INTERACTION OF *ENTEROCOCCUS FAECALIS* TO ROOT CANAL DENTINE; ROLE OF DIRECT ACTION OF CHEMICALS ON DENTINE SUBSTRATE

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Abstract

Several reports have pointed out that *Enterococcus faecalis* (*E. faecalis*) survived chemo-mechanical preparation during root canal treatment, and survived within the root canal when other bacteria were removed by the disinfection of the root canal system. Latterly, several reports from different continents reported that this bacterium was associated with failed root canal treated teeth. It was hypothesised that there may be steps in the process of chemo-mechanical root canal preparation which increases the propensity for this bacterium to adhere and survive as biofilm on root canal dentine.

A step commonly taken during chemo-mechanical root canal preparation is the removal of the smear layer, using EDTA. Reports have shown that application of EDTA on dentine exposes collagen fibrils. There have been reports of increased adhesion of micro-organisms to denatured collagen. Irrigants are commonly used during root canal treatment. In this study different irrigants were used to treat type I collagen membranes and these chemically treated collagen membranes were examined for denaturation, using Circular Dichroism and Differential Scanning Calorimetry. Bacteria adhesion assays to treated collagen were carried out using Confocal Laser Scanning Microscopy using a fluorescent stain. Adhesion force of *E.faecalis* to collagen was assessed using Optical Tweezers. The physico-chemical

changes to chemically treated root canal dentine were monitored using Fourier Transform Infra-red Spectroscopy. The adhesion assay of *E.faecalis* adhesion to treated dentine was assessed using fluorescent microscopy and adhesion force measured using Atomic Force Microscopy. Zeta potential of the chemically treated dentine was also measured to understand its influence on bacteria adhesion to root canal dentine.

It was found that all chemicals applied during root canal treatment denatured type I collagen. The chemicals used made an impact on the bacterial adhesion assays and different chemical treatment sequences led to an increase in *E.faecalis* adhesion. These chemicals altered the surface chemistry of dentine and had an impact on the adhesion assay of *E.faecalis* to dentine. These experiments highlight that different chemicals employed during root canal treatment has specific effects on dentine substrate and can facilitate the adhesion of *E.faecalis* to such chemically modified root canal dentine.

Keywords: *Enterococcus faecalis*, bacteria adherence, bacteria adhesion force, dentine, endodontic irrigants, Type I Collagen.

Table of Contents

ACKNOWLEDGEMENTS ..... **I**

ABSTRACT ..... **III**

TABLE OF CONTENTS ..... ERROR! BOOKMARK NOT DEFINED.

CHAPTER 1 INTRODUCTION **1**

CHAPTER 2 LITERATURE REVIEW **8**

2.1 INTRODUCTION 8
2.2 ENTEROCOCCUS FAECALIS 8
2.2.1 PREVALENCE OF ENTEROCOCCUS FAECALIS IN THE HUMAN MOUTH 9
2.2.2 ENTEROCOCCUS FAECALIS THE BACTERIA MOST OFTEN ASSOCIATED WITH FAILED ROOT TREATED TEETH 14
2.3 THE SUBSTRATES OF INTEREST – DENTINE AND COLLAGEN 20
2.3.1 Dentine Substrate *20*
2.3.2 Type I Collagen *22*
2.4 CHEMICALS USED IN IRRIGATION OF ROOT CANALS, THEIR BACTERICIDAL EFFECTS 25
2.4.1 Sodium Hypochlorite *26*
2.4.2 EDTA - Removing the Smear Layer *33*
2.4.3 The sequence of irrigation in removal of the smear layer – Which of the two, NaOCl or EDTA, should be the final irrigant? *36*
2.4.4 Chlorhexidine – some of its properties *39*
2.4.5 Calcium Hydroxide *44*
2.5 EFFECT OF IRRIGANTS ON DENTINE SUBSTRATE 54
2.5.1 NaOCl – Its effect on Dentine *54*
2.5.2 EDTA - Its effects on dentine *58*
2.5.2.1 Biological Effects of EDTA on Dentine *60*
2.5.3 Other Smear Layer Removing Irrigant – a proprietary product *63*
2.6 ADHESION OF BACTERIA ON DENTINE AND COLLAGEN SUBSTRATE 63
2.7 CLINICAL SIGNIFICANCE OF BACTERIA ADHESION AND BIOFILM FORMATION 67
2.8 BACTERIA FACTORS INFLUENCING ADHESION 68
2.9 Substratum Surface Factors Influencing Adhesion *71*
2.10 PHASES IN BACTERIA ADHESION 73

2.10.1 Reversible Adhesion *74*

2.10.2 Irreversible Adhesion *75*

2.10.3 Theoretical Models of Bacteria Adhesion *76*

2.11 INFRA-RED SPECTROSCOPIC METHODS TO CHARACTERIZE CHEMICAL COMPOSITIONAL CHANGE IN BIOLOGICAL MATERIALS 78

2.11.1 Basis of Infrared Spectroscopy *79*

2.11.2 Advantages and Limitations of FTIR as a Chemical Analysis Technique *81*

2.11.3 Fourier Transform Method *82*

2.11.4 The IR spectrometer Components *83*

2.11.5 Formation of the IR Spectrum *84*

2.11.6 What is Attenuated Total Reflectance (ATR) *85*

2.11.7 FTIR spectroscopy in Dentine Characterization *87*

2.12 SUMMARY OF LITERATURE REVIEW 90

CHAPTER 3 INVESTIGATING THE DENATURING EFFECTS OF EDTA AND NAOCL ON TYPE-I COLLAGEN USING CIRCULAR DICHROISM 92

3.1 THEORY OF CIRCULAR DICHROISM 92
3.2 MATERIALS AND METHODS 94
3.3 RESULTS: 96
3.4 DISCUSSION 99
3.5 CONCLUSIONS: 101

CHAPTER 4 DENATURATION OF COLLAGEN TYPE-I MATRIX MEMBRANE BY VARIOUS ENDODONTIC IRRIGANTS 102

4.1 THEORY OF DIFFERENTIAL SCANNING CALORIMETRY 102
4.2 MATERIALS AND METHODS 107
4.3 RESULTS 109
4.4 DISCUSSION: 111
4.5 CONCLUSIONS: 118

CHAPTER 5 ADHERENCE OF ENTEROCOCCUS FAECALIS TO TYPE I COLLAGEN ... 119

5.1 INTRODUCTION 119
5.2 MATERIAL AND METHODS 121
5.3 RESULTS: 125
5.4 DISCUSSION 129
5.5 CONCLUSIONS 132

CHAPTER 6 ADHESION FORCE OF ENTEROCOCCUS FAECALIS TO COLLAGEN IN THE PRESENCE OR ABSENCE OF CALCIUM DETECTED BY OPTICAL TWEEZERS .. 133

6.1 INTRODUCTION 133
6.2 MATERIAL AND METHODS 136
6.3 RESULTS 148
6.4 DISCUSSION 149
6.5 CONCLUSIONS 153

CHAPTER 7 ENTEROCOCCUS FAECALIS ADHESION AND ADHESION FORCES TO ROOT CANAL DENTINE 154

7.1 INTRODUCTION 154
7.2 E. faecalis adhesion and conditions of the culture 155

7.3 Methods of measuring adhesion 156

7.4 THE ATOMIC FORCE MICROSCOPE 157
Aims of Experiments 163

7.5 MATERIAL AND METHODS 164
Harvesting Dentine and Dentine polishing 164

7.6 EXPERIMENT 1 - AFM MEASUREMENT OF PERPENDICULAR FORCES OF INTERACTION 166
7.7 Results of Experiment 1 168

7.8 EXPERIMENT 2 - AFM MEASURE OF SHEAR FORCE OF ENTEROCOCCUS FAECALIS ADHERING TO TREATED DENTINE 173
Treatment of Dentine 174

Control of AFM 175

7.9 Results of Experiment 2 176

Surface Roughness after Chemical Treatment 177

7.10 EXPERIMENT 3 - BACTERIA ADHESION TO DENTINE AFTER VARIOUS CHEMICAL IRRIGATION 180
Material and Methods 180

Bacteria Culture and Inoculation of Specimens 181

7.11 RESULTS OF EXPERIMENT 3 182
7.12 EXPERIMENT 4 - MEASUREMENT OF ZETA POTENTIAL 184
Dentine powder preparation. 184

Measurement of Zeta potential 186

7.13 Results 187

7.14 DISCUSSION (INCORPORATING EXPERIMENTS 1 THROUGH 4) 188
EDTA as the last irrigant 188

NaOCl as the last Irrigant 189

CHX as the last irrigant 190

Other factors 192

CHAPTER 8 - FOURIER TRANSFORM INFRA RED (FTIR) SPECTROSCOPY OF CHEMICALLY TREATED HUMAN DENTINE 200

8.1 INTRODUCTION 200
8.2 SPECIMEN PREPARATION 200
8.3 Chemical Treatment of dentine 201

FTIR Instrumentation 204

8.4 RESULTS AND OBSERVATIONS 204
8.4.1 Effects of Chlorhexidine (dilute Hibiscrub) on Dentine 204
8.4.2 Effects of EDTA Treatment of Dentine 205
8.4.3 Effects of CH treatment on Dentine Collagen 208
8.4.4 Effect of the NaOCl Treatment 211
8.5 Discussion 212
CHAPTER 9 - DISCUSSION 215
CHAPTER 10 CONCLUSIONS AND FUTURE WORK 230
BIBLIOGRAPHY 234

List of Figures

Fig. 2.1	Bar chart of the prevalence of the 40 test bacterial species in all 30 root canals	13
Fig.2.2	Scanning Electron Micrograph of Dentine With Adherent *E.faecalis* ATCC 29212 on the Smear Layer	57
Fig.2.3	Photomicrograph from Texeira et al, mid-root section, 1 minute using 1% NaOCl and 15% EDTA	58
Fig.2.4	Cartoon depicting the electric double layer on a bacterium	69
Fig.2.5	Bacteria adhesion to dentine conditioned by body fluids	72
Fig. 2.6	Types of Interaction between Bacterium and substratum	74
Fig. 2.7	Diagram showing Evanescent Wave	86
Fig.2.8	Schematic Diagram showing the relationship of IRE and sample	88

Chapter 3

Fig.3.1	Far-UV CD reference spectra of an unordered protein, and of proteins with a prevalent α-helical or β sheet content	93
Fig.3.2	CD spectrum showing effect of NaOCl concentration on Type-I Collagen	98
Fig. 3.3	CD spectrum showing the effect of time of exposure using 0.03M NaOCl on Type-I Collagen	98
Fig. 3.4.	CD spectrum showing the effect of variation of EDTA concentration on Type-I Collagen	99

Chapter 4

Fig. 4.1	Diagram of Heat Flux DSC	103
Fig. 4.2	Heat Transition Peaks of Lysozyme	105
Fig. 4.3	DSC Curves of Collagen type-I after various chemical treatment	110
Fig. 4.4	Schematic Diagram showing DSC Curve	112

Chapter 5

Fig.5.1.	Bacteria count of chemically treated collagen after one hour	125

Fig 5.2.	*E.faeecalis* adhering to collagen after one-hour incubation	127
Fig 5.3	Bacteria count of chemically treated collagen after two-hour	128
Fig 5.4	Clumps of cells (arrowed) were also seen at two hours on CH treated collagen membranes	128

Chapter 6

Fig 6.1	Diagram showing Optical forces acting on a particle	135
Fig 6.2	Schematic diagram of Laser Tweezers setup	138
Fig 6.3	The linear relationship between power and force of laser trap	142
Fig 6.4	a – Optical view of a trapped bacteria b – Centroid Position of a bacterium during analysis c – Stiffness vs. Power plot of laser trap	145
Fig 6.5	Digitized images of bacteria on Type-I collagen matrix	146
Fig 6.6	Schematic diagram showing interaction force measurement using the displacement (Δx) of the bacterium from its mean position in presence of collagen	148

Chapter 7

Fig 7.1	Physico-chemical forces are the fundamental forces of adhesion	157
Fig 7.2	Components of the atomic force microscope	158
Fig 7.3	Diagram of Atomic Force Microscope setup	159
Fig 7.4	Diagram showing a typical AFM force vs. displacement curve	160
Fig 7.5	Typical force curve from AFM experiment	169
Fig 7.6	Force curves after various chemical treatment	170
Fig 7.7	Force Histogram Control	171
Fig 7.8	Force histogram after NaOCl treatment	171
Fig 7.9	Force histogram after NaOCl, EDTA and CHX treatment	172
Fig 7.10	Force histogram after NaOCl and EDTA treatment	172
Fig 7.11	Force histogram after NaOCl, EDTA, CHX and NaOCl	172
Fig 7.12	Mean Vertical Force of Adhesion	173
Fig 7.13a	AFM pictograms of *E.faecalis*	178
Fig 7.13b	The stages of AFM cell detachment	178
Fig 7.14	Mean Shear Adhesion Force to Chemically Treated Dentine	179
Fig 7.15	AFM image of dentine	179
Fig 7.16	Number of bacteria adhering to chemically treated	183

dentine after one hour
Fig 7.17 Photomicrograph showing dentine powder particle size for Zeta potential measurement 186

Chapter 8

Fig 8.1 FTIR Spectrum of CHX treated dentine 205
Fig 8.2 FTIR spectrum of EDTA treated dentine 206
Fig 8.3 FTIR spectrum of EDTA treatment of dentine 207
Fig 8.4 FTIR spectrum of Effect of CH on Decalcified Dentine (Dentine Type I Collagen) 208
Fig 8.5 FTIR of CH Treated Dentine 209
Fig 8.6 FTIR of dentine collagen from 600 to 1650 cm^{-1} 210
Fig 8.7 FTIR-ATR treated in sequence by NaOCl, CH and EDTA 211

List of Tables

Chapter 2		
Table 2.1	Hierarchical Structure of Proteins	24
Chapter 3		
Table 3.1.	Volumes of Chemicals used in assessment of effect of NaOCl concentration on Collagen	95
Table 3.2.	Volumes of Chemicals used in assessment of effect of EDTA on concentration Collagen	95
Chapter 4		
Table 4.1	Heat parameters of test groups	111
Chapter 5		
Table 5.1	Treatment groups of type I collagen membranes for CLSM	123
Chapter 6		
Table 6.1	Treatment groups studied by optical tweezers	140
Table 6.2	Effect of treatment of collagen membranes with calcium on interaction force	150
Chapter 7		
Table 7.1	Treatment Groups for AFM to measure shear force of adhesion	174
Table 7.2	Means and Modes of adhesion force	176
Table 7.3	Surface roughness of dentine after various chemical treatment	177
Table 7.4	Results of ZETA potential measurements	187
Chapter 8		
Table 8.1	Schedule of Chemical Treatment of Dentine for FTIR-ATR	202

LIST OF ABBREVIATIONS

American Type Culture (ATCC)

Atomic Force Microscopy (AFM)

Attenuated Total Reflectance (ATR)

Bovine Serum Albumin (BSA)

Calcium Hydroxide (CH)

Chlorhexidine gluconate (CHX)

Circular Dichroism (CD)

Colony Forming Unit (CFU)

Confocal Laser Scanning Microscopy (CLSM)

Differential Scanning Calorimetry (DSC)

Enterococcus faecalis (*E.faecalis*)

Ethylene diamine tetra acetic acid (EDTA)

Fourier Transform Infrared Spectroscopy (FTIR)

Hydroxyapatite (HA)

Iodine Potassium Iodide (IKI)

Infrared (IR)

Lipo-polysaccharide (LPS)

Matrix Metalloproteinase (MMP)

Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM)

Non-Collagenous proteins (NCP)

Polymerase Chain Reaction (PCR)

Sodium Hypochlorite (NaOCl)

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WHAT WE LEAVE BEHIND IN ROOT CANALS AFTER ENDODONTIC TREATMENT: SOME ISSUES AND CONCERNS
Chee Peng Sum, Jennifer Neo, Anil Kishen, 2005 AUSTRALIAN ENDODONTIC JOURNAL 31(3):1 – 6

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1. INFLUENCE OF ENDODONTIC CHEMICAL TREATMENT ON ENTEROCOCCUS FAECALIS ADHERENCE TO COLLAGEN STUDIED WITH LASER SCANNING CONFOCAL MICROSCOPY AND OPTICAL TWEEZERS: A PRELIMINARY STUDY

   Chee Peng Sum, Samarendra Mohanty, P.K.Gupta and Anil Kishen 2008 JOURNAL OF BIOMEDICAL OPTICS 13(4): 044017-1 to 044017-9

2. INFLUENCE OF IRRIGATION REGIMENS ON THE ADHERENCE OF ENTEROCOCCUS FAECALIS TO ROOT CANAL DENTIN

   Anil Kishen, Chee Peng Sum, Shibi Mathews and Chwee Teck Lim 2008 JOURNAL OF ENDODONTICS 34:850-854

Chapter 1 Introduction

Root canal treatment is undertaken to disinfect the pulp space of a tooth which has become infected. Pulp spaces are usually infected as a result of caries, breaks in teeth or bacteria spreading through dental foramina from an infected periodontal space. Infection in the pulp spreads in time to the periapical area of the tooth, causing apical periodontitis. Bacteria had been shown to be the main cause of apical periodontitis.[14]

There is little epidemiological data about apical periodontitis. One study reported that one in two adults above 50 years of age would have experienced the disease whilst in those above 60 years old, it is reported to be 62%.[15] In recent publications, retreatment of teeth that had failed root canal treatment, constituted about 3 to 5% of all endodontic procedures.[16,17,18] This compares with a failure rate of root canal treatment reported at 13%.[5] The singular study of outcome of root canal treatment undertaken at the teaching clinic in Singapore, puts the failure at about 10%.[19]

The retreatment and apicectomies of such a large number of teeth would incur considerable costs to these patients even though they may not have other observed associated adverse health effects from these failed root canal treatment; the pain and suffering involved with these failures and their further treatment and management would itself be a burden.

What is the meaning of success in endodontic treatment? Many studies use the modified criteria from Strindberg[20] to define success. Hence, a treated tooth was classified to have completed healing when the tooth was found to be clinically free of symptoms and the radiograph showed complete disappearance of the pre-existing radiolucency. Those cases showing a decrease in size of the periapical radiolucency were placed in the incomplete healing category. If there was an expansion or no change in size of the observed pre-existing lesion, the treatment was recorded as a failure[21].Decision making in retreatment is thus very dependent on the projection of x-rays and interpretation of radiographs. Unfortunately interpretation of radiographs is subjective and is very variable amongst practitioners.[22]There are many variables that affect the outcome of root canal treatment.[23]Disappointingly, there was no discussion of species and mix of bacteria at primary endodontic infection and the variability of the root canals, the degree of the patients' immuno-competency, effectiveness of chemo-mechanical treatment and healing, in that report. The variables discussed mostly are related to the physical nature of the tooth, tooth position and technical quality of the treatment.

The bacteria flora isolated from primary root canal infections constitutes a small group of the total flora of the mouth, selected by an anaerobic environment, lack of nutrition as well as competition between cells of different species inhabiting the root canal. The bacteria, in root canals probably exist as biofilms of co-aggregated communities in an extracellular matrix, are made up of roughly equal proportions of Gram-positive and Gram-negative bacteria and are largely dominated by obligate anaerobes, and usually comprise more than 3 species.[24] However, when the root canal treatment has not been successful, the bacterium commonly isolated from these teeth was *Enterococcus faecalis* (*E. faecalis)*.[25,26] In addition, the study by Molander[25] concluded that the flora in obturated canals differed from those found in untreated canals, both qualitatively and quantitatively. This is a worrisome problem, as it had been reported that *E. faecalis* isolated from root canals are resistant to a variety of antibiotics and intra-canal medicaments, including calcium hydroxide, a commonly used intra-canal medicament [27]. *Enterococcus* have been implicated as the causative organism in a variety of ailments, including endocarditis.[28]

E. faecalis was first noticed within root canals in 1964[29]; it was pointed out in 1975[30] that *Enterococcus* should be of special importance to those interested in studies on the influence of infection at the time of filling of root canals on the prognosis or outcome of root canal therapy. From about 1998 onwards, there have been numerous studies on *E. faecalis* in the endodontic literature.[31] Reports of refractory periapical lesions associated with *E. faecalis* from different continents have

been published [26, 32, 33, 34]. It seems therefore, that it is not a mere coincidence that this bacterium is so prominent in failed root canal treated teeth. Even more worrying is that this bacterium had been reported to up-regulate the adhesin ACE when grown in the presence of type I and IV collagen[35]. Another study[36] observed *E. faecalis* eroding dentine when it forms a biofilm on root canal dentine whilst another study [37] reported that it remained viable after being entombed by root fillings for one year.

Together these studies suggest that if *E. faecalis* were to remain in the root canal, it is not only going to survive in the root canal, but likely to thrive. In addition, there is also information that if the root canal is not well filled, fluids can move in and out of the root canal, from and to the periapical region. Presently, its link to failed root canal treated teeth is not clear .[38] A thriving *E. faecalis* biofilm in a root canal may then have very serious implications on the health of an individual harbouring this infection. This was so because *E. faecalis* has been associated with a variety of diseases, including but not limited to, infective endocarditis, urinary tract infections, biliary tract infections, burn wounds, in dwelling medical devices [39] and funisitis.[40]

Very few studies have examined the physical and chemical changes mediated by root canal medicaments on root canal substrate. Since *E. faecalis* was associated with failure of root canal treatment in many instances,[26, 32, 33, 34] we hypothesized that some chemical and/or chemical sequences used during root canal treatment mediated

changes that increased the adhesion force and adhesion of *E. faecalis* to dentine substrate.

The critical first step of the successful establishment of a bacterium in the root canal is the adherence of bacteria to the luminal dentine surface of root canals or to other micro-organisms that may already be adhering to that surface (co-adhesion). Adhesion to a substrate offers bacteria a number of advantages, including but not limited to resisting dislodgement by hydrokinetic forces. Adhering bacteria are better able to access nutrients and have more protection from deleterious effects of antimicrobial agents in the surrounding environment.[41] Different endodontic irrigants have been applied routinely within the root canal. These irrigants have specific functions that are known to produce specific changes to the root canal dentine. However, there are very few systematic studies today that examined the influence of such substrate changes on adhesion of *E. faecalis*.

The objectives of the study were:

1. Monitor the changes to physico-chemical characteristics of dentine after treatment by irrigants and medicaments commonly used during root canal procedures.

2. Measure the changes in adherence and adhesion force of *E. faecalis* on dentine substrate after treatment with different endodontic irrigants and medicaments commonly employed during root canal treatment.

The significance of these studies was:

1. To increase the understanding of factors that may lead to the persistence of *E. faecalis* in teeth in which root canal treatment had failed
2. To increase the understanding of how chemically treatment of root canal dentine may promote adhesion of *E. faecalis*.

The scope of the study was:

1. To measure the changes in the chemical composition of dentine after the application of various irrigants/medicaments on root canal dentine using Fourier Transformed Infrared Spectroscopy –Attenuated Total Reflectance (FTIR –ATR)
2. To measure the Zeta potential of dentine substrate before and after treatment by various endodontic irrigants/medicaments
3. To measure the ability of chemicals to denature type-I collagen using Circular Dichroism(CD) and to compare the extent of denaturation of a collagen membrane after various chemical treatment using Differential Scanning Calorimetry(DSC)

4. To measure the adhesion force of *E. faecalis* to type-I collagen substrate using an Optical Tweezers.
5. To measure the adhesion force of *E. faecalis* to dentine substrate before and after treatment with endodontic irrigants/medicaments using the Atomic Force Microscopy (AFM)
6. To measure the *in-vitro* adhesion of *E. faecalis* to dentine, using Confocal Laser Scanning Microscopy (CLSM), before and after the application of various irrigants/medicaments.

Chapter 2 Literature Review

2.1 Introduction

The literature survey was conducted on the following areas:

- *E. faecalis*: The bacterium most commonly related to root canal treatment failure, as it presents itself as a commensal and as a pathogen in endodontics
- the substrate of interest - Type I collagen and dentine
- the types of chemicals used in root canal treatment: Irrigants, their antibacterial effects and their effects on dentine substrate
- the chemical effects on the biomaterials: Dentine substrate and bacteria
- the types of bacteria substrate interactions.

2.2 Enterococcus faecalis

E. faecalis is a commensal in the intestines of humans and they play a vital role in modulation of inflammatory processes in the gut where specific strains of *E. faecalis* may have evolved to maintain colonic homeostasis.[42] There is also evidence to show *E. faecalis* is able to cross talk with immature gastrointestinal tract cells to regulate

peroxisome proliferators-activated receptor-gammal activity which affects expression of interleukin-10 that in turn prevents enterocolitis.[43] The *Enterococcus* are a diverse group of complex bacteria, which are important, not only because of their interactions with humans, but also because some strains are used in food manufacturing whereas others are pathogens known to cause severe diseases in humans. They can grow in temperatures ranging from 10 to 45^{o}C, and some strains at even higher temperatures, and in media with high salt concentrations over a wide pH range.[44] This gram-positive cocci, occurring singly or in pairs or as short chains, begin to colonize the intestines of newborn infants in the first weeks of life.[45]

E. faecalis has emerged as a common cause of nocosomial infections and has inflicted infections ranging from septicaemia, endocarditis to urinary tract infections in humans. The ability of *E. faecalis* to readily exchange DNA by conjugation is probably the reason for the observed increase in multi-drug resistance among many clinical enterococcal isolates.[46]

2.2.1 Prevalence of Enterococcus faecalis in the Human Mouth

Its prevalence in the mouth is reported to be between 60 -75% in the mouths of three groups of people – laboratory technicians, children with high caries rate and patients who have had root canal treatment.[47] However, more recently, among patients with periodontal disease, its prevalence was reported to be about 41 to 48%

respectively in saliva and sub-gingival plaque, and was significantly related to an increase in pocket depth, attachment levels and other parameters of periodontal disease. In controls, the prevalence was much lower at 15 to 17% respectively.[48] Another more recent study using molecular techniques reported that the prevalence of *E. faecalis* was about 68% in mouths, more so in patients with periodontal disease. However, it was found in only about 5% of the root canals of these patients.[49] Interestingly the same group published an earlier paper that in those who have had no history of root canal treatment, the prevalence of *E. faecalis* in mouths was only about 1%, whereas in those who had root canal treatment the prevalence was 11%.[50] These studies show that the oral rinse method may have underestimated the prevalence of *E. faecalis*.

Whilst *E. faecalis* is quite prevalent in mouths, its prevalence in primary root canal infections has not been reported to be high. [51,52,53] and its low prevalence is similar among patients in North and South America.[54] Its low prevalence in primary root canal infections compares starkly with its prevalence in root canal treated teeth with persistent periapical periodontitis whether using culturing techniques [25,26,33,34] or molecular techniques [55,56,57,58] across geographical regions. In a review on the strategy to eliminate this bacteria from root canal, the author summarized the available data of *E. faecalis* associated with failed root canal treatment and stated that this bacterium is a micro-organism commonly detected in asymptomatic, persistent endodontic infections; with a prevalence ranging from 24% to 77% .[59]

E. faecalis has an microbial surface components recognizing adhesive matrix molecules (MSCRAMM) for collagen named ACE.[60] The prevalence of this organism in the mouth was studied by Gold *et al.* (1975) in populations of laboratory personnel, schoolchildren with high caries and patients undergoing root canal treatment[47]. Cultures were taken from the tongue, vestibular mucosa, plaque, saliva, carious lesions, root canals, and the prevalence (presence of *E. faecalis* from at least one site) for laboratory workers and children with high caries rate were 60% whilst it was 75% for those undergoing root canal treatment.

Using a polymerase chain reaction (PCR) method for detection of *E. faecalis* within root canals that were resistant to therapy, it was reported that the prevalence was 22%.[61] Furthermore, the same group of workers compared the prevalence of *E. faecalis* in teeth with and without periapical lesions that required root canal retreatment. They reported that the presence of periapical lesions was significantly associated with micro-organisms but by using logistic regression, they found the bacterium *E. faecalis* was associated with normal periapices rather than periapical lesions. Hence they concluded that *E. faecalis* was not associated with periapical disease.[62] They had met their criteria of statistical power of 80% in arriving at the number of subjects selected in each group, and had 2:1 ratio between the lesion versus the no lesion group.

Using a culturing method from oral rinses, the prevalence of *E. faecalis* was reported to be 1% in 100 dental students whereas it was 11% in patients undergoing endodontic treatment [63]. In another study, the same group of authors using PCR and culturing techniques found that the prevalence of *E. faecalis* in 41 patients undergoing endodontic treatment was 68%, with a positive test of tongue, oral rinse, or gingival sulcus. Root canals commencing treatment were sampled and the prevalence was only 5%. There were 21 patients with presence of *E. faecalis* from all four sites tested. In this cohort the tongue was found to be the most common site harbouring *E. faecalis.* [49] These authors concluded that the question remains if *E. faecalis* harbouring in possibly the tongue, enters the root canals after root canal treatment and contributes to the pathogenesis of a periapical lesion. In further work, using both culture and PCR techniques to compare primary versus retreatment cases, they reported that PCR was more sensitive in detecting *E. faecalis* in all cases and *E. faecalis* was more frequently found in retreatment cases than primary root canal treatment.[64] Culture techniques only picked up *E. faecalis* in 10% of cases whereas PCR did so in 79.5% of cases.

DNA detection methods found that the prevalence of *E. faecalis* in sub-gingival plaque and that of oral rinses were not significantly different.[48] The prevalence of *E. faecalis* in primary root canal infections was determined at >75% of canals (though by looking at the bar-chart provided in the article it was closer to 90%) and was one of three bacteria species with the highest prevalence (See Figure 2.1).[13] These authors studied 30 root canals, 15 canals each with and without sinus tract and found that *E. faecalis* was not related to sinus tracts. This is in stark contrast to those reported by

other authors such as Fouad *et al.*,[61] who studied the prevalence of *E. faecalis* in 40 root canals undergoing retreatment . The latter group of workers also used PCR but found a prevalence of only 22%.The vast difference in the prevalence may be due to the different DNA probes used by these groups.

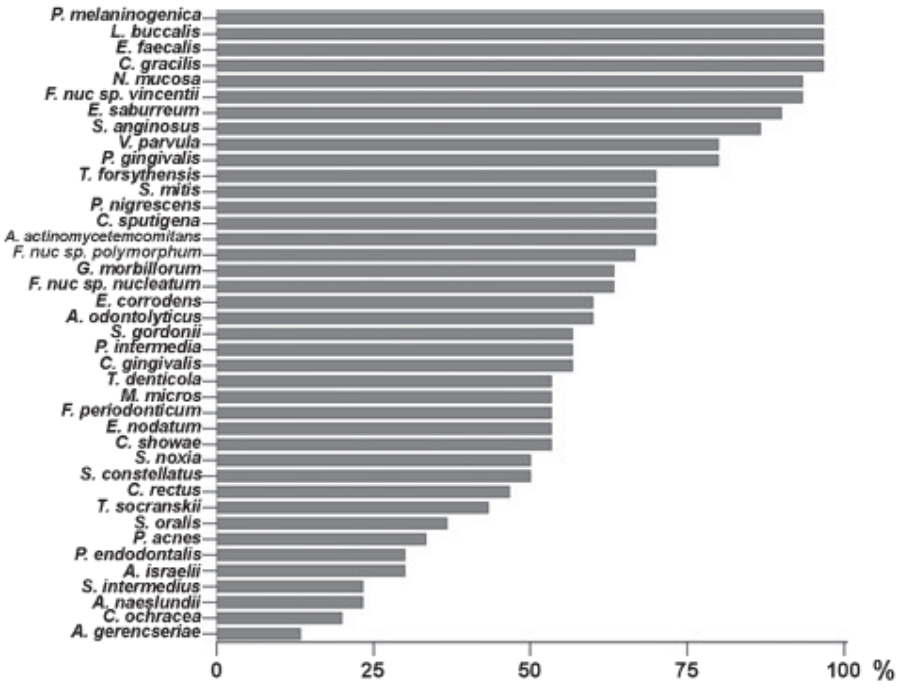

Figure 2.1 Bar chart of prevalence of the 40 test bacterial species in all 30 root canals.[13]

Whilst PCR seems more sensitive and is able to detect the presence of bacteria when culturing cannot, there are some controversies using PCR detection methods in endodontics. Although many would agree that the consortia of bacteria causing persistent symptoms in root treated teeth is highly variable and there are still many

bacteria that are yet to be identified,[65] *E. faecalis* seem to be commonly associated with it yet others take the stand and wonder if we are infatuated with *E. faecalis.* [31] It was pointed out that there was no known permanent bacteria tight seal provided by restorations to deny entry of bacteria from the mouth. In addition, there was no certain way of eliminating all traces of bacteria after they were effectively destroyed within the root canals. Hence, a positive test of presence of *E. faecalis* with DNA techniques may not be taken as proof that living *E. faecalis* were responsible for causing the persistent symptoms. While these are valid comments, they asked if Koch's postulate be invoked before we believe that *E. faecalis* is the main cause of persistent periapical lesions. It must be pointed out that Koch's postulate applies to identifying a single species of bacteria causing a specific disease with specific symptoms. A periapical lesion is hardly a specific disease that is more commonly due to infection by a mono-species of bacteria. In addition, bacteria in root canals have been shown to exist as biofilms[66] and single species biofilms of *E. faecalis* have been shown to be possible *in vitro*;[67] hence by conjecture, possible *in vivo*. Diseases caused by biofilms are chronic diseases and Koch's postulate does not apply. To this end, a new standard for DNA detection of bacteria causing specific disease had been proposed.[68]

2.2.2 Enterococcus faecalis the bacteria most often associated with failed root treated teeth

Clinically, the diagnosis of root canal treatment failure is by use of radiographs or more recently digitized computer tomographic imaging techniques. However, it had

been demonstrated using a risk assessment method that it is essential to follow-up for 4 years before deciding if healing has taken place.[69] One study reported the results of follow-up for between 20-27 years and found that what was considered failures or non-healing lesions at 10-17 years, healed after a further follow-up period of another 10 years.[70] The report also explained that delayed healing was usually related to extruded root filling material. Hence, if there was a periapical lesion that is not healed at 4 years and there was absence of extrusion of root filling material, it can be taken that the failure is likely to be due to the presence of bacteria.

Many species of bacteria are related to failed root canal treated teeth. In one study, 37 species of bacteria were isolated from 60 root canals. No cultivable bacteria were found in 15% of the canals. Though 25% of canals presented with polymicrobial infections, an overwhelming 46.7% had single species of bacteria infecting the canal and 13.3% had two bacteria. 18 of the 60 canals presented with *E. faecalis* as the sole bacterium infecting the canal.[32] Of the bacteria isolated, 57.4% were facultative anaerobes and 83.3% were of Gram-positive species. Other studies[26, 71, 25] had also reported infection by these bacteria in the same range. Sundqvist *et al.*[26] reported 58% facultative anaerobes with 87% of them being gram-positive whereas Molander *et al.*[25] reported 69% facultative anaerobes with 74.3% being Gram-positive. The most commonly isolated species were *Enterococcus, Streptococcus and Actinomyces*, a similar finding to that of Sundqvist *et al.*[21]

Though there are over 300 species of bacteria identified in the mouth, primary root canal infections usually harbour between one to 12 bacterial species. In studying the dynamics of root canal infections in monkeys it was shown that the flora progressively changed from one that involves facultative anaerobes to one that has more anaerobic bacteria over a 6-month period. Further, endogenous bacteria from the mouth in various mixtures if inoculated into a necrotic pulp would over time, also becomes a predominantly anaerobic infection. Selection through nutrient availability, low oxygen-tension and the combination of bacteria present at the outset to initiate the infection, all contribute to selection of the species later identified as being responsible for the infection. Bacteria present at the outset of infection are important, as they interact with each other in terms of adhesion to surfaces and in providing nutrients and other factors to each other [72].A report that studied the bacteria flora of teeth before and after root canal treatment tried to determine whether there was a pattern for certain bacteria to remain after chemo-mechanical treatment of root canals in teeth with apical periodontitis. They reported that in teeth with clinical and radiographical signs of apical periodontitis, non-mutans *Streptococci, Enterococci* and *Lactobacilli* commonly appear to survive following root-canal treatment.[73]

The treated root canal lumen likely provides microscopic ecological niches that are very different to that present in untreated root canals, where there may be a more plentiful supply of nutrients. Hence, the bacteria found in root canals of teeth with failed treatment are mostly Gram-positive facultative anaerobes and are different from those of primary root canal infection that are mainly that of Gram-negative and Gram-

positive anaerobic bacteria[32] examples of which include *E. faecalis*, *Streptococcus* spp*., P. micros, F. necrophorum.*[24] In failed root canal treatment, the bacteria flora associated are usually gram-positive facultative anaerobes.[74,75,26] The species included *Staphlococci, Streptococci, Enterococci, Peptostreptococci* and *Actinomyces* species. However, several papers all point out that the bacterium *E. faecalis* was the most frequently cultured species in failed root treated teeth [25, 26, 32, 33, 34, 58] and since then, there had been a deluge of papers studying various aspects of killing *E. faecalis* in the dental literature. An editorial in the a dental journal[31] thinks that the endodontic community is "infatuated" with this bacterium and it pointed out that presence of *Enterococci* in root filled teeth with periapical lesions are only as common as in teeth that are without periapical lesions.[76]

As had been shown earlier, if *E. faecalis* is one of the strains present before commencement of root canal treatment, it is likely to remain viable after root canal treatment. This shows the resilience of *E. faecalis* to the antimicrobial solutions presented to it during root canal treatment. Indeed numerous papers demonstrate that in an extracted tooth model, when compared to other bacteria species, for the same concentration of an antiseptic solution, *E. faecalis* is not so readily killed.[77] *E. faecalis* was recovered from 20% of teeth irrigated with CHX and root filled in one visit, 25% of teeth that had CHX irrigation and 14 days of CH treatment, 40% of teeth irrigated by CHX and root filled after 7 days. It had also been found to be left within root canals after treatment[73,78] when NaOCl had been used as the irrigant. Hence, its

relative toughness to chemicals used in root canal treatment may be the reason why it survives in the root canal after treatment.

It had been reported that *E. faecalis* can cause caries in an animal model.[47] This means that it is able to make use of dental tissues for survival. The strain of *E. faecalis* reported produced an enzyme that can degrade acid soluble and insoluble type-I collagen.[79] *E. faecalis* had been shown to be able to survive in root filled teeth *ex vivo* for up to one year. Thus *E. faecalis* entombed in the root canal by a root filling could provide a long-term nidus for further infection.[37] The micro-environment in which *E. faecalis* survives in can be further impacted upon by the thin film of root canal sealer used.[80] Together these studies show that *E. faecalis* can survive in the root canal and possibly make use of the dentine substrate as a source of nutrients.

Another reason why *E. faecalis* is so often related to failed root treated teeth may be because it modulates inflammation. Not only does it modulate the inflammatory process of gut lining epithelial cells,[42] sonicated extract of *E. faecalis* (SEF) had been shown to induce irreversible cell cycle arrest in phytohaemagglutinin-activated human lymphocytes. Using Caspase assay, the authors demonstrated that SEF-treated cells exhibited significantly increased apoptosis (56.7%) compared with phytohemagglutinin alone (28.1%). The authors concluded that if the irreversible cell cycle arrest induced by *E. faecalis* occurs *in vivo*, it may result in local

immunosuppression within the vicinity of the periapex and contribute to the persistence of periapical lesions.[81]

Hence, *E. faecalis* is a survivor after root canal treatment. If any calcium depleting agents had been used, collagen would be exposed on the surface of dentine. If the adhesion force between the substrate and *E. faecalis* was increased, we can further argue that it would be more difficult to remove *E. faecalis* by the flushing action of irrigants on bacteria from micro-niches. Hence, any *E. faecalis* introduced into the root canal during treatment would be left in the root canal after treatment .[82] It could also be as Sedgley *et al.* has proposed [64], that somehow after root canal treatment, there is leakage through the tooth. This is possible as many dentinal tubules can be exposed if the restoration has a marginal discrepancy with the dentine preparation and does not seal the tooth completely, exposing dentinal tubules near the margin.[83] Another possible rationale is that *E. faecalis* can migrate deeper into dentine in the presence of unmineralized collagen.[84] Once there, the surface tension of irrigating solutions within the lumen of the root canal can prevent the movement of the irrigant into such narrow confines readily.[85]Bacteria can bind to various forms of fibrillar collagen both through the recognition of the triple helical and denatured forms of collagen in a conformation-independent manner. Along the collagen molecule and triple helix, there are multiple sites for bacteria attachment.[86]Hence study of *E. faecalis* adhesion to dentine and type-I collagen after treatment by various irrigants commonly used in root canal treatment as well as adhesion after a sequence of irrigation would be useful in understanding the nature of *E. faecalis* infection.

