



UNIVERSITY OF
BIRMINGHAM

*OPTIMIZATION OF THE MATCHED IRRIGATION TO
OBTURATION WITH HYDRAULIC CEMENT SEALERS
STRATEGY*

BY

SWETA SURANA BHANDARI

*A thesis submitted to
the University of Birmingham
for the degree of
MSc by Research in Dentistry*

*Institute of Clinical Sciences
School of Dentistry
College of Medical and Dental Sciences
The University of Birmingham
December 2024*

UNIVERSITY OF
BIRMINGHAM

University of Birmingham Research Archive

e-theses repository

This unpublished thesis/dissertation is copyright of the author and/or third parties. The intellectual property rights of the author or third parties in respect of this work are as defined by The Copyright Designs and Patents Act 1988 or as modified by any successor legislation.

Any use made of information contained in this thesis/dissertation must be in accordance with that legislation and must be properly acknowledged. Further distribution or reproduction in any format is prohibited without the permission of the copyright holder.

ABSTRACT

Tricalcium silicate sealer materials are vastly used in root canal obturation, mainly due to the calcium hydroxide release which is responsible for their biological and antimicrobial activity. The current study discusses the importance of selecting appropriate irrigation protocols for dentine preparation during root canal treatment for the choice of sealer and improve the adhesion and sealing ability of sealers with dentine. The study investigated the effect of different irrigation techniques, including continuous chelation, on dentine microstructure and aimed to choose the best irrigation protocol for endodontic treatment using Hydraulic calcium silicate cement (HCSC) sealers for obturation. The research also explored changes in the antimicrobial properties of HCSC sealers on interaction with dentine after irrigation with different chemical solutions using a split tooth model. Sterilisation of sample materials were also checked during the project.

Irrigating solutions can cause structural and chemical alterations of root dentin, affecting the mechanical and chemical adhesion of sealers to dentin and the antimicrobial properties of both dentin and sealer. Sodium hypochlorite (NaOCl), most widely used irrigant, being antimicrobial, eliminates the microbial load but can cause detrimental effects on the collagen. The most employed calcium chelator, Ethylenediaminetetraacetic acid (EDTA) can dissolve the inorganic debris and clear the smear layer produced by instrumentation but reduces the root dentine microhardness due to demineralization of the hydroxyapatite components of dentin. Hydraulic calcium silicate sealers interact with dentin more chemically and do not benefit from the property of calcium chelation and smear layer removal. The study used BioRoot RCS, TotalFill BC, and AH Plus jet as the test materials, and NaOCl, EDTA & Hydroxy ethylidene Di phosphonic Acid (HEDP) as the irrigation solutions. The results showed that in comparison to EDTA, irrigation with

HEDP resulted in a larger mineral concentration zone at the interface for hydraulic cements with less erosion of dentine. Furthermore, continuous chelation with HEDP improved the antimicrobial properties of the HCSC sealers without erosion of dentine making it the best irrigation protocol for endodontic treatment using tricalcium silicate sealers for obturation. Another finding of this project highlighted that hydraulic cement sealers are impacted by sterilization processes used prior to biological testing.

Regarding the method for studying the dentine sealer interface this study also concluded that when employing hydraulic cements, scanning electron microscopy and confocal laser scanning microscopy are not acceptable methods for studying the interface, Fourier-transform infrared spectroscopy can be used instead.

DEDICATION

I dedicate this thesis to my husband Mayank and daughter Sia. Their selfless support, encouragement and patience has been instrumental in this process. I would also like to dedicate it to my parents, Mr Ashok, and Mrs Lalita Surana for showing me the importance of challenging work and giving me the self-belief to be anything I want to be. I would not be where I am without their love, support, guidance, and inspiration. Finally, to my grandparents, Shivalji and Santosh Devi Surana for their eternal love and teaching me values of spirituality, kindness, and patience that I will carry forward in my life.

Om Namah Shivaya.

ACKNOWLEDGEMENTS

I would like to express my deepest sense of gratitude for my main supervisor Prof Josette Camilleri for supporting and believing in me while always pushing towards success. Thrilled to have known and working with my mentor in dentistry. There was never a time you turned me away when I needed some advice. I would not have reached the end without your support.

I would also like to thank Dr Sarah Kuehne and Prof William Palin, for their warm encouragement, thoughtful guidance throughout my study, and critical comments & corrections of the thesis. Dr Sarah, thank you for sailing me through the troughs of microbiology with ease in which I was a new bee.

