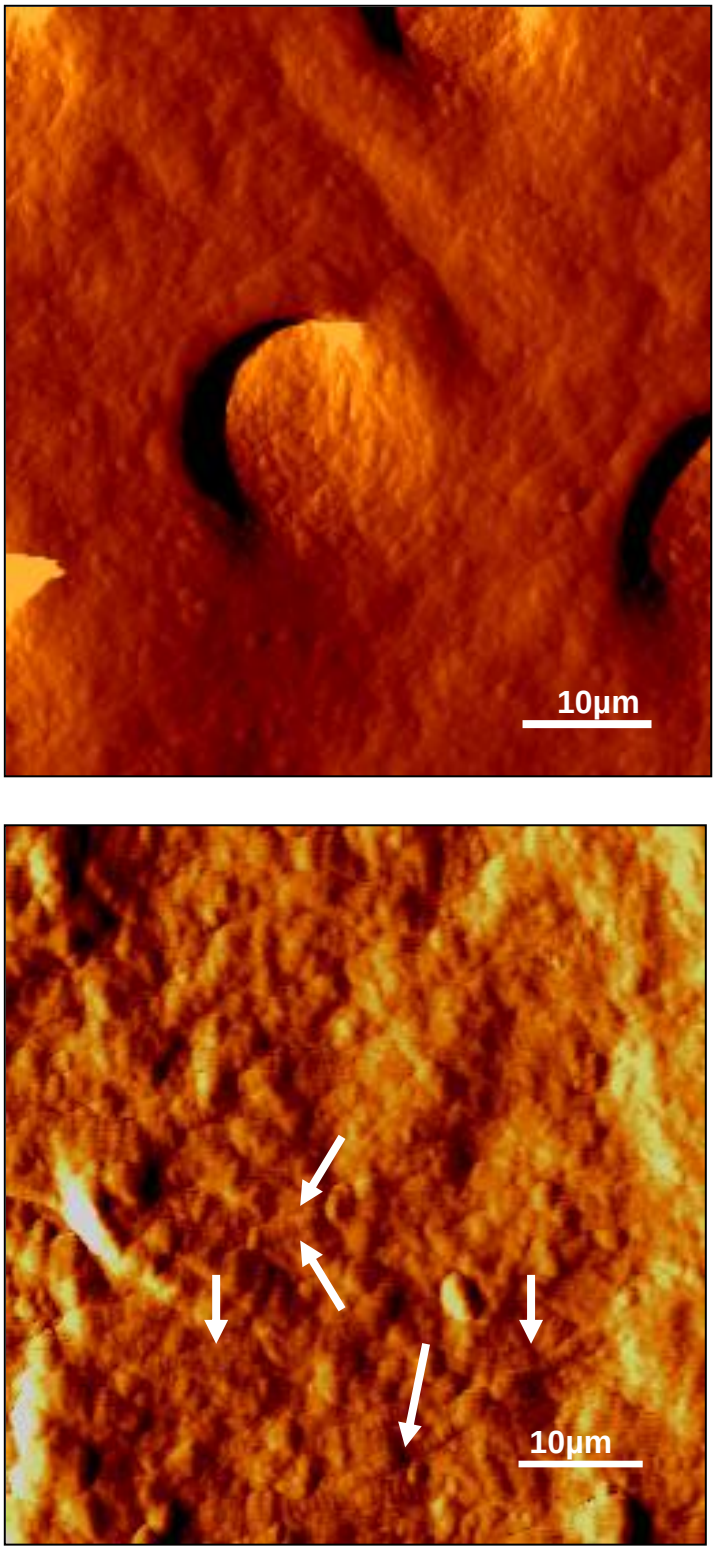


Figure 4.8: AFM images of demineralised dentine surface using commercial etchant at 2 minutes with deproteinisation treatment in 6.5 vol% NaOCl. Topological surface at 20 seconds (above picture) and at 80 seconds (below picture)

Partially demineralised control primary teeth were treated with 6.5 vol% sodium hypochlorite at time intervals of 20 seconds. Figure 4.8 shows generalized particles surrounding the dentinal tubules (above image) at 20s and collagen fibrils network appeared visible at 80s. All images were obtained under contact mode.

In the next step, partially demineralised control primary teeth treated using 6.5 vol% NaOCI for 80 seconds were observed at lower scan size.

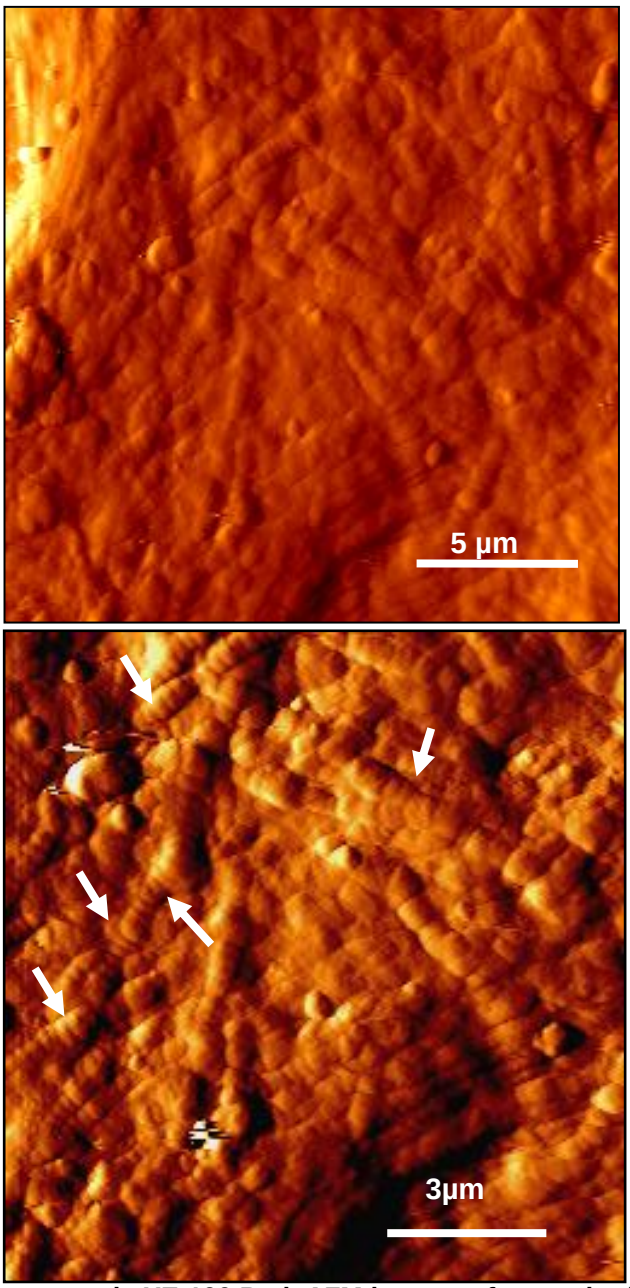


Figure 4.9: Contact mode XE-100 Park AFM images of control primary teeth after partial demineralisation by 37%phosphoric acid for 2 minutes and subsequently treated with 6.5 vol% NaOCI for 80 seconds. Collagen fibrils can be observed showing the repeat pattern and random distribution of fibrils in the intertubular dentine (arrows).

At lower scan size, interlocking pattern of dentinal collagen can be observed. Collagen fibrils show repeat pattern and random distribution at intertubular dentine. The arrangement of dentinal collagen fibrils were in accordance with previous studies (Kramer, 1951, Habelitz et al., 2002).The image contrast of the collagen fibrils in this group was greater than the previous group (treated using 10vol% citric acid). Therefore, the images were suitable for measuring D-banding distances of fibrils. Further details on D-banding measurement in control primary teeth will be discussed in the following chapter.

4.3.3 Demineralisation using 17% EDTA for 12 months

In this group, the effect of demineralisation using 17% EDTA for 12 months on one control primary tooth was observed. Protocol in section 4.2.3 was followed.