2.3 The Substrates of Interest – Dentine and Collagen

2.3.1 Dentine Substrate

Dentine is a mineralized composite connective tissue with two phases – the organic and inorganic phases. The mineralized phase forms about 70% and the organic components forms about 20% of the weight of dentine. The remaining is water. On a volume basis, this would be about 50% and 30% respectively of inorganic and organic components.[87]

The mineral crystals of carbonated nano-crystalline apatite mineral phase is laid on a tight mesh of randomly oriented type-I collagen fibrils of about 50-100 nm in diameter. The mineralized crystals occupy both the sites on the surfaces as well as those within collagen fibrils.[88] Regularly spaced in the tissue are dentinal tubules. These run continuously from the dentin-enamel junction to the pulp in coronal dentin, and from the cementum-dentine junction to the pulp canal in the root. Many other proteins and enzymes important during the formation of dentine are trapped and are enmeshed in the mineralized mass. These non-collagenous proteins (NCP) which constitutes about 10% of the organic matrix, fall into several categories: phosphoproteins, Gla-proteins of the osteocalcin type as well as matrix Gla-protein, proteoglycans, different acidic glycoproteins, and serum proteins.[89]

It must be appreciated therefore, that a bias for the removal of the inorganic components of the mineralized composite material during root canal irrigation will expose the organic phase; and the organic phase loses the "protection" that it gets from the inorganic phase.[90] The proteinaceous organic phase now left devoid of the protection offered by calcified phase have many active functional groups such as carboxyl, hydroxyl and thiol groups. With the exposure of these reactive groups, surface charges, known to affect bacteria adhesion to dentine,[91] can be readily modified by chemicals applied on dentine. Proteins so exposed may also be hydrophobic and take part in hydrophobic interactions with bacteria. All these can act together to increase adhesion of bacteria. In addition, the NCP also include matrix-metalloproteinases (MMPs), enzymes that aid in breakdown of extracellular matrix proteins. MMPs found in dentine include MMP2, MMP8 and MMP20. Recently, it was reported that MMP8 is the major collagenase in dentine.[92] As both collagen as well as MMP8 may be exposed by removal of the inorganic phase such as when removing the smear layer of dentine, it could well mean that in so doing we may be helping to feed remnant bacteria in the root canals. The dentine collagen exposed by EDTA may be accessible to be broken down by dentine collagenase into smaller fragments and amino acids that provide nutrients to some species of non-saccharolytic bacteria and contribute to their survival.[71]

In addition, material had been shown to be extruded from the root canal during root canal treatment.[93] Hence, if there had been a biased removal of inorganic material from the surface of the root canal dentine, leaving organic substances bare on the root

canal surface, further instrumentation following such biased removal of inorganic substances may well mean that if there were any material extruded from the root canal foramen into the periapical area, it will most likely be organic in nature. The extrusion of organic substances such as enzymes released from dentine,[94] as well as the extrusion of EDTA, may impact periapical healing negatively.[95]

2.3.2 Type I Collagen

Collagen is a family of proteins in two main divisions, extracellular matrix molecules and non-extracellular matrix molecules. The common understanding of the term collagen are of fibrillar structural collagens of type I,II,III, IV,V, VI, VII,VIII,IX, XI , XII and so on. It is difficult to define exactly what constitutes collagen. There are 27 distinct genetic types of collagen.[96] Type I collagen, a hetero-trimer composed of two α 1(I) chains and one α 2(I) chain, expression of which are controlled by the genes Col1 A1 and Col 2 A2 respectively. They have a common repeating unit which comprises 3 polypeptides that form triple-helical domains: repeating triplets of Gly-x-y, high in imino acids, X – is often proline and Y- is often hydroxyproline; hence glycine is the third amino-acid throughout and 95% of the α-chains and large amounts of proline and hydroxyproline (up to 22% of all residues).[97] The collagen matrix of dentine is type-I collagen.[98]

Type-I collagen is the most abundant form of collagen in humans and provides mechanical strength to tissues such as skin, tendon, bone and dentine. It is a quasi-crystal or crystalloid because it is highly symmetrical and has essentially identical subunits[97]. Unlike other α–helices, the axial distance between one residue and the next is 0.286nm instead of 0.15nm. The dimensions of collagen however vary with the method of study, varying from 30 to 500 nm in diameter.[97]

Each of the helical trimer is left-handed, with 10 residues in 3 turns giving a pitch of 3nm. The three helical chains are coiled about a central axis to form a right-handed helix[99] with a repeat distance of about 10 nm. Each α-chain is just over 1000 residues and has a molecular weight of about 95kD.[99] Hydrogen bonding between the closely packed trimer is a major stabilizing force in stabilizing the secondary and tertiary structure of collagen.[100] There is only one amide bond per Gly-x-y, involving the X residue. The side chains of remaining amino acids in the remaining X & Y positions protrude from the main chain and can take part in many reactions including acid, basic, hydrophobic reactions and so on. The collagen triple helix is resistant to enzymatic degradation, pepsin digestion, below its denaturation temperature, though it is acid soluble.[101]

According to the online Medline Plus dictionary (available from http://www.nlm.nih.gov/medlineplus/mplusdictionary.html) to denature a protein is "to modify the molecular structure of (as a protein or DNA) especially by heat, acid,

alkali, or ultraviolet radiation so as to destroy or diminish some of the original properties and especially the specific biological activity". There are four levels of protein stereo-chemical structure.[102]

Table 2.1 Hierarchical Structure of Proteins

Level I	Primary Structure	linear sequence of amino acids
Level II	Secondary Structure	local, repetitive spatial arrangements of molecules
Level III	Tertiary Structure	three dimensional structure of native fold
Level IV	Quaternary Structure	non-covalent oligomerization of subunits into protein complexes

The four levels of protein structure as shown in Table 2.1 are stabilized by inter and intra molecular bonds as well as interchain hydrogen bonds. Denaturation of collagen disrupts these bonds and result in the loss of the fibrillar structure and the production of gelatine. (For a more complete review of collagen please refer to Collagens – Structure, Functions and Biosynthesis by Gelse *et al.*)[103] Changes to the structure of collagen can be monitored by various methods including mass spectrometry,[104] X-ray crystallography[105] and nuclear magnetic resonance amongst others. Structural change in soluble proteins however, can be monitored readily by circular dichroism (CD).

2.4 Chemicals used in Irrigation of Root Canals, their bactericidal effects

Instrumentation of the root canal is necessary to widen the root canal to allow chemicals into all parts of the root canal to disinfect all surfaces, as well as to shape the root canals to receive a root filling.[106] As root canal treatment is time consuming and there can be substantial re-growth of bacteria between the treatment sessions, an inter-appointment dressing with an antimicrobial medicament is usually applied.[107]

Many chemicals have been tried as inter-appointment medicaments including formocresol, eugenol, propylene glycol.[108] More recently, calcium hydroxide had become the chemical of choice and its use had been recommended to rid the root canals of bacteria and render them bacteria free, by culturing methods, before root filling[109]. With instrumentation of dentine during root canal treatment, a layer of adherent detritus called the smear layer, comprising dentine chips, pulpal tissues, bacteria among other material, form on the surface of the lumen. The smear layer had been found to allow more *E. faecalis* to adhere to instrumented root canal surfaces[110]. Although removal of the smear layer is considered to be essential for the root filling to abut against dentine directly, others consider its removal to be still controversial.[111] To remove the smear layer ethylene diamine tetra-acetic acid (EDTA), citric acid[112] and a proprietary solution Biopure MTAD (a mixture of a tetracycline isomer, an acid, and a detergent) [113] and other chemicals have been evaluated.

Many chemicals have been evaluated for irrigating and medicating the root canal during treatment including saline[114](when evaluating effects of mechanical preparation), sodium hypochlorite (NaOCl) in various concentrations,[115] Chlorhexidine (CHX),[116] iodine,[117] quartenary ammonium compounds,[118] electrochemically activated anolyte and catholytes,[119] among others. The purposes of using these solutions are to disinfect the root canal, flush out debris and dissolve pulp tissue. In this section, endodontic irrigants such as NaOCl, EDTA and CHX will be reviewed.

Irrigation of root canals with NaOCl and the use of calcium hydroxide (CH) as an inter-appointment dressing are common teachings in a large majority of dental schools from Europe, Scandinavia and the United States of America that participated in a survey[120]; as well as in Singapore. Use of EDTA alternately with NaOCl was also advocated in some of the schools that participated in that survey. The use of other irrigants, such as iodine, is uncommon and the use of electrochemically activated anolyte and catholytes is still experimental. Recently, one dental school in the United States of America advocated the use of CHX as their final irrigating solution.[121]

2.4.1 Sodium Hypochlorite

Sodium hypochlorite is a strong oxidizing agent and is a hydrolyzing agent.[122] Known to have a broad spectrum of anti-bacterial activity as well as proteolytic

activity, it had been in use in medicine as early as 1915 for treatment of infected wounds[123]; as Dakin's solution. NaOCl is toxic to human cells *in vitro*[124]; and can cause severe tissue reactions if injected beyond the tooth root into bone; including lip injury,[125] facial nerve damage[126] and life threatening events.[127]

Sodium hypochlorite exists as a balance of Na^+, OH^-, H^+ and OCl^- radicals in solution:

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

With tissues, it has three different reactions viz saponification, neutralization and chloramination. It degrades fatty acids into fatty acid salts, neutralizes amino acids into salts and water and combines with the amine group to form chloramines.[128] Usually made by bubbling chlorine gas through a solution of sodium hydroxide,[129] it has a high pH of 11-12, even when diluted. Its high pH together with its reactive oxidizing nature accounts for its effectiveness against a wide spectrum of bacteria found in root canals,[118] as many of the enzymes that keeps the intracellular environment of the bacteria constant are located on its membrane, and are easily accessible to it.[128]

NaOCl is the most commonly used chemical irrigant during root canal preparation in Australia[130] and USA.[131] It has many of the desired properties of an ideal irrigant:[106]

- broad spectrum of bactericidal activity
- dissolves pulpal tissue
- lubricate instrumentation
- low surface tension with high penetrability to areas inaccessible to instruments
- non-toxic and stable
- inexpensive

NaOCl had been used in endodontics for many years, most likely since before 1920[132] and firmly established in the 1940's with Grossman's publications of two papers "Solution of Pulp Tissues by Chemical Agents" [133] and Irrigation of Root Canals" [134]; both in the Journal of American Dental Association.

In 1957, Ostby published the first paper on irrigating the root canals with EDTA[135] and since then, the two solutions had been used in studies concerning root canal cleanliness.[136,137,138] An important physical property that has impact on its use in endodontics is its surface tension. This was reported to be 43 dynes/cm for the 5.25%

sodium hypochlorite and 41 dynes/cm for the 2.5% solution of NaOCl as compared to distilled water, which was reported to be 70 dynes/cm. Cetridexine, a surfactant containing 0.2% chlorhexidine gluconate solution had a surface tension of 32 dynes/cm.[139] Though the surface tension of NaOCl is lower than water, it is relatively high, such that it is less effective in killing bacteria established at a distance from the lumen of the root canal. Increased penetration into dentinal tubules has great implications on the amount of dentine removal necessary during mechanical preparation, to remove the zone of bacteria infected dentine around the lumen of the root canal as well as the duration of soaking of the root canal with disinfectants and other strategies necessary to disinfect it thoroughly.[140] In the latter study however, the surface tension of sodium hypochlorite was reportedly higher than that of water, though marginally.

Selection of the appropriate concentration of sodium hypochlorite to use in disinfection is a balance of the following factors: the rate and duration of use, the susceptibility of bacteria to NaOCl, the potential damage to dentine caused by NaOCl and toxicity of the chemical to cells involved in healing. Whilst removal of all the infected tissue and killing of all bacteria are desired goals of disease treatment, this must be balanced by the need for less tissue toxicity, which in turn can influence healing of periapical tissues and maintenance of structural integrity of the remaining tooth structure.

NaOCl solutions of higher pH are more effective at tissue dissolution. However, since it is the OCl^- ion that is effective in destroying bacteria, a lower pH would be more bactericidal.[131] With respect to necrotic tissue dissolution, a 3% solution seems to be sufficient[141]; 2.5% was sufficient to remove predentine to expose the mineralizing front in SEM micrographs.[142] With respect to tissue toxicity however, it was noted that sodium hypochlorite was ten times more toxic than it was bactericidal. A 0.5% sodium hypochlorite solution buffered by sodium bicarbonate to pH8.9 was found to possess good antimicrobial effect commensurate with the level of toxicity to HeLa cells.[143] In another study of periapical repair using histological sections in an animal model, there was poorer repair when canals were irrigated with 5.25% NaOCl as compared to using 2% chlorhexidine digluconate.[144] Clinically, using post treatment discomfort as an indicator, there was no increase in toxicity when using 5.25% NaOCl .[145]

2.4.1.1 Efficacy of NaOCl against Microbes in Root Canals

Waltimo *et al.* showed that *Candida albicans*, which were resistant to calcium hydroxide, when inoculated onto filter paper discs, could be killed by either 0.5% or 5.25% NaOCl within 30s; whereas 0.05% solution was ineffective. This shows that 5.25% had no real advantage and hence may be too strong a solution to be used.[146]Vianna *et al.* tested 0.5%, 1%, 2.5%, 4% and 5.25% NaOCl on planktonic bacteria in culture plates and found that 5.25% NaOCl killed *Candida albicans* in 15s whereas 0.5% took up to 30mins to be effective. [147]

Using 2.5% NaOCl, *E. faecalis*, which was found in one case before treatment, was eliminated by the end of the session. The duration of treatment was not reported nor was the total volume of the irrigant and the rate of irrigation specified.[147] In contrast, in a study of retreatment of teeth with root canal treatment that had persistent periapical lesions, Peculiene *et al.* found 21 of 33 teeth that was cultured positive with bacteria were infected with *E. faecalis*,. At the end of one visit, using 2.5% NaOCl and 17% EDTA, 6 out of the 21 canals still had *E. faecalis* persisting and 5 canals with other bacteria. Yeasts however, were eliminated.[148] Again, the volume and rate of irrigation and duration of treatment were not reported. These studies show that there are many variables with respect to chemical elimination of bacteria within the root canals, and the use of a bactericidal irrigant does not mean the elimination of all bacteria. Further, bacteria may exist in root canals as biofilms[67] and there are persisters in biofilms. Persisters are bacteria that do not get killed by a bactericidal agent that later produce populations that are indistinguishable from the original strain, within biofilms.[149]

In an earlier study, Bystrom and Sundqvist found that there was no necessity to use 5.25% NaOCl, the most popular concentration used, as a 0.5% solution could render 12 of 15 root canals bacteria free after the fifth treatment.[150] In another study, the same authors compared 0.5% and 5.25% NaOCl with and without use of EDTA in root canals of infected teeth. They found that there was no difference between the

antibacterial activity of 0.5% and 5.25% NaOCl. Use of EDTA was found to be advantageous.[151]

It must be remembered, that the organic load in root canals has a significant impact on the total available chlorine for bactericidal activity,[152] as well as the type of bacteria flora within the canals, cannot be standardized in clinical cases. *In vitro* studies using paper discs and planktonic bacteria in multiwell plates are also not representative of the clinical situation and hence of little value to predict how the chemical will behave within the root canal. Time of exposure and nature of bacterial infection are also important parameters.[153] It should also be noted that *ex vivo* and clinical studies using natural teeth with varied anatomy are very different from the controlled situation of the multiwell plates. These studies are also complicated by the fact that dentine itself is a very good buffer against a multitude of chemicals and its presence in the root canal can negate the effects of many chemicals intended for disinfection of the root canal.[154, 155]

When bacteria are killed in the root canal, gram-negative bacteria leave behind endotoxins - lipopolysaccharide (LPS), the major constituent of the external membranes of gram negative bacteria. These are potentially harmful if it leaches out of the root canal into the periapical area and sustain a periapical lesion, preventing healing.[156] LPS has been found in root canals as well as luminal walls of root canals.[157,158] NaOCl (2.5%) had been found to be more effective against bacteria but

not so effective in removing and/or neutralizing LPS.[159] Calcium hydroxide had been found to be effective in various studies in neutralizing LPS in dog teeth.[160,161] Irrigation regimes using NaOCl alone, without the use of calcium hydroxide had been found to be ineffective in removing LPS.[162] *With so many variables affecting the effectiveness of root canal irrigation, it is not surprising that there is little agreement about the appropriate NaOCl concentration to use.*

2.4.2 EDTA - Removing the Smear Layer

EDTA is a molecule for complexing metal ions. It is a polyprotic acid containing four carboxylic acid groups and two amine groups each with a lone pair of electrons. Many salts of EDTA are available but most commonly the di-sodium salt of EDTA is used as a 15 - 17% solution is used.[163]

Removal of the smear layer aids in providing a surface against which a water tight root filling can be placed.[164] The commonly used chemical is EDTA in solution and paste forms (proprietary products RC Prep and Glyde). Recently, a liquid irrigant that removes smear layer BioPure MTAD has become available. EDTA is often used as a 15% solution at neutral pH7. However, the mechanism of action of EDTA pastes may be different as it does not open dentinal tubules though it removes smear layer.[165] They are more effective in the cervical and coronal thirds of the root. Another study compared three different proprietary chelator pastes Calcinase-Slide (lege Artis,

Dettenhausen, Germany), Glyde-File (DeTrey/Dentsply, Konstanz, Germany), RC-Prep (Premier, Norristown, USA), and found that they affected the root canal dentine hardness to the same extent with no significant differences between them. However, there were significant differences between Calcinase-Slide and the other two in their ability to remove smear layer. Calcinase-Slide removed the smear layer in the coronal and mid-root sections more effectively. In the critical apical third however, there was no significant difference.[166] This may be related to the size to which the canal was widened (ISO size 50-60) and only sufficiently widened apical sections will allow the paste to penetrate to the apical extent of the canal. Clinically however, especially in posterior teeth with narrower canals, it is uncommon to achieve such large apical sizes. It leaves one wondering if such chelator pastes used with the belief of removing the smear layer; ever get to work where it mattered most – the apical third of the root canal.

Although about 500 articles (by electronic search in PUBMED) have been published about the smear layer, there is currently no clinical evidence that the removal of smear layer leads to an improvement of the outcome of root canal treatment, based on randomized clinical trials. There is only one histological study of *in vivo* removal of smear layer. The objective of which was to compare the debridement efficiency of a hand/rotary cleaning and shaping technique versus a hand/rotary cleaning and shaping /ultrasound technique as well as the use of a preparation technique with and without use of a one-minute ultrasonic irrigation. The ultrasonic irrigation group had significantly cleaner isthmuses; improvement of a

mere cleanliness range of 15-38% area cleaned to one with a cleanliness range of between 73-96% after one minute of ultrasonic irrigation.[163, 167] However, many authors continue to view the removal of the smear layer as reasonable to bring about a more thorough disinfection of the root canal system.[111] EDTA is also recommended in textbooks as a final irrigant to remove the smear layer.[163]

2.4.2.1 Susceptibility of Bacteria to EDTA

Whilst EDTA is used in the removal of smear layers, it is bactericidal when applied on some micro-organisms. EDTA effectively kills *Candida albicans* in the root canal.[168] However, EDTA is not bactericidal to *E. faecalis*.[169] The proprietary product RCPrep (3M Premier) contains a combination of urea peroxide, EDTA and glycol. In one study, specific combinations of the components were tested against *Streptococcus sobrinos* and were found to have synergistic bactericidal activity.[170] However, for killing the bacteria *Staphylococcus aureus* or *Streptococcus peltzer* within dentinal tubules, the application of the individual chemical urea peroxide for 45 minutes was more effective than 10 minutes. It was found that EDTA was shown to have differing bactericidal activity on these bacteria, with *S. aureus* being more susceptible.[171] EDTA was however not effective against *Micrococcus luteus* in dentinal tubules in another study .[172] EDTA also had been reported to render gram negative bacteria more permeable and hence more sensitive to antimicrobials containing chlorine, and hence their susceptibility to NaOCl.[173] By removing the divalent cations of proteins of the gram-negative bacterium outer membrane, EDTA

removes the stabilizing effect of these divalent cations[174] between neighbouring lipopolysaccharide molecules. The divalent cations neutralize the destabilizing repulsive electrostatic forces between these molecules.[175]

2.4.3 The sequence of irrigation in removal of the smear layer – Which of the two, NaOCl or EDTA, should be the final irrigant?

Ciucchi *et al.* studied the effects of NaOCl, ultrasound with NaOCl, EDTA following NaOCl and the sequence NaOCl with ultrasound followed by EDTA. They found that EDTA as an irrigant, after using 3% NaOCl during instrumentation, consistently removed most of the smear layer and that the additional use of ultrasound with EDTA did not bring about better results. Although they did not categorically state that EDTA should follow NaOCl use, the sequence of chemical use is nonetheless NaOCl followed by EDTA.[176]

Yamada *et al.* in a study of smear layer removal recommended final flush with NaOCl following use of EDTA.[177] Vassiliadis *et al.* found that removal of smear layer in the coronal part reduced microleakage.[178] In their study, the sequence of irrigation given was NaOCl, EDTA and final rinse with water. Barkhordar *et al.* in an *in vitro* study comparing the use of EDTA to doxycycline hydrochloride, used the sequence NaOCl-EDTA.[179] Gambarini in his study of the ability of a tensioactive (Triton 100) compound to aid in the removal of smear layer, used the sequence of solution 5%

NaOCl and a final flush with 17% EDTA were used for the control group. The test group specimens were irrigated using 17% EDTA, followed 15 s later by 1% TRITON X-100 (tensioactive agent) and then by 5% NaOCl.[180] Gilhooly *et al.* in their study comparing lateral condensation and thermomechanically compacted warm alpha-phase gutta-percha used EDTA followed by NaOCl to remove smear layer.[181] Peters and Barbakow in a study comparing smear layer removal and irrigation regimes for different rotary instruments , irrigated the controls with water and the test group alternately with NaOCl and EDTA but always finished with NaOCl as a final wash.[182] Froes *et al.* in their study used EDTA followed by a final flush of NaOCl.[183] Scelza *et al.* studied smear layer removal using three irrigation regimes and found that a citric acid group and NaOCl followed by EDTA gave cleaner dentine surfaces in scanning electron microscopy (SEM) than using a combination of NaOCl and hydrogen peroxide.[184] Al-Dewani *et al.* studied leakage of different root fillings and used the sequence of EDTA followed by NaOCl to remove smear layer.[185] Villegas *et al.* studied different irrigation regimes to see which combination of volume and duration of solutions of NaOCl and EDTA would clean more accessory canals so that they can be filled by root filling material.[186] Torabinejad *et al.* investigated various concentrations of NaOCl using a new chemical MTAD (a mixture of a tetracycline isomer, an acid and a detergent) as a final rinse.[187] Perez used a variety of EDTA mixtures as an irrigant following use of NaOCl as irrigant during preparation and found that an 8% EDTA mixture was as effective as 15% EDTA.[188] Tinaz *et al.* used SEM to study smear layer removal. They used NaOCl to irrigate canals during instrumentation and used passive ultrasound agitation of EDTA thereafter. In the

negative control group, they followed the EDTA irrigation with NaOCl rinse again, whereas their positive control group was irrigated exclusively by water.[189]

From the foregoing literature review, it seems that sequence of irrigation by NaOCl and EDTA is not fixed, though it is common to rinse the canal with NaOCl again after EDTA. Though all the studies above are *in vitro* studies, these are studies done to understand how the chemicals affect the cleanliness of the substrate. There is no clear understanding from these studies that the sequence of irrigation may play a role in root canal treatment outcome. Hence, it is unlikely that specialist endodontists who during their training read these articles are likely to pay much attention to the likely importance of sequence of root canal irrigation. The probability that collagen would be left exposed if EDTA was the last irrigant in the sequence of irrigation had been pointed out,[94] and this had been shown to be so by Tay *et al.*[190] If EDTA was the last irrigant, not only would the root filling be abutting against a bed of collagen, but also any bacteria left in some uncleaned portions of the root canal may get displaced onto collagen during root filling, collagen could well be a source of nutrients to these entombed bacteria. Once EDTA had been used in the root canal, about 3.8% remains in the root canal, despite efforts at drying the root canal.[191] Logically, in order to prevent the progression of the chelating action of remnant EDTA, irrigation with NaOCl after EDTA should reduce that likelihood, by NaOCl dilution and reaction with EDTA. However, recent research has shown this to be untrue. In a study using nuclear magnetic resonance, Grande *et al.* reported that though there was reaction between NaOCl and EDTA, the reaction was very slow and not clinically

meaningful[192]; and not expected to negate the effects of EDTA. Hence, once EDTA had been applied, we can expect that in some part of the root canal, collagen would be exposed. Dentine collagen can be dissolved by NaOCl.[193,194] Hence, even if EDTA might continue to work within the immediate future after dilution by NaOCl during the first visit of root canal treatment, at a subsequent visit, if no EDTA were used, fresh NaOCl would have an opportunity to dissolve any exposed collagen.

2.4.4 Chlorhexidine – some of its properties

Chlorhexidine digluconate is a clear liquid, colourless to very faint yellow in colour, with a molecular formula: $C_{22}H_{30}Cl_2N_{10}.2\ C_6H_{12}O_7$ and a molecular mass of 897.8. As a broad-spectrum bactericidal solution, it is commonly used for skin antisepsis. Used as a 0.12% solution, it had been effective in reducing total aerobes, total anaerobes, oral streptococci and actinomyces species in oral biofilms.[195] Chlorhexidine digluconate is more water soluble than chlorhexidine.

Chlorhexidine digluconate cannot be isolated as a solid, and is supplied commercially as a 20%w/v solution, though it is possible to prepare solutions of up to 50% w/v, the 50%w/v solution is usually too viscous for use. It is most stable in the range pH 5 – 8. Above pH 8, the chemical gets precipitated and below pH 5, the compound is less stable and there is likely deterioration of its bactericidal activity.[196] When compared to several irrigants commonly used in root canal irrigation, CHX

with cetrimide, had lower surface tension when compared to 17% EDTA, 5.25% NaOCl and the proprietary irrigants MTAD and Tetraclean.[197] Having lower surface tension has distinct advantages; it means that this chemical can diffuse more quickly and further into dentinal tubules. This would aid in the overall effectiveness of this chemical against dentinal tubule infection, as bacteria further away from the lumen and deeper within dentinal tubules may be killed.

2.4.4.1 Substantivity of CHX

This cationic bisbiguanide, is known to have a wide spectrum of activity against both gram positive and gram negative bacteria, yeasts[198] but not effective against mycobacterium and spores.[199] It is also not effective against viruses.[200] On bovine root dentine, chlorhexidine had been reported to have substantivity by up to 21 days.[201] Substantivity is the presence of activity which effectively prevents bacteria plaque accumulation on a surface. In another study however, the substantivity was reported to be 12 weeks .[202] CHX at high concentrations (at > 200μg/ml) fixes the cell and is bactericidal. Cells fixed by CHX are not lysed by lysozymes. CHX is bacteriostatic at low concentrations (< 90μg/ml). Cell membranes treated by low concentrations of CHX are quickly lysed by lysosymkes.[203],[196] CHX is cytotoxic to human fibroblasts[204] and concentrations as low as 0.12% may have effects on cell collagen synthesis.[205]

When placed directly in contact with human cells, chlorhexidine displays cytotoxic activity against osteoblastic, endothelial and fibroblastic cell lines in a dose and time dependent manner. It induced apoptosis by altering mitochondrial function, intra-cellular Ca^{++} increase and oxidative stress.[206] Using a fluorescence assay on cultured human periodontal ligament cells, 0.1% CHX solution showed corresponding cytotoxicity to 0.4% NaOCl .[207]

2.4.4.2 Use of CHX as a Root Canal Irrigant

It has been investigated as a root canal irrigant in recent years and about 70 studies had been published. In their study, Portenier *et al.* found that both MTAD (100%) and CHX (0.2%) were adversely affected by a marked delay in killing *E. faecalis* in the presence of bovine serum albumin(BSA) and dentine powder, though both these irrigants were effective against the tested strain of *E. faecalis*. [208] This implies that in weeping canals and canals with large periapical lesions where there may be accumulation of tissue fluids, use of chlorhexidine may not be effective in removing bacteria, as the presence of tissue fluids which contains serum proteins may interfere with the bactericidal properties.

Menezes *et al.* found that 2.0% CHX was more effective than 2.5% NaOCl in killing *E. faecalis* in root canals.[209] In root canals irrigated with 1% NaOCl during instrumentation, a final rinse with chlorhexidine left one of 12 root canals with

culturable bacteria whereas in the control group that did not receive chlorhexidine irrigation, 7 of 12 teeth had culturable bacteria at the end of treatment. The difference was statistically significant and may have clinical implications.[210] This shows that final irrigation with chlorhexidine may bring about a better outcome in cases infected with *E. faecalis*.

In *in vitro* experiments that compares the effectiveness of CHX against NaOCl, 1% CHX was as effective as 5% NaOCl.[211] Other *in vitro* studies comparing agar diffusion test and direct contact tests, found that the effect of CHX and NaOCl were affected by the means used to test its effectiveness, indicating that factors other than bactericidal effectiveness of the chemicals alone were being tested .[212]

Heling and Chandler in their study did not find any significant differences between the two chemicals in bovine teeth [213]. Vahdaty *et al.* however, found that at the same concentrations, CHX and NaOCl were equally effective against bacteria tested. [214] Bucks *et al.* however found that 0.5% NaOCl was more effective than 0.12% CHX in eliminating bacteria from dentinal tubules of root canals that had been exposed to those liquids for one minute.[85] Hence, ex vivo testing of CHX against NaOCl gave conflicting results. This may be due to variations in experimental protocols, rate of irrigation, canal anatomy and other confounders.

CHX however, cannot be used alone during root canal treatment, as it does not have tissue-dissolving capabilities. Without this capability, using it alone may be ineffective and residual tissues and proteins may have a detrimental effect on its bactericidal activity. Although *in vitro* studies seems to show that it is more effective against *E. faecalis* when compared to NaOCl solutions of the same concentrations, there is insufficient clinical studies to show that this is so.

2.4.4.3 Chlorhexidine and Dentine Collagen

Recently, chlorhexidine had been reported to have stabilizing effects on exposed dentine collagen. These studies mostly relate to bonding to dentine. Pashley *et al.* reported that 0.2% chlorhexidine inhibited the collagenolytic activity, probably brought on by matrix metalloproteinases, inherent in demineralised dentine.[215]

Phosphoric acid exposed dentine collagen in hybrid layers, not stabilized by application of chlorhexidine in the control group, degraded over 6 months in clinically functioning primary molars. Those treated with chlorhexidine displayed normal structural integrity of the collagen fibrillar network. Using 1% phosphotungstic acid and 2% uranyl acetate to stain for collagen without the use of CHX in transmission electron microscopy (TEM) sections could not detect collagen.[216]

This was confirmed in another study in permanent teeth. The authors used contra lateral pairs of resin-bonded Class I restorations in non-carious third molars, which were kept under intra-oral function for 14 months. Micro tensile tests and TEM imaging was carried out to assess dentine bonding. They found that using chlorhexidine improved the bond strength at 14 months and TEM showed that with use of chlorhexidine, normal architecture of the collagen fibrils remained whereas those not treated with chlorhexidine had undergone disintegration.[217]

Some reports also suggest that CHX may be more effective on bacteria that had been exposed to EDTA. Harper reported that by the addition of EDTA and Tris, a buffer, the concentration of chlorhexidine could be lowered to 0.01% and it was still effective against gram negative and gram positive bacteria including *E coli , P aeruginosa and S. faecalis* (now named as *E. faecalis*).[218] To this end, it would be beneficial to use chlorhexidine after use of EDTA to remove smear layer. Not only would chlorhexidine stabilize any exposed collagen, using chlorhexidine following the use of EDTA may make the chlorhexidine final rinse even more effective than anticipated.

2.4.5 Calcium Hydroxide

CH is a popular medicament or dressing of the root canal; used as an inter-appointment dressing, when there is insufficient time or when a clinician feels that an

infected root canal had not been disinfected sufficiently to be filled [219]. Many roles have been assigned to an intra-canal medicament. According to Chong and Pitt-Ford,[220] these include:

- Elimination of any remaining bacteria after canal instrumentation;
- Reduce inflammation of periapical tissues and pulp remnants;
- Render root canal contents inert and neutralize tissue debris;
- Act as a barrier against leakage from the temporary filling;
- Help to dry persistently wet canals.

CH, also known as slaked lime; is made by adding water to calcium oxide. Its chemical formula is $Ca(OH)_2$, it is a white powder or colourless liquid, has a molecular mass of 74.093 g/mol and a rather low solubility of about 0.185g/100 cm^3 at room temperature (25^oC). It has a pH of about 12 -12.5. CH had been used in many applications in dentistry since early in the 20th century. Other than as an intra-canal medicament, it is also a pulp-capping agent; it is used for apexification - to allow maturation of root apices of pulpless teeth, in traumatized teeth to prevent resorption and in pulpotomies. Its use is related to its bactericidal high pH and ability to stimulate repair and mineralization[221]; many micro-organisms of the endodontic flora are unable to survive in the alkaline environment .[222] *In vitro* tests of CH in contact with bacteria showed that the hydroxyl ions released by CH damaged the cell membranes, denatured proteins of bacteria [223] and damaged DNA.[224]

2.4.5.1 Perfusion of hydroxyl ions through dentine

Hydroxyl ions from CH diffuse through root canal dentine. In an *in vitro* study, Norwich *et al.* using microelectrodes in small cavities buried in the apical and coronal parts of the root, both near the lumen of the root canal and on the exterior of the tooth, found that the pH at the lumen was raised to pH10.8 at the cervical and pH 9.7 at the apical root. It took from 1 to 7 days for the pH on the exterior of the root to rise and it reached a peak after about 2 to 3 weeks to pH 9.3 at the cervical and pH 9 at the apical portions of the root.[225] Hosoya *et al.* studied the diffusion of calcium and hydroxyl ions through the apices of the roots of teeth by measuring the calcium concentration and pH change of distilled water in which the test sample was deposited. They found that mixtures of CH with distilled water at 38%, 44% and 50% had quicker and higher pH changes. With the mixtures, calcium peak concentration was demonstrable at 3 days when compared to the powder only group, which took 7 days. Hydroxyl ions however took longer to peak; for the mixtures, it was at 14 days, whereas for the powder it was 49 days.[226] They suggested that CH dressing should be kept in the root canals for about 14 days. The peak pH reached by the 44% mixture of CH was pH 11.24. Hence, a mixture of at least 50% should be used for duration of 14 days. A similar study using different calcium hydroxide preparations by different workers has also confirmed that the pH change reaches these levels.[227] The perfusion of these ions through the apices is advantageous to endodontics as infection has been reported in and near the apices in 10 of 12 teeth with periapical lesions.[228]

The carrier medium in which CH is mixed with can affect the effectiveness of the chemical. The study by Hosoya *et al.*[226] showed that presence of water was important and another by Alacam *et al.* [229] showed that water together with glycerine facilitated ionic diffusion better than water alone. In the latter study even when the dentine thickness was about 0.66mm from the lumen, the pH was only raised to 10.1 and does not reach the pH 12 of the CH pastes or solutions.

2.4.5.2 Root canal contents may inactivate effects of CH.

Studies have also shown that dentine itself can negate the effects of CH. In an *in vitro* study Haapasalo *et al.* mixed 50 µl saturated CH solution, 0.5% and 0.05% chlorhexidine acetate and iodine potassium iodide (IKI) in 2/4% and 0.2/0.4% (I:Ki) solutions and 5.25% NaOCl solutions with a suspension of 28mg/µl of dentine powder in 50 µl distilled water and 50 µl of bacteria suspension. They found that dentine powder had an inhibitory effect on all medicaments. If the medicament had been preincubated with dentine powder before bacteria was added, the effect of inhibition was dependent on the concentration of the solution and the time in contact with dentine preincubation. Saturated CH solution was completely inhibited. That of 0.2/0.4% IKI was also lost whereas the effect of 0.05% CHX and 1% NaOCl was reduced but not fully negated and no inhibition of 2/4% IKI solutions and 0.5% CHX was measured.

In a subsequent study, Portenier *et al.* studied the effects of dentine, hydroxyapatite (HA) and BSA on bactericidal activity of CH , CHX and IKI solutions; using *E. faecalis* as the test bacteria.[155] They found that all the three test inhibitors preincubated with the test solutions for one hour prior to addition of the test bacteria, abolished the effects of saturated CH solution. The completely inorganic HA had little effect on CHX, but dentine powder affected the solution such that at one hour after addition of bacteria, there was no reduction in the number of bacteria in the solution. Over 24 hours however, the bactericidal effect of CHX was marked and no bacteria could be cultured. BSA had the most profound effect on chlorhexidine – after 24 hours, the colony forming units (CFU) of the solution was about 20% of controls. BSA and HA did not adversely affect IKI, but it was completely negated by dentine powder.

These reports highlight the importance of the presence of proteinaceous substances that affect different chemicals used in irrigation. Of the three tested inhibitors, serum and dentine powder should be expected in instrumented root canals, strategies at eliminating bacteria with irrigants in root canals should take this fact into consideration, and the use of more than one bactericidal solution may be a good policy.

Sigurdsson *et al.* studied three methods of filling the root canals of molars with CH. They evaluated the quality of the CH filling using radiographs. They found that using a Lentulo spiral gave the best quality fill to the full extent of the root canal.[230] Twisting a K-file in a counter-clockwise direction did not achieve a good result.

Deveaux *et al.* in a separate study using Gutta-Condensor, MecaShaper, K-type ultrasonic file, Lentulo, and Pastinject (a proprietary injector) to place CH in premolars, found no significant differences between the methods. They too had used radiographs, but had in addition, used an image analyzer software to assess the quality of the filling by CH. [231]

Torres *et al.* using plastic block canals with curvatures of about 40 degrees tested 3 techniques – injection only, injection and Lentulo and Lentulo only. They assessed the quality of fill using radiographs and image analyzer at 1, 3, 5, and 7 mm from the canal terminus and found that at 1mm Lentulo spiral gave the best fill whereas at 3mm from the apex, the Lentulo only and the combination technique were not significantly different.[232]

It seems that using a Lentulo would be helpful in filling the root canal with CH. Other methods may not give such a good filling and it may affect the outcome of the treatment. These studies also point to the importance of taking radiographs to check

the fill by CH, especially in cases where the chemicals are used intentionally to disinfect the canals in between visits.

In a histopathological study of periapical lesions in dogs' teeth, Leonardo *et al.* compared two methods of treatment; one using 5.25% NaOCl followed by CH for one week the other used 0.5% NaOCl and root filled immediately. They reported a better outcome with use of 5.25% NaOCl than 0.5% NaOCl.[233] This study however cannot tell us if it was the higher concentration of NaOCl alone that brought about the better success.

Weigar *et al.* in their study recalled 67 of 71 patients (92%) after a 5-year follow-up period. They compared two groups of teeth, one in which 1% NaOCl had been used together with CH for at least 7 days to another in which the canals were prepared and filled in the same day. If CH was used, it was packed into the canals with paper point and no radiographs were taken to check for the fill of CH in the teeth. They reported that there was insignificant difference between the two treatment methods on outcome, but the co-variable – size of periapical lesion >5mm made a difference. They concluded that one visit root canal treatment was a viable alternative to two-visit treatment that included use of CH, since both methods, over an observation period of 5 years, gave a probability of success of 0.9.[234] This conclusion however, is interpreted by accepting that the CH had been effectively placed, by means of compacting the paste with paper points.

Katebzadeh *et al.* evaluated the extent to which CH alone, without the use of NaOCl, could influence the outcome of root canal treatment in dogs' teeth. There were sixty teeth in 4 groups - instrumentation using saline irrigation and then root filled, instrumentation with saline and CH for one week before root filling, positive and negative controls. They found that the group treated with CH were mostly in the improved (healing incomplete) and healed groups. Comparing the healed groups – there was no statistical significance between CH use or not. Mere irrigation with saline had a failure rate of 90% whereas instrumentation with saline had failure rate of about 40%.[235] This study shows that instrumentation alone without the use of any disinfectant can bring about healing. It also shows that when CH was used in the two-step treatment more healing takes place, though not significantly different as the power of statistic in this instance was 43, when in each group they had only 24 roots.[235]

Sjogren *et al.*[52] used 0.5% NaOCl irrigation in a clinical study to evaluate the effect of the presence of bacteria at the time of root filling on outcome. All teeth were filled in one visit without use of CH. At the end of one visit, 22 of 55 root canals had been rendered bacteria free. Of those without bacteria at the end of the visit, 94% had healed. In the group that had bacteria at the end of the visit, only 68% had healed. Though there was statistical difference, it does bring to our notice that the presence of bacteria does not mean failure. Perhaps, the type of bacteria and host response may have confounded the outcome. In one of the case that had a negative culture, but

which failed, histology of the root tip later showed the presence of bacteria at the apical part of the root canal – demonstrating that the sampling technique for bacteria was not foolproof.

In contrast were the results of Peters *et al.* [231] who evaluated if the use of CH made a difference to the fate of the bacteria remaining in root canals. They sampled the canals for bacteria at the beginning and end of each of two visits. They found that there was no significant difference between the number of bacteria remaining within root canals after the first and after the second visit. There were some canals, of the 43 teeth studied, that had negative cultures after one visit. During instrumentation, 2% NaOCl was used. At the second visit however, before the commencement of treatment, they discovered that the number of bacteria positive canals had increased. They concluded that the use of CH for 4 weeks, inserted into root canals using Lentulo spiral instruments, suppressed but did not prevent the regrowth of bacteria.

Peters and Wesselink in a follow on study undertook an *in vivo* clinical study to observe the effects of one visit (without) or in two visits with an interappointment dressing of CH on healing of periapical lesions; as well as influence of the presence of bacteria at the time of root filling on healing. Healing was observed over a period of 4.5 years. They found no significant difference between use and non-use of CH. The presence of infection at the time of root filling did not affect the treatment outcome[236]. However, removal of infection before root filling was important. If the root canal

remains infected at the time of root filling, it is incumbent upon the root filling to entomb the bacteria within dentine as well as within the mass of the root filling, both gutta-percha and sealer .[237] The quality of the root canal filling, especially the extent of it in relation to the apex of the root, becomes important.