Gratitude to all laboratory technicians, especially, Jianguo Liu, Helen, Tzu and Alessandro, for their technical guidance and endless cooperation.

Finally, thanks to my friends Diana, Nesma, Lauter, Maria, Sandeep (Doctoral Students) for discussions and valuable critics throughout this project. It not only helped me in developing of ideas but also cleared my doubts - thank you.

Table of Contents

LIST OF FIGURES	11
LIST OF TABLES	19
LIST OF ABBREVIATIONS	22
CHAPTER 1: REVIEW OF LITERATURE	26
1.1 Background to the study	26
1.1.1 Human Tooth	26
1.1.2 Microbiology of Root canal	29
1.2 Root Canal Treatment	31
1.2.1 Mechanical instrumentation	32
1.2.2 Irrigation	32
1.2.3 Obturation/ Placement of root canal filling	36
1.2.4 Coronal restoration	54
1.3 Interaction of irrigation solutions with dentine and sealers	55
1.4 Interaction of dentin with hydraulic calcium silicate cements	57
1.5 Aims of the study	58
CHAPTER 2: MATERIALS AND METHODS	59
2.1 Materials	59
2.2 Sample Preparation	61
2.3 Characterisation of Set Materials	62
2.3.1 Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray Spectroscopy (EDS)	62
2.3.2 X-ray diffraction (XRD) analysis	63
2.3.3 Fourier transform infrared (FT-IR) spectroscopy	64
2.3.4 Leachate analysis	64
2.4 Sterilisation Requirement of Sealer Discs	65
2.4.1 Characterization of set materials before and after sterilization	66
2.4.2 Macroscopic imaging of sealer discs	67
2.4.3 Sterility Test	68
2.5 Assessment of antimicrobial properties of endodontic sealers	68

2.5.1 Sample Preparation	68
2.5.2 Microbiological assays	69
2.6 Characterisation of dentine	72
2.6.1 Sample preparation and Instrumentation	72
2.6.2 Irrigation protocols	74
2.6.3. Scanning electron microscopy (SEM) and Energy Dispersive Spectroscopy (EDS) analyses	76
2.6.4. Fourier transform infrared spectroscopy (FT-IR)	76
2.7 Preliminary investigation of the dentine sealer interface using fluorescent dyes	77
2.7.1 Sample preparation	77
2.7.2. Confocal laser scanning microscopy (CLSM)	79
2.7.3. Scanning electron microscopy (SEM) and Energy dispersive spectroscopy (EDS)	80
2.7.4. Fourier transform infrared spectroscopy (FT-IR)	81
2.8 Investigation of the dentine sealer interface using an alternative method	82
2.8.1 Sample preparation	82
2.8.2. Scanning electron microscopy (SEM) and Energy dispersive spectroscopy (EDS)	84
2.8.3. Fourier transform infrared spectroscopy (FT-IR)	84
2.8.4 Intratubular infection test - pilot study	85
2.8.5 Attachment Assay/Direct Contact Test for Dentine Sealer Interface	91
2.9 Statistical analyses	93
2.9.1 Studies on antimicrobial properties of eluates	93
2.9.2 Studies on Sterilisation of sealer disks and Dentine Sealer Interface	94
CHAPTER 3: RESULTS	95
3.1 Characterisation of set materials	95
3.1.1 Scanning electron microscopy and Energy dispersive spectroscopy	95
3.1.2 X-ray diffraction analysis	100
3.1.3 Fourier transform infrared spectroscopy	102
3.1.4 Leachate Analysis	104
MICROBIOLOGICAL ANALYSIS	106
3.2 Sterilization of Sealer Disk	107
3.2.1 Characterization of set materials before and after sterilization	107
3.2.2 Macroscopic imaging of sealer discs	114
3.2.3 Sterility Test	115
3.3 Assessment of antimicrobial properties of endodontic sealers	116