Light microscopy for control primary histopathology section

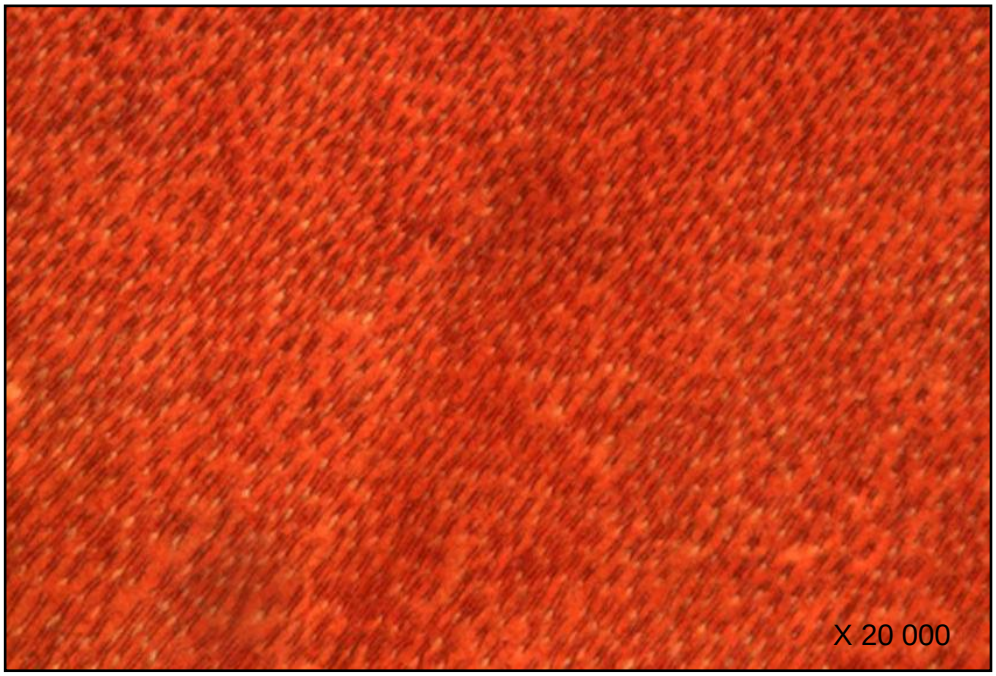
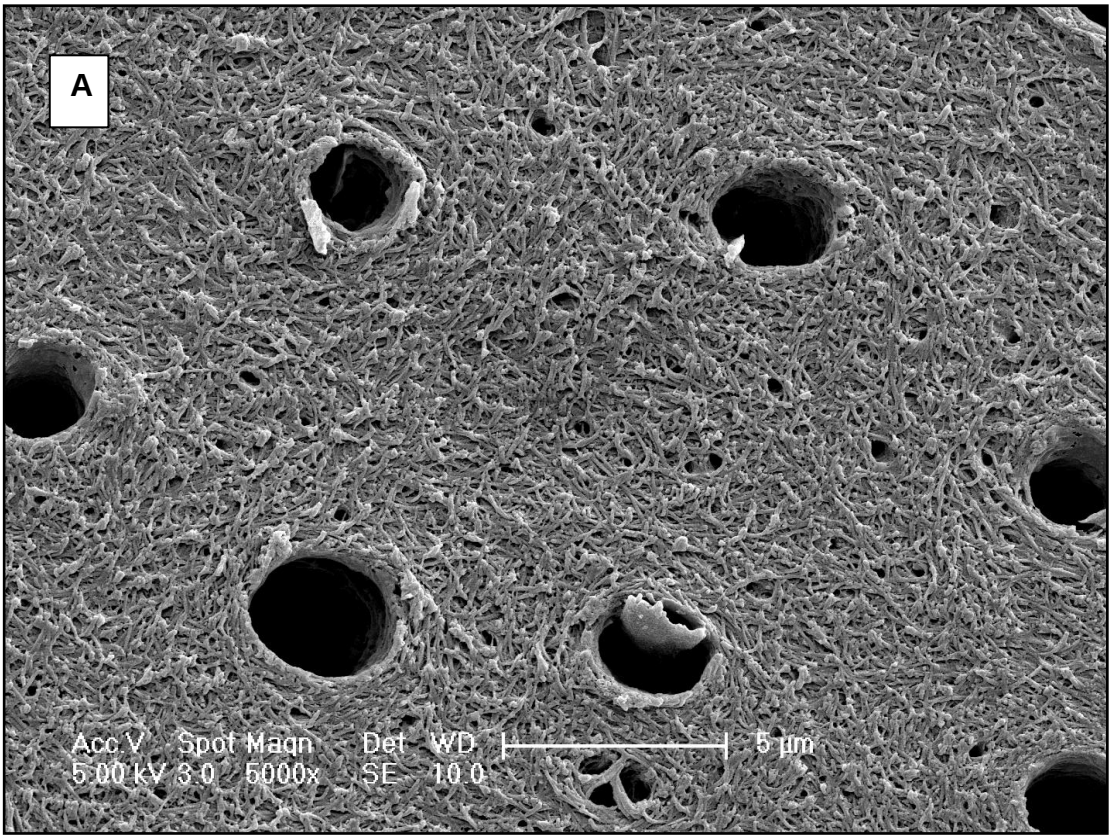


Figure 4.10: Histology section of control primary tooth under light microscopy

Representative histopathology section of primary teeth is displayed in above figure after stained with Picrosirius red (PSR). The PSR stain was viewed under light microscope resulting in orange fibers immediately beneath dentinal tubules (red colour) and appeared in straight aligned pattern.

Imaging control histopathology section using SEM

In order to obtain images from SEM, histopathology section was prepared using protocol in section 2.4.2. Images were recorded in two scan sizes; 5µm at 5000x magnification and 2µm at 15000x magnification as shown in Figure 4.11.



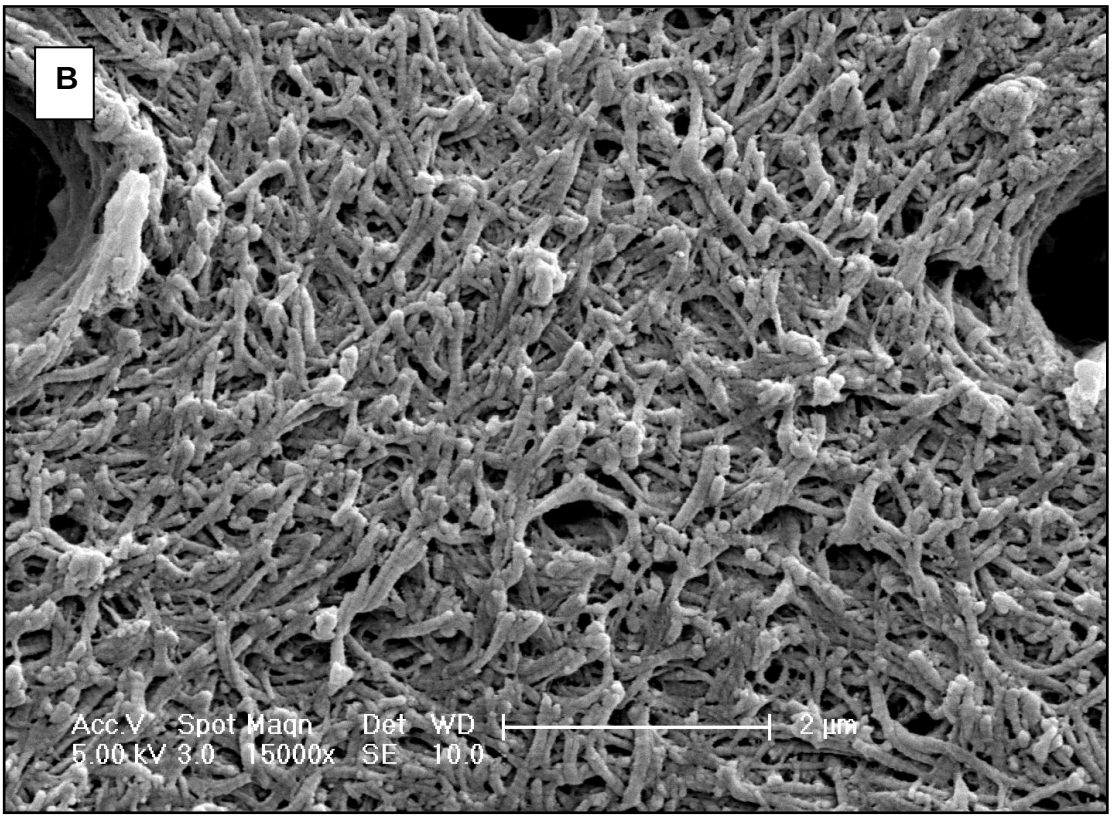


Figure 4.11: Experimental procedure of control primary teeth stored in 17% EDTA for 12 month (A) Enlargement of dentinel tubule openings with sign of erosion of peritubular and intertubular dentine. (B*)* Higher magnification of this specimen showing localised collagen scaffold that surrounded by irregular variation structure which scattered almost at intertubular dentine

SEM observation on control primary tooth after 12 months of storage in 17% EDTA revealed presence of collagen fibril in intertubular dentine. In this specimen, there were also sign of slight erosive effects at peritubular dentine which lead to increased diameter of dentinal tubules. The quality image of fibrillar network improved at higher magnification. However, the morphology of some collagen fibrils appeared to show sign of irregular pattern or disarrangement.

4.3.4 Demineralisation using 17% EDTA for 10 minutes

In this group, one of the control primary tooth was prepared in epoxy resin as described in 2.2.3. Then, the prepared sample was treated with 17% EDTA for 10 minutes and imagings were obtained using SEM.

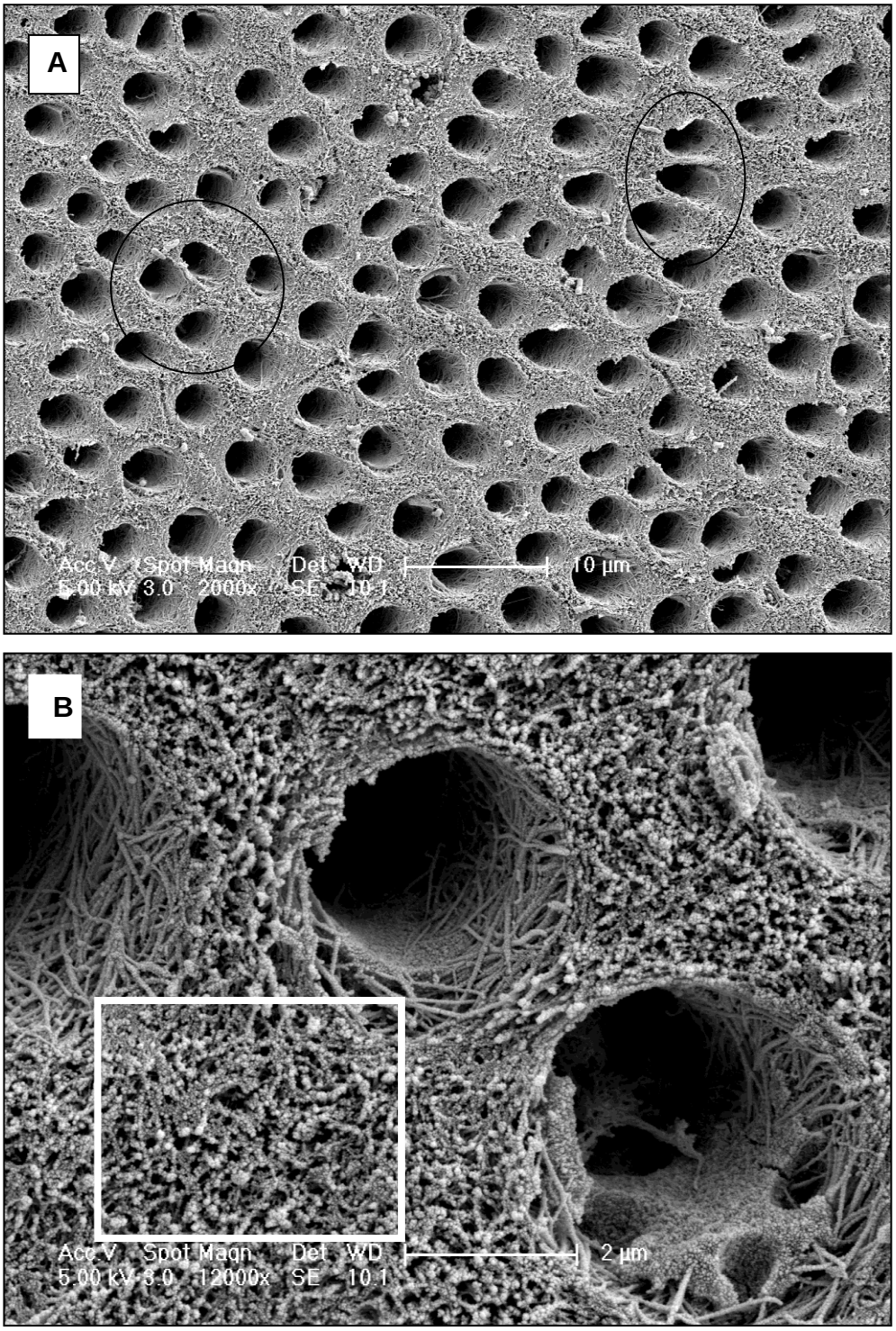


Figure 4.12: Effect of 17% EDTA for 10 minutes on the control primary tooth. Excessive erosion of intertubular and peritubular dentine was seen which lead to widening of

tubular diameters. The demineralisation effects in some extent cause the intertubular dentine to completely disappear (indicated in circle).At 2µm, the collagen network only can be seen at peritubular area

At 10µm, the dentinal tubules opening of control primary tooth demonstrate increased in diameters which were in agreement with the previous study (Calt and Serper, 2002). Although smear layer was removed and the dentine contents were dissolved by the action of EDTA, the collagen networks were still cannot be revealed. It was hypothesised to be due to calcium ion binding to EDTA (indicated by box).