Though important, it has not been consistently shown that with irrigation by NaOCl and followed by the use of CH, we can obtain a root canal free of bacteria. The studies mentioned above demonstrated that culturing at best gives an inaccurate assessment of the sterility of the root canals. The complexities of root canal anatomy are likely to contribute to this inaccuracy in the culturing process using paper points. Canal complexities also make it difficult for clinicians to effectively apply intracanal irrigants and medicaments evenly within them, to obtain the desired outcome.[224] In addition, some species of bacteria such as *E. faecalis* have been shown to creep deeper into the dentinal tubules than others[238,239,240]; where the diffusion of chemicals may not take place sufficiently to kill them and these can seed new growth into the root canal to confound the results of cultures.

Some bacteria may be more difficult to kill by using the strategy of a high pH. *E. faecalis* is one such bacterium. It has been reported that this bacterium has a proton pump that helps maintains its internal environment,[241] so that a pH of < 11.1 would not kill it. Hence, despite antimicrobial treatment during instrumentation and use of intra-canal CH medicament; bacteria can remain in the root canal. Those bacteria

most often found after root canal disinfection procedures are usually gram positive and include Enterococci, non-mutans Streptococci and Lactobacilli.[73]

2.5 Effect of Irrigants on Dentine Substrate

Mechanical instrumentation of the root canal alters its physical form for the purposes of irrigation and root filling. Chemical irrigation for the purpose of killing bacteria however is also going to alter the surfaces irrigated chemically. Changing the physical and chemical nature of the luminal surface of dentine, the fundamental substrate of endodontic treatment and root canal irrigation, is certain to alter the niche environment of the root canal in which infective bacteria live. Together with root canal anatomy complexities, these are likely to form the key determinants of nearly all disease and post-treatment disease processes in root treated teeth.

2.5.1 NaOCl – Its effect on Dentine

Going by the argument of balancing toxicity versus bactericidal activity,[143] the operator should also understand that there is a need to balance bactericidal activity against the extent of damage to dentine. Soaking dentine in sodium hypochlorite removes protein from it.[242] This leads to a significant loss of indentation strength, by about 50%, after 20s etching and deproteination for one minute with sodium hypochlorite 6.5% .[243] Consequently, if root canal treatment were undertaken over two visits, we should ask whether we really need to use EDTA to remove smear layer

after the first visit, and consider the care we need to take in handling dentine in the subsequent visit should we choose to use EDTA in the first. As NaOCl treatment removes the organic portion of the substrate,[242] it can bring about changes in the physical properties of dentine leading to a loss of flexural strength, elastic modulus and micro hardness of dentine. It could also come about by increasing the permeability of dentine making it more permeable to fluids, which can aid in survival of bacteria remaining in the root canal after treatment. It could possibly increase the roughness creating, niches for bacteria to survive in where they are not readily removed by the flushing of irrigants and changes in surface energy that allow bacteria to become more adherent.

Mechanical property testing of dentine bars after treatment with various chemicals, including groups treated with 3% and 5% NaOCl for two hours had been reported. The authors had carefully chosen this duration, as this was the time required to finish the root canal preparation of a molar. They reported that there was highly significant loss of flexural strength as well as loss of modulus of elasticity, using the three point bending test.

There was greater loss with use of 5% than with 3% though the difference was not statistically significant.[244] Marending *et al.* investigated the mechanical, chemical and structural alterations to human root canal dentine after exposure to 1%, 5% and 9% NaOCl for one hour. They used the three point bending test to study the mechanical

property change. Immersion in 1% NaOCl did not cause a significant drop, by half, in elastic modulus or flexural strength values in comparison to water, whilst immersion in 5% and 9% hypochlorite reduced these values by half. They also reported that micro elemental analysis showed that there was significant loss of the elements C and N with 5% and 9% NaOCl but there was little loss with use of 1% NaOCl solution. Using light microscopy, they showed that there was marked increase of surface porosity to basic Fuchsin dyes after dentine specimens were treated by 1% NaOCl. Using backscattered SEM micrograph, they reported that at all concentrations tested, there was no damage to the inorganic phase of dentine.[245] The increase of micro porosities that allowed dyes to penetrate dentine makes us realise that in the sequence of irrigation, irrigation using EDTA after NaOCl would likely allow EDTA to penetrate these porosities quickly, very likely increasing the effect of that chemical on the substratum. Barbosa *et al.* also reported the increased in permeability of dentine by a factor of greater than two. They had studied the hydraulic conductance of human dentine discs before and after soaking in NaOCl and concluded that NaOCl affected the characteristic of the whole dentine.[246]

Perhaps also because the micro porosities have increased in dentine following NaOCl irrigation, Vickers hardness measured using a Vickers diamond micro hardness tester showed that dentine was affected by 1% NaOCl used for a duration of 15 minutes, significantly.[247] In a follow-on study, Marending *et al.* studied the effects of 24 minute treatment using 2.5% NaOCl and reported that there was significant loss of flexural strength.[248] Sim *et al.* also tested dentine samples after 2 hour treatment

with NaOCl. They found that 5.25% NaOCl lowered the resistance of dentine to strain test significantly when compared to 0.5% NaOCl and saline irrigation regimes. For the latter two, strain tests remained largely unchanged. They also tested whole tooth samples that had been decoronated and had the enamel removed. The use of 5.25% NaOCl also altered the flexural strength and strain response of the entire tooth.[249]

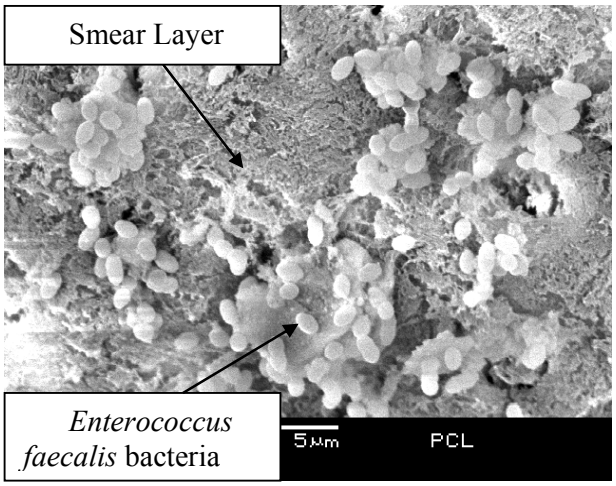

Figure 2.2. Scanning Electron Micrograph of Dentine with adherent *E. faecalis* ATCC 29212 on the smear layer. Few tubular openings are just visible.

Dentine instrumented and irrigated by NaOCl alone, was reported to show a smear layer. Figure 2.2 is an SEM micrograph that shows cut dentine with *E. faecalis* cells on smear layer. There are two zones to the smear layer, what is on the surface and the other part in the dentinal tubules. The surface layer is usually 1-2µm thick whereas the tubules are filled with the debris up to a depth of 40µm.

2.5.2 EDTA - Its effects on dentine

When EDTA is used to irrigate the root canal, the smear layer is removed. Many studies also report that when the concentration and duration of application is increased, the effect on the substrate is also increased. Texiera *et al.* [12] studied the effects of variation of time of exposure of irrigants on cleanliness after EDTA and NaOCl irrigation to remove smear layer, and showed that 1 min treatment using 1% NaOCl and 15% EDTA could provide smear free dentine surfaces as shown in Figure 2.3.[12] They showed that using 3mls of 15% EDTA and 3mls of 1% NaOCl each over a one-minute period removed the smear layer completely in the coronal and mid root sections. Many sections in the apical sections had some smear layer left. According to Nygaard Ostby, [250] who used polarized microscopy to look at cross sections of roots irrigated with 15% EDTA over different durations, there was a clear zone around root canal lumen irrigated by EDTA . He obtained a zone of penetration of about 20-30 μm after 5 minutes.

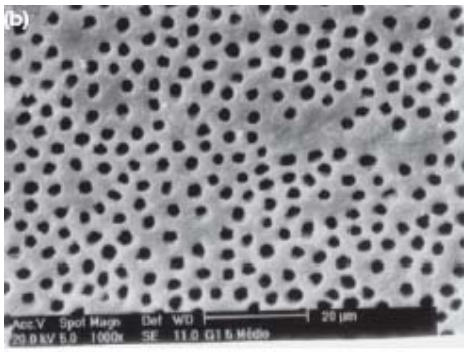

Figure 2.3 Photomicrograph from Texeira *et al.*[12] mid-root sections after 1 minute irrigation using 1% NaOCl and 15% EDTA. Micrograph shows dentine without smear layer.

Most of the studies of EDTA on dentine relate to cleaning of the smear layer and effects of EDTA on the mechanical properties of dentine after EDTA treatment. Oconnell *et al.* [251] and Calt and Serper [252] are recent papers that discuss the effectiveness of EDTA removal of smear layer. Calt and Serper however, noted that extending the time of EDTA use in the root canal would cause erosion and they do not recommend irrigation of the root canal for more than one minute. Ari *et al.* [253] and Marending *et al.*[248] are examples of papers that discuss the effect of EDTA on mechanical properties of dentine after irrigation.

Few if any paper relate to the possible effects of EDTA to the biology of the "root-canal- periapical tissue complex". Segura *et al.* however reported that with use of EDTA, there is some extrusion of the solution and even the very small amounts extruded are in concentrations high enough to affect the binding of macrophages to substrate[95]. As adhesion is the first step in the phagocytic process and in antigen-presentation, any leakage of EDTA to periapical tissues during root canal preparation may inhibit macrophage function and reduce periapical inflammatory reactions and thus has implications on periapical healing.

Whilst most agree that the removal of the smear layer is controversial, some feel that the use of EDTA as an irrigant is questionable. This was due to the extent which it affects dentine permeability, its predilection for removal of peritubular dentine; its

ineffectiveness in softening dentine at the apical end of root canals for easier instrumentation to the apex of the canal.[254]

2.5.2.1 Biological Effects of EDTA on Dentine

Recently, immunohistochemistry techniques have demonstrated that matrix metalloproteinases -2 and 9 (MMPs), a family of peptidases involved with degradation of the extracellular matrix components, are found in mineralized dentine[255]. MMP 8 has also been demonstrated to be the major collagenase in non-autoclaved dentine[92]. Mild self etching adhesives that demineralises dentine to expose collagen, was also shown to activate MMPs and increase the collagenolytic activity of these enzymes by >14 times.[256] Hence, dentine may apparently be inactive, but it contains biologically active substances that can be unleashed by chemical treatment.

Understanding the chemical nature of irrigated dentine would give us a better understanding of how *E. faecalis* may persist on dentine after chemical irrigation. Even a one minute application of 3% EDTA (0.1M) would expose collagen.[257] Though we may follow up with NaOCl irrigation following EDTA use, it is unlikely that the effects of EDTA would be negated completely. [192] Remnant collagen is likely to remain in the lumen of the root canal and together with any activated MMPs not removed, these could have implications on the survival of bacteria remaining in the

lumen of the root canal. Exposure of collagen also has implications on invasion of dentinal tubules by bacteria. Love *et al.* had demonstrated that collagen aided *E. faecalis* penetration of dentinal tubules in the presence of serum.[258] Further *E. faecalis* has been reported to have a specific virulence factor, a collagen binding protein named ACE[60] . This specific binding protein ACE has been shown to aid adhesion of *E. faecalis* to particulate dentine. [259] Hence exposing any collagen in dentine would introduce haptotaxis – attraction by surface bound chemical factors into the equation of *E. faecalis* colonization of dentine. In addition exposure of collagen may increase the adhesion of other Streptococci species to dentine[260]; and some Streptococci species responded by adapting to growing long chains which aided their penetration into dentinal tubules[261]; contributing to failure of root canal treatment failure by other bacteria species.

Exposure of collagen in the root canal also has implications on the long-term outcome of restored teeth. Ferrari *et al.* reported that collagen unintentionally exposed by the acidic nature of zinc phosphate cements used to cement posts and cores, deteriorates over time.[262]

Though both, EDTA and NaOCl, are used routinely during root canal treatment, little is known of their effects on dentine collagen[263], although it is commonly known that NaOCl deproteinates[242] and EDTA demineralises dentine[264], and can cause damage[12]. As more microorganisms had been reported to adhere to denatured

collagen, knowing if irrigants used during root canal treatment denatured collagen would enlighten us if denaturation of collagen may have contributed to why there may be more *E. faecalis* associated with failed root canal treated teeth.

Studies have reported that though dentine irrigated by EDTA may no longer have smear plugs in the dentinal tubular openings, the surfaces of root canal dentine, particularly in the mid-root and apical third portions, are not clean.[265] In fact the micro-Raman spectroscopy spectra of the EDTA-treated smear layer, was found to be similar to demineralised dentine but without contribution of the mineral phase. This indicated that the disorganized collagen within the smear layer was never removed but was not denatured; the mineral that was trapped within the gelatinous mass had shielded it from detection. [266] Therefore, contrary to popular belief, EDTA does not remove smear layer. It is important therefore to understand the interaction of *E. faecalis* to collagen. Our interest in denaturation of collagen stems from the fact that it had been reported that adhesion of certain micro-organism to denatured collagen was more than to native collagen.[263] Further, *E. faecalis* had been reported to have an adhesin ACE which binds to collagen,[267] and the binding is in a hug model.[46]

Of the commonly used root canal irrigants, NaOCl is a known oxidative reagent. Oxidative damage denatures proteins and causes diseases in man.[268] On the other hand, EDTA had been used in protein extraction and had been shown not to alter the enzymatic properties of certain proteins[269] though it had been shown to alter the structures of proteins that contains zinc.[270]

2.5.3 Other Smear Layer Removing Irrigant – a proprietary product

Recently, a new proprietary product aimed at both disinfection as well as smear layer removal have become available – Biopure MTAD. MTAD is an acronym of a mixture of a tetracycline isomer, an acid, and a detergent.[271] It is proposed that this solution should be used as a final rinse on the surface of instrumented root canals at the end of instrumentation to remove the smear layer just before root filling. Use of low concentration of NaOCl during instrumentation is encouraged.[187] and effects on mechanical properties of dentine are reported to be minimal and not significantly different from dipping dentine into saline.[272] Its use is reportedly able to condition dentine in such a way that there was no further need to apply dentine conditioners before the application of dentine adhesives.[273] However, with regards to elimination of *E. faecalis* infection, there is no difference in antimicrobial efficacy for irrigation with 5.25% NaOCl/15% EDTA versus irrigation with 1.3% NaOCl/Biopure MTAD in the apical 5 mm of roots infected with *E. faecalis* .[274]

2.6 Adhesion of bacteria on dentine and collagen substrate

There is little work done on the effect of chemical treatment and bacteria adhesion to dentine. Bjorvatn studied the effect of aqueous solutions of doxycycline HCl on plaque formation in seven volunteers. Dentine samples were impregnated with

doxycycline HCl and ligated to buccal surfaces of teeth of the volunteers. There was less plaque formed on dentine after doxycycline impregnation.[275]

Weerkamp *et al.* studied the effect of salivary pellicle on Zeta potential of dentine and the adherence of bacteria to the coated or uncoated dentine. They found that there was wide variation in adherence of different bacteria as well as differences of the same bacteria type when the dentine was coated.[276]

Calas *et al.* treated root canal dentine samples with citric acid of differing durations and use or non-use of NaOCl after citric acid. They reported that 6% citric acid for 2 min followed by 6.25% NaOCl for 1 min and 6% citric acid for 10 min reduced the adherence of *Streptococcus sanguis* on bovine dentine.[277] In another report, Calas *et al.* reported that removal of the smear layer reduced the adhesion of *Prevotella nigrescens* .[278] The latter results was also reported by Yang and Bae .[279] The foregoing papers suggest that whether bacteria adherence increases or reduces in the presence of smear layer, may be specific to the species. *These reports emphasizes again that using a single idea – removing or not removing smear layer, is not sufficient to deal with all the bacteria that may be present in the root canal.*

Drake *et al.* reported that root canals with smear layer had less *Streptococcus anginosus* (*milleri*) colonizing them than those that had smear layer removed by EDTA followed by NaOCl irrigation, as blocking the dentinal tubules may be a

mechanism in reducing colonization.[280] A similar report by Love,[281] using *Streptococcus gordonii* on smeared and non-smeared dentine supported the finding and the mechanism – that patent tubules retained more bacteria.

MacGrady *et al.* reported that collagen-binding strains were found to adhere to collagen-coated surfaces, while strains unable to bind collagen adhered to a much lesser extent when testing different collagen binding and non-binding strains of bacteria to collagen in micro titre plates.[280] Hubble *et al.* reported on the binding of *E. faecalis* to dentine of root canals. They tested the hypothesis that the *E. faecalis* proteases, serine protease and gelatinase, and the collagen-binding protein (ACE) contribute to adhesion to the root canal. They concluded that ACE as well as serine proteases helped the binding of *E. faecalis* to dentine but could not decide if gelatinase had contributed to it. [282] Yang *et al.* studied the binding of *E. faecalis* to bovine dentine blocks and reported that removal of the smear layer reduced adherence of *E. faecalis* and CHX helps prevent adhesion of *E. faecalis*. [110]

There is no study on the force of adhesion of *E. faecalis* to dentine after various irrigants and after a sequence of irrigation. Understanding the adherence of *E. faecalis* to dentine after various irrigants and the sequence of chemical treatment will contribute to a better understanding of why *E. faecalis* is associated with root canal treated teeth with persistent periapical lesions. To this end, it has been shown that it is possible for a reference strain of *E. faecalis* to form biofilms on dentine *in vitro*.[67] The

effect of different body fluids on the propensity for *E. faecalis* to form biofilms on dentine has also been reported. The adherence of *E. faecalis* to dentin was increased after starvation and when dentin was coated with saliva indicating that coronal leakage of saliva and the physiologic state of microbes might play an important role in their adherence and biofilm formation of bacteria to root canal dentin.[10] Coating with serum lead to moderate adherence whereas dentine blocks kept in water and phosphate buffered saline did not have *E. faecalis* adherence.

We did not find any study that characterizes the chemistry of the dentine surface after various irrigants and sequence of irrigation with chemicals commonly used in the clinic and bacteria adherence. These changes in chemistry by irrigation of different chemicals during root canal chemo-mechanical preparation, alters the environment that may be selective for a certain species of bacteria, perhaps *E. faecalis*. It had been pointed out that the root canal lacks oxygen and lack nutrients,[283] one avenue of selection pressure. The changing available nutrient source offers one avenue of selective pressure.[71]

Since collagen would be exposed following use of EDTA, it should be useful to determine how type-I collagen would behave if it is irrigated by chemicals used in root canal treatment. Interaction between irrigated collagen and *E. faecalis*, would give us a better idea which irrigant we should use to reduce the propensity of *E. faecalis* adherence on exposed dentine collagen. Having this knowledge would give

us a better understanding of how we can use existing chemicals in such a way as to reduce *E. faecalis* colonization of dentine. This was because adherence of bacteria is the first step to infection.[284] Whilst this may be considered somewhat reductionistic, it would give one perspective if selection for *E. faecalis* may be present. It is acknowledged that selection by changes in bacteria consortia within the root canal may be one of the other perspectives which should be seriously considered. [285]

2.7 Clinical Significance of Bacteria Adhesion and Biofilm Formation

Whilst the study of planktonic bacteria cells is a valid way of studying the physiology of bacteria, it does not accurately reflect the way in which bacteria interacts with the real world. Recent studies have shown overwhelmingly, that bacteria associates and adheres to surfaces as a survival strategy.[286] In that paper the authors reported that in all aquatic systems, where there were sufficient nutrients, bacteria form glycocalyx-enclosed adherent biofilms to surfaces. Within the biofilms, the populations of bacteria attain physiological and numerical predominance.

A biofilm is a community of micro-organisms irreversibly attached to a surface. They produce extracellular polymeric substances (EPS), and exhibit an altered phenotype compared with corresponding planktonic cells. These biofilms are not

unstructured, homogeneous deposits of cells and accumulated slime, but rather they are complex communities of surface-associated cells enclosed in a polymer matrix containing open water channels.[284] The glycocalyx polymeric matrix serves as a trap as well as a storage facility for nutrients and minerals.[287] The cells within the biofilm have altered physiology as compared to those in the planktonic state. They function in a co-ordinated manner through quorum sensing; in some ways – mimicking multicellular organisms.[288]

2.8 Bacteria Factors influencing Adhesion

Surface charges are inherently important to bacteria adhesion and the net charge on *E. faecalis* has been reported to be negative. Deletion of D-alanylation in a mutant bacteria removes D-alanine from the lipoteichoic acid of the cell wall of *E. faecalis* and increased the net negative charge that reduced its propensity to biofilm formation compared to wild type strains and increased its susceptibility to cationic bactericidal agents.[289] Particles, including bacteria, acquire charges in aqueous solutions due to ionization of the chemical groups on its surface. Whatever the charge is, it attracts ions of opposite charge in the medium forming an electric double layer. Bacteria are always negatively charged.[290] Hence we expect that a layer of positively charged ions to form the double layer over it as shown in Figure 2.4.[4] In general, a high charge means that the bacteria are hydrophilic, but that does not rule out other causes for it to be hydrophobic in relation to the substratum. *E. faecalis* has been found to be hydrophilic.[10]

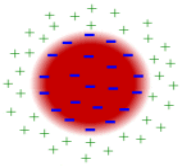

Figure 2.4 Cartoon depicting the electric double layer on a bacterium from Anon2[4]

Electric double layers arise because the net charge of a particle affects the distribution of ions in the media in the immediate interfacial region. If a particle is negatively charged, like bacteria are, they would induce positively charged ions to be on its surface. The immediate layer of induced charges are tightly bound to the particle forming the Stern layer, whereas those slightly further away are more diffuse and less firmly attached. Within this there is a notional boundary called the slipping plane, within which the particle acts as a whole. The charge at this slipping plane is called the Zeta potential and has been determined to be measurable on phospholipids membranes. [291] The surface charge of bacteria is a complicated matter, as there are gel-like structures on the outer surface of the cell with ionogenic groups. As the ionic strength in the suspending medium is reduced, the ionic atmosphere of the bacterium increases. Ionic groups further from the shear plane will not possess counterions.[292]

The value of the surface charge is dependent on the species, as surface chemical groups in differing species of bacteria are different. In addition, as phenotypic expression in bacteria may be affected by culture media , nutrients and age, the

surface charge would also be dependent on these variables.[293] Culture media has been reported to affect the hydrophobicity of *E. faecalis*. [10]

Whilst it is logical to believe that long range electrostatic forces can influence the initial phase of bacterial adhesion there are studies that show adhesion to be unaffected by these long range electrostatic forces. Harkes *et al.* found that there was no correlation between the Zeta potentials and contact angles of the bacteria with the adhesion values found.[294]

There are few studies relating to Zeta potential and adhesion of bacteria to mineralized structures and dental tissues. Weerkamp *et al.*[276] studied the effect of Zeta potential on bacteria adhesion on saliva coated and uncoated enamel and found that there was great variability in adhesion of various bacteria to the same surface. Olsson *et al.*[295] studied the interference of bacteria adhesion on hydroxyapatite treated with alkyl phosphates and non-ionic surfactants. It was reported that hydroxyapatite treated by alkyl phosphates reduced the Zeta potential to highly negative, but when the mixture of alkyl phosphate and non-ionic surfactant was used, it reduced the Zeta potential to near zero. This may reduce the propensity of bacteria adhesion to hydroxyapatite.

Another study looked at the relationship between Zeta potential and cariogenic potential of seven species of acidogenic streptococcal bacteria. The authors reported that the few species of bacteria that were more cariogenic, besides being more acidogenic were all having smaller negative Zeta potential, making them more highly adherent on dental tissues which were naturally negatively charged.[296] Hence, Zeta potential has a direct bearing on the adhesion of bacteria to mineralized structures and dental tissues.

2.9 Substratum Surface Factors Influencing Adhesion

As has been pointed out, in the root canal, the changing nutrients available to a mixed primary infection puts selective pressure on them such that some type of bacteria cultured from the root canal die out with time[297]. In the next, lower scale order, at the substratum surface of chemo-mechanically treated dentine, new niche environments in which remaining bacteria would reside would be created after irrigation. Presence of exposed collagen, remnant smear layer and exposed non-collagenous proteins, would present a very different chemical composition (proteins exposed after chelation of calcium by EDTA), surface charge, hydrophobicity, surface roughness (after chelating action of EDTA) and configuration (more open tubules after EDTA, more porosity after NaOCl) to bacteria. These changes would logically also alter the surface charges, hydrophobicity of the substratum and alter the adhesion of bacteria. Substratum surface charges has been shown to affect the rate that bacteria

would accrete on it [1] and so has the hydrophobicity of substrates. [290] The adherence of *E. faecalis* to dentine coated and or treated with water, PBS, serum and saliva had been reported. The number of bacteria adherent to water and PBS treated dentine was negligible whereas there was moderate numbers adhering to serum-coated dentine. Figure 2.5 is from the work of George and Kishen and shows that saliva coated dentine had the most bacteria adhering.[10] The surface charge of dentine powder had been reported to be negative in low ionic strength.[276]

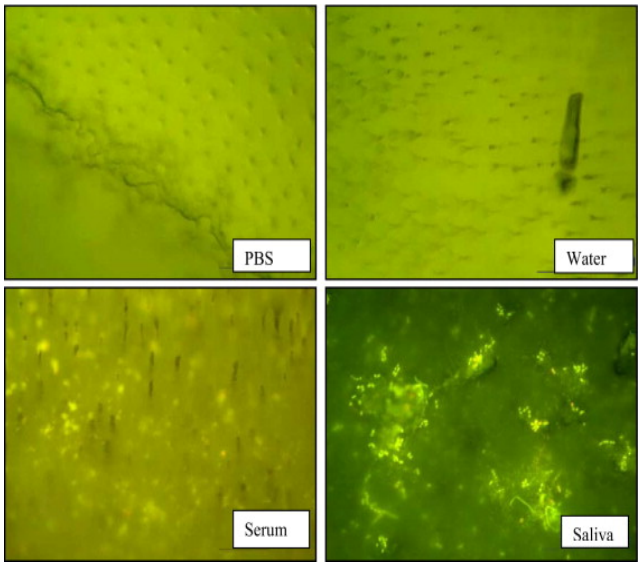

Figure 2.5 Bacteria adhesion to dentine conditioned by body fluids. Dentine conditioned by saliva had most *E.faecalis* adhering. From George and Kishen 2007[10]

2.10 Phases in Bacteria Adhesion

At the outset, we should clarify the use of the terms. Adhesion comes about when a bacterium is firmly attached to a surface by complete physicochemical interactions. Initial adhesion is reversible and with increase of time becomes irreversible; during the conversion from reversible state to the irreversible state, energy is involved.

Adherence on the other hand, refers to the general presence of attachment of bacteria on a surface and is a less scientific term. Attachment is the initial phase of bacterial adhesion, referring more to the physical contact than complicated chemical and cellular interactions and is mostly reversible[298]. There are three main phases of bacteria adhesion. In phase one bacteria is moved to the substratum surface by Brownian motion, van der Waals attraction forces, gravitational forces and the effect of surface electrostatic charge and hydrophobic interactions.[1] On dentine, these physico-chemical forces are likely to be altered when differing chemicals treat its surface. Chemotaxis [299]and hence haptotaxis too, contribute to the rate of bacteria adhesion to surface contacts.

2.10.1 Reversible Adhesion

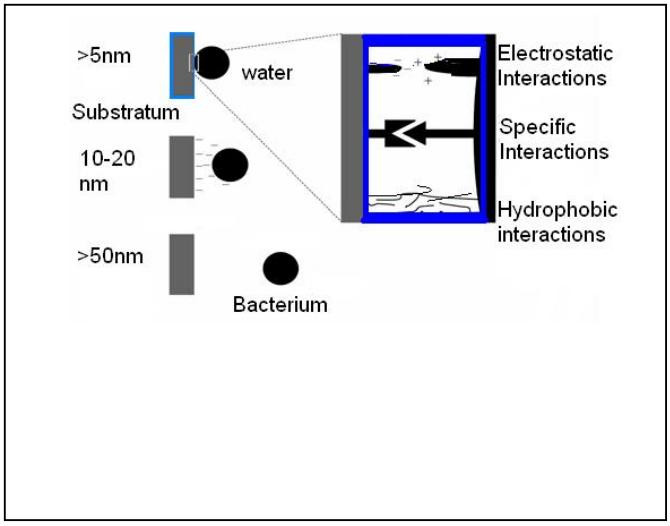

Figure 2.6 Types of Interaction between Bacterium and substratum. Modified from Gottenbos *et al.*[1]

The initial interaction of the surface is reversible and the forces can be classified into long and short-range forces. In Figure 2.6, at a separation distance of > 50nm, long range van der Waals forces only apply. At this distance, only macroscopic cell surface properties play the dominating role in adhesion. At distances of between 10-20nm, both van der Waals and electrostatic charges apply and at distances <15nm, specific molecular force in addition to electrostatic and van der Waals forces apply to adhere the bacteria to the surface. Specific interactions are those that are between stereochemically complementary surface components allow ionic, hydrogen and other chemical bonds whereas non-specific interactions are those due to the overall surface properties such as charges and surface free energy. In addition, specific interaction may require specific metabolic processes to have occurred, such as secretion of

substances.[300] Cell surface appendages however, would contribute to facilitating the adhesion since when the cell body is very close at about 10-20nm, many such appendages are longer than the range of interactive forces and should be able to literally bridge the gap. Even when bacteria appear to be in contact, there is likely to be a thin vicinal layer of water between the cell and the substratum. If bacteria does come so close to the substratum as to squeeze out the thin film of water, the interaction between the cell and the substratum are likely to be hydrophobic in nature. The area in direct contact is likely to be very small. However, both types of contact are likely to be present at the same time. It must be realized that a decrease in bacterial concentration in suspension by a factor of ten can desorb adhering bacteria from a solid surface.[301]

2.10.2 Irreversible Adhesion

In this phase, molecular specific interactions between the bacterium and the substratum surface become predominant. The attachment is likely to be firmer and since many of the adhesions of bacteria are located on or near to appendages [302,303]these appendages including fimbriae, pili, capsule amongst others, are likely to participate in the interaction. To this end, the adhesin for collagen found in *E. faecalis*, ACE, is probably in the cell wall, as are most microbial surface component recognition adhesive macromolecules (MSCRAMM) on Gram-positive pathogens. These MSCRAMMs contain an N-terminal signal peptide followed by a non-

repetitive region called the A region, which in most cases is responsible for ligand binding, in this instance in the Collagen Hug model for formation of adhesion-ligand complex[46]. Once the bacteria is adherent on the surface, it goes about the business of forming a biofilm – as adhesion is the first step to pathogenesis.[304]

2.10.3 Theoretical Models of Bacteria Adhesion

As a bacterium arrives near the surface of the substratum, whether attachment is the outcome depends on the complex interplay of the surface chemistry of the two and the aqueous phase in between, none of the three models proposed explain the adhesion of bacteria adequately. These are the Thermodynamic theory, the Derjaguin, Landau, Verwey, Overbeek (DLVO) Theory and the extended DLVO theory.

The thermodynamic approach takes into account the various types of attractive and repulsive forces acting on the bacterium and expresses the relationship between the two surfaces in terms of surface energy, a thermodynamic term, as energy is required to disrupt bonds between molecules to create a new surface. In addition, this method assumes direct contact between the bacterium and the substratum, with the formation of a new bacterium–substratum interface. This is not entirely true as bacteria have appendages and hydrated polymers are often present at biological interfaces. With a thin film of water in between, contact is hard to define. Furthermore, the contact area used for the footprint of the bacterium, essential in calculation of the energies

involved, may be an over-estimation, as only appendages are in direct contact.[305] It has been reported that *E. faecalis* produces a pleomorphic surface pili that was demonstrated using immunogold electron microscopy,[306] and had been implicated as virulence factor in endocarditis. Fine hairs had also been reported when the species had been exposed to pheromones.[307] Further, this theory does not allow for kinetic interpretation, as it is an equilibrium model.

Within this approach, there are three theories: 1) Neumann's theory, 2) polar dispersion theory and 3) electron donor-electron acceptor approach. The adoption of one is to the exclusion of the others. The electron donor-electron acceptor theory is the most advanced however, and demonstrates that permanent dipole contributes little to the equation of adhesion, that rather it is acid-base and hydrogen bonding is likely to be the more responsible party in the process of adhesion. However, the correctness of the quantitative outcome of this approach is still being debated.[308]

The DLVO theory has been used to describe the interactions between colloid particles and substratum surfaces. Considering the bacterium as a colloid particle, the theory explains adhesion by taking into account the total attractive and repulsive forces (between cell surface and substratum as both are negatively charged). Further bacteria are similar to colloid particles mainly in relation to the size. For DLVO theory to apply to bacteria, many unknowns on the surface chemistry of bacteria that contributes to the electric double layer and the Stern layer that are very different from

colloid particles have to be accounted for.[309] Although this theory can account for the low levels of bacteria adhering to a negatively charged surface, it does not account for attachments to a variety of other surfaces.

The extended DLVO theory was suggested to account for the hydrophobic and hydrophilic interactions that DLVO theory ignores. However, there are still limits to using extended DLVO theory to explain bacteria adhesion to a variety of surfaces.[310]

2.11 Infra-red Spectroscopic methods to Characterize Chemical Compositional change in Biological materials

Infrared spectroscopy (IR spectroscopy) is useful to obtain information about molecular structures by measuring the frequency of IR radiation needed to excite vibrations in molecular bonds.[311] It is used for analysis of a wide range of organic, inorganic and polymeric substances with several advantages:

- simplicity of experimental procedure with minimal sample preparation
- ability to analyze all the three states of matter: gases, liquids and solids
- non-destructive nature of the test, so that sequential analysis of the surface chemical changes can be done
- adequate accuracy in identification of the structure of an unknown material giving quantitative measurements of the components

Modern infrared spectroscopes are also much faster in acquiring spectra than previously available instruments.

2.11.1 Basis of Infrared Spectroscopy

The region of infrared radiation that is most useful for the purpose of IR spectroscopy of organic compounds is the mid-infrared region (4,000 cm^{-1} to 400 cm^{-1}) which corresponds to changes in vibration energies within organic functional groups and molecules.[312] The far infrared region of 400cm^{-1} to 10cm^{-1} is useful for investigation of heavy atoms in inorganic compounds. Wavelengths in IR spectroscopy are measured in reciprocal centimetres – as the numbers are more manageable than using frequencies. The reciprocal centimetre (cm^{-1}), is the number of wave cycles in one centimetre of distance traversed; whereas, frequency in cycles per second or Hz is equal to the number of wave cycles in $3X10^{10}$cm (the distance covered by light in one second). Only those vibrations with a change in dipole moment within a molecule will result in an absorption band in the infrared region. However, dipole moment (charge X distance) need not be permanent to be infrared active. Periodic asymmetrical stretches of linear bonds can also generate infrared bands.

When considering light as a wave, we see that it consists of electric and magnetic fields that are perpendicular to each other, and they oscillate in sine waves as they

propagate through space. The electric field is the one that interacts with the molecules. Infrared energy is absorbed by the functional groups present in molecules, and they would vibrate in specific wavenumbers in any compound.

The IR Spectroscope is an apparatus designed to measure the transitions and plot of the wavelength versus absorbance (transmittance) of energy in a spectrum. Most commonly, the spectra are obtained between the wavelengths 0.78mm to 1000 mm or between wavenumbers 4000 to 400 cm^{-1}.[313]

Energy, wavelength and frequency are related and mathematically this is expressed as:

$$E = h\nu = hc/\lambda \qquad (2.1)$$

The relationship between frequency and wavelength is by the formula:

$$\lambda = c/\nu \qquad (2.2)$$

In equations 2.1 and 2.2 above, h is Planck's constant (6.626 X 10^{-34} J sec), C is the speed of light in vacuum (3.00 X 10^{8}) $msec^{-1}$.

Even within relatively simple molecules there are many different vibrations. It means that the infrared spectrum of a compound usually contains a large number or

peaks, many of which will be impossible to confidently assign to vibration of a particular group or bond.

As a functional group within any compound absorbs a specific wavelength, most compounds would therefore have a complex pattern of peaks between 1500 cm^{-1} to 600 cm^{-1} that are very difficult to assign. However, this complexity has an important advantage as it can serve as a fingerprint for a given compound. A compound can have a typical fingerprint spectrum and these can be compiled into databases for reference to aid in identification of unknown substances. Further as functional groups has specific wavenumbers the chemical nature of a compound can also be identified.[313]

2.11.2 Advantages and Limitations of FTIR as a Chemical Analysis Technique

The spectra are rich in information. The positions of the peaks tells us what type of bonds, the specific functional groups, are present, a bond with large force constants will absorb at high wave numbers. Peak intensities (height) relate to the concentration of a substance present. The quantitative information about the concentration of a molecule in a sample is based on the Beer Lamberts' Law according to which amount of absorbance is related to the concentration of the analyte in a given sample. Hence:

$$\mathbf{A = a\ l\ c} \qquad (2.3)$$

where A is the absorbance, a is the absorptivity, l is the pathlength and c is the concentration of the analyte. Absorbance can be measured from the height and area of the peaks, and peak to height ratio as well as peak to area ratio from an FTIR spectrum.

The absorptivity is a proportionality constant and varies from molecule to molecule and wavenumber to wavenumber for a given substance. Hence, wavelength and absorptivity are fundamental properties of a molecule.[314] The widths of the peaks tell us of the presence of certain functional groups. The overall shapes of the spectra are useful in providing information about the substance. It is a sensitive technique and amounts of material as little as micrograms can be detected.

One of the main limitations of infrared spectroscopy is its use in analyzing complex substances. It is a technique best suited to pure substances.[313] Another limitation is that it cannot detect single atoms in a diatomic compound for example carbon dioxide.[313]

2.11.3 Fourier Transform Method

Fourier transformation is a mathematical method that transforms complex waveforms to its frequency components; in the process converting a spectrum in the

time domain plot to a frequency domain plot. This is now carried out using a computer programmed for the purpose.

The advantages for using a Fourier transform method are:

- increased optical throughput (also called Jaquinot advantage) due to which an increased power reaches the light detector improving the noise to signal ratio
- High resolution and good wavelength reproducibility
- Increased process speed of collection of spectral data (by a factor of 10 – 1000).

2.11.4 The IR spectrometer Components

The IR spectrometer is made up of three main components:

a. Radiation source: emits energy in the infrared range of the electromagnetic spectrum
b. Interferometer : This consists of three active components – a beam splitter, a moving mirror and a fixed mirror
c. Detector: This element is connected to the spectrometer electronics and acts as a transducer, turning IR signals into electric signals, which are then converted to voltage. This is than amplified and digitized before being converted into a spectrum by Fourier transformation.

2.11.5 Formation of the IR Spectrum

IR energy is collimated and directed into an interferometer; most commonly, a Michelson interferometer is used. There are three active parts to the interferometer a beam splitter, a moving mirror and a fixed mirror. The IR radiation is collimated and is directed into the beam splitter towards the interferometer. The beam splitter splits the IR beam into two, allowing only half the beam to strike the fixed mirror and the other half the moving mirror. When these two halves of energy return to the beam splitter, they combine. At the zero path difference, the moving and fixed mirrors are equidistant from the beam splitter. These two beams are therefore totally in phase and when they recombine, they interfere constructively leading to a maximum in detector response. When the moving mirror in the interferometer is translated to point the beam away from the beam splitter, the returning beams to the beam splitter are out of phase when they recombine and hence there is destructive interference. When the moving mirror is rotated at a constant velocity in a back and forth manner from the ZPD, it gives rise to alternating constructive and destructive interferences. The intensity of the signal arriving at the detector would vary in a sinusoidal manner to produce an interferogram output.[314]

The interferogram is based on the time domain. It records the detector's change in response versus time within the mirror scan. When analyte is placed in the path of the beam, it absorbs energy of the emitted beam and the amplitude of the sinusoidal wave is diminished by the amount of energy taken up. In contrast to this simple change in a

single beam at a specific wavelength, when a broadband of IR energy is beamed through a sample, the interferogram produced would be complex and have many peaks and extensive interference patterns. It is a complex summation of superimposed sinusoidal waves, each produced at a different frequency. The interferogram contains information over the entire IR region to which the detector is responsive. Fourier transformation converts the interferogram to the final IR spectrum.[314]

2.11.6 What is Attenuated Total Reflectance (ATR)

Attenuated total reflectance refers to attenuation of energy of a reflected evanescent wave, by a substance which had interacted to absorb some energy from the evanescent wave, just slightly beyond the point of total internal reflection.

In an optical system, when light passes from a denser to a less dense medium at an angle greater than the critical angle, internal reflection occurs. At the point of reflection, a standing wave perpendicular to the reflecting surface forms in the denser medium and an evanescent wave forms in the rarer medium. The electric field amplitude of the evanescent wave decays exponentially with distance from the reflecting surface. [315]

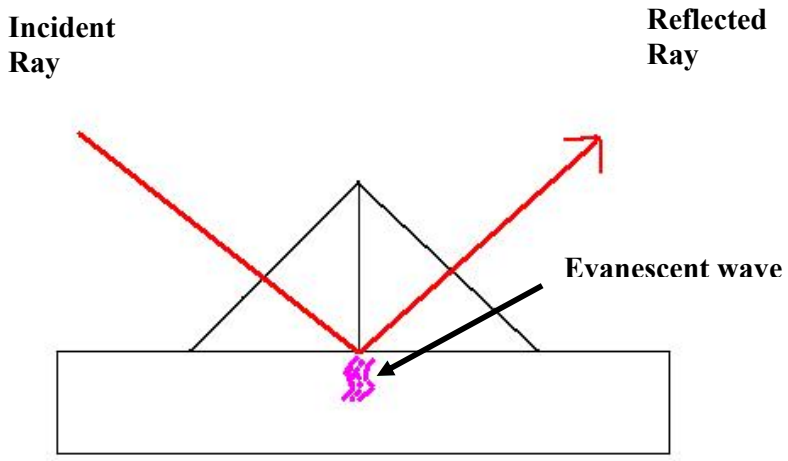

Figure 2.7 Diagram showing Evanescent wave.