3.3.1 Attachment Assay/Direct Contact Test for Sealer	116
3.3.2 Minimum Inhibitory Concentration (MIC)	117
3.4 Characterisation of dentine	120
3.4.1 Scanning electron microscopy (SEM) and Energy Dispersive Spectroscopy (EDS) analysis	120
3.4.2 Fourier transform infrared spectroscopy	127
3.5 Preliminary investigation of the dentine sealer Interface using fluorescent dyes	129
3.5.1 Confocal laser scanning microscopy (CLSM)	129
3.5.2 Scanning Electron Microscopy (SEM)	131
3.5.3 Energy-dispersive X-ray spectroscopy (EDS)	134
3.5.4 Fourier transform infrared spectroscopy	137
3.6 Investigation of dentine sealer interface using a Fourier transform infrared spectroscopy method	140
3.6.1 Scanning Electron Microscopy (SEM)	140
3.6.2 Energy-dispersive X-ray spectroscopy (EDS)	143
3.6.3 Fourier transform infrared spectroscopy	147
3.6.4 Intratubular infection test pilot study	150
3.6.5 Attachment Assay/Direct Contact Test for Dentine Sealer Interface	153
Chapter 4: DISCUSSION	156
4.1 Scope of the project	156
4.2 Selection of materials	157
4.2.1 Selection of endodontic sealers	157
4.2.2 Selection of Irrigants	159
4.2.3 Testing samples for experimental studies	160
4.3 Sterilisation of the endodontic sealers	160
4.4 Methodological design	162
4.4.1 Pilot studies	162
4.5 Chemical characterisation of set materials	167
4.6 Leachate Analysis	169
4.7 Microbiological Analysis of Sealer materials	170
4.7.1 Characterisation of set materials before and after sterilisation	170
4.7.2 Assessment of antimicrobial properties of endodontic sealers	173

4.8 Characterisation of dentine	177
4.9 Investigation of dentine sealer interface using an alternative method	179
4.9.1 Scanning electron microscopy (SEM)	179
4.9.2 Energy-dispersive X-ray spectroscopy (EDS)	180
4.9.3 Fourier transform infrared spectroscopy (FT-IR)	180
4.9.4 Attachment Assay/Direct Contact Test for Dentine Sealer Interface	182
CHAPTER 5: CONCLUSIONS	187
CHAPTER 6: FUTURE WORK	189
APPENDIX	190
LIST OF REFERENCES	190

LIST OF FIGURES

Figure 1.1: Structure of Tooth: Tooth is divided into (a) Crown - enamel, dentine, pulp and (b) Root- cementum, periodontal ligament, alveolar bone proper, pulp root canal, apical foramen. _____ 26

Figure 1.2: Structure of Dentine : Dentine near the DEJ showing tubule lumen, intertubular and peritubular dentine. _____ 28

Figure 1.3: Scanning electronic microscopic image of dentin showing dentine tubules, peritubular dentin and intertubular dentine. _____ 28

Figure 1.4: Flow chart demonstrates the root canal treatment steps and topics discussed in each step. _____ 31

*Figure 2.1: Schematic representation of the assembly for Direct Contact Test of different sealer groups : (a) A sterile petri dish was placed on top of the slide which was then inoculated with 20 μ l of the diluted *E. faecalis* culture and a coverslip was gently placed on it. (b) After overnight incubation, complete disc with the cover slip assembly was immersed in 10ml of BHI in a falcon tube and vortexed for 30 secs.(c) Miles and Misra was performed and Colony Forming Units/ml were calculated. _____ 71*

Figure 2.2: Schematic representation of specimen preparation for dentine characterisation. (a) the tooth sample was stuck to an acrylic block with the help of green stick and were decoronated to produce specimens with a root length of 14 mm using hard tissue microtome. (b)The roots were then split longitudinally to obtain 2 root halves. (c & d) The cleaved roots were reassembled using a light curing resin. (e)This tooth assembly was then placed in an alginate casing to simulate a closed apical system. _____ 74

Figure 2.3: Schematic representation of teeth preparation. a) All teeth were decoronated (b) and their roots were horizontally sectioned to form root blocks with a standardized length of 4 mm, using a microtome (c & d). The root blocks were then sectioned longitudinally to get two halves (e & f) one of the halves was polished from all sides to get a flat dentine specimen of 4x2x2 mm. _____ 78

Figure 2.4: A silicon putty mould was created; dentine specimen was then placed in it and irrigation was performed. _____ 78

Figure 2.5: Schematic representation of EDS line scans A, B, C at three distinct locations at dentine sealer interface. _____ 80

Figure 2.6: Schematic representation of points selected for FT-IR analysis for dentine sealer interface : 1 to 4 points in material, 5 at interface, 6 to 9 in dentine. _____ 81