4.4 Discussion

This study represents the different demineralisation protocols to investigate the feasibility to image the dentinal collagen fibrils in control primary teeth. In the clinical indication of dentine adhesion restorations, the same protocols of acid etching have been used for primary and permanent teeth. However, there were evidence suggesting significant chemical and morphology differences between the permanent and primary teeth which have been discussed in previous literature. Prior to this, the objective were to simply test different demineralisation protocols that were used in chapter 3, to evaluate whether specific demineralisation protocol required for the control primary teeth and to determine the most effective methods to image the dentinal collagen in primary teeth.

4.4.1 Demineralisation using published protocols

In regards to the citric acid reaction, the results of this study as shown in section 4.3.1 was similar to the phosphoric acid that suggests further chemical treatments of dentine substrate were required to reveal collagen fibrils.

4.4.2 Demineralisation of experimental group using commercial etchant

With less mineralised dentine in primary teeth, the chemical reaction seemed to be more intense causing deeper demineralisation of intertubular and peritubular dentine (Angker et al., 2003). Based on this fact, it was hypothesised that etching time for control primary teeth in this experimental study would be shorter compared to the control permanent teeth. This was in agreement with the results in section 4.5, that showing reduction of nearly 70% of phosphate minerals in primary teeth after been treated for 3 minutes with commercial etchant (37% phosphoric acid). Due to high reactivity to etchant, optimum periods of etching time was determined based on the ratio of phosphate band to amide III. This can allow significant changes of mineral in control primary dentine without causing denaturation or severe collapse in collagen fibrils framework. The results showed that the control primary teeth were more susceptible to acid etching. However, at suggested time of 2 minutes, increased diameters of dentinal tubules due to recession of peritubular dentine under AFM can be observed. At this point, acid etching dissolves the mineral content and leaves the partially demineralised layers which consist predominantly of collagen and other non-collagenous matrix. It was suggested that the composite natures of collagen fibrils that have been covered by the non-collagenous proteins may hindered the topological imaging of the fibrils as have been described in previous work by Marshall (Marshall et al., 2001).

On the other hand, the significant removal of mineral phase in the primary dentine at a shorter time can contribute to the clinical indication for dentine bonding protocols in dental restoration. The results stated that there was a different reaction between primary and permanent teeth during the application of etching as shown in Raman spectra in section 3.3.2 and 4.3.2. This was in concurrence to the published study that suggested primary teeth required shorter etching time than permanent teeth (Nor et al., 1996). From clinical point of view, this protocol can benefit the performance of

the restoration especially in paediatric patient. Moreover, it have been established that longer etching times in primary teeth results in formation of thicker hybrid layer that leads to unstable demineralised zone and consequently increased the susceptibility of failures (Nor et al., 1996, Sardella et al., 2005, Gateva and Rossitza, 2012).

Further experimental protocol with 6.5 vol% NaOCl

Due to different reaction of acid etching in primary teeth, it can be postulated that it was likely that the application of sodium hypochlorite solution to control primary dentine would produce different alteration when compared to control permanent dentine. In this chapter, the significant effects on micromorphology aspect of primary dentine after the application of sodium hypochlorite solution were identified. It could be noticed that the collagen fibrils became visible just after the control primary dentine was treated with 6.5vol% sodium hypochlorite for 20s. When the application of sodium hypochlorite was gradually increased to 80s, the collagen fibrils became more obvious. The deprotenisation time was limited to 80s as some studies demonstrated total removal of collagen on primary dentine after the application of 5 vol% sodium hypochlorite was at 120s (Perdigao et al., 1999, Correr et al., 2006). Improvement in the quality of images of the collagen fibrils in this method exhibit the characteristic structure of collagen fibrils and is suitable for measuring the dimension of collagen fibrils features. The latter chapter will discuss in detail about the topological findings and D-banding features of control primary teeth.

4.4.3 Demineralisation using 17% EDTA for 12 months

Based on the literature on permanent teeth and on the results achieved in chapter 3, the highly mineralised peritubular dentine on control permanent teeth was dissolved by the action of EDTA and resulting in increased diameters of dentinal tubules. These can be showed by the appearance of excessive dentinal tubular erosive

effects on the dentine surfaces (Calt and Serper, 2002). Despite the lacked of published data on primary teeth, the micro morphology differences between primary and permanent teeth should logically reflected the reaction towards EDTA. It might be reasonable to assume that once the smear layer was removed, the dentinal tubule openings could created more easily compared to permanent teeth (Kennedy et al., 1986). The findings observed in control primary tooth which was stored in 17% EDTA for 12 months was quite interesting as the dentinal tubules showed less erosive effects and the collagen networks were still intact. It was hypothesised that with less mineral composition in the primary teeth, the collagen may be denaturated and resulting in a degradation of collagen fibrils and disarrangement of the collagen network. In contrast, the collagen networks in this study were preserved. Further works need to be carried out with more samples size in order to discover the reproducibility of the findings.

4.4.4 Demineralisation using 17% EDTA for 10 minutes

Observations in control primary teeth during demineralisation protocol using 17%EDTA for 10 minutes were in accordance to the previous study (Calt and Serper, 2002). The results showed erosive effects of the dentinal tubules. In this group, the collagen network can only be seen at peritubular dentine. Lower collagen content makes peritubular dentine more quickly to dissolve than intertubular dentine. These results were in agreement with the findings observed in control permanent teeth. It was suggested that the dentine surface did not demineralised completely up to the extent where the collagen scaffold can be revealed and the presence of precipitation over the dentine surface may hinder the collagen network.

4.4.5 Potential issues

In this chapter, some potential issues that relevant to the findings will be discussed.

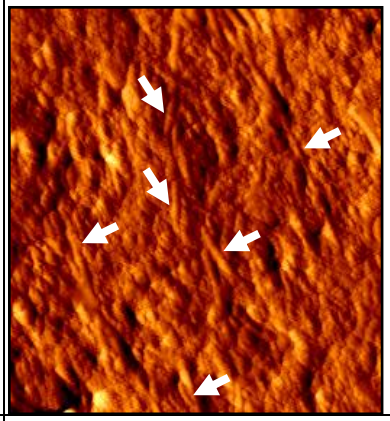
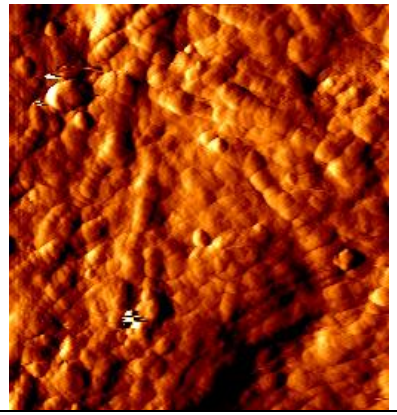
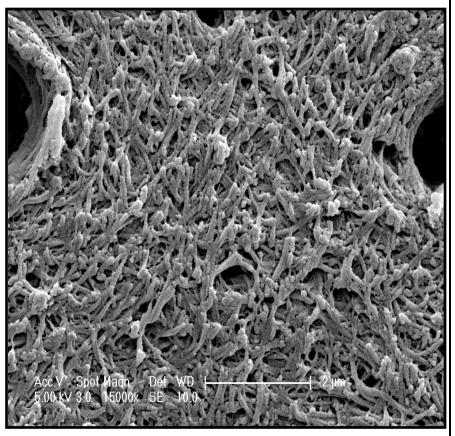
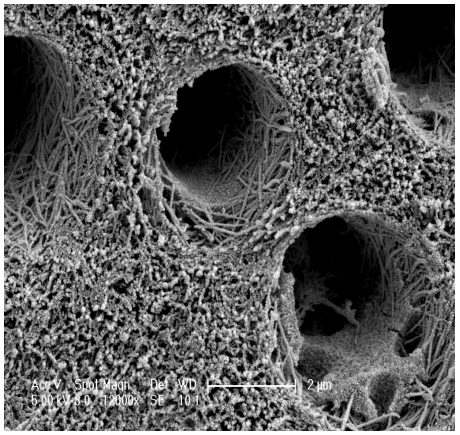
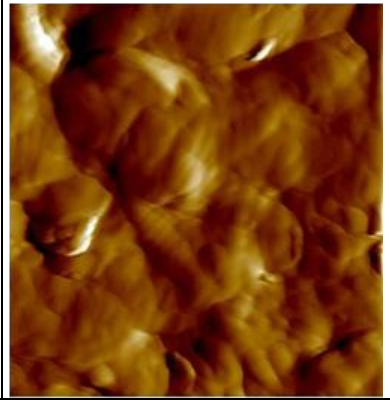
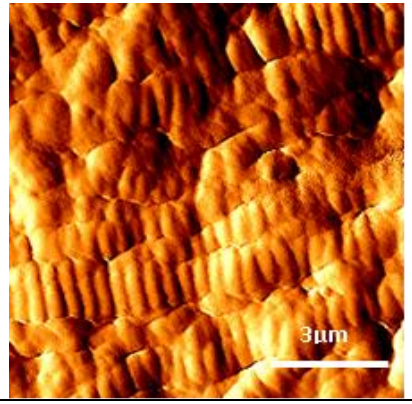
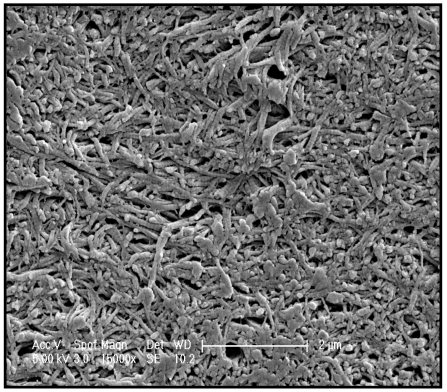
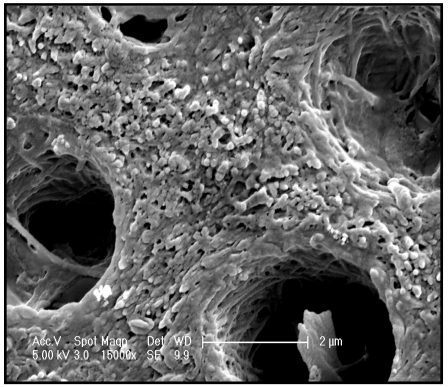
1) **Etching time**