The physical explanation for the existence of an evanescent wave is that the electric and magnetic fields cannot be discontinuous at a boundary (the reflecting surface), as would be the case if there were no evanescent field. In the diagram above, the evanescent wave decays exponentially (and precipitously) in the distance d_e from the interface, at the point of internal reflection of a beam passing from the denser material n_1 to the rarer material n_2. In IR spectroscopy using ATR, the sample is placed in contact with an internal reflection element (IRE) of high refractive index. IR radiation is focused at the edge of the IRE crystal and is then directed into a suitable detector.

Total internal reflection occurs at the sample/IRE surface, but the evanescent wave penetrates a short distance into the sample. Hence, an absorption spectrum of the sample can be obtained. ATR provides a useful means of investigating the solid/liquid interface without altering the surface characteristics of the sample. A schematic diagram of the relationship between sample (N_2) and IRE (N_1) the denser material is shown in Figure 2.8.

2.11.7FTIR spectroscopy in Dentine Characterization

FTIR had been used to study various aspects of dentistry. One of the earlier studies of dental tissues by LeGross (1978) was focused on precipitated apatite and enamel. In that study it was demonstrated that loss of water below 200^{o}C was reversible and does not alter the crystal structure of the apatite crystal whereas above 400^{o}C, there was contraction of the crystal lattice and irreversible loss of water.[316]

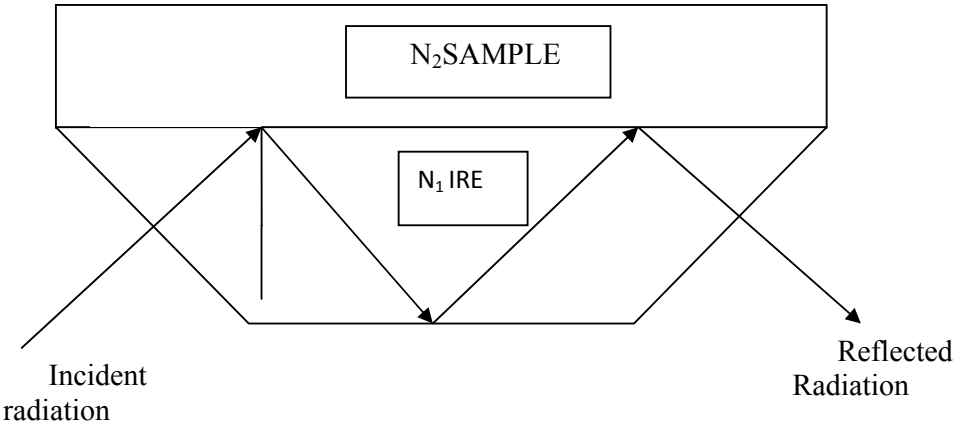

Figure 2.8 Schematic Diagram showing the relationship of IRE and sample

The analysis of dentine composition using FTIR had also been published. White *et al.* (1994) used FTIR to show that sterilization of dentine by gamma radiation did not cause appreciable changes.[317] Strawn *et al.* (1996) used FTIR to study the effects of storage solutions on dentine. In that study they showed that storage solution altered the surfaces of dentine in the mineral component of dentine whereas collagen, the organic component, remains largely unaltered.[318]

FTIR coupled with photoacoustics, was used to study the effect of dentine adhesives on phosphates, amide I and amide II bands of dentine. [319] FTIR was used to study fluoridated carbonatoapatite in the intermediate layer formed between dentine and glass ionomer cement.[320] It was used to show that gamma irradiation of teeth did

not change the nature of human dentine.[317] Specular reflectance infrared Fourier transform spectroscopy was used to study the changes in surface chemistry and optical properties of dentine occurring as a function of storage solution and time. They however reported that microstructural changes in collagen, expected when dentine was soaked in formalin, may not be detectable by FTIR.

Sasaki *et al.* investigated the effects of Er:YAG and CO_2 laser irradiation of dentine as compared to non-irradiated dentine. They found that laser ablated more of the organic components of dentine than the inorganic components. Using FTIR, they concluded that Er:YAG laser used in conjunction with water used as coolant, did not cause major compositional changes or chemically deleterious changes in root cementum or dentine.[321] A study by Lee *et al.* (2004) came to a similar conclusion. [322]

Bachmann *et al.* using FTIR studied the bound energy of water in dental tissues from 100 – 1000^{o}C. Both enamel and dentine were studied. They reported that below 400^{o}C, only the loosely bound or adsorbed water was lost. For the structural water that is tightly bound, this could only be removed between 700-1000 ^{o}C.[323] The same group did another study investigating the effect of temperature rise on collagen bands in heated and rehydrated dentine. They reported that changes to collagen heated below 175 ^{o}C was reversible by rehydration but when heated above 200 ^{o}C, the structural changes to collagen became irreversible.[323]

FTIR was also proved effective in demonstrating demineralization of dentine by phosphoric acid[264] as well as deproteination of dentine by NaOCl .[242] Hence, FTIR is a method which can help in non-destructive testing of a specimen and much information can be gleamed from the spectrum regarding the chemical nature of the treated specimen.

2.12 Summary of Literature Review

From the foregoing, we can see that there is increasing number of reports of *E. faecalis* infections associated with failed root canal treated teeth. It had been reported that chemo-mechanical preparation of the root canal changes the flora of a mixed infection to one with a bias for gram-positive facultative anaerobes. Whilst the main aim of using chemical disinfection of the root canal is to kill off all the bacteria within the space of the root canal, this cannot be achieved due to anatomical complexities within root canals giving rise to concentration gradients. It must also be realized that all the chemicals used had been reported to have detrimental effects on dentine, both in a concentration and time dependent manner.

Chemicals used during root canal treatment have been shown to alter the physical nature, as well as chemical nature of dentine, offering a new environmental niche to

bacteria. These changes may bring about an increase in adhesion of *E. faecalis*. It has been shown that *E. faecalis* trapped within filled root canals *ex vivo*, can survive for long periods,[37]implying that the niche environment offered after chemo-mechanical treatment and root filling, is suitable for their survival.

Hence, it is important to understand the chemical nature of the treated substrate, its influence on adherence and adhesion force of *E. faecalis* so that we can develop strategies of irrigation of root canals to reduce this phenomenon. Studying the adhesion of *E. faecalis* to chemically treated substrate as well as the adhesion force related to that surface may contribute to our understanding why there is an increased in *E. faecalis* associated with failed root canal treatment better.

Since there is no defined sequence of root canal irrigation, dentists can irrigate the root canal using EDTA as a final irrigant. As mentioned earlier, even if NaOCl is used following EDTA use, it does not stop the action of EDTA within a clinically meaningful period. Hence, invariably, collagen would be exposed. Due consideration should be given to exposed collagen. Further irrigation with NaOCl may dissolve away some collagen, but can also leave denatured collagen in the root canal, since there are concentration gradients within the root canal. The adherence of several species of bacteria to collagen and denatured collagen has been shown to be different.[263] It would be useful to learn if there is any difference in adherence of *E. faecalis* to collagen and denatured collagen.

Chapter 3 Investigating the Denaturing Effects of EDTA and NaOCl on Type-I collagen Using Circular Dichroism

3.1 Theory of Circular Dichroism

There are two groups of active chromophores on proteins. These are the side chains of aromatic amino acid residues and peptide bonds. Each of these known structures absorb left and right polarized light between the wavelengths of 170-700nm.[324]

As light passes through an absorbing optically active substance, left and right polarized rays travel at dissimilar velocities and these rays are also absorbed to different extents. The difference of absorption between the left circularly polarized light and that of the right circularly polarized light is the circular dichroism (CD). After the light has passed through the optically active medium, one of the polarized rays would trace out an electrical vector that is smaller than the ray of opposite polarization. When these two rays are recombined after passing through the medium, the circular trace would be replaced by an elliptical trace. CD is thus also a measure of ellipticity of the emergent light.[325]

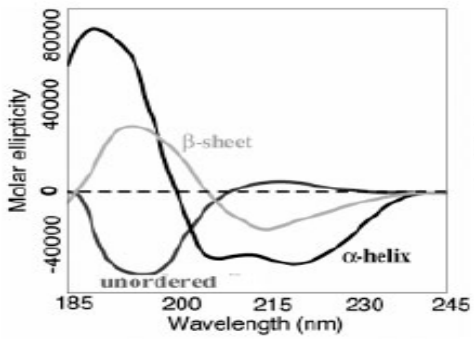

Figure 3.1 Far-UV CD reference spectra of an unordered protein, and of proteins with a prevalent α-helical or β sheet content.[7]

The far-UV CD range (180-250nm) reflect the symmetry of the peptide bond environment and allows us to estimate the secondary structure content of a protein in solution. Hence proteins have been found to present characteristic CD spectra.[325] A change in the spectra shows the change in chirality and represents a change in the secondary structure of the protein dissolved in the solvent.[326] This technique requires dissolved proteins in the microgram range and would provide an assessment if proteins are denatured. The typical CD of proteins with prevalent α-helix, β sheet and unordered proteins are shown in Figure 3.1. For monitoring of peptide bonds, the spectrum around 210 to 220nm is near zero if the protein is unfolded or denatured. Any loss of the secondary structure of a protein is considered denaturation, and this brings about a shift in the peak around 220nm.

The aim of this experiment was to determine using CD if the endodontic irrigants NaOCl and EDTA would denature type-I collagen.

3.2 Materials and Methods

Kangaroo tail Type-I collagen (Sigma Aldrich, USA) was dissolved in 0.1M acetic acid to a concentration of 2mg per ml. This method is recommended by Sigma-Aldrich for coating cell culture plates and acetic acid dissolved collagen type I had been used to study collagen denaturation.[327] The experiments were conducted to assess the effects of variation of concentration of EDTA and NaOCl on type-I collagen and the influence of duration of application of NaOCl (0.03M) on the conformation change of Type-I collagen. A low concentration was selected as it was envisaged that a higher concentration of NaOCl would completely breakdown the acid solubilised collagen. The experiments done were 1) effect of EDTA, 2) effect of NaOCl and 3) the effect of variation of time with NaOCl. The volumes of liquids used are shown in Tables 3.1 and 3.2 below:

Table 3.1 Volumes of Chemicals used in assessment of effect of NaOCl concentration on Collagen

Collagen (μl)	NaOCl (0.175M; 1.25%) (μl)	DD water (μl)	NaOCl Molarities
50	57	93	0.05M
50	45.7	104.3	0.04M
50	34.2	115.8	0.03M
50	22.8	127.2	0.02M
50	11.4	138.6	0.01M

Table 3.2 Volumes of Chemicals used in assessment of effect of EDTA concentration on Collagen

Collagen Vol.(μl)	EDTA (0.25M;8.5 μl)	DD water (μl)	EDTA- Molarities
50	48	102	0.06M
50	40	110	0.05M
50	24	126	0.03M
50	16	134	0.02M

The spectropolarimeter (JASCO J810, USA) was purged with nitrogen for about 15 minutes prior to commencement of experiments. A scan speed of 20nm $min^{-1}$was used; with a slit width of 1nm and a time constant of 1s through a quartz curvette of

1mm path length. Five readings were taken and the average taken for plotting of the curve. All readings were taken at room temperature.

3.3 Results:

Typically, collagen-like triple helical structures show a large negative peak near 195 nm and a weaker positive peak around 220 nm.[328] In all the curves we obtained for controls, these peaks were present but were altered drastically in the curves obtained after addition of NaOCl and EDTA. Loss of peak height around 220nm had been reported as a characteristic feature of denaturation of collagen by heating [329] and loss of positive peak as well as red shifting of negative peak had also been reported for denaturation of type I collagen.[330] Alpha-helix transiting to random coil show changes around 200nm in the CD spectrum.[331] Reductions in the positive and negative peaks were taken to mean the loss of conformity of the collagen.[332]

Representative CD spectra of NaOCl and EDTA treated collagen are shown in the Figures 3.2 and 3.3 (those of effects of NaOCl) and 3.4 (the effects of EDTA) below. The CD spectra of native type-I collagen has a positive peak around 225 nm and a negative peak around 195 nm. The peak around 225 nm is characteristic of the triple folded helix.

As can be seen from the curves in Figure 3.2, there were reductions in the positive peaks as well as reduction and red shifting in the negative peaks. Figure 3.3 shows that just after 5 mins there was almost complete flattening of the positive and negative peaks; and Figure 3.4 shows that at all concentrations starting from 0.01M, there was significant flattening of the positive and negative peaks. Hence, there was denaturation of acetic acid solubilised collagen by 0.2M EDTA and higher concentrations.

There was also denaturation of collagen by 0.01M NaOCl and those of higher concentrations. With the application of the 0.03 M NaOCl solution to study the effects of time on collagen, there was denaturation of type I collagen at all time-periods. Our results are therefore similar to those reported by others.[330, 331] Both these chemicals altered the secondary and higher order structure of collagen.

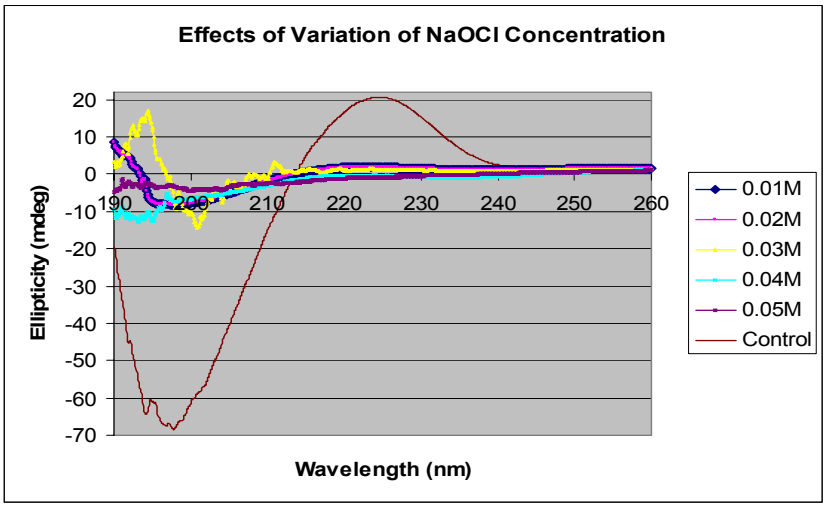

Figure 3.2. CD spectrum showing effect of NaOCl concentration on Type-I Collagen

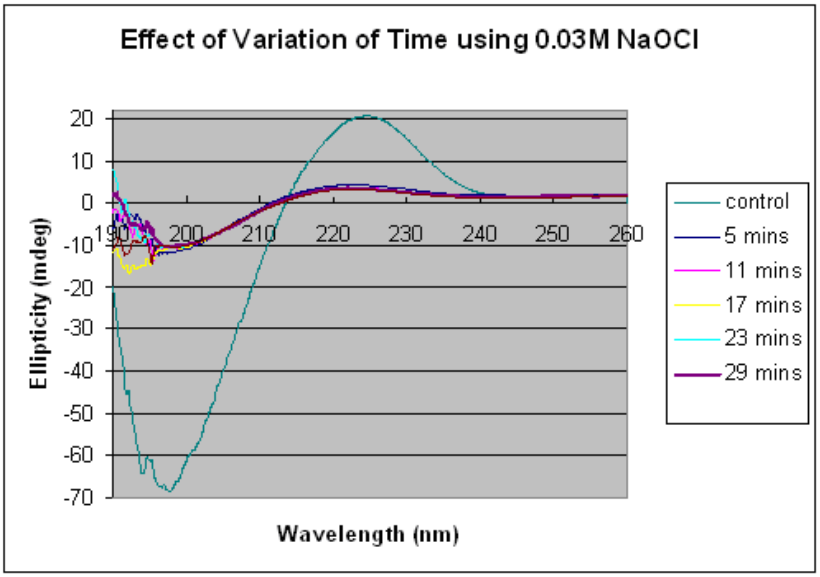

Figure 3.3 CD spectrum showing the effect of time of exposure using 0.03M NaOCl on Type-I Collagen

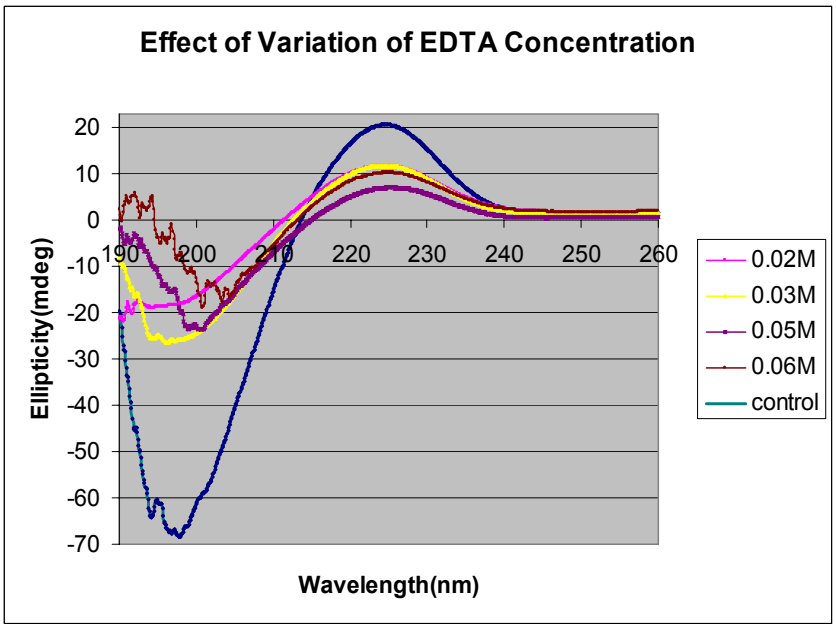

Figure 3.4 CD spectrum showing the effect of variation of EDTA concentration on Type-I Collagen

3.4 *Discussion*

CD can be used to measure changes to low concentrations (< 0.1mg to 1mg of protein) of proteins whereas techniques such as nuclear magnetic resonance requires a larger concentration (0.5mg). CD is a versatile technique and can be used to monitor protein denaturation under a wide range of experimental conditions, is non-destructive and requires less time than techniques such as nuclear magnetic resonance.[333]

There are however limitations to use of CD to assess protein denaturation. In general several assumptions have to be made when using CD. These assumptions include:

(1) the secondary structure composition of the crystalline protein is retained in solution;

(2) the effect of tertiary structure is negligible (i.e. individual secondary structural elements do not interact and their contribution to the overall CD spectrum is additive);

(3) only the amide chromophores are responsible for the far UV CD spectrum (contributions from side chain chromophores are assumed to be zero for all proteins);

(4) the geometric variability of secondary structural units is assumed to be negligible, i.e. a single CD curve is sufficient to describe each type of secondary structure (e.g. one for $\tilde{\alpha}$ helices, one for all β-sheets, etc.)[334]. Although these assumptions can affect the data, we feel that these have not affected ours, as the peak shifts were very definite, and the trend was very consistent. Our data shows very definite loss of ellipticity of the peak at 220 nm to nearer zero which is interpreted as denaturation .[335]

Ultraviolet irradiation can also cause denaturation of collagen. In our study, all the effects of NaOCl had taken place within 5 minutes and concentrations of NaOCl as

low as 0.01M had caused denaturation. The effect of exposure of collagen fibrils to UV irradiation for 35 minutes during the experiment therefore should have no bearing on the outcome.[336]

Most CD data are analysed for the percentage changes to the amount of α-helix and β -sheet or β -bends present. We did not analyze CD data for these, as our main purpose was to see if both these two chemicals denatured collagen. We have used kangaroo tail collagen, as it is an acid soluble type I collagen that is easy for CD to detect denaturation in. Though both dentine collagen and kangaroo tail collagen are type I collagen, dentine matrix is collagen is interwoven and is not easily soluble in acids such that acid etching of dentine is the main method of exposing collagen for dentine bonding,[337] our results using acid soluble collagen fibrils cannot therefore be directly extrapolated to dentine collagen.

3.5 Conclusions:

Within the limitations of this study, using kangaroo tail type-I collagen as a model of type-I collagen , the findings of this experiment demonstrated that EDTA and NaOCl, both commonly used irrigants during root canal treatment, caused secondary structural changes to collagen. Even low concentrations of 0.01M of NaOCl and 0.2M EDTA will denature type-I collagen.

Chapter 4 Denaturation of Collagen Type-I matrix membrane by various endodontic Irrigants

Differential Scanning Calorimetry (DSC) can be used to examine conformational change.[338] It has also been shown that a denatured protein has significant higher heat capacity or specific heat than that of native protein.[339] Macromolecular structures are stabilized in their secondary and tertiary conformations by weak forces. Changes to these weak forces can be studied by using high sensitivity DSC.[340]

4.1 Theory of Differential Scanning Calorimetry

This technique measures the energy needed to maintain the temperatures between a reference substance and the specimen of interest at zero. The specimen could be cooled or heated and the specific heat transiting from one phase to the other is measured. There are two types of DSC – power compensation DSC or heat flux DSC. In power compensation DSC, the temperature of the reference and test specimens are in separate but identical furnaces. The electrical power to the furnaces is varied to maintain the two at the same temperature. A heat flux DSC makes use of a reference cell and a test cell within one furnace. Both cells are heated and a computer continually monitors their temperature and the rate of heating. If there were a slower

rise in the temperature of the test cell because of an endothermic process in the specimen, a higher heating rate would be applied so that the temperatures of the two cells are maintained to be identical for the duration of the experiment. A diagram depicting the heat cells of the DSC instrument is shown in Figure 4.1. The data collected were used to plot the heat flow versus temperature curves.

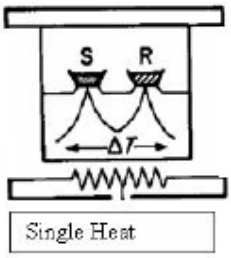

Figure. 4.1 Diagram of Heat Flux DSC;
S=specimen, R=reference.Adapted [3]

DSC analysis provides a large amount of thermodynamic information. Equation 1 below shows that integrating the experimental heat capacity curve yielded the calorimetric transition enthalpy (ΔH_{cal}):

$$\int C_p \, dT = \Delta H^o \qquad (4.1)$$

where C_p is the measure of heat capacity and the change over a temperature range is *T*. The enthalpy thus determined is model independent and does not depend on the

nature of the transitions. The temperature at which the excess heat capacity is at the maximum is denoted T_m and is the transition temperature. The transition temperature is the temperature at which the specimen changes from one phase to another. For example, as a solid turns to a liquid it will require more heat flowing to the sample to keep the increase of its temperature to match that of the reference. This is due to the absorption of heat by the sample as it undergoes the endothermic transformation from solid to liquid (also known as the latent heat of fusion).

The difference in the initial and final baselines of the curve provides a measure of the heat capacity change that is specific for the transition. The ΔH value, calculated from the area under the transition peak, is correlated with the content of ordered secondary structure of a protein[341]. This value includes a combination of endothermic reactions, such as the disruption of hydrogen bonds determined as 1.7kcal per mole of hydrogen bond [8]and exothermic processes, including protein aggregation and the break-up of hydrophobic interactions.[342] The direction of the curve, having a peak above or below the x-axis, denotes whether the reaction is endothermic or exothermic. In interpreting DSC curves, whether peaks pointing above the x-axis are endothermic or exothermic depends on the setting of the software. The height of the peak tells us the heat capacity of the protein. The height was reported to be higher in denatured proteins than native proteins,[343] as more heat is required to heat denatured proteins. In making comparisons between thermograms however, it was necessary to check if the scan rates are comparable, as this affects the melting temperature.[344]

The sharpness of the transition peak can be measured as the width at half-peak height and is a good measure of the cooperative nature of the change from native to denatured state. A narrower peak shows a more cooperative transition. [345] The diagram below extracted from Privalov and Khechinashvilli 1974,[8] shows three peaks. The lysozyme has different transition temperatures in solutions of differing pH. In addition, the heights of the peaks, as well as the co-operativity of the transitions under the three conditions are also different.

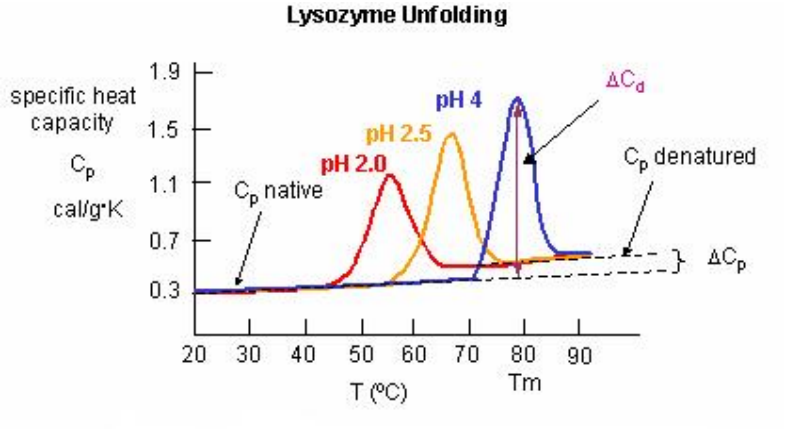

Figure 4.2. Heat Transition Peaks of Lysozyme. [8]

As can be seen, the height of the peak is a measure of the specific heat capacity (Cp) of the substance, at the transition temperature or melting temperature (Tm). Hence, a heat flux thermogram provides information on the temperature dependence of the specific heat capacity of a substance over a range of temperatures. Note from the above diagram that there is a difference (Δ C p) in the specific heat capacity of the substance after denaturation. From the thermogram, it can be observed that

throughout the range of temperature that the substance is heated to melting, the specific heat capacity increases drastically due to the large increase in energy required for unfolding of the protein before the temperature of the mass can be increased. The transition temperature or melting temperature is dependent on the molecular weight and can be used to aid in determining the purity of substances.

Analysis of a DSC thermogram enables the determination of two important parameters. The enthalpy of denaturation (ΔH) a measure of the total energy required to make the transition through the melt and the peak height of the transition temperature (or melting temperature peak) the specific heat change, which is a measure of the extent of disorder within the structure of protein.[346] From the diagram above note that denatured proteins have a higher specific heat capacity.

The aim of this experiment was to characterize the effects of NaOCl, EDTA, CH, CHX, and the combination of CH followed by EDTA on type-I collagen, using an eggshell collagen membrane model and DSC to examine if the chemicals used during endodontic treatment would denature an interwoven type I collagen membrane.

4.2 Materials and Methods

Chicken eggshells obtained were cleaned with detergent and the membranes were obtained using the method of Carrino *et al.* .[347] Briefly, the cleaned eggshell was placed in 1M Hydrochloric acid. After one hour, the decalcified collagen membrane was carefully removed and washed in distilled water. The membrane was then soaked overnight in an acid solution of bovine pepsin (Roche, Switzerland) to remove all non-collagenous proteins. The collagen membrane was then carefully cut to size, about 10mm X 8 mm using sterile surgical scissors, and then placed in a sterile covered petri dish under UV illumination of a laminar flow chamber for one hour to kill any bacteria. The membranes obtained through this method were about 100 ± 30μ thick. The membranes were stored in 50ml of deionised water in sealed tubes until used. The prepared membranes were then soaked in selected chemicals commonly used in root canal treatment. All membranes in this experiment were obtained from three eggs, and a piece of membrane cut from each of these eggs, were put into one sealed test tube to constitute one treatment group.

Group 1 was the control group, in which the collagen specimens were left untreated but soaked in an inactive solution (deionised water). Collagen specimens in Group 2 were soaked in saturated CH solution (CH group) for 24 hours. Group 3, collagen specimens were treated with 17% EDTA pH 7.2 for 1 hour. Group 4, collagen specimens were treated with Calcium Hydroxide for 24 hrs followed by EDTA for 1 hour (CH EDTA group). Group 5, collagen specimens were treated with

CHX for 1 hour (1: 10 Hibiscrub v/v, 0.4% Chlorhexidine w/v). Group 6, collagen specimens were treated with CH (CH 50% group) for 24 hours and Collagen specimens in Group 7, was treated with NaOCl 0.05% for 1 hour. All the chemical treatments were carried out in 15ml Falcon tubes with 10mls of solutions. A square shaped specimen, roughly 3mm X 3mm was prepared from each eggshell membrane from each treatment group from a tube. The three specimens from the same treatment groups were treated by reagents from the same stock solution and for the same duration.

After treatment, the specimens were vortexed for one minute in 10 ml deionised water in a 50ml-Falcon tube, air dried for 48 hours at 37^{o}C in a humidified incubator and then kept enclosed in a screw cap plastic bottle until testing. Care was taken to ensure that all specimens were exposed to the same heat experience and are kept in the same environment until testing with the DSC system. The baseline of the heat–flux type DSC apparatus (Pyris 6, Perkin Elmer, USA), calibrated using Indium, was measured at the start of the experiment and this was subtracted from the experimental data recorded. Samples of each treatment groups were accurately weighed and then placed in a pan. Each pan was sealed by crimping the edges. Thus, the thermogram for each treatment group is an average of each of the three different eggshell membranes in each group. The experimental temperature range, determined by a series of pilot experiments, was set from 20^{o}C through 230^{o}C and heat application was at the rate of 10^{o}C per minute. The heating chamber of the DSC apparatus was purged throughout the experiment with nitrogen.

4.3 Results

All the specimens yielded only a single denaturation peak in the DSC thermograms. Group 4 (CH-EDTA) had an increase in melting temperature compared to control, whereas all other groups gave a reduction of melting temperature. Of the collagen membranes used, all their melting temperatures were about 120^{o}C. Groups 3, 4 and 7 gave rise to the most increases in specific heat change (see Table 4.1) as compared to the other groups. There was a similar trend in the change in ΔH, the enthalpy, of the groups (see Table 4.1 below). The highest enthalpies were from groups 3 and 7. The ΔH was raised to 288.597J/g and 270.998 J/g respectively, after treatment, from 224.250J/g of untreated collagen. This represents an increase of 28.69 % and 20.84% respectively. It is also interesting to note that 50% saturated CH solution had altered the ΔH about 6.3% less than that caused by saturated CH solution. Further, the treatment of collagen by EDTA after CH treatment had a ΔH lower than those treated by saturated calcium hydroxide alone, but was lower than that treated by EDTA alone.

Statistical analysis was carried out using pairwise comparisons with application of Bonferroni's correction, with $p < 0.05$. There was significant difference between the control (no treatment) group and all other groups, except with the CHX groups. It is interesting to note that all chemicals used in irrigation of the root canals, including 50% saturated CH denatured collagen. From the thermograms in Figure 4.3, we note

that there is very little difference between the collagen membranes treated by saturated CH solution and that treated by 50% saturated solution in terms of enthalpy as well as entropy. CHX treatment altered the heat parameters the least. Figure 4.4 helps with the interpretation of thermograms.

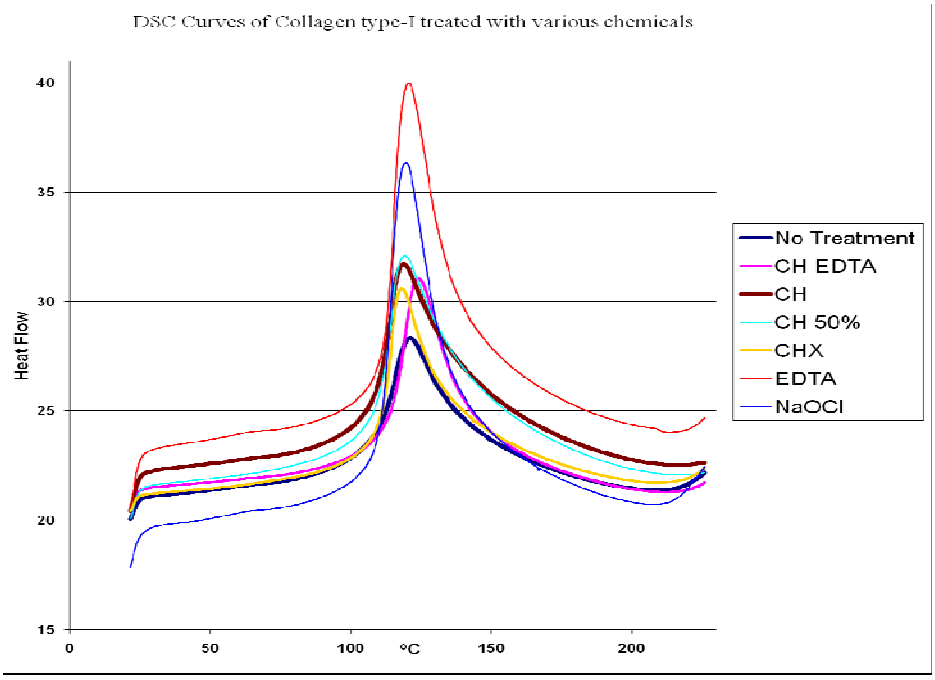

Figure 4.3 DSC Curves of Collagen type-I after various chemical treatments

Table 4.1 Heat parameters of test groups

	Group 1 Control	Group 2 CH	Group 3 EDTA	Group 4 CH-EDTA	Group 5 CHX	Group 6 CH 50%	Group 7 NaOCl
Peak Temp ^{0}C	121.16	118.9	120.4	124.3	117.9	119.3	119.4
Peak height mW	6.28	8.29	15.39	8.59	8.16	9.08	15.14
ΔH Jg $^{-1}$	224.3	267.6	288.6	278.6	225.1	253.5	271

4.4 Discussion:

Mineralized tissues such as dentine are composite structures of an organic matrix that had become mineralized. Collagen is exposed after irrigation with EDTA. In the case of dentine, this collagenous matrix is a network of collagen fibres. Some studies have indicated that denatured collagen allowed adhesion of certain species of micro-organism to a greater extent.[263] It was necessary to compare the extent of denaturation of type-I collagen matrix by different chemicals, as this may have an implication on the nature and extent of bacteria adhesion to it.

Collagen types are formed with the aid of several different classes of proteins, including other collagens, glycoproteins and proteoglycans.[348] Collagens of the same type have similar domain types and structures. The extracellular matrix collagen of dentine is type I collagen.

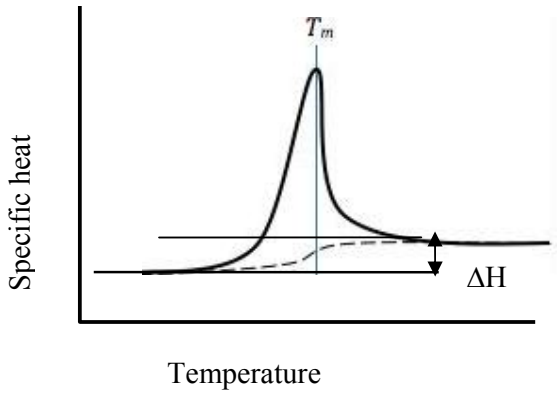

Figure 4.4 Schematic Diagram showing DSC Curve (Tm=melting temperature)

It had been shown that UV illumination of collagen using a UV transilluminator which emits light mainly at a wavelength of 302 nm carried out in a quartz curvette at a distance of 1 cm from the light source increased the melting temperature of collagen by 2^{o}C, from 64 ^{o}C to 66 ^{o}C, whereas further exposure to 20 hours reduced the melting temperature to 39 ^{o}C, similar to that of acid soluble collagen[349]. In our case, exposure to UV was at about 60 cm from the light, in the laminar flow chamber. As the intensity of light diminishes with distance following the inverse square law, exposure

of the membranes to UV light for sterilization was not expected to affect our results, though it cannot be entirely ruled out.

The chicken eggshell collagen is also type-I collagen with a interwoven matrix pattern similar to that of dentine collagen and hence it was decided to use this as a model of dentine collagen.[347] Further, egg-shell collagen matrix was a thin, soft and pliable collagen fabric which can be easily cut to a defined size and tested. Although we tested collagen from decalcified eggshell, all our DSC thermo grams gave rise to a single peak, indicating the presence of a single material phase. If a mixture or impurities were present, there would be multiple peaks in the DSC thermo gram. [350] It confirms that this method of obtaining type I collagen provides specimens that are free of other organic impurities. The conformational stability of a collagen is important in our study from the point of view that the bacterium of our interest, *E. faecalis*, has a microbial surface component recognizing adhesive matrix molecule (MSCRAMM) called ACE that recognizes and binds to collagen. The adhesin binds in a collagen hug mode.[46]

The DSC experiments were conducted to determine if there was denaturation by monitoring changes in the enthalpy (ΔH) and the change in the heat capacity of collagen specimens.[339] Although visual and touch sense could not detect any difference in the chemically treated collagen membranes, DSC clearly demonstrated that the specific heat (peak height) of the collagen membranes had changed. An

increase to the protein specific heat is due to denaturation.[343] The DSC thermogram represents the energy required to break all the bonds that gives protein its secondary and tertiary structures without disruption of the covalent bonds of the primary structure .[351] The transition or melting temperatures of all the membranes hovered around 120^{o}C. The transition temperature of collagen is very much affected by the state of hydration. Varying the state of hydration, can have profound effects on the transition temperature of collagen ; even the dehydration process of cross linking collagen with a chemical with loss of some intrafibrillar water leads to an increase in melting temperature.[352] Hence, careful management of the moisture content and heat experience of the collagen membranes was necessary. A change in melting temperature may be related to the heterogeneous local stability of the collagen triple helix and variations in the extent of structural disruptions of the protein. [353] In the context of this experiment, the change in melting temperature would be due to disruptions and changes in the secondary and tertiary structure of the protein arising from chemical treatment. A higher melting temperature implies that the remaining protein in the substrate had become more stable, though it may be denatured. Changes to the melting temperatures were observed in all treatment groups. These changes can only have come about by the chemical treatment that the collagen membranes were subjected to.

As the moisture content of the membranes was carefully controlled, it is unlikely that there would be much variation in the melting temperature of our specimens due to free water. It was not known however, if there had been dehydration due to loss of

structural water of the membranes when treated by the different chemicals. If there had been such dehydration, it would still contribute to the slight changes in melting temperatures. It is generally accepted that a folded protein is in a state with the lowest entropy. The entropy of a protein is related to several factors: the hydrophobic effect, electrostatic charges, hydrogen bonds, van deer Wails forces, dipole-dipole interactions, salt bridges and conformational entropy – the number of is-energetic conformations possible without any change in heat capacity .[354] From our results, all the chemical treatment carried out in our study altered the entropy of collagen membranes. Hence, after the chemical treatment, some or all of the above factors could contribute to the increase in entropy. Taking hydrophobic groups, which is thought to contribute the most to the overall Gibbs free energy of a protein,[355] as an example, this may mean that after chemical treatment, more hydrophobic groups are exposed and these could change the interaction of bacteria to the substrate, as hydrophobic interactions is a method of bacteria adhesion.

The "all or none" character of protein denaturation had been generally accepted[339], as only in accepting this definition can we describe the stability of any protein. Therefore, even if one hydrogen bond were to be disrupted, we can say that the protein had been denatured. EDTA, CH-EDTA and NaOCl treatment altered the enthalpy the most. Previous studies have also shown that both EDTA and NaOCl treatment can denature collagen[356]. Perivale had reported that in double stranded coiled coils, the energy required for disruption of a hydrogen bond would be about 25 Jg^{-1} and that of van deer Waals contacts for stabilization of the structure was reported

to be about 5 Jg^{-1}. Judging from the enthalpy change in our specimens, if we assume the energy requirements to be similar in triple helices, the chemicals used would have disrupted many hydrogen bonds or the chemicals had broken bonds of higher energy in our collagen membranes. The enthalpy change was the smallest for CHX at 225.1 Jg^{-1}, an increase of just 0.8 Jg^{-1} more than untreated collagen membranes, accordingly this may not have caused any denaturation, as the energy difference is less than required to break a hydrogen bond or disrupt any van deer Waals forces acting within the structure. It is however incorrect to come to such a conclusion. This was because unfolding a protein is a multi-event phenomenon in which some effects of the unfolding protein might be cancelled by other events; breaking a hydrogen bond is an endothermic event,[8] whereas disruption of a hydrophobic bond is an exothermic event.[357] Thus, when there is a very small change to the enthalpy, it could be due to multiple exothermic and multiple endothermic events cancelling out each other to give only a very small difference.

The enthalpy and the entropy of collagen transition from coiled-coils to random coils are characteristics of the given collagen molecule and are specific to the species they are drawn from[358]. Great care should be taken in making any extrapolation of these results directly into the clinic. Our results are in contrast to that of Spencer *et al.*, who used micro-Raman spectroscopy and reported that phosphoric acid treatment for 15 secs. denatured dentine collagen but EDTA treatment of dentine collagen for four hours does not.[359] In that study, the volume and method of EDTA applied was not specified. However, since they were studying the smear layer of a section of

fractured dentine which showed "smear layer-demineralised-undisturbed dentin interface ", it is logical to presume that the EDTA was applied on the surface of the dentine section with the smear layer only, and it is likely that the volume applied was very small. This would mean that despite the application for 4 hours in that experiment, the EDTA would have been fully reacted after a short time. In our experiment, the thin collagen membrane piece was completely immersed in a relatively large volume of EDTA. This may account for the contrast in the results. The method used in this study simulated the use of a large volume of irrigant in the small volume of a prepared root canal.