Figure 2.7: Schematic representation of EDS point scan for dentine sealer interface (3 points in material, 3 points in dentine, 3 points in material towards dentine sealer interface, and 3 points in dentine towards dentine sealer interface). _____ 84

Figure 2.8: Schematic diagram of sample preparation for intratubular infection test. 3 The cylindrical root segments were sectioned longitudinally into halves. Each of these dentine specimen was placed in centrifugal filter with the canal side up for dentinal tubule infection. After centrifugation, the dentine specimens were removed, outer surfaces of the specimens were closed with nail varnish; irrigation followed by different sealer groups (medic) were applied on the canal side. After incubation, samples were vertically cut into two halves, stained with live and dead stain, and viewed under confocal microscope. _____ 86

Figure 3.1: Indicative backscatter Scanning Electron Microscopy (SEM) (1,000 X magnification) showing microstructural components of AH Plus, BioRoot RCS, BioRoot Flow and TotalFill sealer after immersion in HBSS for 0 day ,7days and 90 days. _____ 97

Figure 3.2: Indicative Corresponding Energy-Dispersive X-Ray Spectroscopy (EDS) analyses showing elemental composition of AH Plus, BioRoot RCS, BioRoot Flow and TotalFill sealer after immersion in HBSS for 0 day ,7days and 90 days displaying main peaks of calcium, zirconium, silicon, carbon, and oxygen. _____ 99

Figure 3.3: X-ray diffraction plots of AH Plus, BioRoot RCS, BioRoot Flow and TotalFill at 0 ,7 and 90 days showing the crystalline phases present after immersion in Hank's balanced salt solution. _____ 101

Figure 3.4: Fourier transform infrared spectroscopy plots of the AH Plus, BioRoot RCS, BioRoot Flow and TotalFill at 0-day,7 days and 90 days. nitrogen to hydrogen bond (N–H) peaks were detected in AH Plus and Carbonate (CO₃), calcium silicate hydrate (CSH), & calcium hydroxide (Ca (OH)₂) peaks were detected in other tricalcium silicate sealers. _____ 103

Figure 3.5: Secondary electron Scanning electronic micrographs of AH Plus ,BioRoot RCS ,BioRoot Flow and TotalFill sealers after sterilisation with Ethanol & Ultraviolet (EU), Ultraviolet alone (U) and Autoclave(A) at 500 x magnification. In ethanol plus ultraviolet group, red arrow indicates areas with deposits interspersed with areas that are darker and without deposits in BioRoot RCS, green arrow indicates cracking and increased porosity in BioRoot Flow, blue arrow indicates some surface deposits in Total Fill. In autoclave group red arrow indicates deterioration to the material surface of AH Plus with channels devoid of radiopacifier. Yellow arrow indicates some surface deposits in BioRoot RCS. _____ 110

Figure 3.6: Fourier transform infrared spectroscopy of AH Plus, BioRoot RCS, BioRoot Flow (BRF) and Total Fill (TF) after different techniques of sterilisation: No sterilisation as control (C), Ethanol (E), Ethanol + Ultraviolet (EU), Ultraviolet (U) alone and Autoclave (A). Changes in water (H-O-H) and alcohol peaks could be seen at 3421cm^{-1} in AH Plus and BioRoot RCS specimen. No changes could be seen with BRF and TF. _____ 113

Figure 3.7: Macroscopic view of all four sealer discs (BRF-BioRoot Flow, TotalFill, AH-AH Plus, BR-BioRoot RCS) before and after sterilisation with different techniques (C-control, E-ethanol, EV-ethanol +ultraviolet, U-ultraviolet, A-autoclave). _____ 114

Figure 3.8: (a) Agar plates showing growth when disks of AH Plus, BioRoot RCS, BioRoot Flow and TotalFill were prepared in open lab. (b) Uncontaminated agar plate. _____ 115

Figure 3.9: Antimicrobial activity in CFU/ml for four different sealers (AH Plus, BioRoot RCS, BioRoot Flow and TotalFill) at 0-day, 7 days and 90 days with the Direct Contact Test. Results of two-way ANOVA test with $p < 0.0001$: statistically significant difference. The limit of detection was at 50 CFU/ml. ANOVA: analysis of variance; (n=3). _____ 117