Different etching times using the same acid etching concentration result in different morphological changes in demineralised dentin surface. In 1993, Marshall reported that prolonged etching time causes morphological changes to dentin surface. Such changes have, in turn, a detrimental effect to the properties of the dentine (Marshall et al., 1993). The effects will results in excessive demineralisation of peritubular and intertubular dentine which may leads to collapse of dentinal collagen (Nor et al., 1996). In this study the etching time was determined according to the protocols provided in previous studies and new protocols that were generated based on experimental study design as explained before.

2) **pK_a of the acid**

pK_a is the ability of the acid to dissociate the hydrogen ion [H^+]. The higher the pK_a value, the greater the potential proton will be available and making the solution more effective. The strong acid will have negative pK_a values whilst the weak acid will have a larger positive value. With the ability of the acid to generate the hydrogen ion, the pK_a can reflect the efficacy of certain acids to dissolve the enamel. Optimum pH for dentine demineralisation is important as different pH and concentration will results in different demineralising effect (Cury et al., 1981, Marshall et al., 1997c)

Figure 4.13: Comparing results between primary and permanent teeth

Teeth	10vol% of citric acid and 6.5vol% of NaOCl	37 %phosphoric acid and 6.5vol % of NaOCl	17%EDTA for 12 month	17% EDTA for 10 minutes
Primary	15s for citric acid+ 80s NaOCl 	2 minutes phosphoric acid+80s NaOCl 		
Permanent	15s for citric acid+ 120s NaOCl 	5 minutes phosphoric acid+ 100s NaOCl 		

4.5 Conclusion

On the basis results obtained in this study, the images of collagen were feasible irrespective of the demineralisation protocols used. In summary, the effective protocol to allow in situ imaging of the collagen both in control primary and permanent teeth in this study were the chemical treatment with 37% phosphoric acid (commercial etchant) followed by controlled deproteinisation protocols with 6.5vol% of sodium hypochlorite. A shorter protocol for chemical treatment in primary teeth showed a significant different in chemical reaction between primary and permanent teeth. Comparison results with EDTA demineralisation protocols were restricted as this study unable to expose collagen under AFM, however microscopic observation under SEM suggested that there were differences between primary and permanent teeth when the teeth were exposed at same time and concentration. The hypothesis of EDTA still need to be confirmed in the future works. Despite the limitation of the results, the protocol appeared to be an effective tool to gain understanding of the collagen structure and for further analysis of the mechanical properties of the collagen in the future works.

CHAPTER 5

CHARACTERISATION OF DENTINAL COLLAGEN OF OI AND DI USING AFM

5.0 Characterisation of dentinal collagen of OI and DI using AFM

5.1 Introduction

OI is a clinical and inherited disorder results from the quantitative and/ or qualitative defect of type I collagen (Byers, 2000). OI type I has been shown to cause the mildest abnormalities in bone ultrastructure whilst OI type III has a distinctive structural abnormalities in collagen (Sarathchandra et al., 2000)

The range of severity of dental phenotype in OI is extensive. Although the varieties of clinical, radiographic as well as genetics findings have been described in details in chapter I, the ultrastructure findings of the dentinal collagen structure is still limited. While considerable attention has been focused on characterizing collagen bones in OI, the dentinal collagen of the teeth remain poorly characterised. One available method to study the ultrastructure characteristics of the dentinal collagen is by using the AFM (Marshall et al., 1993, Kinney et al., 1993, Habelitz et al., 2002)As expected in DI type I in which the collagens is defective, AFM imaging of the dentinal collagen in these patients would be expected to show similar changes seen in the bone in the dentine as well.

To further delineate the range of dentinal collagen in DI type I, in this chapter, observation on primary teeth of DI type I using the AFM was carried out. The aim of this study was to investigate the morphological appearance of the dentine in primary teeth of DI type I from normal control teeth using AFM. In addition to understand the abnormalities of these affected teeth, the demineralisation protocols which enable the imaging of the collagen fibrils (citric acid/commercial etchant) in chapter 4 were used as a baseline guideline in this chapter.

5.2 Materials and methods

5.2.1 Tooth preparation

One exfoliated lower primary tooth DI type I (OI type III) was prepared in epoxy resin as been described in section 2.2.2.

5.2.2 Demineralisation protocols –Effect of 10vol% of citric acid for 15s

The prepared sample was etched with 10vol% citric acid for 15 seconds and was washed under steady flow of ultra high quality (UHQ) water for a few seconds. Then the sample was ultrasonicated in UHQ water for 10 seconds. The image of the sample was obtained by using AFM.

5.2.3 Measuring the D-banding

Collagen fibril D-banding distances were measured from the obtained images. The average of the D-banding distance was obtained by taking the profile of the fibrils parallel to their length from various height images. The lengths of these profiles were recorded and divided by the number of bands present. The mean of the D-banding was calculated and recorded using WSxM analysis programme.

5.3 Results

5.3.1 Assessment of dentinal structure of DI type I of primary teeth before demineralisation

Images of the dentine in DI type I tooth were obtained using AFM. Since the tooth had exfoliated naturally, only the crown of the tooth was available for sample preparation as described in 2.2.2. After preparing the sample, the surface obtained was not clean enough to show the collagen fibrils as shown in Figure 5.1

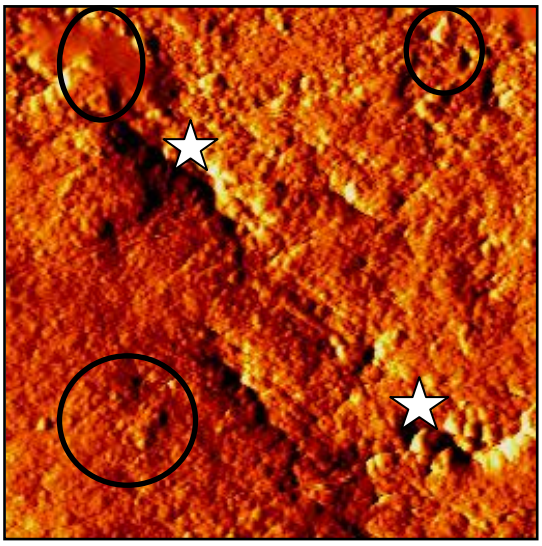


Figure 5.1: AFM images of lower primary tooth of a patient with DI type I. The dentinal tubules (indicated by star) were obstructed with the smear plugs. The dentine surface was covered by smear layer (indicated by circle area).

The image of the dentine surface showed that the surface was covered by a smear layer and smear plugs. At this point, it was not possible to identify the collagen fibril network.

5.3.2 The effect of 10vol% citric acid for 15 seconds on primary DI type I

The purpose of the demineralisation protocol with 10 vol% of citric acid was to remove the smear layer in the dentine surface. The cross section sample of DI type I tooth was treated with 10vol% of citric acid for 15 seconds and the images were obtained in two different area of the dentinal surface as shown in Figure 5.2.