Calcium had been shown to play a role in the stability of collagen.[360] Our results are also similar to that of Freudenberg *et al.* who used DSC to characterize the changes with collagen type-I using calcium chloride ($CaCl_2$), and found that calcium binding to negatively charged carboxylic acid moieties influenced the conformity of collagen.[361] Knott *et al.* however, have reported that in turkey tendon, EDTA removes the cross linking between collagen in calcifying tissue and this reduced the stability of the collagen.[362] Hence, it is not surprising that CH affected the collagen in our study. CH can participate in the water bridging between collagen fibrils as well as in other proteins.[363] This calcium however is not likely to be the same as that which calcifies collagen to form calcified tissues. The calcium calcifying collagen had been reported both in the extra-fibrillar space as well as intra-fibrillar spaces.[364]

The finding from this experiment also shows that enthalpy and entropy of collagen was altered the least by CHX. CHX had been reported in several studies to in fact stabilize collagen against attack from matrix metalloproteinases left exposed on dentine, by EDTA or acids, and is considered a synthetic protease inhibitor [216, 217].

4.5 Conclusions:

DSC is a useful method in assessing protein denaturation and can be used to assess the denaturation of fibrillar type I collagen membranes. The present study shows us that all chemicals used during root canal irrigation increased the enthalpy and entropy of eggshell type-I collagen membranes. Chemical treatment reduced the melting temperature of collagen membranes, destabilizing them.[365] It was demonstrated that the specific heat of type I collagen was increased by the chemicals used during root canal irrigation, as these chemicals denatured it. This study confirms our CD data that EDTA denatures collagen. Within the limitations of this study, it can be concluded that chemicals commonly used during root canal treatment can denature collagen type-I interwoven in three dimensions to form a membrane

Chapter 5 Adherence of Enterococcus faecalis to type I collagen

5.1 Introduction

Removal of the smear layer formed on the root canal wall during instrumentation lowers the propensity of retention of some species of bacteria after root canal treatment and hence reduces the likelihood of root canal reinfection by these bacteria.[279] Removal of the smear layer has been reported to reduce microleakage of root fillings[366] though it increases dentine permeability.[163] In addition, up to 3.8% of originally applied volume of EDTA has been reported to remain within the dentinal tubules.[191] This is likely to continue to demineralise dentine and affect the final seal of the root filling[367] which suggests indeed that a layer of collagen may be exposed. It had been established that a mere two minutes exposure to 0.5M EDTA, similar in duration to the clinical use of EDTA to remove smear layer, would result in exposed dentine collagen. [368] The exposed collagen may not be removed by follow on irrigation with NaOCl, as the complexities[369] within the root canal system may mean that NaOCl irrigation need not traverse over those areas of exposed collagen fibrils, leaving them exposed.

Collagen is a substrate to some species of oral bacteria including *E f.* [267] Exposing collagen may not be desirable in the root canal, especially when it is known that complete disinfection of the intricately complex anatomy of the root canal system is difficult[370,371,370-372]. Anatomical irregularities, such as cul-de-sacs and isthmuses within teeth, have been reported to harbour necrotic tissue as well as bacteria, even after root canal treatment is completed[369]. Although NaOCl, rapidly dissolves necrotic pulp tissue[373] it is commonly known to denature collagen. EDTA is also known to denature proteins rich in cysteine [270] and type-I collagen as previously shown. Denaturation of collagen, in the form of gelatine, had been shown to encourage the adhesion of *Candida albicans*.[374]

The species of bacteria tested was *E. faecalis* ATCC 29212 a reference strain that is vancomycin susceptible.[375] In their work on the genome of the *E. faecalis* bacterium, Paulsen *et al.* reported that there were 41 putative cell-wall anchored surface proteins in this species[376]; 17 proteins resembled the microbial surface component recognizing adhesive macromolecule (MSCRAMM) of staphylococcus. They studied nine of these. Two were found to be cell-wall-anchored enzymes. The other seven are structurally related proteins that are likely to interact with host proteins, such as the collagen adhesin ACE. Further, it was found that the genes encoding these seven proteins were found in all species of *E. faecalis*. [376] ACE is a collagen adhesin expressed in *E. faecalis*.[60] Although the collagen binding MSCRAMM, ACE, is reported to be much better expressed at 46^{o}C, *E. faecalis* has been found to also express ACE at 37^{o}C.[259] The presence of an appropriate ligand has

been shown to stimulate the up-regulation of ACE.[35] This same adhesin had been reported to be important to *E. faecalis* binding to particulate dentine.[259] This suggests that *E. faecalis* can and would after a period of incubation on extracellular matrix proteins, adapt to the substratum by expressing genes which help them to survive on the new substratum surface.

In order to study the bacterial interaction with chemically treated type-I collagen substrate, we used an eggshell membrane collagen model.[347] The collagen type-I membrane obtained through this method is consistently about 100 ± 30 μm thick and is interwoven in a network much like collagen obtained from decalcified dentine and may help to provide a reasonable estimation of the extent of bacteria adhesion to collagen exposed by EDTA treatment of dentine. The aims of the present study was to enumerate the number of bacteria adhering to type-I collagen and to see if there were differences when the membranes were treated by chemicals routinely used during root canal treatment.

5.2 Material and Methods

Eggshell type-I collagen membrane was obtained as described in chapter 3. The chicken eggshells obtained were cleaned. The cleaned eggshell was placed in 1M hydrochloric acid for one hour; the resulting decalcified collagen membrane was

carefully removed and washed in distilled water. The membrane was then soaked overnight in an acid solution of bovine pepsin (Roche, Switzerland) to remove all non-collagenous proteins. The collagen membrane was cut to size, about 10mm by 8 mm using sterile surgical scissors, and then placed under UV light for one hour to kill any bacteria. The prepared membrane sections were treated by chemicals commonly used in root canal treatment as shown in Table 5.1.

Whilst in conventional light microscopy, there is parallel and simultaneous transformation of all object points to the image, in confocal laser scanning microscopy, this transformation is done by directing a laser light or by moving the specimen relative to the laser beam, point by point serially, over the entire specimen to obtain information. The confocal beam path is created by using a confocal aperture (usually called the pinhole), arranged in a conjugate image plane between the light detector and the objective. Light has to pass through the pinhole (infinitely small) to get onto the detector. The laser is focused tightly onto a spot on the specimen at a time, the point illuminated, and the point observed is focused into each other giving a confocal beam. It is hence possible to make optical sections (about 100μm) to examine the vertical plane (Z axis) of the specimen. The fundamental advantage of CLSM is the ability to get controlled depth of focus for the entire specimen, whereas in optical fluorescent microscopy stray light from out of focus planes also get into view, reducing image contrast. In addition, if more than one chromophore is present, colour mixing, due to light from out of plane of focus would also occur. In addition, with the ability of making controlled focus along the Z axis, it is possible to make

optical sections at different depths and reconstruct them into a three dimensional image, giving much more information than a merely two dimensional image. Although the first commercially available microscopes came on the market only in 1987, the concept and setup of confocal microscopy was invented by Minsky in 1961.[377] Confocal laser scanning microscopy (CLSM) is a useful tool for assessing the presence of bacteria in the entire thickness of the collagen membrane. The quality of the fluorescent microscope images would be affected by the auto-fluorescence of collagen making the enumeration of the adherent bacteria difficult. CLSM is thus a superior technique for this purpose.

Table.5.1 Treatment groups of type I collagen membranes for CLSM.

Group	Chemical	Concentration	Duration
1	CH	Saturated solution	24 hours
2	CH	Saturated solution	24 hours
	EDTA	17% pH7.2	1 hour
3	EDTA	17% pH7.2	1 hour
4	CHX	0.4% pH6.8	1 hour
5	NaOCl	0.05% pH 11	1 hour
6	Control	Deionised water	1 hour

After chemical treatment, the membranes were rinsed thoroughly in 10 ml of deionised water for five minutes and put into an overnight culture of *E. faecalis*

ATCC 29212 in AC media, with the number of bacteria adjusted to an optical density equivalent to10^8 cells per ml was used. The culture was grown at 37^{o}C in a shaker incubator, shaking at 100 revolutions per minute. It had been shown that *E. faecalis* undergoing doubling in about 83 mins.[378] Further, in pilot experiments of the AFM experiment, it was realised that by the time the AFM was set up, it was about two hours. Hence, it was decided that the membranes be incubated for two durations – either one or two hours. At one hour therefore, the number of adhering bacteria is what is physically attached to the collagen membrane and the number of bacteria enumerated at two hours would also include those of daughter cells; and we would see the early biofilm forming capacity. Further if there was any Substantivity of any of the chemicals at two hours it should not allow an increase in bacteria adhesion count over that of the count at one hour. Substantivity had been reported for CHX on dentine.[202] After incubation, the membranes were removed from the culture and placed in 5 ml of deionised water in a 15 ml Falcon tube and vortexed for 30s at 3000rpm (Barnstead MaxiMix II, Type 37600, USA) to remove the loosely adherent bacteria cells. The maximum relative centrifugal force (RCF) exerted by the vortex mixer was calculated to be 450G. The membranes were then mounted onto a microscope slide and stained with LIVE/DEAD BacLight bacteria viability stain (Molecular Probes, USA) , following manufacturer's instructions and observed in a CLSM immediately. The CLSM (Olympus FV 500, Japan), was set to monitor FITC and PI. Confocal illumination was provided by a Kr/Ar laser (488nm laser excitation) fitted with a long-pass 514nm emission filter. A 580nm beam splitter was used together with a long-pass 520nm filter (green fluorescent signal) and a long-pass 590nm filter (red fluorescent signal). Simultaneous dual channel imaging using

pseudo-colour was used to display green and red fluorescence. Specimens were imaged using 60 times oil immersion lens. The experiment was repeated three times. In each experiment, three similarly treated specimens were observed by randomly selecting a single field of about 250μmX250μm. The total number of adherent bacteria on the nine fields of each group was counted manually, the average calculated and plotted.

5.3 Results:

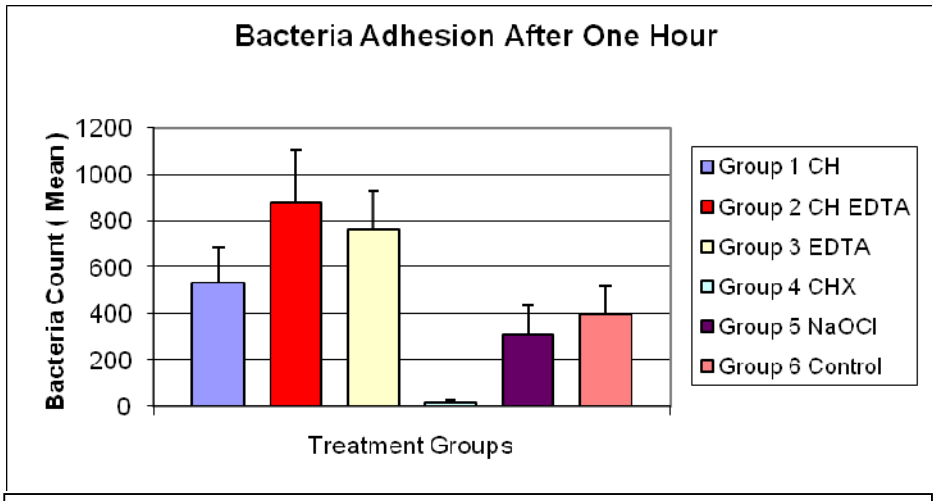

Figure 5.1 Bacteria count of chemically treated collagen after one hour

Figure 5.1 shows the number of bacteria adhering to the membranes by treatment groups. Different chemicals affected bacteria adherence to collagen type I differently. One hour after inoculation and incubation at 37^{o}C, CLSM showed that bacteria adhered to all samples of type-I collagen membrane treated.

Statistical analysis by one-way ANOVA using Multiple Comparisons, with $p < 0.05$, showed that there was significant difference between all groups, except that there was no difference between the (CH) group 1 and the (CH-EDTA)group 2 treated groups. In percentage terms, the increase in number of bacteria adhering to the CH-EDTA and EDTA groups was about 200% when compared to the control.

In the CH-group, there were clumps of *E. faecalis* cells on the surfaces of the collagen fibrils. These clumps of *E. faecalis* cells were not seen in any membranes treated with CH and then soaked in EDTA. As these clumps were bigger than *E. faecalis* cells, it could not be determined if these comprised only bacteria though it seemed to be of bacterial origin. Such clumps were counted as a single cell; hence, in the CH-group there may be an underestimation of the number of bacteria present. Accordingly, in percentage terms, the CH-group had 30% more adhering bacteria when compared to our controls.

Collagen treated by NaOCl and CHX had less bacteria adhering to them than controls. The NaOCl treated group had about 25% less bacteria than controls. Consistently, after one hour of incubation, the collagen membranes treated by CHX had the least number of bacteria adhering.

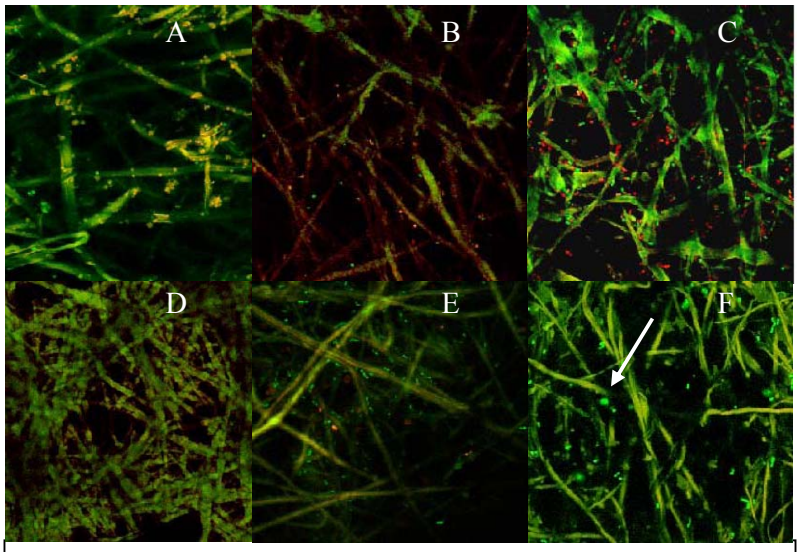

Figure 5.2. *E.faecalis* adhering to collagen after one-hour incubation time at 37°C. A – control; B – NaOCl; C-EDTA;D- CHX;E- Ca-EDTA;F-CH, note clump of *E. faecalis* cells arrowed.

Figure 5.3 below is the mean number of bacteria adhering two hours after inoculation and incubation at 37° C. As was in the one-hour results, CLSM showed that bacteria were present in all specimens. There was also variability within groups. The total number of bacteria had increased in each group. The clumps of *E. faecalis* cells, which we saw earlier in the one-hour CH specimens, were also seen in the two-hour CH specimens (Figure 5.4 below). Statistical analysis by one-way ANOVA using Multiple Comparisons, with $p < 0.05$ showed that there were significantly more bacteria in the control and CH groups than the other groups. There were no significant differences between the other groups.

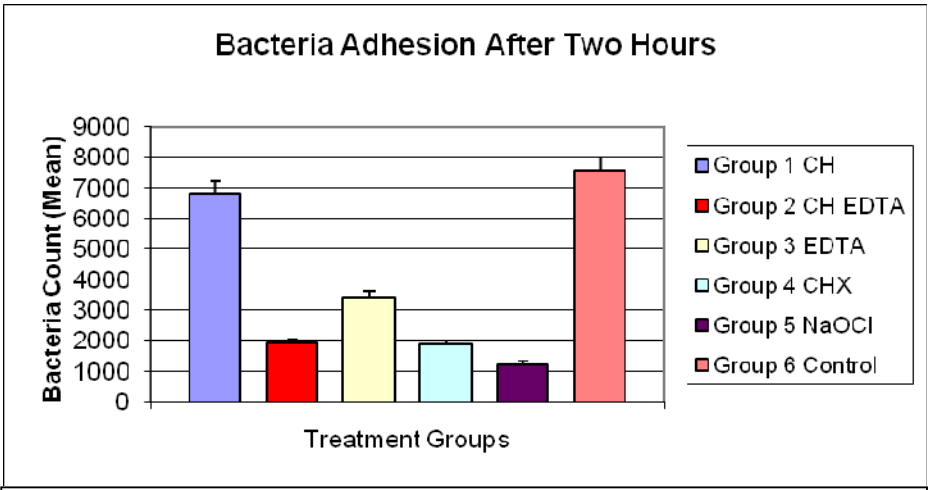

Figure 5.3 Bacteria Count of Chemically Treated Collagen after Two Hours

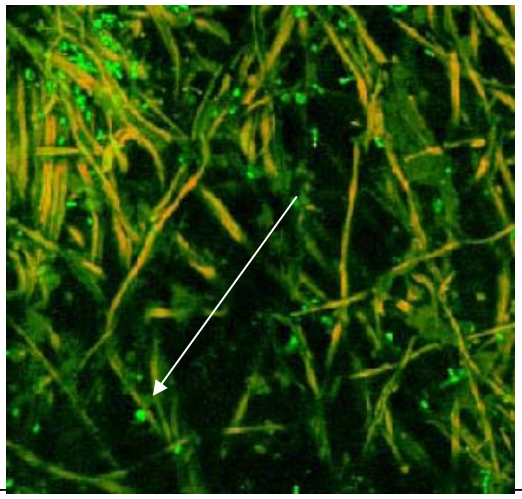

Figure 5.4. Clumps of cells (arrowed) were also seen at two hours on CH treated collagen membranes.

After one hour, the least number of bacteria were present in the CHX treated collagen membrane and the most bacteria on the CH EDTA treated membranes in the order CHX<NaOCl<Control<CH<EDTA<CH EDTA. After two hours, group with the least bacteria adhering were the NaOCl treated membranes whereas the most

bacteria were found on controls, in the order NaOCl<CHX<CH EDTA <EDTA <CH <control. Hence after one and two hours, the two treatment groups that had the least bacteria adhering were either NaOCl or CHX.

5.4 Discussion

E. faecalis was reported to secrete a hydrophobic gelatinase with a prominent ability to attack collagenous materials and certain bioactive peptides.[79] The two hour enumeration of bacteria represented the early biofilm forming potential of the treated membranes. After two hours however, we found no significant differences between CHX and other treatment groups, except that control and CH group had more bacteria than all other groups. We suspected that adaptation to the substratum collagen may be a factor for the increase in numbers of bacteria adhering to all groups. This was because, as indicated earlier, the ligand that *E. faecalis* is adhering to would contribute to up-regulation of enzymes that contribute to its survival.[35]

There seemed to be substantivity of CHX, evident at the one-hour experiment, however, this did not carry over into the two-hour experiment. We did not find any study dealing with substantivity of CHX on collagen. CHX substantivity had been reported to be effective on dentine to 12 weeks after application.[202] Other groups had reported that substantivity last for only about 48 hours[379] or 72 hours.[380] It is clear however, that CHX substantivity comes from its interaction with phosphate groups of

hydroxyapatite.[381] Since there is little of phosphate groups within collagen, it is no wonder that at two hours, the substantivity effect observed at one hour was missing. Our experiments showed therefore that there is no substantivity of chlorhexidine on collagen except for a very short duration of perhaps an hour. However, this effect was sufficient to reduce the presence of bacteria such that at the end of two hours, there was still less bacteria in specimens treated by CHX than all other chemicals except NaOCl.

In both the one and two hour experiments, we saw aggregations of *E. faecalis* in the CH treated groups. Venegas *et al.*[382] had earlier reported that an increase in Ca^{++} in the medium contributed to an increase in oral bacteria (*Lactobacillus* and *Streptococcal* species) adhering to hydroxyapatite. There may have been an increase in Ca^{++} concentration in the membranes treated by CH despite our washing them. This increase in concentration may have contributed to the aggregation as well as the higher number of adherent bacteria at two hours. Calcium bridging had been reported among bacteria to cause flocculation. [383] Calcium is also known to participate in stabilizing complex bacterial exo-polysaccharides in a gel state that enhances cell to cell as well as cell to surface aggregation and adhesion.[384] It is speculated that the calcium ion is somehow responsible for the presence of aggregates which are not seen among other specimens. However, the relationship between collagen and calcium had been shown to be complex, as pre-treatment of *E. faecalis* with collagen renders them more susceptible to CH.[385] In addition, with CH treated membranes had more bacteria adhering to them than those that had been treated by EDTA. It is speculative, but it is

suggestive of calcium bridging being an important method by which *E. faecalis* adheres to collagen as it had been reported that calcium-dependent junctional integrity might play a role in augmenting bacterial translocation in enterocytes of the intestines, the natural habitat of *E. faecalis* .[386]

Further, it had been previously reported by Kolenbrander that the use of EDTA breaks up co-aggregating pairs. [387] Although we have vortexed the membranes, we wonder if there are any remnants of EDTA on the membranes. If there had been, this could have contributed to an increase in the bacteria count, as *E. faecalis* are often found in pairs, breaking up the co-aggregation would increase the number of singly occurring bacteria in any field. Although this model is a method to see how treatment may affect initial adhesion to exposed collagen matrix, we have not yet fully characterized this membrane. Chemical treatment of the membrane affects not only the chemistry but also the physical dimensions of the interwoven collagen lattice structure, and these differences should be accounted for in a conclusive study of bacteria adhesion using this model. For example, when NaOCl had been used, it could reduce the strands for bacteria to adhere to per unit area, hence reducing the number of bacteria in a field giving an impression that NaOCl reduced bacteria adhesion. Also, the bacteria were counted by counting bacteria trapped in the entire thickness of the membrane. If NaOCl also thinned out the membrane, the depth over which bacteria were counted would be reduced and reduce the overall number counted.

Nonetheless, these results indicate quite clearly that if dentine collagen were to be exposed after EDTA treatment of dentine, if the last irrigants were either CHX or NaOCl, the exposed collagen would harbour fewer *E. faecalis*. In this study, bacteria had been inoculated with culture media onto the collagen membranes. Although we expect little in terms of nutrient value of the media after 8 hours, we cannot rule out that it still provided some nutrition to the bacteria. In further studies, using this model, we should wash the bacteria cells and then re-apply them without any culture media to see how they survive and use collagen as a nutrient.

5.5 Conclusions

The results of this study show that chemicals used in irrigation of root canals can affect the quantity of *E. faecalis* adhering on collagen. Collagen membranes irrigated by NaOCl and CHX had the least number of bacteria adhering, both after one hour and two hours of incubation. Due consideration should be given in selecting the final irrigant of the root canal.

Chapter 6 Adhesion Force of Enterococcus faecalis to collagen in the presence or absence of Calcium detected by Optical Tweezers

6.1 Introduction

Eggshell type-I collagen membrane is a soft membrane of interwoven collagen fibrils. In order to measure the interaction force between *E. faecalis* and the collagen membrane, we can choose either the use of a tipless AFM cantilever, or use an optical tweezers. As AFM cantilevers with tips may be caught in the open weave membrane and give inaccurate readings. We chose to use the optical tweezers.

Ashkin first demonstrated that it was possible to create radiation pressure using by focusing a laser beam using an objective lens of high numerical aperture, to trap small dielectric particles.[388]In addition, the sensitivity of measured forces using AFM is at best in the tens of picoNewton range, whereas for a single bacterium, the force of adhesion may be much smaller. An optical tweezers is capable of measuring forces down to tens of femtoNewtons (10^{-15} N).[389]

When light falls on a particle, it may be scattered, absorbed or transmitted (in the case of transparent and translucent particles). A particle near the focus will experience a force due to transfer of momentum from the scattering incident rays. The optical force may be resolved into two components – the scattering force (which pushes the particle away from the light source) and the gradient force (which draws the particle towards the light source). In most situations away from where the light beam is tightly focused, light is mostly scattered and hence scattering force dominates.

Near the point of focus however, most of the light is not scattered as there is a steep intensity gradient and the gradient force arises as a dipole in an inhomogeneous electric field experience a force in the direction of the electric field gradient. The scattering force is in the same direction as the laser beam, whereas the gradient force is in the other direction. The gradient force arises because there are inhomogeneous electric fields at the focus. The gradient force is proportional to the intensity of the beam at the focus. Stability of the trap is achieved when the restoring force is stronger than the pushing force or scattering force on the dielectric particle. For this reason, the trapped particle is always slightly beyond the focal point. The ray diagram (see Figure 6.1) illustrates the idea of gradient force and scattering force.

In an optical tweezers, the relationship between the wavelength of the trapping beam and the diameter of the dielectric particle are important considerations. Where the diameter of the sphere to be trapped is smaller than the wavelength of the beam,

where the radius (a) of the particle is bigger than the wavelength, that is **a** >>. λ; the Rayleigh regime is assumed. In this regime, the dimensions of the sphere are neglected and the particle is treated as an induced point dipole.

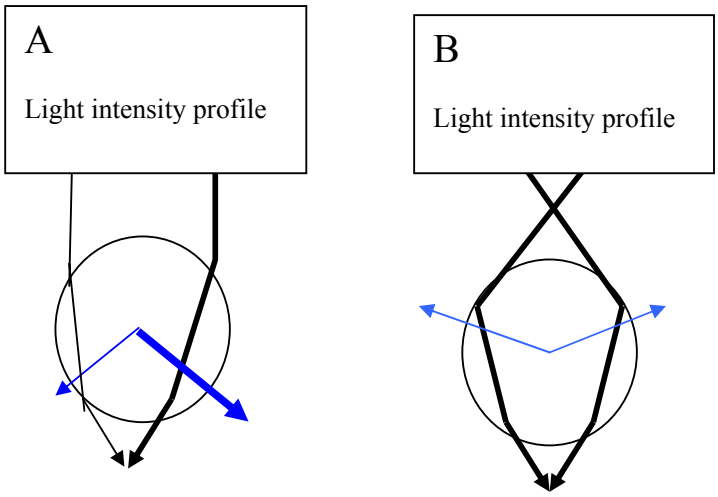

Figure 6.1 Diagram showing optical forces acting on a particle.

A – Roughly parallel beam of different intensity represented by lines of different thickness, gives rise to a net force to the right.

B − A tightly focused beam produces a three-dimensional intensity gradient giving light of about equal intensity on both sides; lateral forces cancel each other out leaving a gradient force towards the focus.

Light incident on a dipole divides naturally into two components, the gradient force and the scattering force. The focus is taken to be a small region roughly equal to the wavelength of the beam. The trapping force is calculated between the laser field and the induced dipole. The gradient force points opposite to the direction of the beam whereas the scattering force points in the direction of the incident light. Further, in

Rayleigh regime, the force is independent of particle shape. Should the diameter of the trapped particle be bigger than the wavelength of the beam, that is **a <<̣ λ̣;** conditions of Mie scattering are met, than optical forces can be simply computed from ray diagrams as shown in Figure 6.1. By Newton's third law, an equal and opposite change in momentum is imparted on the sphere by light. The force exerted on the sphere is proportional to the light intensity and is given by the rate of change of momentum.[390]

The aims of the present experiment were to determine the adhesion force of *E. faecalis* to collagen in the presence or absence of calcium, since we had determined in the adherence assays of *E. faecalis* to collagen experiments, reported in chapter 5, that the groups treated with calcium had the highest numbers of bacteria adhering.

6.2 Material and Methods

6.2.1 Laser tweezers set up

A schematic diagram of the laser micromanipulation set up is shown in the Figure 6.2. The output of a 1064nm cw Nd: YAG laser (SSLD, CAT, India) operating in fundamental Gaussian mode was expanded using a beam expander (BE) and coupled to the objective of an inverted microscope (Axiovert 135TV, Carl Zeiss) through its

base port via a folding mirror (M2) that retains its regular fluorescence imaging configuration and capability. Another convex lens (SL, f = 200 mm) was placed into the optical path of the beam external to the microscope. This lens along with the tube lens (TL) of the microscope behaves as a 1:1 telescope and thus a collimated beam is sent to the 100X microscope objective. A 100X Plan Neofluor oil immersion phase objective (MO) was used to focus the laser beam to diffraction limited spot. The dichroic mirror (DM) reflects the UV-visible light from the excitation source (mercury lamp, HBO) through the excitation filter (EX) and transmits the fluorescence. The dichroic beam splitter (DBS) transmits the cw 1064 nm and reflects the emitted fluorescence through emission filter (EM) to the CCD. An infrared cut off filter (CF) was placed before the CCD to reject the back-scattered laser light. The fluorescence images were captured using a cooled CCD and processed using Q-Fluoro standard image analysis software (Leica). The bright field trapping sequences were recorded using video CCD on to a VCR, and digitized using frame grabber (FG).

The CW Nd: YAG laser trapping beam power was adjusted to attain powers up to 200 mW at the focal plane of the objective. The trapping laser beam power at the back aperture of the objective was monitored with a power meter (Scientech, USA). Furthermore, for estimating the transmission factor of the objective, the dual objective method was adapted in order to correct for the total internal reflection losses at the objective-oil-glass-water interfaces. The transmission factor of two cascaded objectives was found to be 0.33. From this, the transmission factor of a single l00 X

Plan Neofluor phase objective (Carl Zeiss, Germany), was estimated to be $(0.33)^{1/2}$ i.e. 0.57.

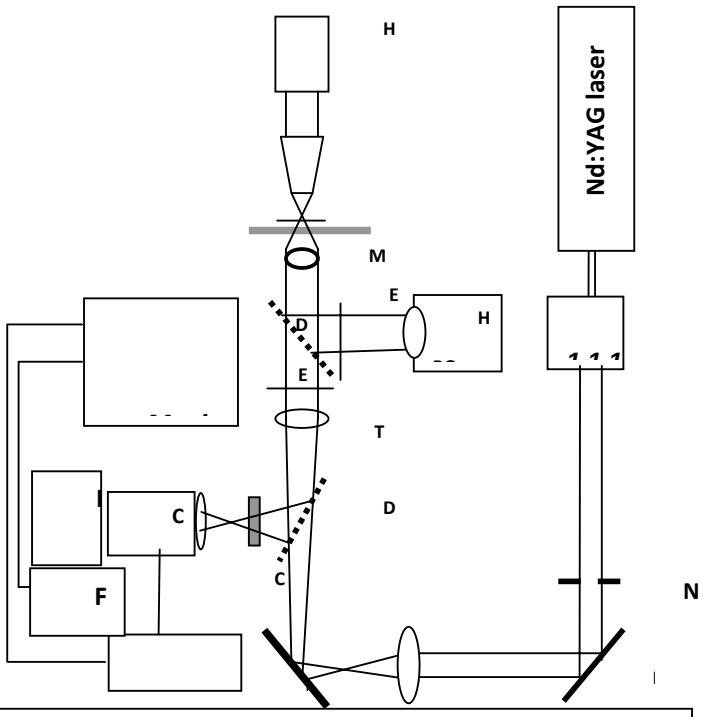

Figure 6.2 Schematic diagram of Laser Tweezer setup.

In order to determine the position of a bacterium in a video frame, the method of centred detection was used. A software program based on the LabView platform was developed for quick analysis of large number of images. The region of interest selection and thresholding was carried out in order to reject background images of other objects (such as collagen fibre) outside the trap. For determination of tracking resolution, a bacterium was fixed on the coverslip and the position of its centre was measured using the software for ~1000 time-lapse (40 ms) digitized frames. For *E. faecalis*, the tracking resolution obtained was better than 50 nm (standard deviation of position histogram).

For force measurements using optical tweezers, eggshell membranes were obtained as described previously. *E. faecalis was* grown overnight and diluted using AC broth (Sigma Aldrich, USA) to about 10^4 cells per ml. The prepared collagen membrane, whether treated by CH or not, was carefully spread onto a clean glass slide and the edges teased under magnification to obtain frayed edges with single strands of collagen fibres. A small volume of AC broth with the adjusted bacteria concentration, about 200µl, was applied onto the membrane, and a cover clip was placed over it and the entire system was sealed. A single bacterium was trapped and moved in order to measure the trapping force and stiffness. Then the bacterium was brought to a single collagen fibber with the optical-tweezers and manipulated to ensure that it was in plane with the fibber. The bacterium was then held in place for varying durations and then its mean position was measured in order to estimate the interaction force. At the end of approximately ten minutes, the rupture force was measured by pulling the

trapped bacterium away from the fibre using the optical-tweezers of varying laser power.

For this experiment, type-I collagen membrane was soaked for 5 mins in a media that contained 50% AC broth and 50% saturated CH. The membrane was spread on a glass slide, onto which diluted bacterial suspension was added. The force of laser tweezers on the bacteria was estimated at different trap power levels using escape force method and trapping stiffness was calculated using Equipartition theorem method. Different treatment groups of collagen membranes, studied using optical tweezers, are listed in the Table 6.1.

Table 6.1 Treatment groups studied by optical tweezers

No. of Bacteria Studied	**Treatment groups**
3	Collagen soaked with 75 % CH for 15 min.
4	Collagen soaked with 50 % CH for 15 min.
4	Collagen soaked with 50 % CH + 50 % AC media for 5 min.
5	Collagen not soaked with CH
3	Collagen overnight with 50% CH and 50 % AC media
4	Collagen overnight with 25% CH and 75 % AC media

6.2.2 Force calibration using escape force method:

In this method of force calibration[391, 392], a single bacterium was trapped at different power levels and translated. The velocity was progressively increased until it escaped from the optical trap. The limiting velocity was derived using the method described above and the corresponding viscous drag force was calculated. The *E. faecalis* bacterium was assumed to be spherical in shape with diameter ~ 0.5 μm.[393] When the bacterium, is more than a few diameters away from the sample cell walls, the viscous drag exerted by moving the stage or the bacterium (of diameter d*)* at a velocity *v* and is given by Stoke's law:

$$F = 3\pi\eta dv \qquad (6.1)$$

Here, η is the viscosity of the medium (0.001 Kg m^{-1} s^{-1}). For a specified laser power, the critical velocity at which the trapped bacterium comes out of the trap determines the trapping force. For a trapping power of 50 mW, the limiting velocity was found to be 90μm/s and this corresponds to a viscous drag force of 0.52 pN. Repeating this experiment for other laser powers gave a "force vs. laser power" calibration curve and it scales linearly (Figure 6.3) with the laser power for a particular size and refractive index of the object (bacterium). The slope of the linear

fit (Figure 6.3) provides an estimate of the trapping efficiency for an *E. faecalis* bacterium.

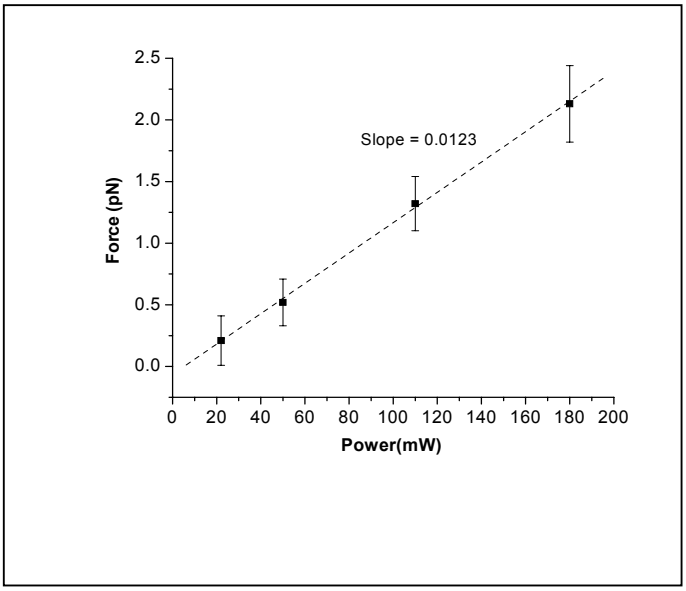

Figure 6.3 The linear relationship between power and force of laser trap.

The trapping efficiency of the optical tweezers was described in terms of a dimensionless parameter *Q*. The fraction of momentum transferred to the trapping force from the trapping laser beam, which is related to the force on the bacterium, *F*, the power of the laser, *P,* and the refractive index of the surrounding medium (n = 1.33*)*, through the equation:

$$F = n\,Q\,P/c \qquad (6.2)$$

Using the slope of the linear fit (~ 0.0123, Figure 6.3) the lateral trapping efficiency of optical tweezers *Q (lateral*) for the *E. faecalis* bacterium was found to be as low as ~ 0.3%. For dielectric particles, the trapping force (hence efficiency) has been seen to decrease as linearly with r as particle size increases beyond the focused spot size (~0.8 μm). For Rayleigh (size of particle $<<\lambda$) particles, trapping efficiency decreases more rapidly (~ r^3) since the gradient force is proportional to polarizability (α), which depends on the particle radius (r) as r^3. In addition to very small radius of the *E. faecalis* bacterium (~ 0.25μm), since the relative refractive index of the bacterium with respect to the surrounding medium is also quite small, the theoretical values for *Q* is expected to be very small. Due to this small value of trapping efficiency, the maximum trapping force that can be applied to the *E. faecalis* bacterium was limited to ~ 2pN at the maximum trapping power of 180 mW. Application of larger trapping power was limited since it can lead to loss of viability of the bacterium.

6.2.3 Measurement of trap stiffness using Equipartition theorem method

Forces acting in a trap are described by Hooke's law: $F = -K_{trap}. X$. Using the trap to determine the force exerted by a bacterium therefore required calibration of its stiffness K_{trap}. The trap stiffness was determined by use of the Equipartition theorem,

$$½ K_{trap} \langle x^2 \rangle = ½ K_B T \qquad (6.3)$$

Where, K_B is the Boltzmann constant and T is the temperature of the medium in which the bacterium was suspended. Measuring the variance of the position of the trapped bacterium provides an estimate of trap stiffness,

$$K_{trap} = K_B T / Var \langle x \rangle \qquad (6.4).$$

The chief advantage of this method is that knowledge of the viscous drag coefficient is not required and therefore of the bacterium shape as well as fluid viscosity. The transverse positions of a bacterium in the trap are detected using centroid detection method and a representative track over 50 frames (i.e. for 2 seconds) is shown in Figure 6.4b. The bacterium can be seen to be around one most probable position, which is centre of the trap focus (here, pixel number 11). Frequency count at different positions of the bacterium inside the trap is done and the variance of the position of the trapped bacterium is calculated. The stiffness of the optical trap was then calculated using Eq. (6.4) and this experiment was repeated at different trapping power levels. The stiffness vs. laser power graph was plotted as shown in Figure 6.4 C.

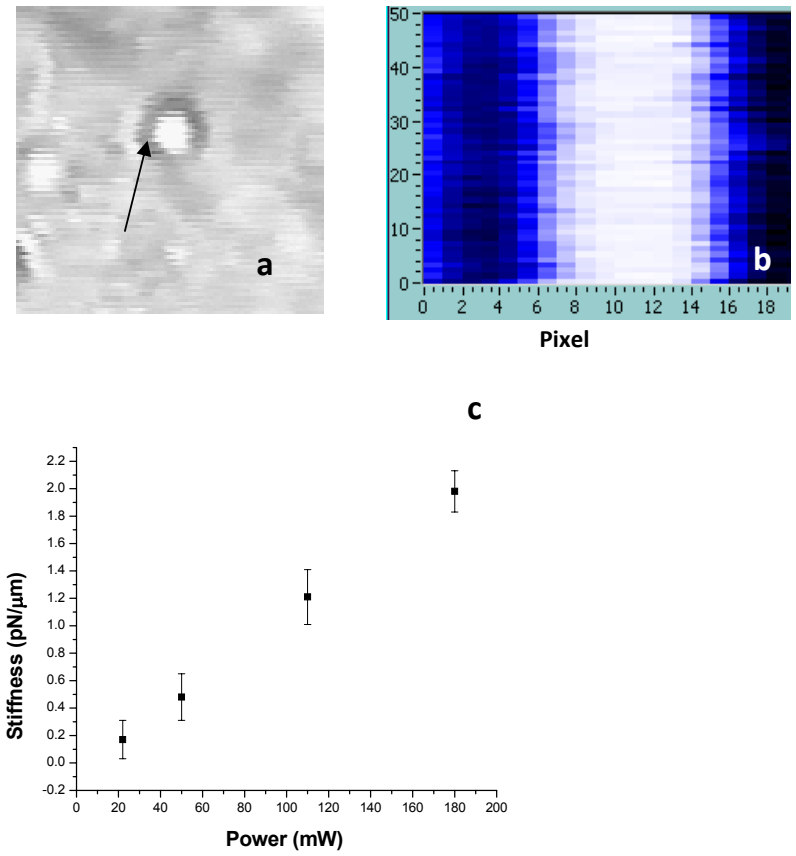

Figure 6.4 a – Optical view of a trapped bacterium (arrowed)

b – Centroid Position of a bacterium during analysis

c – Stiffness vs. Power plot of laser trap

6.2.4 Optical tweezers based study on interaction between bacteria and collagen

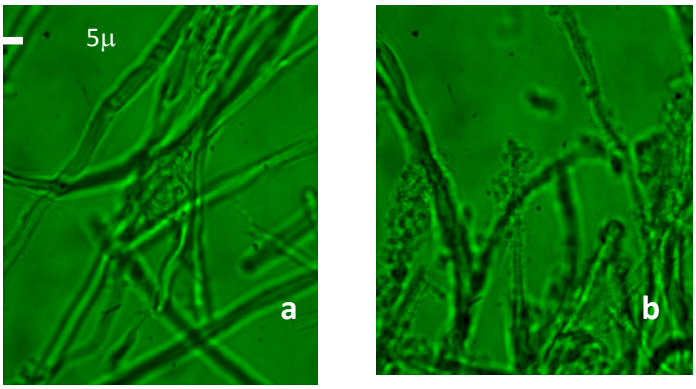

Figure 6.5 Digitized images of bacteria on Type-I collagen matrix

Figure 6.5 above shows digitized images of bacteria on collagen matrix. Large numbers of adhering bacteria were found (b) on collagen membranes treated with calcium as compared to those collagen without calcium treatment (a). In order to assess the adhesion of bacteria with collagen under various treatment conditions, optical tweezers was employed. During experiments with the use of the optical tweezers, a single bacterium was brought near to a collagen fibril and the time taken by the bacterium to attach with the collagen fibril was measured. Studies on the different treatment groups listed in Table 6.2 (based on varying fraction of calcium, duration of immersion etc) showed that maximum bacterial adhesion occurs for

collagen soaked with 50% CH as compared to 0%, 25% or 75% saturated CH solution. Hence, it was decided to study the adhesion force of *E. faecalis* to collagen in the presence of 50% saturated CH solution.