*Figure 3.10: This graph represents optical density of various sealer eluents of a time frame of (a) 7 days and (b) 90 days frame. Bacteria only are the control. Results of one-way ANOVA / Dunnett's multiple comparisons test. Bar graph and error bar (mean $\pm$ SD). Differences between means are indicated. ****, $p < 0.0001$. ns, not significant. ANOVA: analysis of variance; (n=9).* ____ 119

Figure 3.11: Photomicrographs indicative secondary electron Scanning Electron Microscopy (2,000X magnification) showing microstructural components of human

root dentine from the coronal (C), middle(M) and apical(A) thirds after irrigation on surface with Distilled (DW)- Group A, Hypochlorite (NaOCl)- Group B, EDTA -Group C and Hypochlorite followed by EDTA (NaOCl-EDTA)- Group D, Etidronic acid mixed with hypochlorite(HEDP+NaOCl)- Group E and Etidronic acid mixed with Distilled water (HEDP+DW)- Group F. Yellow arrow indicates smear layer, red arrow indicates erosion of dentinal tubule. _____ 123

Figure 3.12: Corresponding Energy Dispersive Spectroscopy plots showing elemental components of human root dentine from the coronal (C), middle(M) and apical(A) thirds after irrigation on surface with Distilled Water(DW)- Group A, Hypochlorite(NaOCl)-Group B,EDTA-Group C, Hypochlorite followed by EDTA (NaOCl-EDTA)-Group D, Etidronic acid mixed with hypochlorite (HEDP+NaOCl)- Group E and Etidronic acid mixed with Distilled water(HEDP+DW)- Group F. Increased intensity of Sodium (Na) & Chloride (Cl) and reduced Carbon (C) with the NaOCl-treated dentin as compared to control. Increased carbon(C) levels and decrease in the level of oxygen (O) and calcium (Ca) was seen with EDTA groups as compared to controlling and Cl levels increased in HEDP+NaOCl. _____ 126

Figure 3.13: Fourier transform infrared spectroscopy (FT-IR) of dentine plain and dentine exposed to different irrigation protocols: Distilled Water (DW)- Group A, Hypochlorite (NaOCl)-Group B, EDTA -Group C, Hypochlorite followed by EDTA (NaOCl-EDTA)- Group D, Etidronic acid mixed with hypochlorite (HEDP+NaOCl)- Group E and Etidronic acid mixed with Distilled water(HEDP+DW)- Group F. _____ 128

Figure 3.14: Representative fluorescence-mode CLSM images of sealer-dentin interface of BioRoot RCS and AH Plus with Rhodamine B (R) or Orange G (O) added to the sealer; showing a richly dye-infiltrated dentinal tubules. _____ 130

Figure 3.15: Back-scatter scanning electron micrographs of AH Plus, BioRoot RCS, BioRoot Flow and TotalFill at dentine sealer interface (2,000×

magnification) showing microstructural components of three groups: Sealer with No dye (N), Sealer mixed with Rhodamine B dye (R) and Sealer mixed with Orange G dye (O). _____ 133

Figure 3.16: Fourier transform infrared spectroscopy point scans of AH Plus, BioRoot RCS, BioRoot Flow and TotalFill at 3 points in dentine(D), 1 point at interface (Inter/I) and 3 points in sealer/material(M) at interface (Inter/I) and 3 points in sealer/material(M) at 300 μ m, 200 μ m and 100 μ m distance from interface. [ID3, ID2, ID1=point at 300,200,100 μ m distance from interface in dentine. [IM3,IM2,IM1= point at 300, 200, 100 μ m distance from interface in material]. Results were assessed within the spectral range of 700 to 2000 of three groups : Sealer with No dye(N) ,Sealer mixed with Rhodamine B dye (R) and Sealer mixed with Orange G dye (O). Increase in intensity in the 803 Si-O peaks of AH Plus when the dye is incorporated into the resin sealer. Carbonate and CSH peaks intensify with the addition of dyes in the BioRoot RCS, BioRoot Flow, and TotalFill while the phosphate peak fades towards dentin at the interface.[Silica(Si-O), Phosphate (PO₄) ,Hydroxyl (OH), Calcium Silicate Hydrate (CSH), and carbonate(CO₃). _____ 139