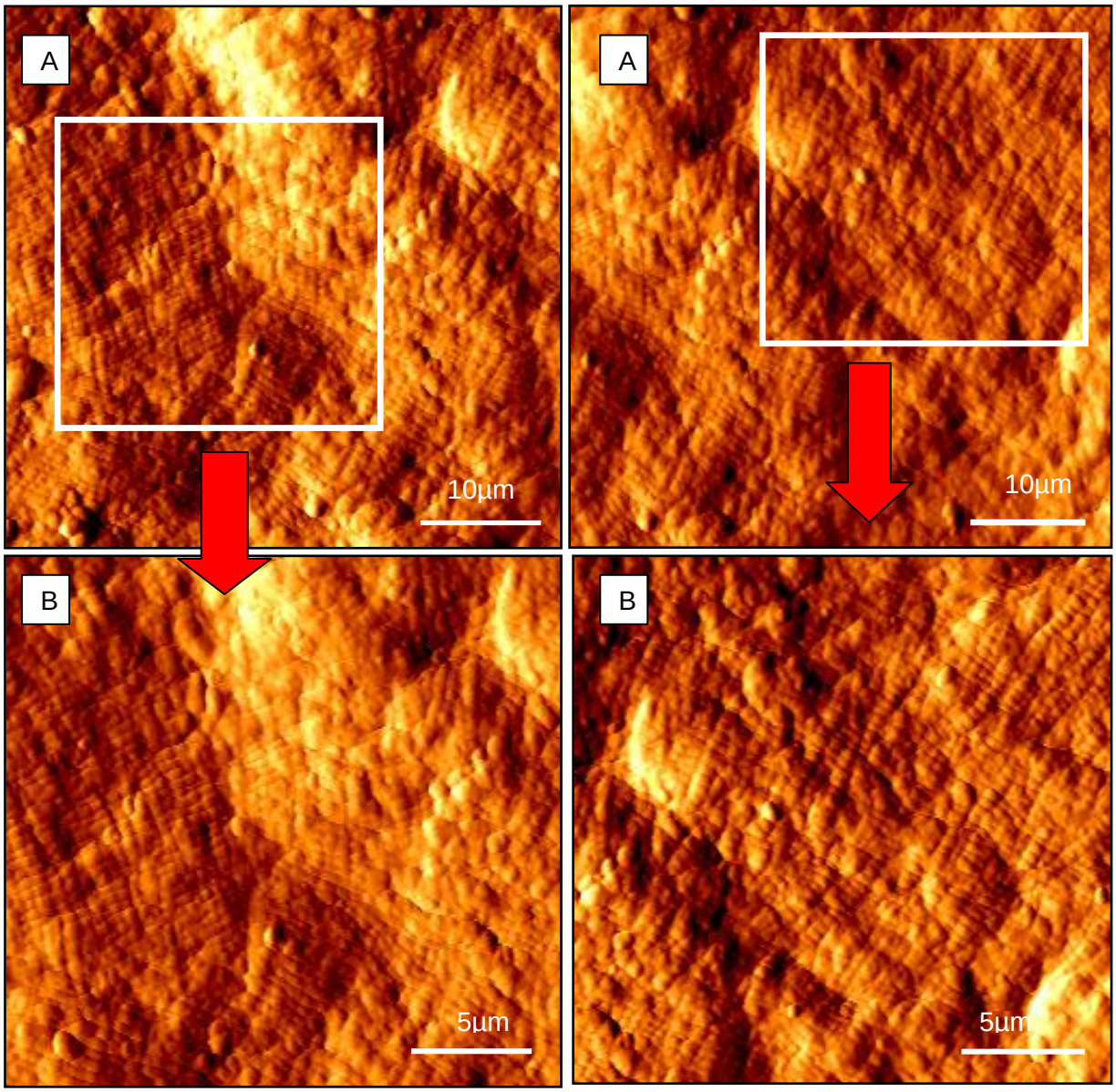


Figure 5.2: DI type I dentine treated with 10vol% citric acid for 15 seconds at two different areas (A) at 10µm, collagen fibrils was densely packed in one orientation (B) The image showing the distribution of the collagen fibrils (indicated in arrows) in the intertubular dentine at lower scan size; 5µm.

For a comparison, a prepared control primary tooth in epoxy resin also was treated with the same protocol. Images were shown in Figure 5.3.

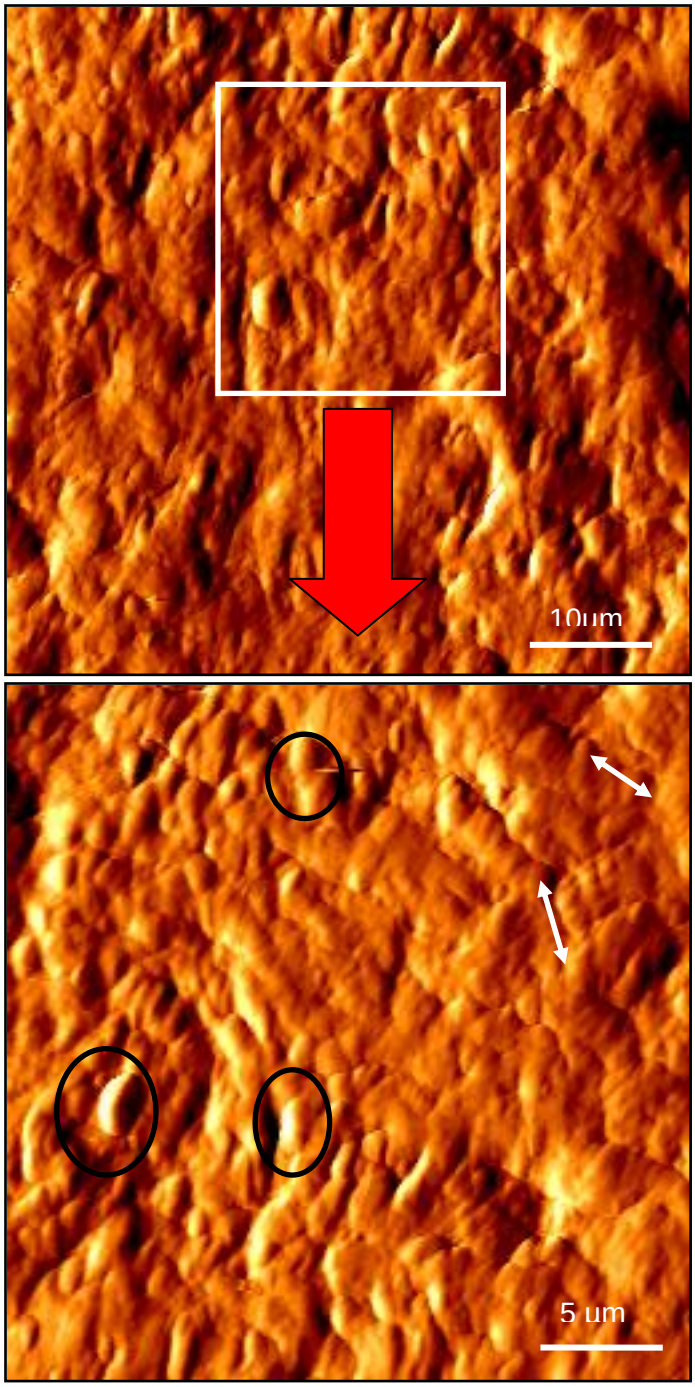


Figure 5.3: Control primary tooth images of intertubular dentine treated with 15s of 10 vol% of citric acid (A) The partially demineralised dentine did not reveal any collagen fibrils (B) At lower scan size, the image showing the smear plugs (indicated in circle). and smear layer covering the dentine and hindered the collagen network. Arrows suggesting the possibility of collagen fibril.

The findings showed that the dentine surfaces can be influenced by the treatment of 10 vol% citric acid for 15 seconds. The test sample in figure 5.2 resulted in thickened dentinal collagen fibrils that were distributed in only one direction. No obvious interlocking areas were noticed. At different areas of the dentinal surface of the same tooth, the same morphology was noted. However, in the control primary tooth, the partially demineralised dentine was covered by hydroxyapatite and non collagenous proteins suggesting that the smear layer and smear plugs had not been fully removed. This increased the difficulty of imaging using AFM.

5.3.3 Measuring the D-banding distance of primary DI and control

D-banding distance of the collagen fibrils were measured from the profile of the fibrils in the images. Analysis was made by determined the step height of individual fibrils as an example shown in Figure 5.4.

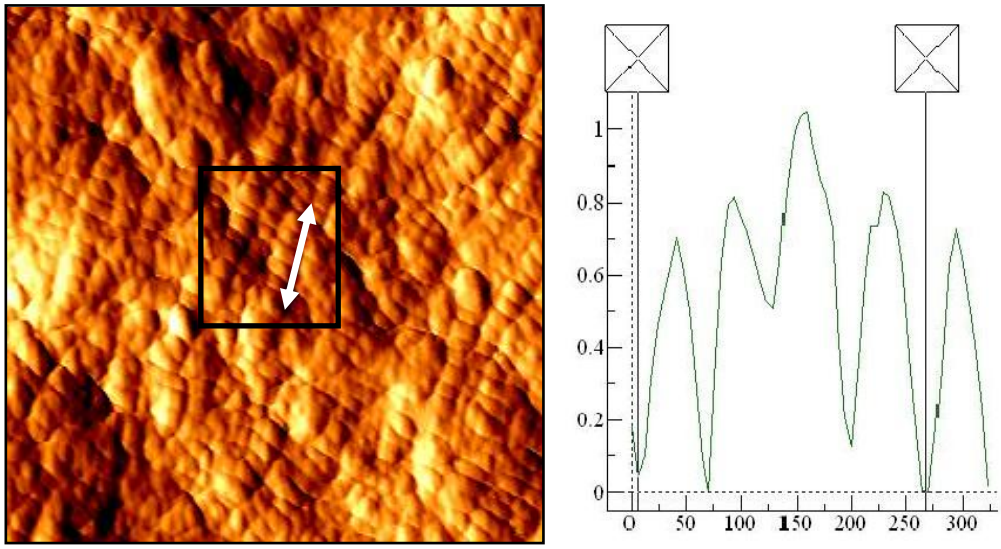


Figure 5.4: Measurement of D-banding distance along the collagen fibril in DI type I by WSxM analysis.

The distribution of the D-banding distances for the DI type I and control primary teeth are shown in Tables 5.1 and 5.2 respectively.

DI type I	Total banding length (nm)	Bands	Banding length (nm)
1	550.4	8	68.8
2	485.5	6	80.9
3	427.6	5	85.5
4	240.2	3	80.1
5	310.0	5	62.0
6	341.0	5	68.2
7	208.2	3	69.4
8	308.2	5	61.6
9	184.1	3	61.4
10	425.2	6	70.9
Mean			64.7
Standard deviation			8.4

Table 5.1: Distribution of D-banding length of DI type I in primary teeth

Table 5.1 shows the measurement of the D-banding distance of collagen fibrils in the DI type I primary tooth. The D-banding measurements were taken from the collagen fibrils at different area in the test sample. The distribution of D-banding distance appeared in the range of 61nm to 85nm with the mean value was hence found to be 64.74nm ± 8.4.

Control primary teeth	Total banding length (nm)	Bands	Banding length (nm)
1	390.2	6	65.0
2	210.7	3	70.2
3	138.4	2	69.2
4	638.9	10	63.9
5	140.4	2	70.2
6	206.1	3	68.7
7	177.9	3	59.3
8	192.4	3	64.1
9	279.6	4	69.9
10	254.0	4	63.5
Mean			66.42
Standard deviation			3.7

Table 5.2: Distribution of D-banding length of control primary teeth

Table 5.2 shows the measurement of the D-banding distance of collagen fibrils in the control primary tooth. The distribution of D-banding distance appeared in the range of 59nm to 70nm with the mean value was hence found to be 66.42nm ± 3.7.