6.2.5 Force measurement using laser tweezers

Since with collagen soaked with 50% calcium for 15 min, the bacteria was found to firmly attached in ~6.5 min, detachment of the bacterium could not be accomplished at the highest laser power used (180 mW). Therefore, instead of rupture force measurement, continuous measurement on interaction of bacteria was made using laser tweezers. Figure 6.4 shows the time-track of interaction of *E. faecalis* bacterium with collagen. After a duration of interaction, the mean position (black lines in Figure 6.4 b) of bacterium was found to shift towards the collagen fibril. A cartoon depicting interaction force measurement using the displacement (Δx) of the bacterium from its mean position in presence of collagen is shown in Figure 6.6.

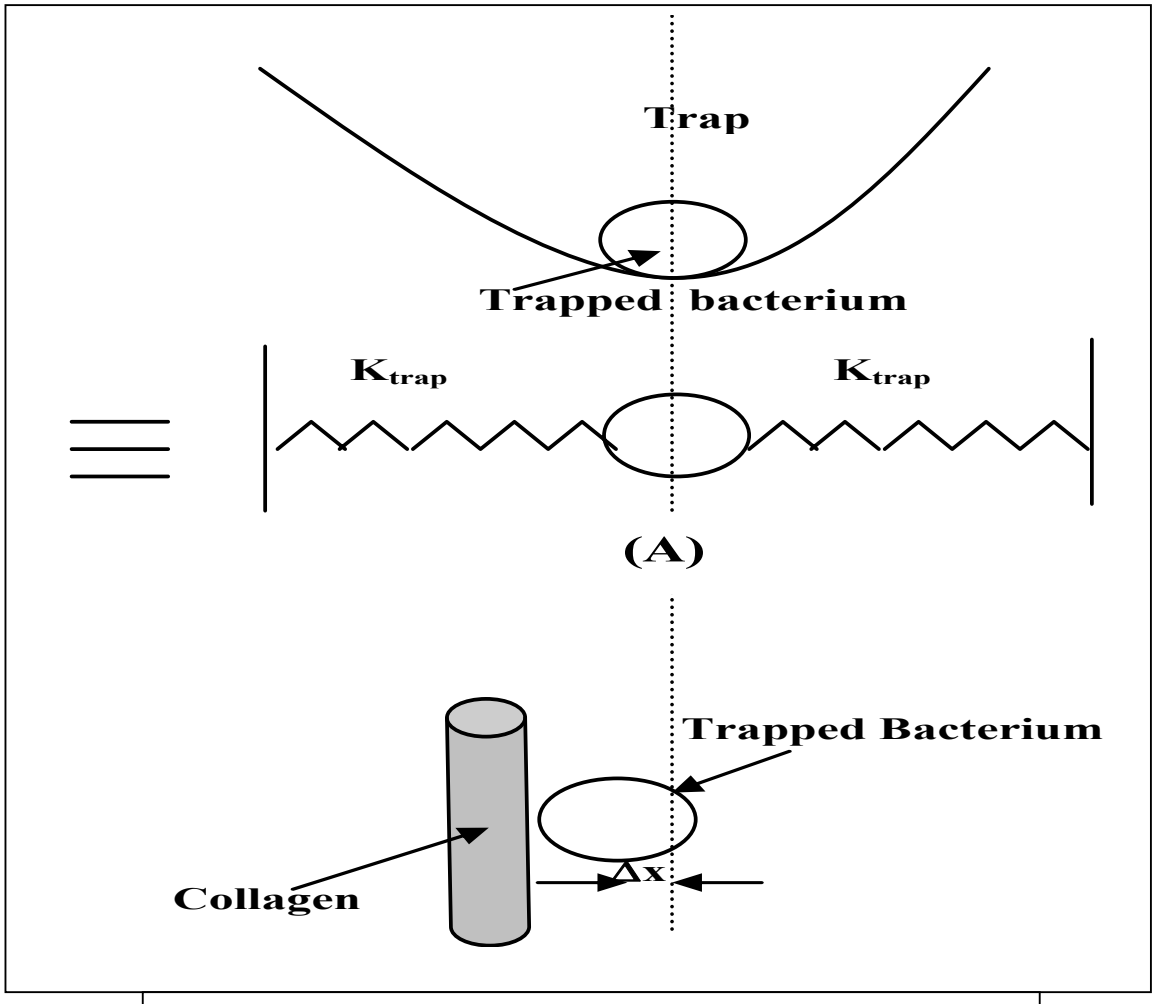

Figure 6.6 Schematic diagram showing interaction force measurement using the displacement (Δx) of the bacterium from its mean position in presence of collagen

6.3 Results

From the pre-determined stiffness of trap (K_{trap}) for *E. faecalis* bacterium in absence of collagen (see Figure 6.4), the interaction force was determined using,

$$F = K_{trap} \cdot \Delta x \qquad (5)$$

For a fixed interaction period of 5 min, the interaction force was found to increase from 0.03 pN when the collagen is not treated with calcium to 0.9pN in case of calcium treated collagen fibre. Further, in calcium treated collagen, the interaction force increased with increase in time of interaction of bacterium with collagen (Table 6.2). In some cases, at small interaction durations, the bacterium could be detached from the collagen fibril by increasing the laser power to 180 mW and thus rupture force could be measured for short interaction durations (~5 min). The mean rupture force was significantly lower (0.38pN corresponding to a trap beam power of 36 mW, obtained from Figure 8.3) in absence of calcium as compared to the case of the calcium treated ones (2.1 pN, at 180 mW, see Figure 6.3).

6.4 Discussion

Optical tweezers based experiments highlighted that CH treatment increased adhesion forces of *E. faecalis* to type-I collagen. Cations are essential to help bacteria to adhere to surfaces. It is no wonder that in the presence of calcium, the adhesion force is higher.[394] Though calcium bridging had been reported for many species of oral bacteria, [395] there had been no specific report of the importance of calcium to *E. faecalis* adhesion to surfaces and co-adhesion.

Table 6.2 Effect of treatment of collagen membranes with calcium on interaction force.

Condition	**Mean displacement of bacteria (µm)**	**Power (mW)**	**Force (pN)**
After 5 min near collagen (No Ca)	0.26	22	0.03± 0.02
Rupture of bond after 5 min near collagen (No Ca)	Away from collagen	36	0.38± 0.11
After 1 min near collagen (50 % Ca)	0.25	22	0.04± 0.01
After 5 min near collagen (50 % Ca)	0.43	22	0.9± 0.17
Rupture of bond after 5 min near collagen (50 % Ca)	Away from collagen	180	2.1± 1.05

In this study, there seemed to be a maximum force of adhesion at 50% saturation with calcium. More studies are required to understand the increase in the adhesive force of *E. faecalis* to type-I collagen in 50% saturated CH solution.

This finding may have a bearing on the outcome of endodontic treatment and retreatment. During primary root canal treatment, if a clinician did not manage to complete the treatment within one seating, an inter-appointment dressing, commonly CH would be placed to prevent regrowth of bacteria .[224] Similarly, when a tooth has a periapical lesion and needs retreatment, after removal of the root filling material, the clinician is likely to place an interappointment dressing in the attempt to kill remnant bacteria within the root canal system. Regular culturing is not the current clinical practice, nor is culturing able to inform the clinician accurately of all the species of bacteria present in the root canal. The clinician therefore does not know which species of bacteria caused failure. Further, though *E. faecalis* has been shown increasingly as a bacteria involved with failed root canal treatment, it is but one of many species of bacteria to cause failure. Hence, clinicians are likely to continue to use CH as an inter-appointment medicament.

It is important for clinicians to realize that they should try to ensure the complete removal of CH within the root canal. Not removing it completely has implications on *E. faecalis* adhesion and may affect treatment outcome if *E. faecalis* was present in the root canal. In addition, NaOCl in combination with EDTA, have been reportedly useful in removing CH paste debris from root canals[396]; though none of the techniques used, including passive ultrasound, could remove CH remnants entirely.[397,398] In our study, we did not use CH paste but saturated CH solution. The CH paste used in clinical practice is made by mixing CH powder into a thick paste and contains much more calcium hydroxide than the saturated solution, which is only a 0.14% solution.

We hypothesize that the effect of CH paste on *E. faecalis* retention is likely to be more pronounced than that with CH solution.

Collagen makes CH even less effective against *E. faecalis*, as strain A197A was found to be more tolerant to exposure to CH after prior exposure to collagen. [399] Hence, during retreatment, if any EDTA was used to remove smear layer in the first visit, collagen that is exposed with this step should be removed by soaking the root canal with NaOCl of appropriate concentration for a sufficient duration. A two-minute soak with 5% NaOCl may be deemed sufficient to remove collagen. [400]

Although bacterial adhesion is known to occur in two phases,[401] it is uncertain what event marks the end of phase one and which the commencement of phase two. In the minutes that had taken the bacteria to move closer to collagen, we assume that this was within the early reversible phase of adhesion. Hence, the forces measured are not likely the stereochemical bonding type of force, such as would be expected between *E. faecalis* ACE and collagen, but rather more likely to be physico-chemical forces, such as electrostatic attractions, hydrophobic interaction, hydrogen bonding and van der Waals type forces. These results should be validated by studies of adhesion assay and adhesion force studies by *E. faecalis* to dentine.

It is interesting to note that whilst we measured the *E. faecalis* adhesion force to collagen was increased in the presence of calcium hydroxide; the two hour adhesion to collagen was not more than controls (see Figure 5.3). It should be remembered that the method we use to measure the force in this experiment was to bring the single bacterium against a collagen fibril to measure the force of adhesion. In any adhesion assay, this does not happen. Essentially, by bringing the bacterium against the collagen fibril, we help overcome the electrostatic repulsion that each colloidal particle has to overcome in Brownian motion before it comes to rest against a surface. In so doing, several other factors that would affect the bacteria adhesion assays, such as gravity, pair particle interactions and collision frequency are no longer operative.[402] Other authors have also found that adhesion force increases could not be linearly related to the desorption rates.[403]

6.5 Conclusions

Within the limitations of this experiment, it was shown that the presence of calcium increases the force of adhesion between *E. faecalis* and eggshell type I collagen. We should however be mindful about relating adhesion forces linearly to adhesion assay numbers as many other factors which affect bacteria adhesion are neglected when the adhesion forces are measured. Laser tweezers was a useful method in assessing the force of adhesion between bacteria and collagen substrate.

Chapter 7 Enterococcus faecalis Adhesion and Adhesion Forces to Root Canal Dentine

7.1 Introduction

In a solution, the bacterium is in constant Brownian motion. It was noted by Zobell in 1943 that bacteria prefer to grow on available surfaces, rather than remain in the aqueous phase.[404] The adhesion of a bacterium to a surface depends first on its transport to the vicinity of the substrate, followed by the attachment to the substrate and then followed by the development of molecular interactions with the substrate to desist dislodgement.[9] Hence, these events can be thought of as occurring in two phases, an initial instantaneous reversible phase or the physicochemical phase also called phase 1 and a time dependent irreversible molecular and cellular interaction phase also called phase 2.[298, 405]

To study the factors that promote the phase 1 of adhesion between bacteria and the substratum adhesion is therefore to study the interaction of physicochemical forces operating between bacteria and the substratum. The term association describes the interaction between the bacterium and the substratum without specifying a mechanism. Adherence is a term many authors use to mean different things and is a

term best avoided.[406,298]. Figure 7.1, modified from Bos et al 1999, show the specific and non-specific forces that affect the adhesion of a bacterium to the substratum.

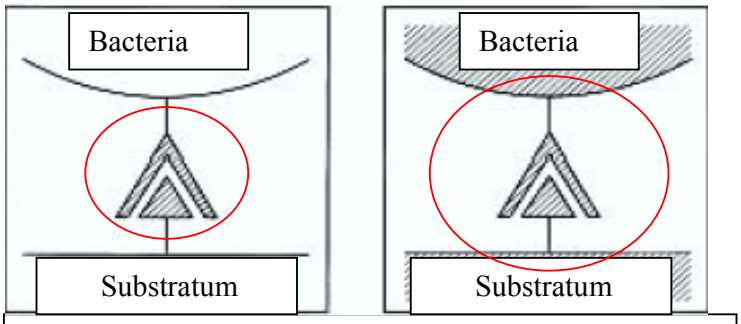

Figure 7.1 Physico-chemical forces are the fundamental forces of adhesion. Left panel, stereochemical forces or specific forces are considered (circled), whereas in the right panel, forces affecting surfaces of the entire body are considered.[9]

7.2 E. faecalis adhesion and conditions of the culture

There had been much variation in the number of bacteria reported in adhesion studies of *E. faecalis*. This may be due to the differences in clinical strains, experimental protocols including values used to differentiate specific binding to non-specific binding[407] and differences in growth conditions that affect surface components in enterococci.[408] Hence in any study of *E. faecalis* adhesion to a substratum, it is important to use the same growth media and the same bacteria culture protocol to minimize variations, so that comparisons between substrates treated by different chemicals can be made. It is also important to define the number of bacteria

per ml of culture used to study adhesion. Other factors such as culture conditions were (log phase or stationary), rocking and flow rates are important and need to be specified and be consistent. Culture conditions, method of rinsing, counting and direct counting need to be specified and the conditions of the substrate surface should be standardized.[409]

7.3 Methods of measuring adhesion

Adhesion of a microbial cell is unambiguously defined in terms of the energy required to remove the cell from a surface. It is important to know if removal leaves a fragment of the cell behind. If this were to happen, smaller energies would be measured as both cohesive and adhesive failure would have happened.

There are in general three different methods to study adhesion of bacteria to a substratum. These are:

1. Measurement of association of microbial populations on a surface can be done by using optical microscopy, confocal or scanning electron microscopy. These methods may be combined with dyes, fluorescence and antibodies and use or non-use of laminar flow techniques. These methods measure the overall results of adhesion events between bacteria and the substratum surface and but do not provide direct evidence of the increase or decrease of the adhesion force resulting from treatment of the substratum.[410]

2. Direct measurement of adhesion force by methods involving separation of cell and substratum shows direct evidence of increase or reduction in adhesion force. It neglects to account for the overall effect on the substratum, but rather takes into account the interaction of specific points of the substratum.[411]
3. Techniques involving measurement of contact angles. This method measures the surface energies and gives an understanding of the potential of an adhesion event.[412, 413]

Recently, the Atomic Force Microscope (AFM) had been used to measure the interaction force between bacteria and the substratum. This can be achieved by attaching bacteria to an AFM tip and test it against the substratum to measure the interaction force[414] or by allowing the bacterium to settle onto the substratum and apply a force through the AFM tip to remove the cell. [411] Both these methods would measure the force of removal of the bacterium from the substrate, and give a direct measure of the effect of chemical treatment on adhesion.

7.4 The Atomic Force Microscope

A surface profiler is used to measure the surface features in a vertical direction. An AFM tip can be thought of as such a profiler. A laser is pointed at the reflective surface, on the back of an AFM tip and a sensor picks up the laser light reflected there from. The reflected laser beam strikes a position-sensitive photo-detector consisting

of four-segment photo-detector. The differences between the segments of photo-detector of signals indicate the position of the laser spot on the detector and thus the angular deflections of the cantilever. Figure 7.2 illustrates the parts of the AFM modified from Daniel et al.

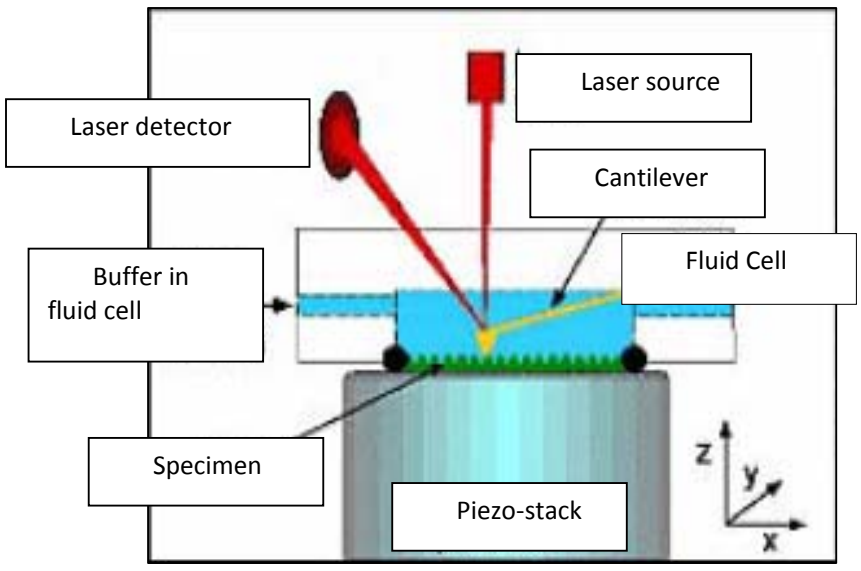

Figure 7.2 Components of the atomic force microscope.[6]

Depending on the set-up, the movement of the tip, which has a sensitive spring constant, can be amplified many times such that even movements over distances as small as a silicon atom can be detected and visualized. Movements of the tip as it traverses over a surface are recorded using software by a computer and is used to make comparisons. [415] The computer also controls the movement of the sample, which can be moved in three dimensions through electrical control of a piezo-electric stack.

A block diagram of the setup would be as in the diagram in Figure 7.3. The similar setup is used for both study of the vertical forces of adhesion and the shear force necessary for removal of a bacterium. A typical force curve is shown in Figure 7.4.

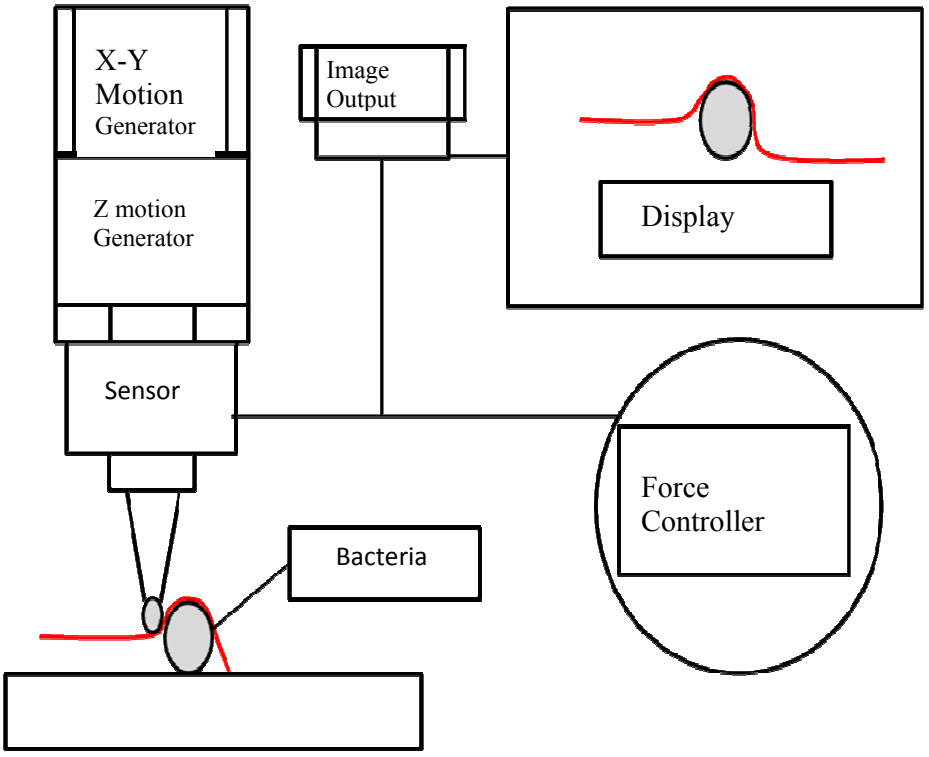

Figure 7.3 Diagram of Atomic Force Microscope setup

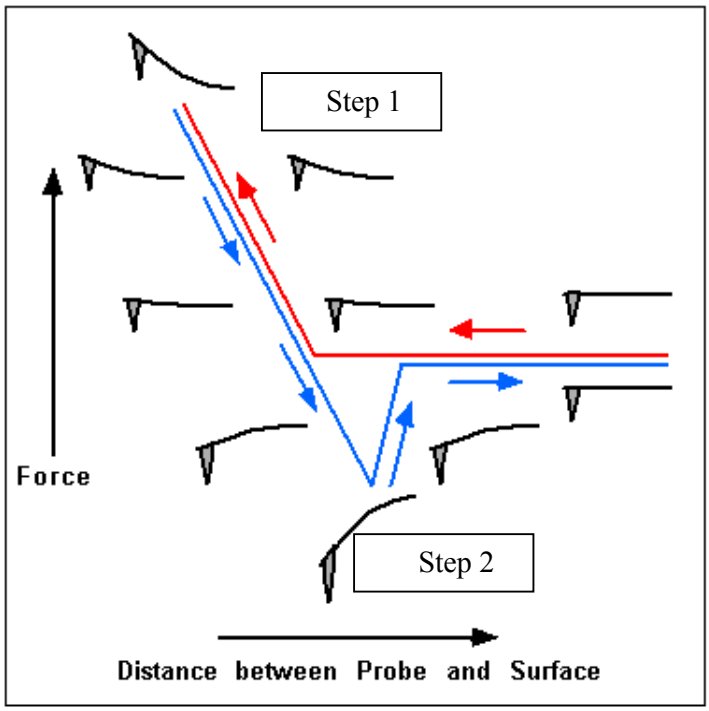

Figure 7.4 Diagram showing a typical AFM force vs displacement curve. The red line is the approach curve and blue line is the retraction curve, and they represent the forces acting on the AFM tip. At step 1, there is repulsion pushing the tip up till movement of the piezo finally gets it into contact with the specimen. As the tip is moved away, adhesion prevents the tip from detaching until step 2, when the adhesive force ruptures giving rise to the step in the retraction curve. Adapted from a webpage.[5]

Raw force curves consist of a voltage measured on the photodetector as a function of the vertical piezo displacement (z) at a given x, y location. The slope of the retraction curve is then converted into deflection force using Hooke's law:

$$F = - kD \qquad (7.1)$$

Where F is the force, D is the deflection and k is the spring constant. The zero separation distance corresponding to the contact point between the cantilever tip and sample, when the tip suddenly snaps into contact as the tip goes past the repulsive forces (step 3 in above diagram). At the pull off from the surface, there is a hysteresis of the curve and the size of the hysteresis is dependent on the area of contact, position of cell in contact, surface energy and time of contact between the tip and the substratum surface.[416]

In an ideal situation there is no separation between the approach curve and the retraction curve, at the straight part of the curve known as the constant compliant region. However, when there is drift in the piezo-electric stack or when the substratum is a little soft and when there is displacement of the substratum, there can be a separation between the two curves in the constant compliant region, which leads to errors in forces measured. The errors that enter into the measurement of forces using this method include:

- spring constant error (if two significant Figures are used a 5% error is assumed)
- error in calculating the deflection of the cantilever from the region of constant compliance
- surface separation -at small forces, the error in surface separation is dictated by the control of the piezoelectric crystal

- error due to the nonlinearity of both the piezo voltage versus distance and the diode-voltage versus cantilever deflection responses. [417]

There is still controversy regarding how forces should be measured from an AFM force distance curve. It has not been determined which represents the point where the forces acting on the probe tip is at zero on the curve. Whether the Ducker and Selden method[417] or contact biomechanics method reported by Emerson and Camesano[418] is more appropriate is at best uncertain. Further authors have used various positions of the curve as the contact point between the tip and the substratum, hence the forces of interaction between the tip and the substratum can take on several interpretations.[419]

There are also two ways to remove bacteria from a surface. A bacterium could be removed by pulling it vertically off the surface or it can be peeled away. It was decided that both these methods be used to make a comparison of the forces measured. The method of Razatos *et al.*[414] measured the vertical forces of adhesion of a bacterium to the substratum surface within seconds of contact probably represents the early events of adhesion and that of Tedjo *et al.*[411] measuring a peeling or shear force after many hours of adhesion representing the shear force of detachment and included the later events of bacteria adhesion such as molecular interactions with the substratum.

Aims of Experiments

The aims of the experiments were: 1) to characterize the number of bacteria adhering and the adhesion forces between bacteria and dentine substrate after various chemical treatments, 2) to obtain a better insight into the process of bacteria substrate interaction, the Zeta potential of chemically treated dentine was also measured, 3) AFM based analysis was used to determine the forces of perpendicular and lateral adhesion *E. faecalis* to dentine. Whilst it may be good to measure the force of interaction between bacteria and the substratum, it is important to assess the impact of such measured forces on the overall numbers of *E. faecalis* adhering to a surface after a period of time. To make this assessment, we decided to use confocal laser scanning microscopy using fluorescent dyes to count the number of bacteria adhering to the surface. Hence, the experiments reported in this chapter included:

Experiment 1 AFM measurement of perpendicular forces of adhesion between *E. faecalis* and dentine substrate after various chemical treatments

Experiment 2 AFM measurement of the shear force of adhesion between *E. faecalis* and dentine substrate after various chemical treatments

Experiment 3 *E. faecalis* adhesion to dentine substrate after various chemical treatments

Experiment 4 Zeta potential measurements of dentine powder after various chemical treatments.

Information from these experiments would give us an understanding in the changing adhesion forces with each type or sequence of chemical treatment and lead to a better insight as to whether increased forces of adhesion are related to increases in number of bacteria adhering to the substratum.

7.5 Material and Methods

Harvesting Dentine and Dentine polishing

Permanent single rooted premolars extracted for periodontal or prosthodontal reasons were collected. The age of these teeth were unknown. Decoronation was carried out using a diamond disc, and the pulpal tissues and cementum were carefully removed using endodontic and periodontic hand instruments. The tooth was then sterilized using a steam autoclave at 121^{o}C and held at that temperature for 20 minutes. Autoclaving teeth had been recommended as the method of choice to disinfect dentine in dentine bonding studies, as it does not affect dentine and dentine bond strength studies[420] and an accepted method of disinfection. Even if dentine collagen were to denature during autoclaving, at temperatures below 175 ^{o}C , the conformation of collagen reverted to normal, [323] and is unlikely to affect the results of our studies.

Slices of dentine roughly parallel to the longitudinal axes of the roots, from the mid root section of the root, about 6mm long, were harvested using a hard tissue microtome. The microtome uses a diamond wafering blade of dimensions about 76mm diameter and thickness of 0.15mm (Series 15 LC, Buehler, Illinois, USA).

The first cut along the root was offset by about 0.15mm from the centre of the coronal root canal aperture, such that the cut surface was about 0.3mm from the centre of the root. The microtome has a micrometer gauge to control the selection of the next cuts. Careful adjustments to the micrometer were made to obtain a second cut about 0.12mm further away from the first, thus harvesting a dentine section of about 0.12mm thick. The dentine section was then freed from the block and used for experiments. Only one section from each side of the root canal was harvested. All dentine sections were polished by hand using sandpaper, progressing from 1200, 2500 to 4000 grit. (Buehler, Illinois, USA). This gives a standardized surface texture for all the experiments. It had also been reported that this process of polishing would remove any smear layer.[421] The external root surface was also trimmed by sandpaper, checked using Vernier callipers, to make it parallel to the test surface. The final thickness of the specimens was about 110μm. Each section was cleaned in DI water in a Falcon tube place in an ultrasonic bath for 10 minutes (Elma S30H, Germany).

7.6 Experiment 1 - AFM measurement of perpendicular forces of interaction

The aim of the present experiment was to determine the vertical force of adhesion of bacteria with chemically treated dentine. A single colony of *E. faecalis* was grown in AC Broth(Sigma Aldrich, USA) and harvested after 6 hours, washed twice in deionised water, and kept overnight in 2.4% electron microscope– grade glutaraldehyde at 4^{o}C until it was applied onto the AFM tip. The AFM tip was treated with 1% polyethyleneimmine (PEI; molecular weight 1200, Sigma Aldrich, USA), according to the method suggested by Razatos *et al.*.[414] A drop of the bacteria suspension was placed on a clean glass slide. The AFM tip was viewed through a microscope whilst a micromanipulator (Leica, Switzerland) was used to move the AFM tip carefully such that the tip of the cantilever was just dipped into the bacteria suspension. Dipping the cantilever far into the drop led to a large area of the gold plated reflective back of the cantilever to be covered with bacteria; which would affect the reflection of laser from it, affecting the signal strength arriving at the photosensor. Following the protocol of Razatos *et al.*, after about 60 minutes,[414] the micromanipulator was adjusted to lift the tip and away from the drop of bacteria suspension.

Pilot studies showed that it was not possible to standardize the force curves of different probes functionalized in the same manner tested on the same specimen. This was probably due to differences in the number of protuberant bacteria from around

the probe tip. Using a single probe and testing different dentine samples also gave different forces of adhesion. This was thought to be due to the differences of the chemicals groups that were exposed for contact. In both instances, it was due to the heterogeneity of the system. Hence, in this experiment each specimen was used as its own control; that is, the force of interaction with the untreated specimen be first recorded before any chemical treatment was initiated. This would further remove any systemic errors that ensue while comparing data from different tips. [422]

A Nanoscope-IV AFM system, with a Multimode Pico-force controller (Digital Instruments, Veeco, Plainview, NY) was employed in the study. The cantilever in the system was a DNP gold-coated silicon nitride tip, with a nominal tip radius of 20–60 nm and a nominal spring constant of 0.06 nano-Newton per nanometre ($nNnm^{-1}$). Calibration of the system was performed and each of the cantilevers used was thermally tuned at the commencement of each experiment and before the attachment of bacteria. It was found that all the probes used had spring constants within the range given by the manufacturer. During force measurement, a ramp size of 2 μm was used with a forward and reverse speed of 1 μm/sec. After contact with dentine, retraction of the AFM tip was delayed by 10 seconds to allow for a short duration of interaction. This was arbitrarily selected as during pilot studies; a one-second interaction time did not give rise to any meaningful force curve. A total of 44 force curves were recorded for each specimen surface. The polished dentine specimen was mounted onto a specimen mount. After the control curves were recorded, the mounted dentine section

was dipped in 15mls of the following chemicals, in sequence, for the specified durations, washed twice in deionised water and further force curves measured:

(1) 5.25% NaOCl , (Chlorox; The Chlorox Co, Oakland, CA) for 1 hour,

(2) 17% EDTA pH 7.2 (Amresco, USA) for 5 minutes,

(3) 0.4% CHX for 5 minutes, and then in

(4) 5.25% NaOCl for five minutes again.

All force curves were recorded whilst the specimen was in a fluid cell (Veeco, Santa Barbara, CA) filled with deionised water with a resistance of 18.2 MΩ·cm as measured by the Ohmmeter of the water purification unit (Nanopure Diamond, Barnstead, USA). This was because increasing salt concentrations will reduce the interaction force and may reduce the relative difference between testing of differently treated substratum for an effect to be noticed. [423] Optical microscopy was used to confirm the presence of bacteria on the tip at the commencement at the end of the experiment. If there was no bacterium adhering to the tip, the data was discarded.

7.7 Results of Experiment 1

Individual force curves were analysed and the data plotted into histograms. A curve was fitted for each histogram and the mean force for the sequence of irrigation is shown in Figure 7.12. Samples of the curves obtained during experiments are shown

in Figures 7.5 and 7.6 below. When the force maxima obtained from the AFM force curves were compared between groups, it was observed that there were more adhesion events and larger maxima for all force curves obtained after EDTA and CHX treatment when compared with control and NaOCl treatment.

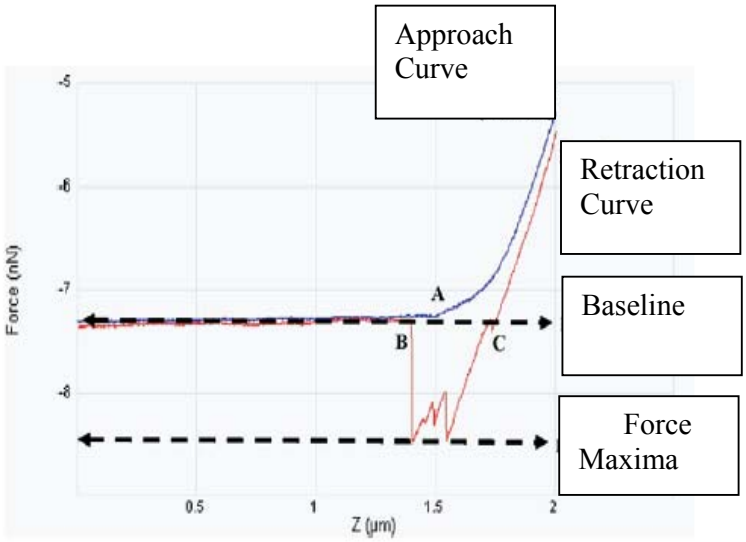

Figure 7.5. Typical force curve from AFM experiment. From Kishen *et al.* Influence of Irrigation Regimes on the Adherence of *E. faecalis* to root canal Dentine"[11]. Force curve was taken after irrigation with EDTA.

Statistical analysis with the analysis of variance and Scheffé test showed that there was no statistical significance between the force maxima observed in the control and NaOCl-treated dentine. There were statistically significant differences between the control curves and those curves obtained after irrigating with EDTA and after irrigation with CHX ($P < 0.05$).

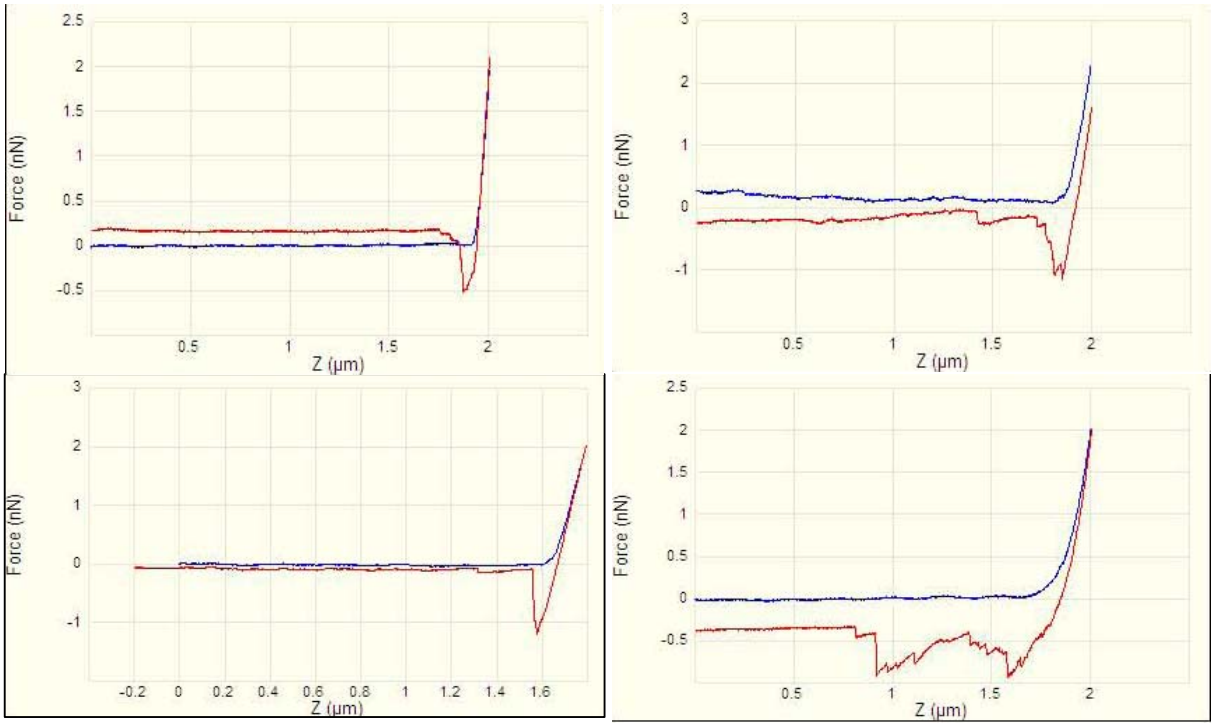

Figure 7.6: Force curves after various chemical treatment: a, After irrigation with NaOCl; b, Control; c,after the sequence of irrigation NaOCl, EDTA,CHX and then NaOCl again; d. after EDTA . Note the multi-adhesion events in d after EDTA exposure.

The mean adhesion force of untreated dentine was 0.35 nN, which reduced to 0.33 nN after NaOCl irrigation. The mean adhesion force increased to 0.97 nN after EDTA treatment, which was about 300% more than that after NaOCl treatment. CHX treatment increased the mean adhesion force further to 2.16 nN. However, subsequent NaOCl irrigation of CHX-treated dentin reduced the mean adhesion force to 0.70 nN. Histograms of the forces are shown in Figures 7.7 through 7.12.