Figure 3.17:Back-scatter scanning electron micrographs of dentine sealer interface of AH Plus, BioRoot RCS, BioRoot Flow and Total Fill sealers (2,000 $\times$ magnification) showing microstructural components irrigated with three irrigant groups; Distilled water-(DW), HEDP mixed with hypochlorite (NaOCl)-(HN) and Hypochlorite (NaOCl) followed by EDTA-(NE). Red arrow depicts the detached and cracked interface of sealer to dentine. _____ 142

Figure 3.18: Fourier transform infrared spectroscopy point scans of AH Plus, BioRoot RCS, BioRoot Flow and TotalFill at 3 points in dentine(D) ,1 point at interface (Inter/I) and 3 points in sealer/material(M) at 300 μ m, 200 μ m and 100 μ m distance from interface. .[ID3, ID2, ID1=point at 300,200,100 μ m distance from interface in dentine. IM3, IM2, IM1= point at 300, 200, 100 μ m

distance from interface in material]. Results were assessed after irrigation within the spectral range of 800 to 1200 with three irrigant groups: Distilled water- (DW), HEDP mixed with hypochlorite (NH) and Hypochlorite followed by EDTA(NE). (Phosphate (PO₄) peak in dentine was reduced with hydraulic cement as compared to that dentine area , while the intensities of the Calcium silicate hydrate (CSH) were reduced gradually towards dentine . _____ 149

Figure 3.19: Representative confocal laser scanning microscope images of *E. faecalis* infected dentinal tubules after irrigation with distilled water (DW), HEDP+NaOCl (hypochlorite with etidronic acid), NaOCl-EDTA (hypochlorite followed by EDTA), followed by placement of BioRoot RCS sealer. The viability staining indicated live cells in green, and dead cells in red. Scanning was performed using 40xwater lens, 1µm step size. Dentinal tubules showed autofluorescence. _____ 151

Figure 3.20: Representative confocal laser scanning microscope image of *E. faecalis* infected dentinal tubules after reducing the dentine autofluorescence. The viability staining indicated live cells in green, and dead cells in red. Scanning was performed using 40xwater lens, 1µm step size. _____ 152

Figure 3.21: Representative confocal laser scanning microscope images of *E. faecalis* infected dentinal tubules after treating with ethanol. The viability staining indicated live cells in green, and dead cells in red. Scanning was performed using 40xwater lens, 1µm step size. _____ 152

Figure 3.22: Number of CFU (Colony Forming Unit) of *E. faecalis* in the presence of AH Plus, BioRoot RCS, BioRoot Flow, TotalFill sealer in contact with dentine at the dentine sealer interface after 3 irrigation protocols (DW= distilled water; NaOCl+HEDP= hypochlorite mixed with etidronic acid; NaOCL EDTA= Hypochlorite followed by EDTA), aged for 7 days and 24h of direct contact: ($p<0.05$). Sealer with no contact with dentine was used as a control. _____ 154

Figure 3.23: Number of CFU (Colony Forming Unit) of E.faecalis in Dentine in contact with (AH Plus, BioRoot RCS, BioRoot Flow, TotalFill sealer) at the dentine sealer interface, after 3 irrigation protocols (DW= distilled water; NaOCl+HEDP= hypochlorite mixed with etidronic acid; NaOCl-EDTA= Hypochlorite followed by EDTA), aged for 7 days and 24h of direct contact: (p<0.05). Dentine in no contact with sealer was used as control. _____ 155

LIST OF TABLES

<i>Table 1.1: Classification of clinically available hydraulic calcium silicate cements</i>	43
---	----

<i>Table 2.1: Table showing different sterilisation methods used for sterilisation of sealer discs</i>	64
--	----

<i>Table 2.2: Irrigants were tested in different sequences. NaOCl (sodium hypochlorite), EDTA (ethylenediaminetetraacetic acid), HEDP (1-hydroxyethylidene-1,1-diphosphonic acid) (mins= minutes)</i>	73
---	----

<i>Table 2.3: The table depicts three groups of dyes mixed with BioRoot RCS sealer mixed with Rhodamine B, Orange G, and no dye as control. Irrigation of samples was done by 5.25% (NaOCl) Hypochlorite for 5 minutes followed by 17% EDTA(ethylenediaminetetraacetic acid)</i>	77
--	----