5.4 Discussion

5.4.1 Characterisation of DI type I

A remarkable finding of this chapter is in the characterisation of the morphology of dentinal collagen ultrastructure in DI type I teeth. The aberrations found were primarily abnormal organisation of dentinal collagen and wide distribution of D-banding distance of the collagen fibrils. The results revealed thickened collagen fibrils in a network. There was no obvious interlocking of fibrils, as the organisation of the collagen appeared to be aligned in the same direction. In a previous study using transmission electron microscopy (TEM), some of the affected dentinal collagen areas were found to be densely thick which supported our findings however the collagen were in haphazard orientation (Waltimo et al., 1996).

The morphology of bone collagen fibrils in OI type III appeared to have a different organization, described as flower-like with some fused, composite fibrils. The collagen fibre bundles were also twisted and spiralled but loosely packed throughout a very thick osteoid (Sarathchandra and Pope, 2005).The findings achieved by Majorana using confocal laser scanning microscopy (CLSM) in OI (OI type I, type III and OI type IV) showed that no histological differences were noted in the teeth among three of types of OI. The dentinal collagen in all of the teeth were randomly orientated when away from the odontoblastic process i.e; resembles in dilated structure in the histological sections, and was parallel inside the channel (Majorana et al., 2010).

In this study, it was assumed that the orientation of the collagen fibrils in primary teeth of DI type I were parallel and less interlocked with each other. Comparison of these findings to the normal primary control tooth revealed that the orientations of the collagen fibrils in the control tooth were more interwoven with numerous crossings of

the fibrils. The dentinal collagen fibrils were reported to show repeat pattern and random distribution of fibrils in the intertubular dentine (Kramer, 1951, Habelitz et al., 2002).

The findings of this study can provide substantial supporting morphological evidence that the dentinal collagen orientation of DI type I tooth are densely packed with an aligned organisation. Less interlocking of collagen fibrils can be observed in the intertubular area. It can be postulated that the differences in the organisation of dentinal collagen between the dentine in normal control and test sample can contribute to the variation of collagen morphology that was affected as the results of mutation of genes in OI.

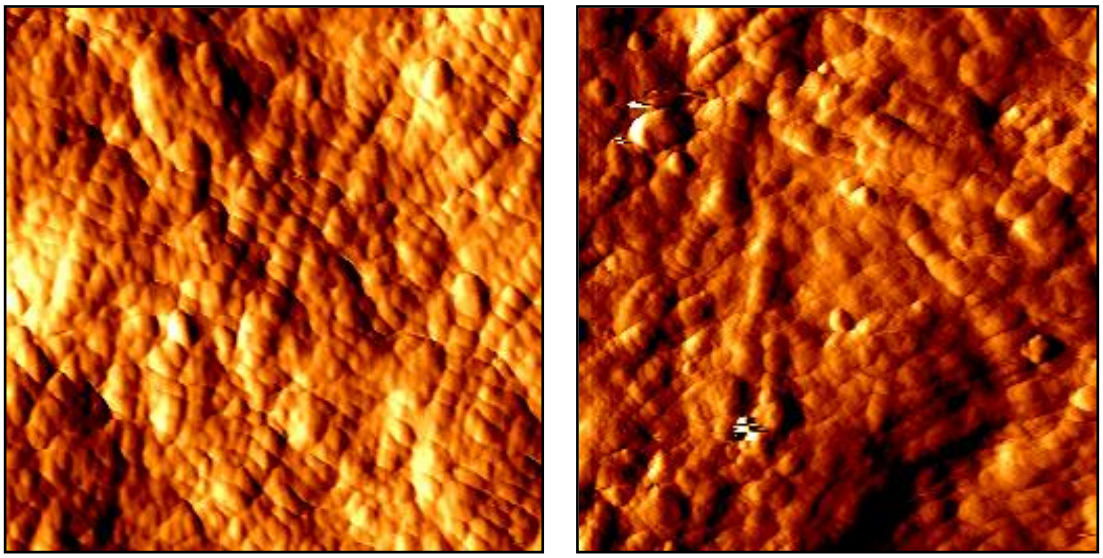


Figure 5.5: Right; showing thick dentinal collagen with repeated pattern that directed in one angle in DI type I whilst Left; showing interlocking fibrils with repeated pattern and random distributionin normal primary teeth

Data on the D-banding distances for the test and control primary teeth were also obtained. Analyses of contact mode AFM images exhibited significant differences in the distribution of fibril D-banding distances in DI type I. The D-banding distance of DI type I varied between 61nm to 85nm with mean value of 64.7nm ± 8.4nm. The

control primary sample showed that the D-banding distances were ranged between 59nm to 70nm with the mean value of 66.42± 3.2nm. This implied to a suggestion that dentinal collagen in DI type I have a wider range distribution of the D-banding distance when compared to the control primary tooth. It was hypothesised that the variability of the D-banding distances results in less regular pattern of fibrils. However, the results cannot be conclusive as this study has a very limited amount of samples.

This was in agreement with the findings in OI affected mice that showed significantly greater variability of D banding distances of bone collagen (ranging from 56 to 75nm) (Wallace, 2012). By characterising the mean fibril D-banding and the distribution of fibril morphologies in the bone of normal mice and mice that were heterozygous for a specific COL1A1 point mutation (G349C), phenotypic changes in the collagen fibril ultrastructure were detected (Wallace, 2012).

The alteration of Type I collagen structure as a result of mutations of genes associated with OI were directly related to the wide distribution of the D-banding distance in the dentinal collagen as shown in Table 5.1.To date, no studies have related the dentinal collagen morphologies in DI type I by measuring the fibril D-banding distance.

Further studies are currently underway to link the features in the ultrastructure morphology to the alteration in the nanosacle mechanical behaviour of type I collagen.

5.4.2 The effect of 10vol% citric acid for 15 seconds on primary DI type I

Despite the large number of studies focused on the mutation of the helical domain of type I collagen in OI, only a few studies have reported on the ultrastructure studies of dentinal collagen in OI/ DI type I. In 1994, Waltimo carried out a TEM study of the dentine matrix and its collagen on six exfoliated teeth of OI type IV and one incisor

tooth of type I OI. In this study, the teeth were partially demineralised by EDTA for eight weeks and samples were prepared for fixation to allow TEM examination (Waltimo, 1994).The ultrastructure findings of large vesicular and hyperfibres were reported in abnormal areas of the dentine. The dentine appeared irregular in shape and the tubules were poorly organized. This hyperfibres may have possibly arisen from uncontrolled growth of collagen.

The same protocol was repeated by Majorana using EDTA as a decalcifying agent to observe the ultrastructural dentine changes in DI type I using optical microscopy and confocal laser scanning microscopy. Collagen defects with randomly orientated collagen fibrils due to odontobast dysfunction was theorized in this study (Majorana et al., 2010). This was also in agreement with another study using polarized –light microscopy, SEM and TEM (Hall et al., 2002). Lygidakis et al reported on SEM observation of twelve teeth from patients with OI type I. Some of the samples were treated with 10% phosphoric acid and some were deproteinised with 5 vol% of NaOCI. The findings did not report on the collagen network however, they marked a reduction in number and irregular dentinal tubules in affected opalescent and non opalescent teeth (Lygidakis et al., 1996).

Most of the dentinal collagen studies in DI type I were carried out using electron microscopy. The results proved that acid etching with 10vol% of citric acid for 15 seconds can expose the collagen fibrils in a DI type I primary tooth. This was not the case for the control primary teeth, where at the same etching time, the fibrils were still been covered by non-collagenous matrix proteins and hydroxyapatite. The effect of the acid treatment may relate to the calcium to phosphorus ratio that has been shown to be lower in OI patients (Cassella and Ali, 1992). Because the hydroxyapatite growth is closely associated to collagen fibril formation, the defect in fibril formation in OI patients may interrupt hydroxyapatite growth (Byers et al., 1988) When there is a reduction in the amount of minerals, then the demineralisation will be

faster. This observation was in agreement with bone collagen of OI type III, where the treatment with 2.5 vol% of NaOCI removed the organic material, leaving no residue of mineral crystal (hydroxyapatite) whilst the same treatment on normal bone, will still leave the minerals on the surface area (Traub et al., 1994).

5.4.3 Potential issue

1) **Number of samples**

The main limitation of this study that needs to be highlighted was the sample number. Much effort was carried out to collect DI teeth from patients as mentioned in 2.1.2. In addition, patients who were seen in Great Ormond Street Hospital by the Paediatric dentistry consultant from EDH were also invited to take part in this study. Still, there were important issues to the number of patients reported here. Firstly, the ethical considerations for patients need to be considered; it was only possible to obtain DI teeth from patients that required extraction as part of their treatment plan, or were happy to donate their exfoliated primary teeth. Unfortunately this meant, that there were very few patients that could contribute teeth during the study period. Second, some of the patients that fit the criteria declined to take part in this study. Third, the sources of sample for permanent affected teeth were restricted with the fact that the DI Type I is more prevalent in primary teeth than permanent.

2) **Selection of demineralisation technique**

With only one primary tooth of DI type I (OI type III), the description of the ultrastructure findings was limited to only the effect of demineralisation using citric acid. Although the established protocol with commercial etchant in control primary teeth was reported in chapter 4, the intention to commence this study with published protocol was carried out first in this chapter to help to clarify the ultrastructure variation of DI type I teeth in response to the chemical treatment. As this is an

ongoing project, further demineralisation protocols for primary teeth as established in chapter 4 will be performed once more sample received from DI patients. Future study was planned to compare the findings between DI patients to the findings in normal primary teeth as described in chapter 4. This study should form the baseline protocols and information for the future work in characterizing the ultrastructure findings of DI type I teeth.

5.5 Conclusions

The ultrastructure findings of dentinal collagen organisation and a suggestion of wide distribution of D-banding distances in DI type I can provide additional understanding of the alteration in dentinal collagen in this disease. The differences in mineral content in test DI Type I and control primary teeth indicate that different demineralisation protocols are required for affected teeth. The marked ultrastructure variations noted can be a basis form to the future work.

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CHAPTER 6

FINAL CONCLUSION AND FUTURE WORK

The work presented in this study was carried out in order to characterise the morphology of the dentinal collagen ultrastructure in primary and permanent control teeth, and affected OI and DI teeth using AFM. Dentine is a calcified tissue where collagen fibrils are covered by hydroxyapatite and non-collagenous matrix proteins. In order to reveal dentinal collagen ultrastructure, it is essential to ensure that demineralisation protocols used does not affect the morphology of the fibrils themselves. At the same time, it is also important that all non-collagenous proteins are removed so that topological assessment of collagen can be performed.