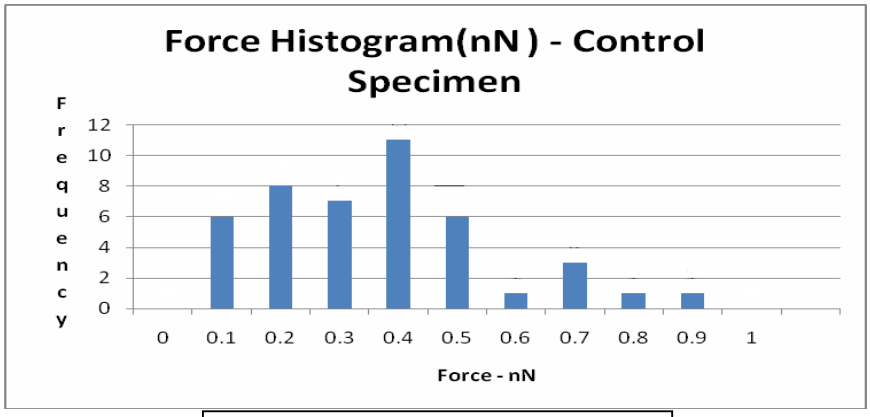

Figure 7.7 Force histogram of control specimen

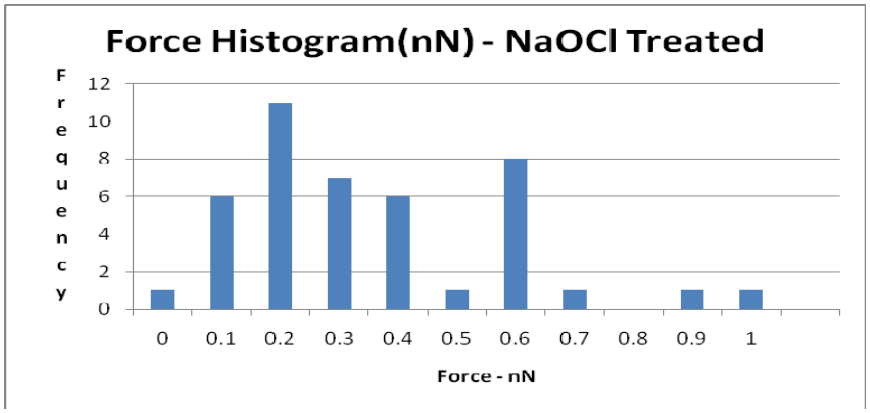

Figure 7.8 Force histogram after NaOCl treatment

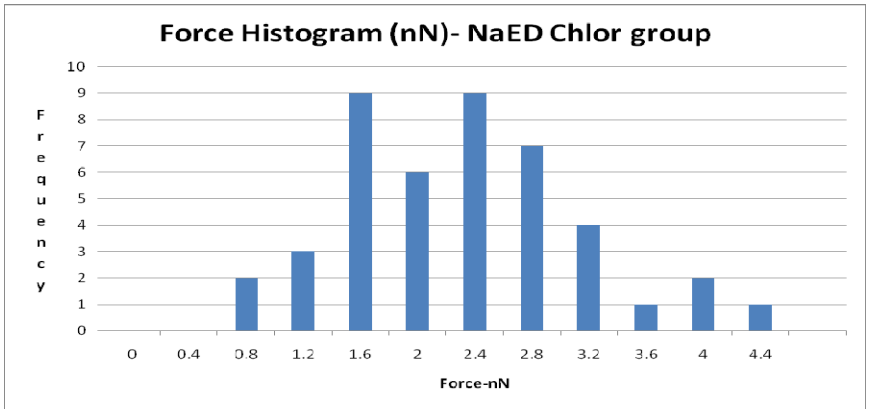

Figure 7.9 Force histogram after NaOCl EDTA and CHX treatment

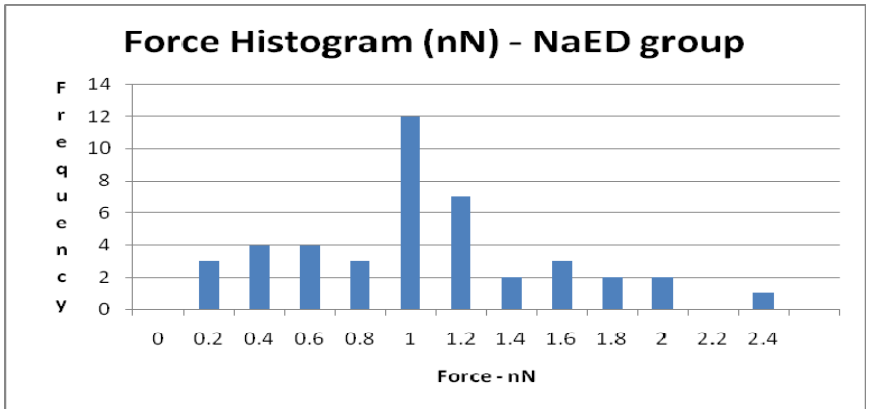

Figure 7.10 Force histogram after NaOCl and EDTA treatment

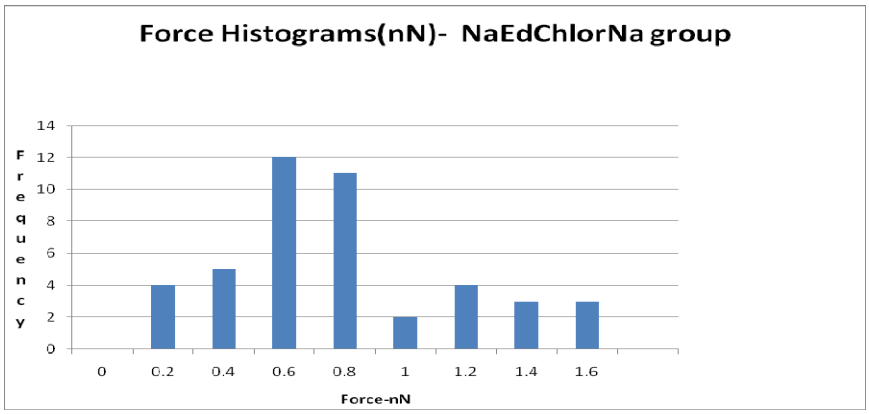

Figure 7.11 Force histogram after NaOCl, EDTA, CHX and NaOCl

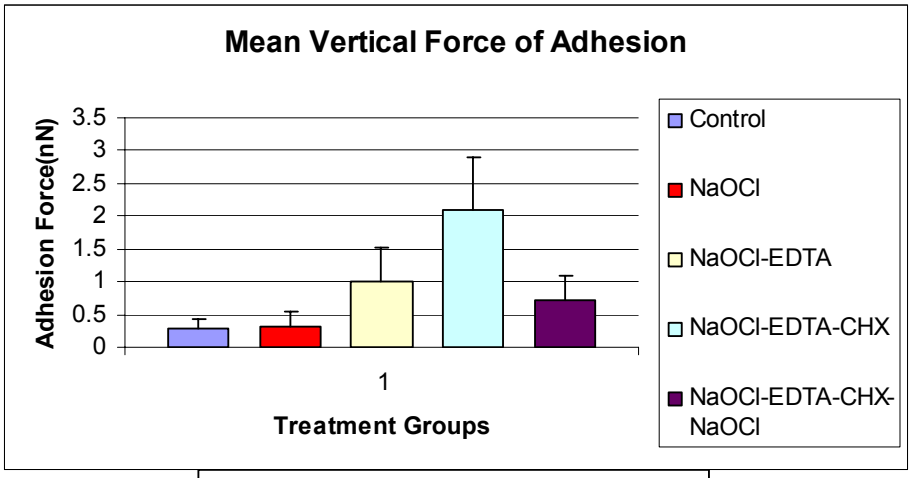

Figure7.12 Mean Vertical Force of Adhesion to Chemically Treated

7.8 Experiment 2 - AFM measure of shear force of Enterococcus faecalis adhering to treated dentine

The aim of the present experiment was to measure the shear force necessary for removal of an adhering bacterium after about 24 hours of adhesion. A single colony of *E. faecalis* was grown in AC Broth (Sigma Aldrich, USA), harvested after 6 hours. After it was washed twice in deionised water, the cells were diluted to an optical density of about 0.3(OD_{600}), equated to approximately 1.12 X 10^8 cells, using a UV-VIS spectrophotometer (Shimadzu, Japan). The Falcon tube containing the bacteria was placed in an ultrasonic bath (ELMA type S30H) at 25^oC, for 10 secs to separate co-aggregating cells. A small volume (10 µl) was then applied onto the treated dentine surface, using a micropipette for about 30 minutes. Excess bacteria culture fluid was then removed using a micropipette. The specimen, with a thin layer of

remnant culture, was placed in a sterile culture plate left ajar in a laminar flow chamber and allowed to dry overnight, for about 16 hours.[411]

Treatment of Dentine

Before the polished dentine sections were inoculated with bacteria they had been treated as follows:

Table 7.1 Treatment Groups for AFM to measure shear force of adhesion

Group	Chemical	Duration
1	Deionised water	1 hour
2	NaOCl 5.25%	1 hour
3	CHX 0.4%	5 minutes
4	EDTA 17% pH7.2	5 minutes
5	CH	7 days
6	CH	1 week
	EDTA 17% pH7.2	5 minutes

Treated dentine specimens were vortexed in Falcon tubes containing 5mls of de-ionized water, air dried and then inoculated with bacteria.

Control of AFM

The atomic force microscope used was made by Asylum Research, (USA), using a Molecular force Probe 3D controller. The method used was that reported by Tedjo *et al.*[18] Briefly, at the commencement of examining each dentine sample, a new AFM tip was thermally tuned and the deflection voltage per nano-Newton calculated. Deionised water was applied on the prepared specimen and allowed to hydrate for 30 minutes. An area about 40 microns by 40 microns was first examined in contact mode using the smallest deflection voltage to locate singly adhering bacteria. The controller was then zoomed in to 5 microns by 5 microns. Starting with the smallest deflection voltage in contact mode, an attempt was made to remove the bacterium. If the bacterium was not removed using 5 nano-Newtons, additional attempts were made using increasingly larger deflection forces, in steps of 5 nano-Newtons per centimetre till the bacterium was removed. The bacteria cells were observed to distort with increased use of force, and the stages it went through is as in Figure 7.13b.

Each group had three specimens harvested from different teeth of unknown age. Specimens in each group were treated in the same way. Each specimen provided data for three or four single randomly located bacterium until a total of ten bacteria were tested for each treatment group. The data from each group was averaged. Only single bacterium that was found on intertubular dentine was tested. Those in depressions and tubular openings were omitted.

7.9 Results of Experiment 2

The mean, mode and standard deviations of adhesion force of each of the test groups are as given in Table 7.2. The units of forces measured are in nanoNewtons (nN). There were statistically significant differences amongst all groups ($p<0.05$), using ANOVA with multiple comparisons and Post Hoc tests with Bonferroni's correction; except between group1 (control), group2 (NaOCl) and group3 (CHX). The modes of these 3 groups which were not significantly different had forces measured at 20, 40 and 20nN respectively. Whereas the modes of the group 4 (EDTA), group 5(CH) and group 6 (CH-EDTA) were increased to 80, 90 and 80 nN respectively.

Table 7.2 Means and Modes of adhesion force.*Units are in nN.

Group	1	2	3	4	5	6
Mean	24*	43	19	79	103	68
Mode	20	40	20	80	90	80
sd	7.26	15.81	1.67	10.54	23.45	16.91

Surface Roughness after Chemical Treatment

The surface roughness of intertubular dentine is shown in Table 7.3. A one micron long line of interest was chosen over the image and the height changes over that distance were averaged to determine the roughness in nanometre scale.

Table 7.3 Surface roughness of dentine after various chemical treatments

Control	NaOCl	EDTA	CHX	CH	CHEDTA
29.82nm	61.19nm	52.86nm	143.26nm	65.04nm	36.96nm

It was noted that the specimen treated by dilute Hibiscrub (CHX) had been coated with a layer of substance, most likely reacted CHX with hydroxyapatite of dentine[381] and it was not possible to measure dentine roughness per se. The roughness measured was therefore that of the coating of chlorhexidine. The coating by CHX on dentine is shown in Figure 7.15.

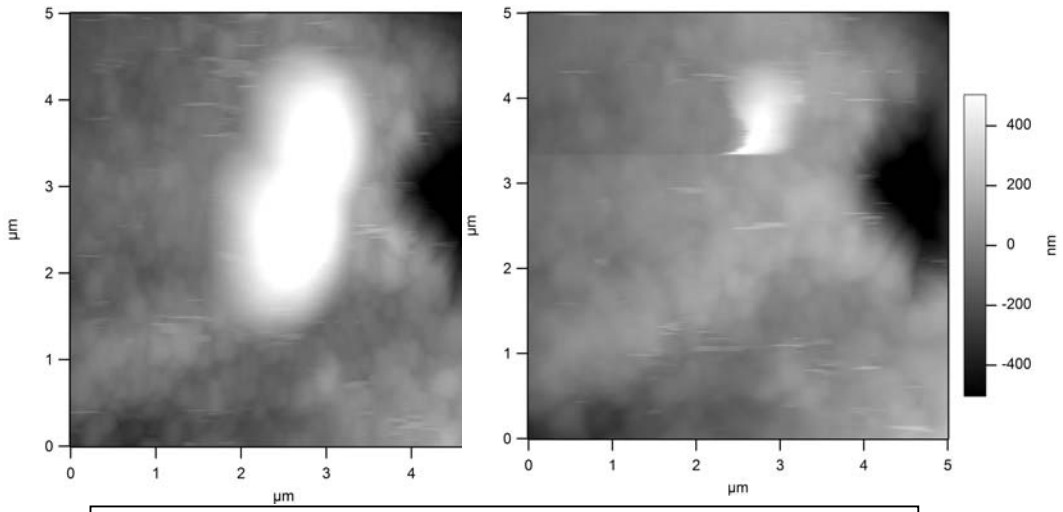

Figure 7.13a AFM pictograms of *E. faecalis* .The left figure shows a single bacterium sitting on intertubular dentine; the right figure shows the bacterium at the time of being removed.

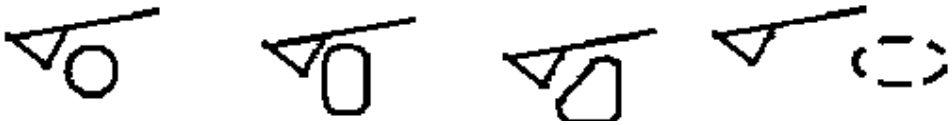

Figure 7.13b. The stages of AFM cell detachment. From left to right at: I. the bacteria is resting squarely on the substratum (as in the left panel in Fig 7.13a above); II.as force was increased, part of the bacteria is lifted such that the height profile becomes bigger; III. Further force increase distorts the shape of the bacteria; IV. the cell was detached from its original position (as in the right panel in Fig 7.13a above).

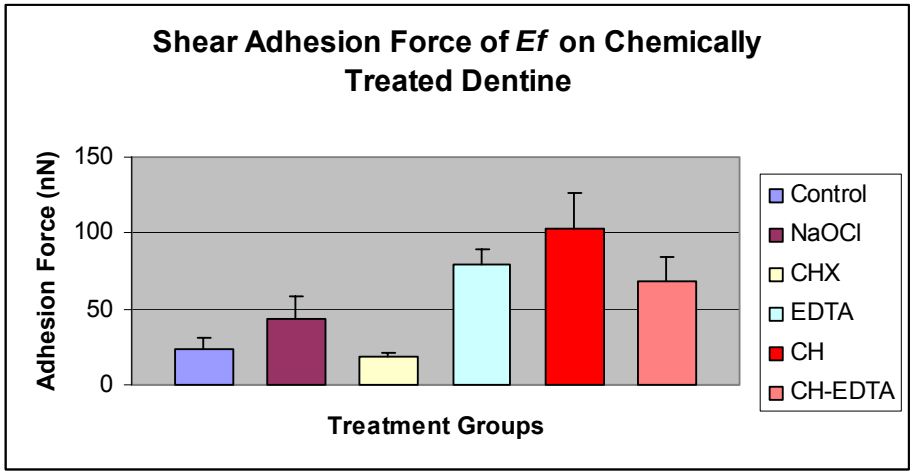

Figure 7.14 Mean Shear Adhesion Force to Chemically Treated Dentine

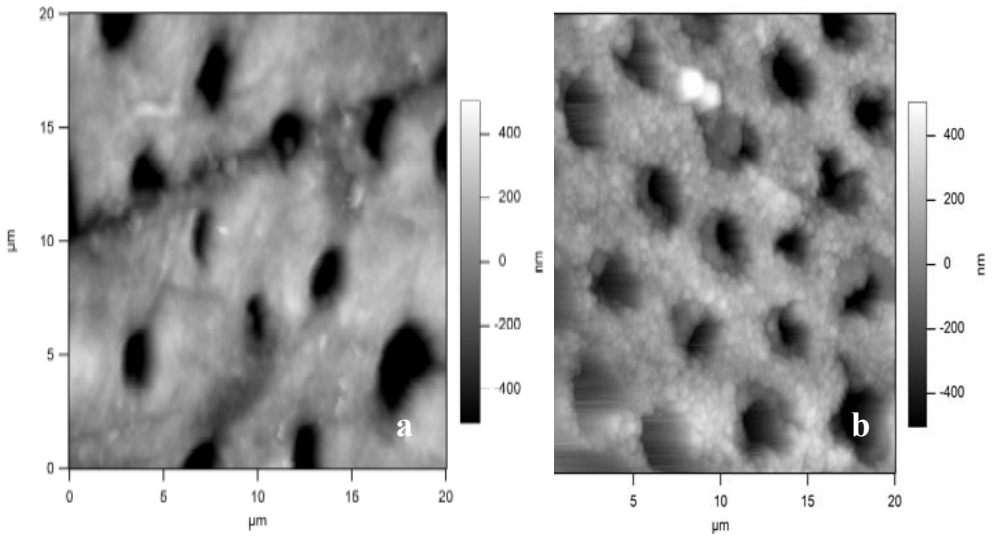

Figure 7.15. AFM image of dentine. a)Control specimen. Note no smear layer, no coating b)treated by CHX specimen coated with a layer of chlorhexidine

7.10 Experiment 3 - Bacteria adhesion to dentine after various chemical irrigation

A bacteria adhesion assay measures the ability of a bacterium to a surface.[424] In order to understand the effects of chemicals on the adhesion of *E. faecalis* to dentine, an adhesion assay of bacteria on root dentine sections was carried out. The initial number of bacteria adhering to a substrate had been reported to be importantly related to the propensity to form biofilms.[425] The aim of the present experiment was to enumerate the number of bacteria adhering to chemically treated dentine. Sequences of chemical treatment were planned to see if the last irrigant in the sequence affected *E. faecalis* adhesion.

One hundred non-carious single-rooted teeth were used. These had been maintained in PBS. The crowns were sectioned off at the level of cemento-enamel junction, and apical portions were ground to obtain uniform root sections of about 8-mm length and dentine specimens harvested as described in section 7.5. The dentine sections were divided randomly into 7 equal groups.

Material and Methods

Group 1 specimens were left untreated (control).

Group 2 specimens were treated with 17% (pH 7.2) EDTA (Merck KGaA, Darmstadt, Germany) for 5 minutes and 5.2% NaOCl for 30 minutes.

Group 3 specimens were treated with 5.2% NaOCl for 30 minutes and 17% (pH 7.2) EDTA for 5 minutes.

Group 4 specimens were treated with 5.2% NaOCl for 30 minutes, 17% EDTA for 5 minutes, and 5.2% NaOCl for 30 minutes.

Group 5 specimens were treated with 5.2% NaOCl for 30 minutes, 17% EDTA for 5 minutes, and 2% CHX for 30 minutes.

Group 6 specimens were treated with 17% EDTA for 5 minutes, 5.2% NaOCl for 30 minutes, and 2% CHX for 30 minutes.

Group 7 specimens were treated with CHX alone for 30 minutes.

Bacteria Culture and Inoculation of Specimens

A single colony of *E. faecalis* (ATCC 29212) which was maintained in Tryptone Bile X-glucuronide (TBX) agar (Merck, Germany) was transferred to 50 mL of All Culture (AC) media (Sigma Aldrich, St Louis, MO, USA). Bacterial cells were allowed to grow overnight (14 hours) at 37°C in an orbital incubator at 120 rpm.

After the designated treatment with endodontic irrigants, the dentine blocks were rinsed with distilled water. Each well was inoculated with 200 μL of *E. faecalis* (10^6cells/mL colony-forming units) in 96-well plates at 37°C for 1 hour. The dentine specimens were subsequently removed, gently rinsed to remove lose cells and stained with BacLight (Invitrogen, Molecular Probes, Carlsbad, CA, USA), and observed with a fluorescence microscope (Nikon Eclipse 80i; Tokyo, Japan) by using a 520-nm filter under oil immersion (100 X objective). The average number of adhering bacteria was recorded by counting at least 9 randomly chosen microscopic fields. The experiment was carried out in triplicates. The results of the bacterial adherence assay were subjected to one-way analysis of variance with post hoc Tukey test to determine if there was statistical significance.

7.11 Results of Experiment 3

Control specimens had higher numbers of adherent bacteria cells than any of the treated specimens. The average number of bacteria found adherent on control specimens was considered to be 100% and bacteria adhering to treated specimens are expressed as percentages in relation to control in Figure 7.16 .

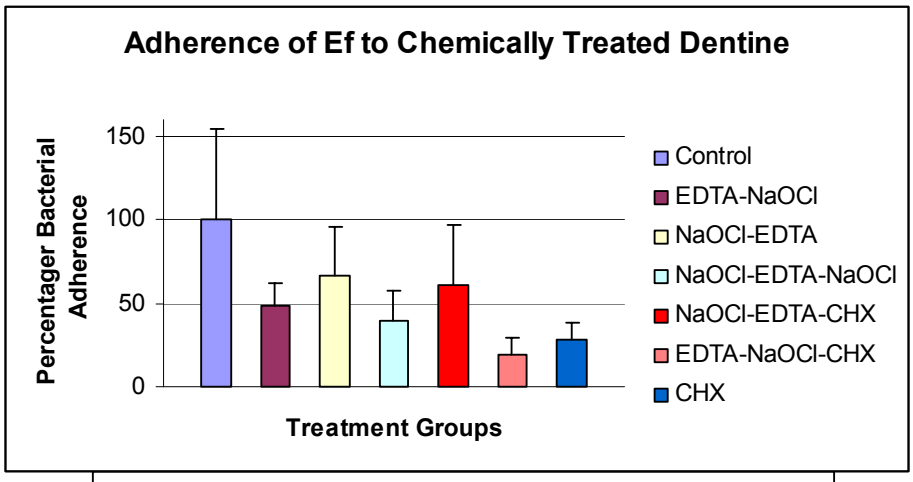

Figure 7.16 Number of bacteria adhering to chemically treated dentine after one hour.

From Figure 7.16 shows that the last chemical in the sequence had a significant effect on the number of bacteria adhering to dentine. Specimens that had been treated with EDTA had a much larger (67%) number of bacteria adhering then when NaOCl was the last irrigant (40-49%). When NaOCl was used as the last irrigant after EDTA irrigation, the numbers of bacteria adhering was reduced by 27%. After CHX irrigation, the number of adhering bacteria was only between 19 – 28%. In addition, after EDTA irrigation, if NaOCl was not used before application of CHX, CHX did not significantly lower the increase in bacteria adhering brought on by the use of EDTA.

7.12 Experiment 4 - Measurement of Zeta Potential

Zeta potential is an abbreviation for the term - electrokinetic potential, normally denoted by the Greek sign zeta (ζ). The Zeta potential is not the surface charge of a particle but rather the electric potential difference at the slipping plane between the dispersion medium and the stationary layer of fluid that is normally present around each dispersed particle.[426] Knowing the Zeta potential will allow us an understanding of the electrostatic charges left on dentine after chemical treatment and further help in explaining phase I interactions between *E. faecalis* and dentine. The aim of the current experiment was to measure the Zeta potential of chemically treated dentine.

Dentine powder preparation.

For Zeta potential measurements, fine dentine powder was generated using a finishing diamond (diamond size about 25μ;102 R Yellow band, Shofu Inc.) in a water-cooled air-rotor-handpiece (Presto Aqua, NSK, Japan) driven at 2.2MPa by compressed air. This method was an adaptation of the method of Weerkamp *et al.*.[276] Four teeth that were extracted for orthodontic reasons were used. Dentine powder was generated only from the root canal aspect of the roots and was pooled together. Both the water and dentine debris were collected and sedimentation allowed to take place overnight. The supernatant was decanted and the dentine powder allowed to dry in air,

at room temperature. Optical microscopy of the fine dentine powder showed the particle size to be mostly about 1μm to 5μm.

As dentine powder was used for Zeta potential measurements, the EDTA solution used was diluted with deionised water to 1.5%. Pilot studies showed that when 17% EDTA was used, the dentine powder became a clear gel like substance and this would be stuck to the sides of the Falcon tube and could not be removed easily. All other chemicals used were as discussed in experiment 1 above. The duration of the chemical treatment was for NaOCl, 1 hour; for EDTA, 5 mins; for CH, 1 week; for CHX, 1 hour, for the first treatment by any of the chemicals. If more than one solution was used in the sequence, the first liquid was carefully decanted and the dentine powder washed with deionised water before the next chemical was applied. All treated samples were washed two times in 10 mls of deionised water centrifuged and resuspended in deionised water for Zeta potential measurements.

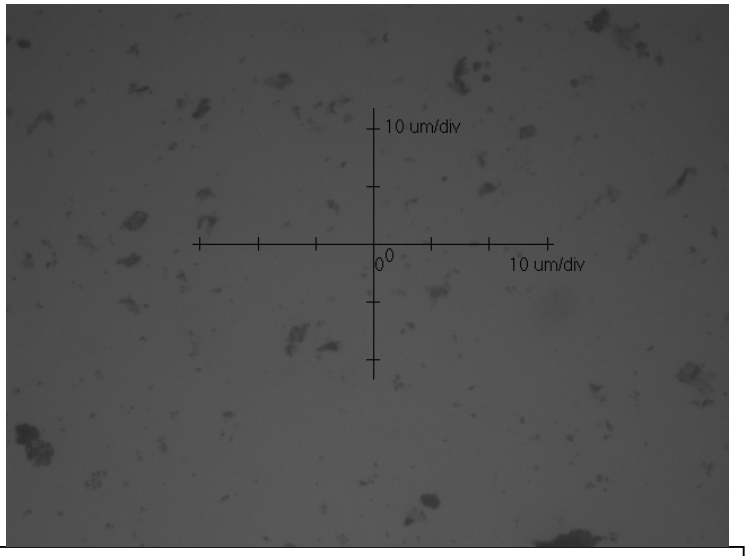

Figure 7.17 Photomicrograph showing dentine powder particle size for Zeta potential measurement.

Measurement of Zeta potential

Zeta potential was measured using ZETASIZER Nanoseries (NanoZS, Malvern Instruments, UK). For each test, the instrument was programmed to make15 runs per sample to provide a digital readout. All the samples were tested in deionised water in a low volume quartz curvette. The concentration of dentine particles in the experiments were adjusted to a spectrometric optical density of 1.5 (OD_{600}), this value amounts to about 300 mg of dentine powder in 1 ml of water.

7.13 Results

Table 7.4 Results of Zeta potential measurements.

Treatment sequence		Zeta Potential	Standard Deviation
Group 1	Dentine untreated	−3.57mV	7.64mV
Group 2	NaOCl	−21.1mV	8.6mV
Group 3	EDTA	−2.18mV	8.85mV
Group 4	CHX	+1.13mV	4.72mV
Group 5	CH	+3.04mV	7.31mV
Group 6	NaOCl- EDTA	−13.2mV	8.64mV
Group 7	NaOCl- EDTA- CHX	+8.8mV	4.09mV
Group 8	NaOCl- EDTA- CHX- NaOCl	−32mV	7.24 mV

7.14 Discussion (Incorporating Experiments 1 through 4)

The main thrust of these four experiments was to study the effects of irrigants on the physico-chemical forces of the surfaces of dentine after chemical treatment and how this affects adhesion of *E. faecalis*. Hence, data from both the AFM studies as well as the Zeta potential study help to explain the increase or reduction in the number of bacteria adhering to chemically treated dentine. The following discussion is thus organized into three sections based on the last irrigant in the irrigation sequence.

EDTA as the last irrigant

The results from experiment 3 (adhesion assay of bacteria to dentine) showed that the number of adherent bacteria to the substratum (dentine) was very much affected by the characteristics of the last irrigant used and whether NaOCl was used to remove the organic phase of dentine, the bulk of which is collagen.[88] The sequence of NaOCl followed by EDTA had the highest number of adhering bacteria after the control group (Figure. 7.16). NaOCl removes the organic phase and leaves a rougher more porous dentine surface of mostly calcified phase.[246] Once EDTA is applied, the protuberant calcified tissue is readily dissolved, as a rougher surface has a higher surface area for contact with EDTA, a powerful chelating agent. The organic phase, mostly collagen and other non-collagenous proteins are then exposed. The data from experiment 4 shows that the Zeta potential of this surface would be about -2.18mV,

similar to one that is irrigated by EDTA alone, but slightly less negative than controls (- 3.57mV). According to Weerkamp *et al.*,[276] an increase in Zeta potential would result in a reduction of bacteria adhesion. Our data from experiment 3 shows that the group treated by the sequence NaOCl-EDTA with a Zeta potential of -13.2mV had less bacteria than controls, which had a Zeta potential of -3.57mV clearly showing that the number of *E. faecalis* adhering was not related to an increase in Zeta potential. The organic phase of dentine contains more than just collagen and includes various other extracellular matrix proteins such as lectins and fibronectins which aid in bacterial adhesion.[427] It is possible that the presence of receptors for bacteria adhesion will mean that electrostatic charges are not the only consideration for *E. faecalis* adhesion.

NaOCl as the last Irrigant

When the sequence of EDTA-NaOCl and NaOCl-EDTA-NaOCl were tested, there were less bacteria adhering to dentine sections again contradicting the findings of Weerkamp *et al.*[276] However, it must be pointed out that they were studying different species of bacteria. When the sequence EDTA-NaOCl was compared to the sequence NaOCl-EDTA, there was significant difference in the number of bacteria adhering. The rate of dissolution of the inorganic phase of dentine by EDTA may be quicker than the dissolution of collagen and organic components by NaOCl, since the organic components are incarcerated by the inorganic components,[88] such that upon

application of EDTA, much of the inorganic phase was removed quickly removed from extrafibrillar sites of collagen and exposing the organic portions. Hence, once collagen and the organic phase were exposed, the number of *E. faecalis* adhering increased significantly.

Following the use of EDTA that exposes collagen, immediate application of CHX without first using NaOCl as an intermediary irrigant, there was reduced bacteria adhering compared to any other group except the EDTA-NaOCl-CHX. Hence, it is advantageous to use NaOCl to remove the collagen and organic phase from the surface beforehand. This result is corroborated by our data from experiment 1, which showed that CHX on EDTA treated dentine, which is when collagen was exposed; the force of adhesion was increased. Experiment 1 also showed that once collagen had been removed from the substrate by the irrigation sequence NaOCl-EDTA-CHX-NaOCl the force of adhesion is reduced, corroborating results from experiment 3. From these data, it seems quite clear that collagen and organic matrix exposure does cause an increase in the adhesion of *E. faecalis* to dentine.

CHX as the last irrigant

The Zeta potential of dentine treated by the sequence EDTA-CHX is estimated at +8.8mV and according to Weerkamp *et al.*,[276] this would increase the adhesion.[276] This was supported by data from the AFM study of perpendicular forces of adhesion

in experiment 1. In that experiment, CHX irrigation following on EDTA use, gave a high adhesion force that was lowered by the use of NaOCl. In the presence of collagen therefore, even after the use of CHX, the force of adhesion was large. This increase in force may be due to hydrophobic interactions.[381] It seems logical therefore that in the clinical situation, it would be good to remove collagen by using 5.25% NaOCl for 5 minutes before application of CHX.

It is apparent from the data of experiment one, the vertical adhesion force study cannot be directly compared to that of experiment two, the shear force of adhesion. We note that the shear force measured in experiment two was much larger in tens of nN, than the forces measured in experiment one, in units of nN. The shear force was measured after about 24 hours of contact between bacterium and substratum whereas the vertical force was the initial adhesion force measured after 10s of contact time as we wanted to measure the initial adhesion force. This interaction time was arbitrarily chosen as it gave a meaningful force curve during pilot studies. The force measured is therefore the adhesion force soon after initial contact and if the force were large enough it would retain the bacteria on the surface of the substratum. In this respect, the vertical adhesion force was a measure of the electrostatic attractive forces of adhesion, whereas the shear force we have measured represented some more stable form of stereo-chemical reaction.[300] We are uncertain why samples irrigated by CHX alone in experiment 2 did not give rise to an increase in adhesion force. In fact dentine irrigated by CHX alone, had the lowest measured shear force (Experiment 2) though this was not significantly different from controls. This contrasted starkly with an

increase in adhesion force after use of CHX in experiment one. However, it had been reported that micro-roughness of two interacting surfaces gives a larger repulsion in lateral interaction than vertical interaction.[428] This may be the reason for the differences between the results of experiment 1 and experiment 2. From Table 7.3 we note that the surface treated by CHX was the roughest and the AFM image of the treated dentine section shows a layer of CHX (Figure 7.15). In the shear force of adhesion study (experiment 2), CHX treated dentine was not significantly different from NaOCl and untreated controls. This further shows that it is likely that exposed proteins and collagen are the cause of the increase in adhesion force.

The Zeta potential data of experiment 4 showed that if CHX was the last irrigant or the only irrigant, the Zeta potential measured was always positive, irrespective of the preceding irrigants. In addition, if NaOCl was used, the measured Zeta potential was always negative, irrespective of the preceding irrigants and when it follows EDTA and CHX, it was the most electronegative in our series of combinations, at −32mV. The most electropositive was dentine after treatment with CH.

Other factors

The bacterium used in this study, *E. faecalis* ATCC29212 is not known to have a collagen binding protein ACE. Even if it were to express this MSCRAMM, ACE is

better expressed if the bacteria were cultured at 46^{o}C, our experiments were done with cultures at 37^{o}C and hence the ACE ligand, even if present, is unlikely to be significantly expressed. [259] Nevertheless, the adhesin could still be expressed at 37 ^{o}C, as this MSCRAMM is highly conserved among isolates of *E. faecalis* with at least four variants and is commonly expressed *in vivo* during infection in humans.[429] We speculate that it may still have a role in adhesion to dentine.

Dentine however consist of different proteins besides type-I collagen, and it could be that some of these other proteins that are exposed when EDTA was used, could have contributed to the increase in adhesion force.[89] EDTA is known to extract certain proteins from dentine including the matrix-metalloproteinase known as gelatinase-A.[430] However, it is not known how much of these proteins would be removed by irrigation with EDTA; except, we know that once the protective calcified phase is removed, the organic phase becomes exposed. Indeed, it had been pointed out by using micro-Raman spectroscopy, that EDTA or other acids does not remove the collagenous material of smear layer although scanning electron micrographs demonstrate a clean smear layer free surface .[431]

In experiment 1, the errors in the AFM measured perpendicular forces are not merely those already discussed in the literature review of AFM (pages 166-167). Errors could also come about because of the topographic nature of dentine. Cut dentine is penetrated by many tubular openings and there may also be tubules cut

along its length and in the method used in experiment 1, it is not clear whether the tip of the cantilever is interacting with these recesses, inflating the forces of adhesion measured. This is in contrast to experiment 2 in which the sample with inoculated bacteria was imaged first to identify single adherent bacteria in a standard orientation on intertubular dentine before the force on the cantilever was increased to remove it. Further, as can be seen from the force-distance curves of vertical adhesion forces in Figure 7.6, there is non-linearity of the piezo-voltage leading to separation of the approach and retraction curves [417]. In addition, there can be multiple combinations to the configuration of the functionalized tip contacting the substrate, because it is not functionalized with only a single bacterium. The tip height is about 2.5 to 3.5 μm and since the *E. faecalis* bacterium has dimensions of 0.38 ± 0.31 μm and 1.29 ± 0.35 μm in stationary and exponential phase respectively [432]; there can be many combinations of arrangements of bacteria at the tip. Therefore, results between differing functionalized AFM tips can only be made with great caution.

The topographical changes to the surfaces of dentine with treatment can contribute to the differences of adhesion forces. From our roughness data, the specimen with least undulations was polished dentine and the roughest surface was that offered by the coating of CHX. Although roughness may cause bacteria adhesion to a surface to increase, it is not the absolute change in roughness that is important, but perhaps the aspect ratio of the roughness in relation to the size of the bacterium that may be important[433]. In our instance, the roughness increase with CHX treatment was still only about 20% of the width of a bacterium of *E. faecalis*, hence the increase in

roughness is unlikely to have a bearing on the adhesion force and numbers. In fact, looking at the positive charge after CHX use, one should begin to wonder if it is not this roughness reducing the overall contact foot-print of the bacterium that makes removal of bacteria adhering to CHX treated specimens easier, that was responsible for the lower number of bacteria adhering. [434]

It is known that when the calcified phase of dentine is removed, the hardness of dentine would be altered.[249] We should also consider the plausibility of mechano-selective adhesion mechanisms in prokaryotes as a parameter that regulates bacterial adhesion and subsequent colonization of the substratum.[435] CH treatment of dentine increased the roughness as well as gave the highest shear force. CH is known to dissolve proteins.[436] It could also be that removal of some of these proteins made the specimens more rough thus aiding adhesion. However, though the roughness was marginally more than NaOCl treated specimens (65nm for CH compared to 61nm for NaOCl), the force of adhesion for CH treated samples was larger by about 2.5 times. Equally, it could be deposits of calcium onto the surface of dentine, which increased roughness.

The increased presence of unbound calcium ions on the specimen could increase the pH; which may not be adequately neutralized by washing. This change to a more alkaline pH had been implicated to increase *E. faecalis* adhesion to collagen[437], and may also contribute to increase in adhesion force to dentine. The role of Ca^{2+} ions itself contributing to increased adhesion cannot be ruled out. Venegas *et al.* reported

that even in the presence of saliva which sequestrates Ca^{2+}, the low Ca^{2+} concentrations in their study, could still cause charge reversal and provide the bridges to link functional groups of hydroxyapatite to those on bacterial membranes to increase bacteria to substrate hetero-coagulation. [382] Though they did not test with *E. faecalis* in that study, it is suspected that Ca^{2+} treatment of dentine may also contribute to increased *E. faecalis* binding force to the dentine substratum. Our Zeta potential data confirms that CH reverses the charge of dentine. Hence, whenever CH is used in the root canal, resources must be employed to remove it. Ideally therefore, EDTA is recommended after CH to remove these ions. NaOCl is then employed to remove any exposed collagen followed by CHX to reduce forces of interaction between *E. faecalis* and dentine. Care however has to be taken to ensure that in between the application of CHX and NaOCl, the root canal has to be rinsed and dried. This was because when these two chemicals mix in the root canal, a toxic precipitate would ensue.[438]

Although CHX is the active ingredient, we did not really use the pure form of CHX in our experiments. The more commonly available form of CHX in any dental surgery was either in the form of a mouth-rinse or a hand-scrub. The choice of using a diluted hand-scrub over a mouthrinses was because mouthrinses are more often used by periodontists and not so likely in the practices of endodontists. Chlorhexidine gluconate 4% containing hand-scrubs such as Hibiscrub, are popular aqueous hand-scrubs and they have been reported in a Cochrane Review to be effective in reduction of bacteria counts on hands.[439] It is also commonly used to clean wounds although

there is controversy if this is a good wound cleanser[440]. The brand of hand-scrub used in a1:10 dilution was Hibiscrub (SSL International PLC, UK). Its ingredients included: 4% chlorhexidine gluconate, polyoxyethylene-polypropylene-block copolymer, lauryl dimethyl amine oxide, isopropyl alcohol, perfume, d-gluconolactone, Isopropyl alcohol 4% (preservative) and purified water.

The fact that it contains surfactants is an added advantage as this would aid in wetting of all the internal surfaces of the root canal and improve its bactericidal effect. The polyoxyethylene-polypropylene-block copolymer is a non-ionic surfactant that aids in transdermal delivery of chlorhexidine and is added as a thickening agent and are used in oral care products.[125] Lauryl dimethyl amine oxide as well as D gluconolactone[441] are both very water soluble. Hence, the surface coating seen in the CHX treated dentine sample in experiment two (Figure 7.15) is that of the reacted chlorhexidine, as CHX is known to bind to phosphate and react with it; so long as the concentration was above 5mmol[381] . The CHX concentration used in this experiment was 0.4% and the molecular weight of CHX was 897.8, a concentration much higher than the minimum required for this reaction. In addition, the chlorhexidine moieties retained react with the phosphate groups of hydroxyapatite. Further, chlorhexidine once adsorbed onto hydroxyapatite, is not readily washed off with water as there are chlorophenyl and methylene moieties that render these molecules hydrophobic and it is unlikely that chlorhexidine gets washed off.[381] This would also explain the AFM data in experiment 1, when after CHX irrigation; there was increase in adhesion force,

which could be due to the hydrophobic interactions between the bacteria on the AFM tip and these chlorophenyl and methylene moieties.

The treatment times of the experiments were not completely synchronized. In experiment 3, we had used a 30 minute treatment time for NaOCl instead of the 1 hour we have used for the rest. This was because all the groups in experiment 3 were sequences using NaOCl and EDTA with another irrigant. The combination using NaOCl for 1 hour and then EDTA made pilot specimens very fragile and broke readily upon handling. Hence, it was decided to use the 30 minute NaOCl treatment. This was not expected to alter the results of the study as it had been shown that deproteination of dentine by 5.25% NaOCl was very fast. In two minutes, deproteination had taken place[442] and collagen removal was more than 3μm deep. [443] With collagen and organic more than 3μm away from the specimen surface, these are not expected to affect the data .[300]

The finding from these experiments showed that there was no clear relationship between adhesion force, Zeta potential and the number of bacteria adhering to the substratum. In addition, we note that the standard deviations to the Zeta potential readings were high and we should be cautious to make interpretations with them. However, these experiments support the findings of Rao *et al.*, who found that bacterial adhesion takes place even when both the bacteria and the minerals are negatively charged. The possible factors in controlling such adhesion of similarly

charged particles (by corollary, less negatively charged bacteria adhering to positively charged surfaces) include surface heterogeneity of the bacteria, hydroxyl, hydrophobic and ionic moieties. Also the flexible fimbriae can possibly regulate the surface charge characteristics, depending on the environment.[444]

These experiments show that dentine sections which were negatively charged and which would have high surface energy and whose surfaces were not made hydrophobic by removal of the inorganic phase had less *E. faecalis* adhering to them. In contrast, dentine sections which had low surface energy, relatively more hydrophobic surfaces due to removal of surface mineral and which had a lower electrostatic charge had an increase in *E. faecalis* adhesion. Further, we observed that the antimicrobial agents CHX and NaOCl reduced *E. faecalis* adhesion to dentine; whereas CH increased *E. faecalis* adhesion.

Chapter 8 - Fourier Transform Infra Red (FTIR) Spectroscopy of Chemically Treated Human Dentine

8.1 Introduction

When chemicals are applied on dentine, chemical reactions should be expected to take place on the surface of dentine. Chemical reactions with the surface could alter the substrate by dissolving a part of the composite material that dentine is or the chemical applied could react to form a new compound on the surface of the specimen. We have selected FTIR-ATR as the method for assessment of dentine specimens treated by chemicals regularly used during root canal treatment. The main aims of this experiment were to examine the effects of chemicals routinely used during root canal treatment on dentine. The effects of sequencing the application of these chemicals on the material characteristics of dentine were also investigated.

8.2 Specimen Preparation

Dentine sections were harvested as detailed in 7.6 earlier, except that the dimensions were 4 X 8 X 1mm. All dentine sections were sonicated for 10 minutes in an ultrasonic bath; in a Falcon tube containing 10 mls deionised water before use.

During experiments, to prepare dentine collagen three dentine sections were immersed in 5mls of EDTA (17%, pH 7.2) solution. The EDTA solution was decanted and refreshed every other day for 180 days. This was to ensure that all non-collagenous proteins would be extracted from dentine[445] and to remove all of the calcified phase from the dentine specimen(dentine collagen).

8.3 Chemical Treatment of dentine

The following chemicals were used to treat dentine sections:

a. CH (Sigma-Aldrich, Germany) from which a saturated solution (0.2%) pH 12.2 solution was prepared.

b. NaOCl (5.25%, pH 11.5 Chlorox, Malaysia), followed by CH and then EDTA

c. CHX (Hibiscrub (SSL International PLC, UK) pH 6.8

d. EDTA (Amresco, USA) from which a 15% solution pH 7.2 was prepared.

The durations of chemical treatment chosen were similar to that used in clinics.

The treatments of the specimens are tabulated (Table8.1):

Table 8.1 Schedule of Chemical Treatment of Dentine for FTIR-ATR

Specimen		Chemical/sequence	Duration
Group 1	Dentine	CHX	1 hour
Group 2	Dentine	EDTA	1,2,3 and 5 minutes
Group 3	Dentine Collagen	CH	1 week
			3 week
Group 4	Dentine	CH	1,2,3,7 and 14 days
Group 5	Dentine	NaOCl	1 hour
		CH	7 days
		EDTA	5 minutes

To understand the effects of chlorhexidine on dentine, three specimens in group 1 were treated in 5mls of the solution for one hour and the FTIR spectrum measured. The effects of EDTA on dentine was studied by soaking three sections of group 2 in 10mls of EDTA solution and FTIR spectrum made after 1,2,3 and 5 minutes. After each immersion in the treatment solutions, the sections were gently rinsed in deionised water gently blown dry and the FTIR spectrum recorded. The specimen

was again rinsed in deionised water gently blown dry before being returned to the EDTA solution for the next period of treatment.