In this study, the first objective was to evaluate the demineralisation protocols for control permanent teeth. Four demineralisation protocols were evaluated;

1) published protocol in which blocks of dentine were treated with 10 vol% citric acid for 15 s and 6.5 vol% NaOCLaq for 120s;

2) experimental protocol in which dentine blocks were treated with commercial etchant (37% phosphoric acid) and subsequent treatment with 6.5 vol% NaOCLaq for 100s. Raman spectroscopy was used to assess the degree of demineralisation.

3) demineralisation protocol where teeth were stored in 17% EDTA for a 12 month period before being histology sectioned and stained with Picrosirius red; and

4) demineralisation protocol in which dentine blocks was treated with EDTA for 10 minutes.

AFM observation revealed well-defined collagen fibril networks during the topological assessment of demineralised dentine using commercial etchant (37% phosphoric acid) and 6.5 vol% of NaOCI. In this particular experimental group, Raman spectroscopy was used to assess the degree of demineralisation of the dentine as a function of exposure time to commercial etchant. The results revealed that at 5

minutes, significant changes in mineral can be obtained. Further controlled deproteinisation protocol was required to allow the feasibility of collagen fibrils by using 6.5 vol% NaOCI. In comparison with the published protocol using 10 vol% citric acid and NaOCI, the images that recorded using AFM were very poor in contrast and we were not able to reproduce details on the dentinal collagen fibrils. Likewise, the results on the control permanent tooth treated by 17% EDTA for 12 months also failed to generate good quality images by the AFM. Therefore, SEM was used as an alternative method to reveal the topological changes on the dentine surface. Direct comparison between the four protocols proved that all of the protocols achieved partial or complete demineralised dentine as verified by AFM and SEM.

Next, the evaluation of demineralisation protocols in primary teeth was carried out. The results in both protocols with acid etching (citric/commercial etchant) and deproteinising agent (NaOCI) enabled imaging of a defined dentinal collagen scaffold with interlocking between collagen fibrils. A shorter protocol for chemical treatment in primary teeth showed a significant difference in the chemical reaction between primary and permanent teeth. When the teeth were exposed to the same time and concentration of EDTA, the microscopic observation under SEM suggested that there were differences between primary and permanent teeth. An interesting finding in the primary teeth stored in 17% EDTA for 12 months was the preservation of the collagen fibrils, as it was hypothesised that they would denature as the result of lower mineral content. This hypothesis still needs to be confirmed in the future work.

It is important to highlight that the intention to evaluate the demineralisation protocols for both primary and permanent teeth was to determine the most suitable method to reveal dentinal collagen fibrils without causing any denaturation of the structure. The established method was applied to obtain further information about the collagen fibrils organisation under high magnification microscope. Chapter 3 and chapter 4

compared the reaction of demineralisation protocols between primary and permanent teeth. The most appropriate method was then used to demineralise the DI Type I teeth and provide a tool to characterise the morphology of the dentinal collagen ultrastructure in affected teeth using AFM.

In the ultrastucture findings of the DI Type I tooth, the results showed that only acid treatment protocol was needed to image the dentinal collagen. The AFM images revealed an area of thickened dentinal collagen fibrils that was only distributed in one direction. No obvious fibrils interlocking were noticed. The D-banding periodicity of these collagen fibres resulted in a wide range distribution between 62nm to 85nm with mean value of 64.7nm ± 8.4nm that can be hypothesised to produce a less regular pattern of collagen fibrils.

The difference in dentinal collagen fibril arrangement and wide range distribution on affected primary tooth can contribute to the diversity of defects in collagen formation in DI type I. This characteristic may lead to an alteration in mineralization. This type of DI has an associated with the mutation of genes that encode collagen type I. In another form of DI, which is not associated with OI, the gene involved appears to be from mutation in the gene encoding dentine sialophosphoprotein (DSPP). In this condition, the differentiated odontoblasts may secrete irregular predentine matrix which is not conducive to form normal mineralisation of tissue and may produce dentine at a faster rate which would explain the frequent pulp obliteration in DI teeth.

With the limitation of the sample size in this study, the interpretation of the analysis must be viewed with caution. However it forms the basis for future work that is currently underway to collect more teeth and identify more ultrastructure features of dentinal collagen in DI Type I, such as the diameter of collagen fibrils, and subsequently linking these features to the alteration in the nanoscale mechanical behaviour of Type I collagen.

Clinical consideration and implication

The utilization of composite resin systems in dentistry has dramatically increased the improvements in physical and mechanical properties of tooth-coloured restorations. Because of these advancements, contemporary restorative materials and techniques allow minimal preparation of tooth structure and improvement in the longevity and aesthetics of the restoration. A key factor for the clinical outcome of bonded aesthetic restoration is the ability of the adhesive system to prevent the opening of the interfacial gaps.

The adhesive system is based on a process that includes two phases. When acid is applied to the enamel and dentine, the inorganic substances and the mineral components are partially removed, exposing the collagen mesh. It is followed by the hybridization phase, where the adhesive penetrates and infiltrates into the dentine to form the hybrid layer. The acid etching is fundamental for effective bonding and times for application, concentration and composition of acidic solution should be carefully controlled. The contemporary method reported that 15 seconds application of 30-40% orthophosphoric acid was required in order to provide a better substrate for bonding to dental adhesives (Buonocore, 1955). Unfortunately, manufacturers do not provide specific instructions for the use of their adhesives in primary dentition, even though some microstructural and micro- mechanical characteristics of dentine in primary teeth differ from those of permanent teeth.

In this study, the results of different demineralisation protocols for acid etching of permanent and primary teeth showed that a longer time is needed in order to expose the collagen of the dentine. Significant differences of demineralisation techniques were observed between primary and permanent teeth. The topography of the dentine surfaces were obtained with AFM and SEM on the samples with different etching times and etching agent. When the commercial etchant was applied, the results

showed that the effective etching time of the dentine surface appears to be 2 minutes for the primary teeth and 5 minutes for the permanent teeth. The inorganic substances changes can be seen after 1 minute of acid exposure for both primary and permanent teeth. Yet, at these points, the smear layers have not been removed completely. These showed that the conventional etching times of 15 seconds prove to be insufficient. Based on the results obtained, it could be suggested that a longer etching time may have beneficial effects on the performance of composite restorations.

During the restorative procedure, it is also necessary to uncover the collagen and expose the dentinal tubules so that resin can infiltrate into the partially demineralised dentine and form a hybrid layer (intercalation between dentine surface and resin). This study prove that etching and controlled deproteinisation of dentine with sodium hypochlorite enabled the in situ imaging of fibrous network of partially demineralised dentine collagen by AFM. The effects of sodium hypochlorite on partially demineralised dentine exposed the collagen fibrils. The results demonstrated that etching alone would not completely open the dentinal tubules since they remain filled with the protein, whereas deproteinisation alone does not open the tubules since mineral binds with the proteins. In relevance to the bonding system, this may lead to a suggestion that deproteinisation may allow better penetration of resin into partially demineralised dentine that has been exposed by etching. This hypothesis still needs to be confirmed through well conducted randomized clinical trials in order to evaluate the effects of deproteinisation of etched dentine on adhesive restoration.

EDTA is generally accepted as the most effective chelating agent and also used as demineralising agent. On the basis of the clinical etching procedures, this study demonstrated the demineralisation effect caused by EDTA on the structure of primary and permanent dentine. The action is not selective for removal of smear

layer but also demineralised the dentine surface. These can provide space for dentine infiltration and so that such hybrid layer can be formed. However, when the samples were stored longer in EDTA solution, excessive demineralisation effects were observed in which it can more likely affect the sealing ability of adhesive material. This study leads to the suggestion that EDTA could be a potential etching agent provided that the dentine were expose for the appropriate time. Longer etching times will result in formation of a thicker hybrid layer, leading to an unstable demineralised zone and consequently increasing the susceptibility of restoration failure.

In summary, this study has elucidated the effects of demineralising agents on the dentine surface. Current dentine bonding systems rely on hybrid layer formation by acid etching procedure that mainly involves 37% phosphoric acid for 15 seconds. In this study, optimal etching time (37% phosphoric acid) to enable imaging of collagen was shown to be at 2 minutes for primary teeth and 5 minutes for permanent teeth. It also showed that controlled deproteinisation facilitates the exposed collagen fibrils. Ideally, the application of etching will remove the smear layer and is followed by demineralisation of the dentine surface so that the network of collagen can be revealed. Although most studies use common acids such as citric acid, phosphoric acid and maleic acid, in this study the use of EDTA as an etching agent was observed. Based on the results obtained, EDTA also can provide the same effects as the other demineralising agents. Further studies are necessary to determine the effect of bond strength of restorative materials for agents that were used in this study.

Future work

The comparison of the demineralisation protocols on both primary and permanent teeth was validated using the direct comparison between AFM (citric acid/ commercial etchant) and SEM (EDTA) images. Future work of gaining images from both tools for each of the demineralisation group is strongly recommended. However the difficulties in obtaining quality images from experimental work in test samples stored in long term application of EDTA would probably necessitate the development of an additional protocol so that the samples were fixed upon the preparation.

Since the comparison of samples using EDTA was generated in two different groups; long term application of EDTA and another group treated for just 10 minutes, an assay of the exposure time of 17% EDTA is highly suggested. Such techniques would prove benefits in order to monitor the topological changes of the dentine surface in achieving collagen fibrils without any detrimental effect of the structure.

Finally, with the limitation expansion of the experimental work due to the restricted sample size, increasing the number of DI Type I teeth would permit an accurate description and understanding on the morphology of the affected dentinal collagen, and allow subsequent impact to the mechanical properties of the collagen. With a better understanding of the collagen structure in OI associated with DI, it is an effective tools to provide information on the importance of structure- properties relationship of collagen fibrils in clinical relevant such as in restorative treatment.

Our findings confirm that etching and controlled deproteinisation protocols enabled collagen imaging for both primary and permanent teeth. On the basis of the experiments that have been performed on the control teeth, it can be summarised that the most effective time for etching using commercial etchant was at 5 minutes for permanent teeth and 2 minutes for primary. The optimum deproteinisation time was

ranged between 80s to 100 seconds. This protocol appeared to prove that there were no obvious sign of collagen fibrils degradation suggesting that the demineralisation protocol will be adequate for DI/OI teeth.

Establishing a demineralisation protocol was the first step required in order to elucidate the dentinal collagen ultrastructure in OI and DI patients. This study found an ultrastucture variation in the DI Type I primary tooth. Further work will establish the ultrastructure features of dentinal collagen using larger sample sizes and relate these features to the mechanical properties of the collagen in the dentine.

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CHAPTER 8: APPENDIX

Appendix 1

Research team:

Dr Susan Parekh,

Dr Agnes Bloch Zupan,

Dr Peter Brett,

Dr Laurent Bozec

MashaelAbdullatif

Nurjehan Ibrahim

Nabilah Harith

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UCL Hospitals cannot accept responsibility for information provided by external organisations.

If you need a large print, audio or translated copy of this document, please contact us on 0207 915 1022. We will try our best to meet your needs.

If you wish to discuss this study with a member of the research team or an independent expert who is not part of the research team, please ask Dr Susan Parekh

Thank you for taking the time to read this leaflet.

University College London Hospitals NHS
NHS Foundation Trust

Publication date: 07/07/11

Date last reviewed

Version number :

Patient's Information Leaflet

A Study of the genotype and phenotype in Amelogenesis Imperfecta& Dentinogenesis Imperfecta

Invitation

You are being invited to take part in a research study. Before you make a decision, it is important that you know why the research is being done and what it would involve from you. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if anything is not clear at any time before or after participating. If you need more information we are willing to spend more time to satisfy you before taking any decision.

What is the purpose of the study?

To obtain and gather more information about dental anomalies, such as Enamel defects (AmelogenesisImperfecta), and dentine defects (DentinogenesisImperfecta). We want to use this information to improve our knowledge of genetics and the properties of the teeth, to provide better support and long term care.

Why have I have been chosen?

We are asking all patients who have been diagnosed with dental anomalies and members of their families with the same or other dental conditions to participate in the study

Do I have to take part?

No. It is up to you to decide. If you do decide to participate we will ask you to sign a consent form. If you change your mind, you are free to withdraw at any time, without giving a reason. The standard of care you will receive will not be affected in any way

What will happen to me if I take part?

We will ask you some questions about your teeth and your medical history, examine your teeth, take photographs, and a saliva sample. The saliva sample will be used to link your DNA with the physical properties of your teeth. We will also measure the colour of the front teeth using a machine called the spectroshade™ micro, which rests gently on the teeth and uses a light to record the shade of the tooth (see information sheet provided). If you require any teeth to be extracted as part of your treatment, these will becollected for laboratory testing of the teeth. You will not need to do anything else. If any member of your family has similarteeth, we will invite them totake part as well, as this will help to detect the common dental genesin families. If you do not want other members of your family to participate, you can refuse and your treatment will not be affected in any way.

What are the possible disadvantages or risks of taking part?

There are no risks anticipated. None of your answers will affect your treatment in any way.

What are the possible benefits?

We cannot promise the study will help you, but the information we get might help treat young people with dental anomalies in the future.

What will happen with the results?

Any samples that we collect will be stored using a study ID number, so that they cannot be directly linked to you. We hope to publish the results of the study on completion.

Will my taking part in the study remain confidential?

Yes. We will keep your information in confidence. This means we will only tell those who have a need or right to know.The safety

and security of the data will be the responsibility of the principal investigator (Miss Susan Parekh). The information will also be stored in a database developed by Strasbourg University (phenodent database), who we work closely with. All information will be anonymised before putting on the phenodent database.

What happens if something goes wrong?

In the event that something does go wrong and you are harmed during the research and this is due to someone's negligencethen you may have grounds for a legalaction for compensation against UCLH NHS Trust, but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you (if appropriate).

Who has reviewed the study?

All research in the NHS is looked at by independent group, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given favourable opinion by the Joint Research Ethics Committee. Thank you for reading this – please ask any questions if you need to.

Appendix 2

University College London Hospitals NHS
NHS Foundation Trust

The Eastman Dental Hospital
256 Gray's Inn road
London
WC1X 8LD

Telephone: 020 3456 7899
Direct Line: 020-3456-1067
Fax: 020-3456-2329
Web-site: www.uclh.nhs.uk

Version 1

Study Number

Patient Identification Number for this trial: ……

PARENTCONSENT FORM

Title of Project:

A Study of the genotype and phenotype in Amelogenesis Imperfecta& Dentinogenesis Imperfecta

Name of Researchers: Dr Susan Parekh, Dr Agnes Bloch-Zupan, Dr Peter Brett, Dr Laurent Bozec, Miss Amanda O`Donnell, MashaelAbdullatif, Nurjehan Mohamed Ibrahim and NabilahHarith.

_____

Please initial box

1. I confirm that I have read and understood the information sheet dated 21/12/10 (version 1) for the study. I have been allowed some time to think about this, ask questions, and have had these answered in a way that I understand. ☐

2. I understand that my child's is voluntary and that I am free to withdraw at any time, without giving any reason , without their medical care or legal rights being affected. ☐

3 . I understand that sections of any medical notes may be looked at by the researchers and responsible individuals from regulatory authorities where it is relevant to my child taking part in research. I give permission for these individuals to have access to my child's records. ☐

4. I give permission to the investigators to pass clinical data collected from my child's examination to my General Practitioner or General Dental Practitioner ☐

5. I understand that the samples taken from my child may be stored and used for the purpose of further research at a later date. I understand that these results will also remain anonymous. ☐

6. I understand that (this project or future research) will include genetic research aimed at understanding the genetic influences on dental defects in children. ☐

7. I agree for my child to take part in the above study. ☐

_____ _____
_____

Name of Patient | Date | Signature of parent

_____ _____
_____

Name of Person taking consent | Date | Signature

When completed, 1 for patient; 1 for researcher site file; 1 (original) to be kept in medical notes

For further information about this study please contact Dr Susan Parekh

Phone : 020 3456 1067 email: s.parekh@eastman.ucl.ac.uk

UCLH welcomes feedback from their patients who have been involved in research. In the first instance, you should inform the Principal Investigator. If you are not satisfied with the response of the research team then you should address your complaints to the UCLH complaints manager at UCLH postal address or through our website http://www.uclh.nhs.uk/Contact+us/. To help us identify the research study you have been involved in, please mention the title and the name of the research doctor or principal investigator. You can find this information on the Patient Information Sheet.

Appendix 3

Dental anomalies proforma

Study ID:.....

Date of clinic:……. Pt sticker:

Clinician name:……..

Ethnicity: White Mixed Black Asian Chinese

Other.....

Referred by: GDP CDS HDS GP

Other:……..

c/o: Nil pain sens appearance

Other:……..

Relevant medical history:……..

.....

.....

.....

Fluoride history: supp Y/N water Y/N toothpaste child/adult

Dental history: restn Y/N ext Y/N LA Y/N sed Y/N GA Y/N

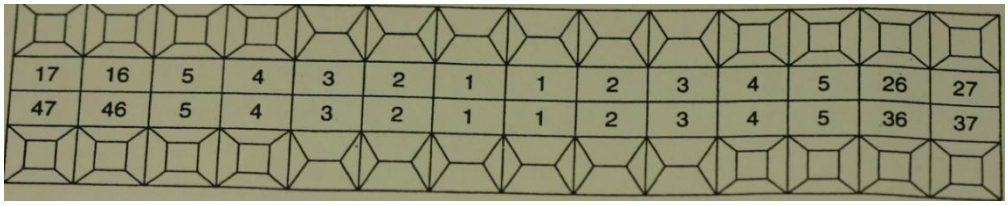
Family history (inc family tree): Plaque score:

Extra-oral features: Skeletal pattern I II III

Hair: normal/sparse skin:……

face:……. hands/nails:…….. Other:……

Introral features: lips gingiva palate m ucosa sali



Eruption: early Y/N delayed Y/N infraoccluded Y/N
impacted Y/N
General/local Mild/mod/sev; teeth:…….
Teeth:…….

Occlusion: Class I Class IIi ClassIIii ClassIII OJ= OB:
complete / incomplete
AOB Y/N

Dentine:
discoloured: Y/N abscess: Y/N tooth wear: mild / mod / sev
(which teeth):

Enamel:

18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28

DDE index: **Location (L): 1** incisal ½; **2** gingival ½; **3** whole surface. **Demarcation of defect (D)**: **1** demarcated;**2** diffuse;**3** both	55	54	53	52	51	61	62	63	64	65	**Extent of defect(E):** **1**<⅓;**2**⅓ – ⅔;**3** at least ⅔. **Wear:mild**mild; **Sev**severe
	85	84	83	82	81	71	72	73	74	75	

48	47	46	45	44	43	42	41	31	32	33	34	35	36	37	37

Type of defect:0 normal;**1** opacity (white/cream);**2** opacity (yellow/brown);**3** hypoplasia (pits); **4** hypoplasia (horizontal grooves);**5** hypoplasia (vertical groves);**6** hypoplasia (missing enamel);**7**discoloured enamel (not assoc. with opacity);**8** post-eruptive breakdown;**9** other defects;

Number /Form /size:

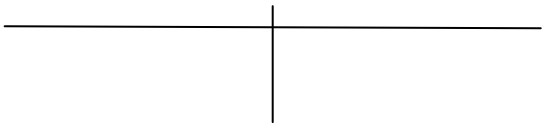
	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
			55	54	53	52	51	61	62	63	64	65			
			85	84	83	82	81	71	72	73	74	75			
48	47	46	45	44	43	42	41	31	32	33	34	35	36	37	37

con conical;**shov**shovel;**dbl**double;**rog** rounded or bulbous;**tap**tapered;**cet** talon cusp;**can** abnormal cusp;**noc**notched;**mic**microdont;**mac**microdont;**inv**invagination;**env**evagination;**mih**enlarged mamelons;**pem** enamel pearls;**sup**supernumerary;**hyp**hypodontia

Radiographic findings: taurodont Y/N thin enamel Y/N

short roots Y/N pulp stones Y/N

apical area Y/N resorption Y/N



Diagnosis:

Proposed treatment plan:

Treatment to date:

Allocated to:

Review on anomalies clinic: Y/N when?

Photographs Y/N **Saliva** Y/N

Consents : Y/N