To study the effects of CH on dentine, three dentine sections from Group 4 were placed in 10 mls of saturated CH solution. FTIR spectra were recorded after 1,2,3,7 and 14 days of treatment. Just before their FTIR spectrum were recorded the specimens were rinsed in deionised water and gently air dried. The same CH solution was used throughout the experimental period. After each measurement, the dentine specimen was replaced in the same Falcon tube after rinsing in deionised water and gentle air blow-drying. The three sections that had been decalcified from group 3(dentine collagen), were placed in 10mls of saturated CH solution and FTIR spectrums measured after 1 week and 3 weeks. The same CH solution was used throughout the period.

To observe the effects of the sequence of irrigation on dentine, the dentine sections of group 5 were soaked in 10 mls NaOCl for one hour, in 10 mls saturated CH solution for one week and then in 10mls EDTA for 5 minutes. FTIR spectra were measured after each irrigant. All sections were carefully washed in 10 mls of deionised water thrice and blown dry after each chemical used in the sequence and the FTIR spectrum recorded.

FTIR Instrumentation

A Fourier Transform Infrared Spectrometer with a window for attenuated total reflectance mode was used (Magna-IR System 850 Series II, Nicolet, Wadison, USA).The region analysed was 4000 cm^{-1} to 400 cm^{-1}, using a resolution of 2. The sample was positioned so that the detector showed the major reflected infrared signal and the spectra acquired after interior of the spectrometer was maintained by dry air for about 20 minutes. Nine readings were made, three from each section at each time interval. Each reading was made up of 124 scans at a resolution of 4. The spectra were examined for the effects of chemical treatment. During the experiment each specimen was maintained wet with deionised water over the FTIR ATR IRE crystal of the apparatus and the specimen holder gently tightened to maintain pressure on the specimen to keep it in good contact with the crystal.

8.4 Results and Observations

8.4.1 Effects of Chlorhexidine (dilute Hibiscrub) on Dentine

The FTIR spectra of dentine before and after soaking in dilute Hibiscrub for an hour is shown in the Figure 8.1. After soaking for one hour in CHX, there were many new peaks formed in the spectra of dentine. The peaks 833, 858, 1234, 1311, 1396 and 1610 cm^{-1} were changes due to CHX treatment of dentine. These peaks are due to

the precipitate that forms on dentine when CHX reacts with hydroxyapatite. The peaks 833, 858 cm^{-1} are related C- Cl stretch, whereas those that are from about 1300 – 1230 cm^{-1} are related to CH_2-Cl bend (wagging). [446] The peak 1510 cm^{-1} is due to the presence of C-C bonds and the peak around 1650 cm^{-1} due to the C=N bonds present in the chlorhexidine molecule. [447] The reacted compound had been shown to be chlorhexidine phosphate.[381]

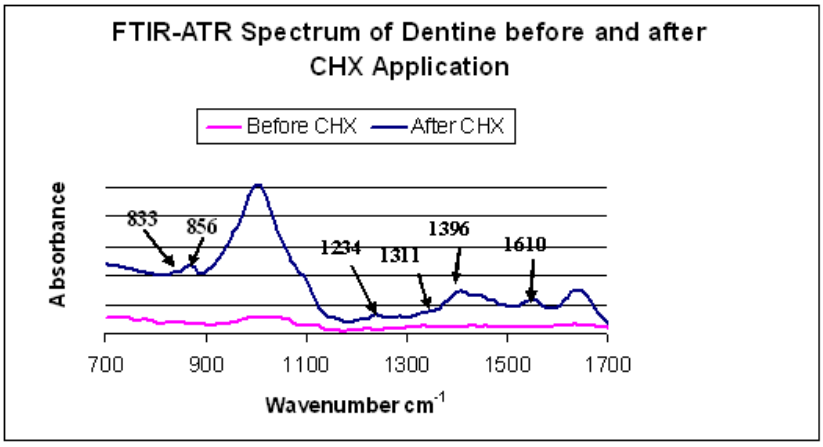

Figure 8.1 FTIR of CHX treated dentine

8.4.2 Effects of EDTA Treatment of Dentine

Figure 8.2 is the spectrum from 1120 to 1820 cm^{-1} after 1, 2, 3 and 5 mins in EDTA. New peaks appeared at 1250 cm^{-1} (Amide III Band),[448] 1540 cm^{-1} (Amide II band) and 1650 cm^{-1} (Amide I Band).[449] These peaks (1120 to 1820 cm^{-1}) also

increased in intensity with the increase in time of exposure to EDTA. The increasing amide I through III bands, are consistent with the increasing presence of these organic bonds due to the increased demineralization of the sample over time and represent exposure of collagen, even after 1 minute. The spectrum after 5 mins appeared noisy and is probably due to surface roughening of the specimen after this duration of treatment with EDTA. Hence, in this experiment, EDTA was shown to have demineralised dentine within one minute of exposure and over 5 mins, more of the calcified phase is removed and more organic matrix is exposed.

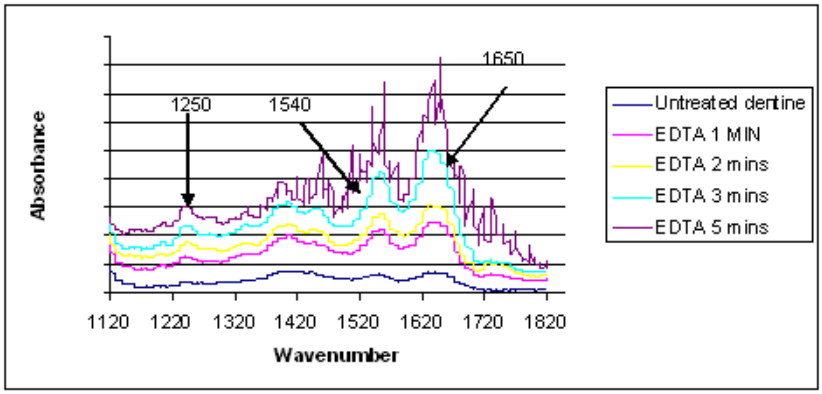

Figure 8.2 FTIR-ATR of EDTA treated dentine

Figure 8.3 is the spectrum from 2700 cm^{-1} to 3700 cm^{-1}. A new peak appeared at about 3300 cm^{-1} and this increased in intensity with increasing duration of treatment with EDTA. This peak is consistent with the appearance of collagen after one minute in EDTA. This peak is that of Amide A and water. This indicates that the dentine

sample probably held more water after decalcification and therefore the peak intensity increased with treatment time of EDTA.

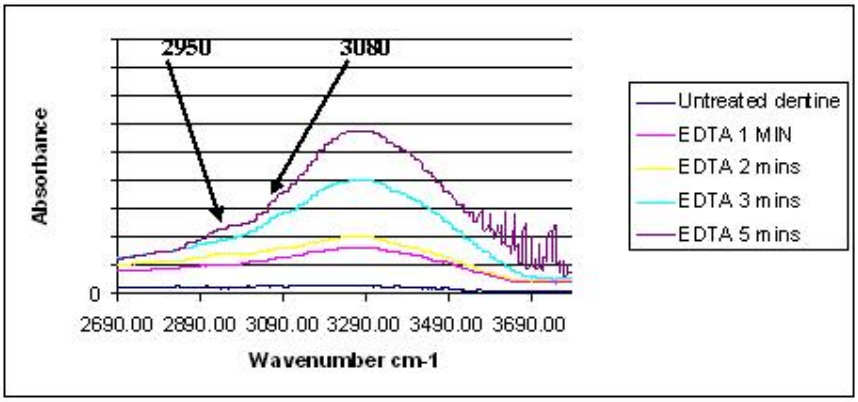

Figure 8.3 FTIR spectrum of EDTA treatment of dentine. The OH^- stretch and Amide B bands at 2950

The amide A band is due to the increasing presence of NH-group involved in interchain hydrogen bonding, whereas the amide B band is due to amide II (N-H bending and C-N stretching) overtones.[450, 451] These bands indicate the presence of proteins. Hence, more protein structures were now present on the surface of dentine.

In Figure 8.7 below, in the specimens treated by a sequence of chemicals, after NaOCl and CH treatment the specimen was rinsed in EDTA for 2 minutes and

immediately, the organic peaks increased to above those of controls, indicating that there were more organic groups on the surface of EDTA treated dentine than the control. In the region 3000 to 3800 cm^{-1}, there was progressive loss of peak height after NaOCl and CH treatment. This was due to the hydroxyl stretch and amide stretch about 3300 to 3450, suggesting that there was dehydration in the process. With EDTA treatment, there was more exposure of the organic matrix and more retention of water and the peak immediately increased to that above that of control. Hence, treatment of dentine by EDTA, even for a very brief 2 minutes, exposed the organic phase, even if some organic material had been removed by prior NaOCl treatment and CH treatment, as in the specimens treated by the sequence of chemicals NaOCl-CH-EDTA.

8.4.3 Effects of CH treatment on Dentine Collagen

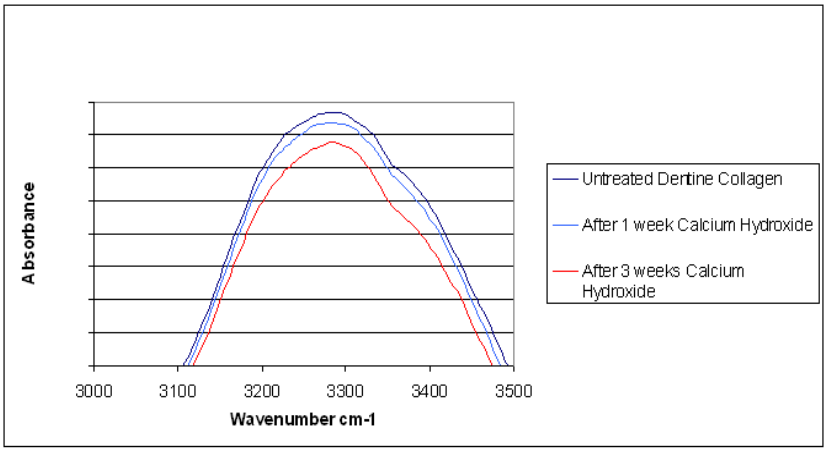

Figure 8.4 FTIR spectrum of Effect of CH on Decalcified Dentine (Dentine Type-I Collagen)

The effects of untreated, 1 and 3 week treatment of dentine collagen by CH is as shown in Figure 8.4 above. The peaks present in the control specimens were also present in the treated specimens, showing that there was minimal alteration of the functional groups after CH treatment. The decrease in the peak at about 3300 cm^{-1} suggests some dehydration, as that peak is the absorption peak of water. Earlier, a report studying the effects of calcium chloride on collagen, reported that calcium bound to collagen over a wide pH range, probably to the carboxyl groups rather than the amide groups and causes denaturation of collagen by shrinkage.[452]

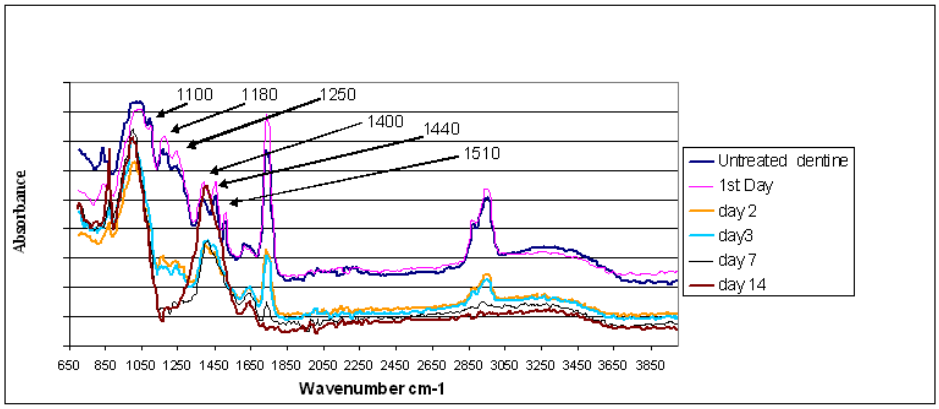

Figure 8.5 FTIR of CH Treated Dentine

The spectra of untreated dentine and after 1 day of CH shows distinct peaks at about 1100,1180,1250,1400,1440 and 1510 cm^{-1}. The increased in intensity of the same peaks show that there is increase in binding of CH to the organic phase of dentine (Amide I band). After two days of treatment, these peaks had largely diminished. After one week (and similarly at two weeks), those peaks had

disappeared, indicative of the solubilisation of the organic phase of dentine after treatment with CH. The peaks 1180 and 1510 cm^{-1} that had disappeared by one week were not present in the spectra of dentine collagen (Figure 8.6). We conclude therefore that these were peaks of non-collagenous proteins that had been solubilised by CH treatment and were removed after one week.

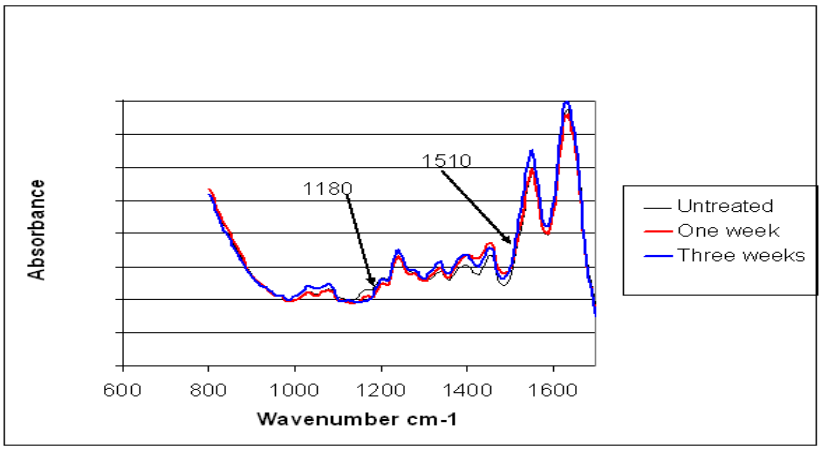

Figure 8.6 FTIR of dentine collagen from 600 to 1650 cm^{-1}

The spectra of dentine collagen after CH treatment highlighted binding of CH with collagen but there were no signs of solubilisation. This was because the non-collagenous proteins had been previously removed by EDTA treatment. The peak at about 1000 and 1400 cm^{-1} increased in intensity after two weeks, suggesting that there was re-crystallization of carbonated apatite and increased re-mineralization. This was not observed in dentine collagen. This was perhaps because the solubilised non-collagenous proteins were present within the CH solution, which was not decanted and refreshed, and which may play a role in remineralisation. A recent publication

had shown that in the presence of non-collagenous proteins, it was possible for dentine remineralisation to take place. [436] In the band between 3000 and 3800cm^{-1}, there was progressive loss of peak height that was indicative of dehydration of dentine matrix. The dentine specimens that were subjected to the sequence of chemicals, also showed that following NaOCl, and then subjected to CH treatment, there was further loss of the organic matrix as organic bands were further flattened (See Figure 8.6). CH therefore, removes organic components from dentine. Its effects are in addition to those that NaOCl has removed.

8.4.4 Effect of the NaOCl Treatment

The spectrum after NaOCl treatment showed very clearly that the Amide II and Amide I band has drastically diminished with dissolution of organic matrix, in this case collagen. There is also characteristic increase in the C-O stretching related to carbonates (~870 – 1450 cm^{-1}) and peaks related to PO_4^{3-} stretch and PO_4^{3-} bend

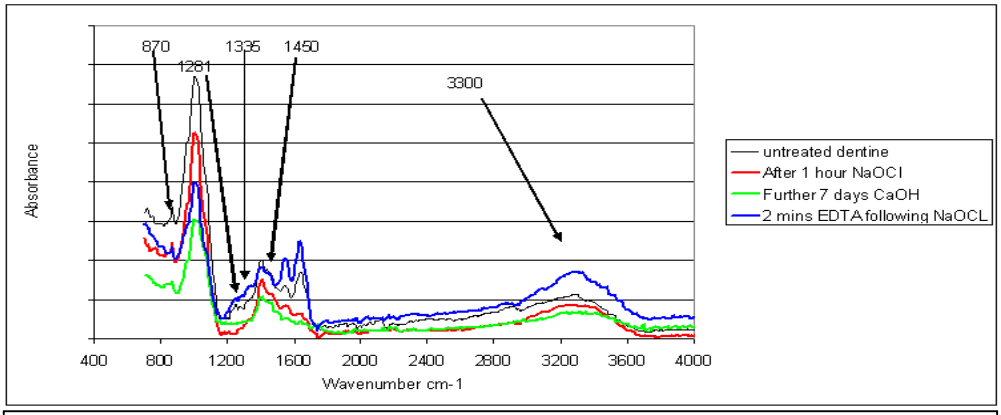

Figure 8.7 FTIR-ATR of Dentine After the Sequence of Chemical Treatment NaOCl, CH and EDTA.

related to phosphate (~900 cm^{-1}) of the dentine surface after treatment with NaOCl. The spectral region, 1400-1100 cm^{-1}, is sensitive to the collagen molecular conformation and is called the "fingerprint" region because changes in bands of this region are attributed to different conformations of the same molecule.

The CH_2 deformation, ~ 1335 cm^{-1}, C-(CH_2) twisting ~1315 cm^{-1}, C-N stretching and NH deformation 1281 cm^{-1} all point to conformational changes in the collagen of the dentine samples after one hour of sodium hypochlorite treatment.

8.5 Discussion

From the above experiments, we can see that each chemical gave rise to different changes to the dentine substrate. It had been reported that CHX has substantivity on dentine; however, we note that the IR absorbance peaks showed that there was reaction of CHX with hydroxyapatite of dentine, probably forming the chlorhexidine phosphate reported previously. [381]

The chemical used last left an impact on the substrate as it determined what chemical groups were left exposed on the surface of dentine. Our FTIR studies show that, if the last chemical applied was EDTA, the surface of the dentine would be left with a higher amount of organic matrix exposed than untreated cut dentine. Presence

of organic material on the surface may contribute to increased nutrient opportunities for bacteria secreting. It could also offer enhanced opportunities to bacteria possessing specific collagen binding proteins such as ACE containing strains of *E. faecalis*. Even a 1 min exposure to EDTA would leave exposed collagen. Collagen had been reported to be important to *E. faecalis* in penetration of dentinal tubules [261] .Once it enters the dentinal tubule, it can by binary fission over time, burrow deeper into dentine. Deeply buried bacteria cannot be readily killed by irrigation of the lumen of the root canal with disinfectants and has implications on the successful outcome of root canal treatment.[453]

The sequence of chemicals used should be a deliberate act to influence the condition of the root canal; such that the chance of survival of bacteria left in the root canal, after the irrigants have been dried out, would be minimal. Exposing dentine to CH also solubilised the non-collagenous proteins of dentine. Whilst the use of CH in root canal is for its bactericidal effect, we have to recognize that we are also altering the substrate. After using CH, upon subsequent instrumentation, these non-collagenous proteins extracted by CH may be extruded into the periapex and they can affect periapical healing. It had been demonstrated that when EDTA extracted dentine protein was used to vaccinate rats root resorption can be reduced. [454]

Longer-term use of CH has caused increased crystallinity of dentine. The possibility of remineralisation within the root canal, with use of CH is raised. There is

possibility therefore that a mineralizing front may trap bacteria in a dentinal tubule, lateral or accessory canal; preventing fluids within the main root canal from coming into contact with them after intracanal treatment using CH as the medicament. From the results of this study, it would seem that CH use as an interappointment dressing, should be used for less than 2 weeks. However, we have only used saturated CH solution and we have not used the CH paste that most clinicians use. The outcome is likely to be different as the physical presence of a slurry of CH should likely bring on a more pronounced effect.

Chapter 9 - Discussion

We hypothesized that some chemical and/or chemical sequences used during root canal treatment mediated changes that increased the adhesion force and adhesion of *E. faecalis* to dentine substrate. Our experiments show that there were indeed certain chemicals and chemical sequences that increased *E. faecalis* adhesion, both adhesion forces as well as number of bacteria adhering in adhesion assays. Our study suggests that indeed we may be selecting for *E. faecalis*. Future studies in this direction should work out the mechanisms that allow this to be so.

The purposes of root canal irrigation are to flush out debris, dissolve remnant pulpal tissue and kill bacteria; and that of intracanal CH treatment is to suppress bacteria growth in the root canal between appointments as well as to kill any remnant bacteria at the end of a session of root canal treatment. Since most primary root canal infections are of mixed flora of several species[24] it is perplexing to learn that *E. faecalis* that is commonly associated with failed root treated teeth[25, 26, 33, 148]. However, *E. faecalis* was not commonly found in primary root canal infections.

The prevalence of *E. faecalis* in the mouth, where it is a transitory rather than a resident, had been reported to be 11% in patients receiving endodontic treatment and was only present in 1% of dental students who had no history of root canal treatment [50]. In another study of prevalence of *E. faecalis*, the same group found that among patients receiving root canal treatment, 68% had at least one tongue, oral rinse, or gingival sulcus sample positive for *E. faecalis* but only 5% of patients had *E. faecalis* in root canals. In 21/41 patients who had *E. faecalis* from all three sites the prevalence of *E. faecalis* in root canal was increased to 10%. It was also found that those with gingivitis and periodontitis – had a prevalence of *E. faecalis* raised to 73%. These authors also raised the possible existence of *E. faecalis* in the viable but non-culturable state, especially in the tongue, and suggested that its presence in the mouth was probably higher than previously thought.

Engstrom in a controlled longitudinal study found that 9% of the 223 cases he reported on, whether root filled teeth or primary root canal infections, had *E. faecalis* [29].He used 0.5% NaOCl during chemomechanical preparation and iodine-potassium iodide as the interappointment dressing. It was reported that of the 20 cases that harboured *E. faecalis*, chemomechanical treatment failed to remove *E. faecalis* in 13/20 (65%). Other teeth infected by different taxa had 45/114 (40%) that remained infected. He linked the presence of *E. faecalis* in the mouth to its presence in root canals.

A more recent study by Ferrari *et al.* [455] of 25 single rooted teeth with intact pulp chambers showed that 24% or 6/25 teeth harboured *Enterococci*. Chemomechanical preparation was carried out with 0.5% NaOCl and EDTA. At the end of the first visit, none of the teeth harboured *Enterococci* though 5/25 teeth was found with other taxa. The root canals were left empty and the tooth sealed with zinc-oxide cement. After seven days, before the commencement of any treatment, the teeth were cultured and 14/25 teeth were found to contain *Enterococci*. The authors reported that the initial number of *Enterococci* was too low for detection in many canals hence the number of canals with *Enterococci* increased after the first visit. (They did not use any medicament in the root canals for the seven days in between the two visits.)

Our experimental data shows that CH plays a big role in *E. faecalis* adhesion to collagen and to dentine. The presence of CH increased the adhesion force to collagen in our optical tweezers study as well as in the shear force study using AFM. In our study of *E. faecalis* adhesion to collagen, the numbers of adhering bacteria increased in the presence of CH and even higher with EDTA, when compared to controls. Our DSC and CD experiments also show that EDTA and NaOCl denatured collagen. It had also been shown that a pH of 8.5 increased *E. faecalis* adhesion to collagen. [437] Further, our FTIR data shows that even a one-minute exposure to EDTA would remove sufficient calcified phase to expose collagen and organic phase. Assuming that Ferrari *et al.*[455] were correct in the assertion that there were too few *Enterococci* for the culturing technique to pick up to give a positive culture; can we use our data to explain the increase in finding more *Enterococci* in the second visit?

After taking the first culture which showed that there were 6/25 teeth with *Enterococci*, they had used 0.5% NaOCl and "final irrigation with 10mL of SDS-EDTA" and then flushed the canal with 20mL of saline before placing a cotton pellet in the pulp chamber and sealing the tooth with a temporary filling material. Final irrigation here was taken to mean the final rinse of active chemical used during chemomechanical preparation of the root canal. As had been pointed out earlier, [192] even if EDTA irrigation was followed by NaOCl, the degradation of EDTA by NaOCl was so slow that it was not clinically meaningful to expect it to limit the effects of EDTA.[192] Since, saline was a much more inert material, it can be assumed to have the same outcome. Hence, we conclude that in the cases treated by Ferrari *et al.*[455] collagen fibrils must have been exposed in the root canal during chemomechanical preparation.

EDTA not only exposes collagen but also denatures it (information from our DSC study), increases the adhesion force of *E. faecalis* to dentine (AFM vertical force and shear force study). If any *E. faecalis* were present in the canal but was not picked up by paper points during the first culturing procedure or during chemomechanical preparation; it could now be dissipated from its original niche, by the higher flush rate used when syringing the canal with 20mL of saline, onto a collagenous bed. It was now slightly more difficult for saline to flush it off, as the adhesion to EDTA treated dentine surface was slightly stronger. It remains alive and flourishes on a bed

of collagen in the 7 days leading to the result of 52% of canals cultured positive with *Enterococci*. Admittedly, this argument is tenuous. We did not in our experiments show that it was possible to dissipate bacteria using a syringe and we did not know the rate at which Ferrari *et al.* syringed the root canals; we also did not do these experiments within root canals but on dentine sections. The scenario is nonetheless possible that exposing collagen and the organic phase of dentine can contribute to a higher survival rate for *E. faecalis* in root canals.

The main purpose of using EDTA as an irrigant during root canal preparation was to remove smear layer. An SEM view of the smear layer typically presents it as an amorphous, irregular and granular layer, more pronounced in some parts of the root canal than elsewhere with bacteria and debris on its surface. It is thought of comprising both organic and inorganic debris and is the detritus formed during instrumentation of the root canal walls. It is about 1-5 μm thick but has been reported to plug dentinal tubules to depths of about 40 μm. [138, 456] Various chemicals have been used to clean off the smear layer, as the smear layer, thought to be only lightly adherent to bare dentine and root filling against it would result in a poorly adherent and poor sealing root filling.[366] As recent as 2008, authors continue to publish papers regarding the smear layer and its evaluation using SEM.[457, 458] However, as pointed out earlier in 2002, using technology that is more sensitive at registering the presence of organic as well as inorganic material, micro-Raman spectroscopy has shown that after irrigation with EDTA, a collagenous material is left on the surface.[431] In fact, even earlier, in 1995, it was reported once dentine is etched, collagen is exposed and

due consideration should be given to the leakage through nanometre sized porosities that may allow hydrolysis of collagen. [459] Further, Ferrari *et al.* reported that collagen exposed in root canals by the acid of zinc phosphate cements used for cementation of posts in root canals left collagen exposed which deteriorated over time. [262] Tay *et al.* also reported that collagen was exposed "when NaOCl was used as initial rinse, followed by the use of 17% ethylenediaminetetraacetic acid or BioPure MTAD as the final rinse. Compaction of conventional root filling materials over a demineralised collagen matrix in instrumented dentin is analogous to performing the same procedure against the predentine collagen network in the uninstrumented parts of root canals" [190] . Endodontists should really begin to consider if they should be using solutions that remove the calcified phase of dentine to just get a visual sense of cleanliness of dentine from SEM photomicrographs.

At the National University of Singapore Faculty of Dentistry, students are taught to use Milton's solution, a 1% solution of NaOCl, for irrigation during root canal treatment. Students are not taught to remove the smear layer using EDTA solution nor are they encouraged to use commercial preparations with EDTA. Whilst there had not been a country wide survey to show that most dentists in Singapore continued with the irrigation and treatment regime they were taught, it was the author's impression that most were using the same concentration of NaOCl. With the advent of the era of nickel-titanium root canal instrumentation, the same cannot be said of the use of EDTA containing pastes. That was because companies introducing these instruments into the market speak of the wonders these pastes do to help instrumentation, and include them in the introductory kits sold to dentists.

Whilst we have tested EDTA solution in our studies, we have not tested the commercially available EDTA pastes and this can be a criticism of the study. These commercial pastes contain many ingredients, of the commercial products RCPrep includes urea peroxide, cetyl alcohol[460], Calcinase slide contains disodium edtate[461], Glyde File Prep contains urea peroxide, propane-1,2-diol[462]. For these products to be studied, pure compounds of each of the ingredients would have to be tested for their effects on collagen and dentine in each of the methods we have used. These should be pursued in future studies by designing experiments using these commercially available EDTA containing pastes.

In our study of *E. faecalis* adhesion to collagen, we observed that there were clumps or aggregates of *E. faecalis* after collagen had been treated by CH, which we suspect to be an effected by the presence of calcium. Assuming that calcium does play a role in the binding *E. faecalis* together into aggregates, we wonder if EDTA use in root canals will not remove the calcium and cause the aggregated bacteria to dissipate and spread to various parts of the root canal, onto beds of collagenous material in various niches, allowing the bacteria to flourish.

There are other reasons why the use of EDTA should not be taken too lightly. Apart from having pointed out that EDTA denatured collagen and caused increased numbers of *E. faecalis* to adhere to collagen, EDTA can release all the other enzymes previously incarcerated by the calcified phase of dentine, such as matrix

metalloproteinase. These proteins are bioactive and hence if extruded through the apices of the root canal during treatment can affect healing of periapical lesions.[463] Other detrimental effects such as decrease in flexural strength,[272] reduction in microhardness, [464] increase in tubular opening dimensions [465] and plausible increased bacteria penetration of dentinal tubules[466] though using a proprietary EDTA containing paste was shown not to increase tubular openings.[389] Smear layer removal had been shown to not to reduce bacterial leakage through the root canal[467] yet in another report the contrary finding was made [468]. Further, it was reported to be also related to the type of sealer used.[469] Yet some others consider the smear layer helpful as it prevents bacteria penetration of dentinal tubules and retention of bacteria[470] and feel that the presence of smear layer does not enhance bacteria adhesion. [281] Hence, smear layer removal is still considered controversial and whether it should be removed is best decided by long-term prospective clinical studies with sufficient power to determine this. In the absence of such information, due consideration should be given to exposing collagen and organic matrices of dentine which increases *E. faecalis* adhesion.

From the *E. faecalis* adhesion to collagen, the optical study of force of attraction between *E. faecalis* and collagen in the presence of CH and the shear force of adhesion using AFM, it is observed that calcium participates in the adhesion of *E. faecalis* to collagen. A similar finding had been previously reported by others. [437] Further, from the FTIR study, the use of CH for two weeks against dentine, the peak at about 1000 and 1400 cm^{-1} showed increased intensity after suggesting that there

was re-crystallization of carbonated apatite and increased re-mineralization. There is plausibility of a mineralizing front trapping a bacterium within lateral canals and dentinal tubules .The mineralizing front that does not incarcerate the bacterium completely would pose additional problems killing the bacterium; whilst its secretions and by products may still have access to the periapical region. Since its alkalinity can also aid in increased adhesion of *E. faecalis,* [437] consideration must be given to as completely as possible, remove any remnant CH from the root canal after it is used. Clinically, this may mean using EDTA and use of ultrasound though even this did not remove CH completely. [397]

CHX irrigation of dentine lowers the adhesion force (AFM shear force, late adhesion forces). This accounts for the reduced *E. faecalis* adhesion dentine. CHX had been known to have substantivity.[202] Within one hour of application of CHX on collagen, there was also a reduction of bacteria adhesion compared to other chemically treated collagen samples. However, after two hours, this effect had been lost. Early adhesion forces studied by AFM suggest an increased force of adhesion after an interaction of tens seconds. Accordingly, there was also higher numbers of bacteria adhering to dentine after EDTA and CHX irrigation. However, if NaOCl had been used to remove collagen and then CHX applied the number of *E. faecalis* adhering reduced. The increase in early interaction force measured is therefore very likely due to the alteration of the charges of collagen and proteins by treatment with CHX. These are supported by data from the Zeta potential study. Zeta potential was positive if CHX was applied on EDTA treated dentine. These experiments show that

CHX was useful on calcified dentine but not on collagen. This is similar to a clinical study which showed that in the sub gingival environment, no substantivity could be achieved. [471] Hence, removal of organic phase of dentine seems essential for substantivity of CHX. In addition, *E. faecalis* that had been exposed to collagen would make it less susceptible to CHX. [385]

As discussed in chapter 7, we have used the commercial product Hibiscrub in this study in place of pure CHX. Though we belief that all the other chemicals have been washed off completely as they are entirely water soluble, the fact that they have not altered the substrate cannot be shown in this study. Hence, future studies should include the individual components of Hibiscrub and report their effects on collagen and dentine using the instrumentation used in this study.

The results of these experiments show the importance of the sequence of root canal irrigation as the number of adherent bacteria to the substratum (dentine) was much affected by the last irrigant used and whether NaOCl was used to remove collagen. If NaOCl was the last irrigant, fewer *E. faecalis* was adhering whereas if EDTA was the last irrigant, even if NaOCl was used before hand, the number of adherent bacteria increased.

By keying in the search terms "sequence of irrigation and root canal" in PUBMED we found only one study that paid any attention to the order of use of irrigants. That paper was concerned with the mechanical properties of dentine after irrigation as it was testing if prior irrigation with EDTA would enhance the effect of NaOCl and affect the mechanical properties of dentine more severely .[248] The fact that authors who use the term "final rinse with EDTA" and then follow that up with irrigation with saline, shows that little thought had gone into the meaning of final irrigation.[455]

This study is likely to be the first to show that the sequence of irrigant use can affect the adhesion and adhesion force of *E. faecalis* to chemically treated dentine and collagen. More studies are required to know if the same will affect other pathogens in a similar way. In some studies of bacteria adhesion, various body fluids including saliva and serum are often added, as it is argued that these are present on dental tissues. Both serum and saliva increased the adhesion of *E. faecalis* ATCC 29212 to dentine. [10] In these experiments, none of these conditioning liquids was used. This was because we felt that in the case of root canal treatment, root fillings are usually done after root canal irrigation, when the root canal was found to be dry, after excess irrigants were removed by paper points. Hence, the canal should be devoid of any conditioning fluids.

Throughout these experiments we were using only a single strain of *E. faecalis* – that of ATCC 29212. There had not been any studies at the Faculty of Dentistry, National University of Singapore that isolates bacteria from clinical cases of failed root canal treatment. We thus did not have any clinically isolated *Enterococcus* species to test in these studies. Future studies should include clinical isolates of bacteria as they may behave very different from the ATCC 29212 strain that we have used as clinical isolates may vary considerably from environmental isolates[472] and hence ATCC 29212. Whilst we have focused our attention on *E. faecalis* as this species was commonly isolated as a single species in failed treatment,[26] in many cases of treatment failure, other species are regularly isolated.[75] Hence, it would be useful also to include other species in similar studies. We have hypothesized that when collagen was exposed, the propensity to retention of *E. faecalis* was increased and hence the common finding of this species in failed root canal treated teeth. However, the presence of a bacterium adhering reversibly on the substratum can be affected by the next bacterium as it comes to rest on the substratum.[428] To mimic the clinical situation, where the root canal has more than a single species of bacteria, the use of more than one species of bacteria to study interspecies interactions would make adhesion studies very complex.

Another variable that could not be controlled in this study was the age of teeth. It is known that teeth of different ages may contain differing components of incarcerated enzymes. This would give rise to more variable results. However, it should be pointed out that dentine is itself a very variable material. It has been shown how the last

chemical in a sequence of irrigation of dentine has an impact on the number of *E. faecalis* adhering to dentine. The last chemical in the sequence altered the Zeta potential of dentine. It is very likely that the changes taking place on the surfaces of dentine during chemical treatment alter the surfaces to change the Zeta potential. This in turn had effects on bacteria adhesion.

It was found that the hypothesis that some chemical and or chemical sequences used during root canal treatment mediated changes that increased the adhesion force and adhesion of *E. faecalis* to dentine substrate was not defeated. The results show that each chemical alters the surface of dentine differently and the interaction with *E. faecalis* altered accordingly. This alteration has a bearing on adhesion of *E. faecalis* and hence the chances of *E. faecalis* infection after treatment. However, due consideration should be taken in interpreting this results for the clinical setting. It should be remembered that living bacteria cell surfaces respond to the external environment. Changes in the surface of the cell, in response to the environment are probably the reason why biofilms continue to grow on surfaces chemically treated with antimicrobial compounds. [473] Further work remains to be done to understand what happens to the *E. faecalis* cell, after it adheres to chemically treated dentine.

What can we apply from these studies to the clinical situation to avoid failure if root canal treatment associated with *E. faecalis*? Several points are relevant. First, use NaOCl after using any EDTA. This was because in the sequence tried on dentine,

fewer bacteria were adhering on dentine so treated in the adhesion assay. This may be related to the fact that both the shear force and vertical adhesion force were smaller. Second, use of CHX on EDTA treated dentine reduced the number of *E. faecalis* adhering, but this was not as low as when CHX was applied in the sequence EDTA-NaOCl-CHX. Hence, in the clinic, it is recommended that whenever EDTA had been used, this should be followed up with NaOCl. Then CHX may be applied. CHX has substantivity on dentine and would prevent infection for a period of time. Third, CH had increased the adhesion of bacteria and adhesion force. Whenever CH is used, it should be completely removed. The use of ultrasound irrigation and instrumentation with irrigation and the use of EDTA may be good means of doing this.[397, 474] Fourth, initial forces of adhesion may not be correlated to number of bacteria adhering to a specimen in an adhesion assay. This was so because in AFM vertical adhesion force study, we helped the bacteria overcome the energy barrier to adhesion to the substratum, unlike in the natural situation. To this end, the application of CHX to bare dentine makes the surface positively charged. Surfaces with cationic charges may attract bacteria but the positive charge threshold upon adhesion of a negatively charged bacteria releases counterions in the electric double layer and this release is responsible for bacteria death[475]. CHX application to dentine therefore, reduces the number of bacteria on CHX treated surfaces even though the initial adhesion force was increased.

However, we have to remember that this study was an *in vitro* study and the clinical situation might not work out in quite the same way and is not on par with the level of evidence that a randomized clinical trial would provide.

Chapter 10 Conclusions and Future Work

- All chemicals used in root canal irrigation, with the exception of CHX, denatured collagen. There was however, no linear correlation between the extent of denaturation of collagen and the number of bacteria adhering to eggshell type-I collagen membrane.

- CHX treatment of dentine has been shown to be effective in reduction of *E. faecalis* adhesion when compared to the other chemicals tested. However, whether it is collagen left exposed on dentine or eggshell type-I collagen membrane, our data shows that CHX use does not reduce *E. faecalis* adhesion. In the case of collagen membrane, the reduction was short lived and the effect was lost at 2 hours.

- When CHX was used, the Zeta potential of dentine was made positive. However, the adhesion of bacteria to CHX treated dentine was the lowest when compared to other chemical treatment. We conclude that the adhesion of *E. faecalis* to CHX treated dentine is not a linear relationship with the change in Zeta potential; as vertical forces of adhesion of *E. faecalis* to CHX treated exposed dentine collagen was increased. Other

factors, such as changes in the surface roughness of treated dentine may have reduced the footprint area of bacteria on dentine and this may have allowed bacteria to be removed more readily.

- These studies show that CH increased forces of adhesion of *E. faecalis* to collagen (measured by laser tweezers), increased numbers of bacteria on type-I collagen membrane (assessed by CLSM) and increased adhesion of bacteria to dentine (shear force AFM and *E. faecalis* enumeration on dentine studies). In addition, the FTIR data shows that CH removes non-collagenous proteins from dentine.

- After EDTA use, NaOCl should be used to remove any exposed collagen. Once we have removed any exposed collagen, CHX may be used effectively.

- If any CH is used, we should endeavour to remove it as completely as possible. If *E. faecalis* was one of the suspected organisms, such as during retreatment of teeth with persistent periapical lesions, greater care must go into removing any remnants of CH. Though CH is known not to be effective against *E. faecalis,*[241] it is effective against many other bacteria species and can also aid in neutralizing remnants of lipopolysaccharide of gram negative bacteria [476]and lipoteichoic acid of *E. faecalis* .[477]

- The last irrigant in the sequence of irrigation affected the number of adhering bacteria on dentine. We should plan the sequence of irrigation

such that the dentine surface would be left as bacteria free and as repulsive to bacteria adhesion as can be clinically achieved.

Future work

1. Mechanical effects of irrigation and bacteria adhesion were neglected in these experiments. Although we have reported that there were changes in surface roughness, we did not assess the effect of change in surface roughness and its effect on bacteria adhesion.

2. In these experiments, in order for AFM work to be less affected by surface rugosities, we had polished the surfaces of dentine sections to 4000 grit, removing the smear, producing a smear free dentine surface. We should attempt to measure if these effects are similar on smeared dentine.

3. There had been increase in adhesion force with CHX application of dentine surfaces with organic phase exposed. We should study the effect of CHX on surface energies of treated collagen to try to understand if the effects observed were due to increases in hydrophobicity.

4. The CHX used in this study was a commercial product formulated with many surfactants in addition to the active ingredient chlorhexidine digluconate. As

the non-chlorhexidine components could contribute to the effect of the chemical used, pure chlorhexidine digluconate should be used in future studies to see if chlorhexidine alone would produce the same effects.

5. Adhesion force between *E. faecalis* ATCC 29212 and collagen was increased in the presence of CH, we should determine if this was due to the presence of Ca++ ions or due to increased alkalinity of the system. Future work using other *Enterococcus* strains and clinically isolated strains should also be carried out.

6. Several brands of EDTA containing pastes are available on the market. These may act differently than the EDTA solution which we have used. Future work should include the use of these commercially available products and see how they affect bacteria adhesion to collagen and dentine.

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