<i>Table 2.4: Three irrigation groups; Group A: Distilled Water, Group B: Etidronic acid mixed with hypochlorite and Group C; Hypochlorite followed by EDTA; for investigating dentine sealer interface with four sealers AH Plus, BioRoot RCS, BioRoot Flow and TotalFill (NaOCl: sodium hypochlorite, EDTA: Ethylenediaminetetraacetic acid, HEDP: 1-hydroxyethylidene-1,1-diphosphonic acid)</i>	80
---	----

<i>Table 2.5: Three groups for intratubular infection test using different irrigation protocols followed by BioRoot RCS as sealer (NaOCl: sodium hypochlorite; HEDP: etidronic acid; EDTA: Ethylenediaminetetraacetic acid).</i>	85
--	----

Table 3.1: Cumulative data of leachate collected after total of 7 days and 90 days: Calcium (Ca) ion concentration in leachate with standard deviation of four sealers: AH Plus, BioRoot RCS, BioRoot Flow and TotalFill. BDL: Below Detection Limit _____ 105

Table 3.2: Data of leachate collected every 15 days for a total of 90 days duration: Mean calcium ion concentration in leachate media of four sealers: AH Plus, BioRoot RCS, BioRoot Flow and TotalFill with standard deviation. ____ 106

Table 3.3: Energy-dispersive X-ray spectroscopy (EDS) mean values with standard deviation for sterilisation of sealers. Ratio of calcium with silicon and ratio of calcium with zirconium of distinct groups of sterilisation methods on four sealers AH Plus, BioRoot, BioRoot Flow and Total Fill: no sterilisation as control. _____ 112

Table 3.4: Association between optical density and CFU/ml conducted in a preliminary experiment. _____ 114

Table 3.5: EDS point scans of mean ratio of calcium with silicon (Ca/Si) and calcium with zirconium (Ca/Zr) of BioRoot RCS mixed with Rhodamine B and Orange G dye ,with no dye as control; at four different points, one point in material, two points at interface and one point in dentin. BDL:below detection limit; Inter-M: interface towards material; Inter-D: interface towards dentine. 135

Table 3.6: EDS point scans of mean ratio of calcium with silicon (Ca/Si) and calcium with zirconium (Ca/Zr) of BioRoot Flow mixed with Rhodamine B and Orange G dye ,with no dye as control; at four different points, one point in material, two points at interface and one point in dentin. BDL:below detection limit; Inter-M: interface towards material; Inter-D: interface towards dentine. 135

Table 3.7: EDS point scans of mean ratio of calcium with silicon (Ca/Si) and calcium with zirconium (Ca/Zr) of TotalFill mixed with Rhodamine B and Orange G dye ,with no dye as control; at four different points, one point in material, two points at interface and one point in dentin. BDL: below detection limit; Inter-M: interface towards material; Inter-D: interface towards dentine. _____ 136

Table 3.8: EDS point scans of mean ratio of calcium with silicon (Ca/Si) and calcium with zirconium) after irrigation with three groups and using sealer as BioRoot RCS at three distinct locations, material, interface, and dentin at 4 different points. One point in material, two points at interface and one point in dentin, with standard deviation. (NaOCl+HEDP= hypochlorite mixed with etidronic acid, NaOCl-EDTA= Hypochlorite followed by EDTA, DW=distilled water), BDL: below detection limit; Inter-M: interface towards material; Inter-D: interface towards dentine. _____ 144

Table 3.9: EDS point scans of mean ratio of calcium with silicon (Ca/Si) and calcium with zirconium) after irrigation with three groups and using sealer as BioRoot Flow at three distinct locations, material, interface, and dentin at 4 different points. One point in material, two points at interface and one point in dentin, with standard deviation. (NaOCl+HEDP= hypochlorite mixed with etidronic acid, NaOCl-EDTA= Hypochlorite followed by EDTA, DW=distilled water, BDL: below detection limit. Inter-M: interface towards material; Inter-D: interface towards dentine. _____ 145

Table 3.10: EDS point scans of mean ratio of calcium with silicon (Ca/Si) and calcium with zirconium) after irrigation with three groups using sealer TotalFill at three distinct locations, material, interface, and dentin at 4 different points. One point in material, two points at interface and one point in dentin, with standard deviation. (NaOCl+HEDP= hypochlorite mixed with etidronic acid, NaOCl-EDTA= Hypochlorite followed by EDTA, DW=distilled water, BDL: below detection limit; Inter-M: interface towards material; Inter-D: interface towards dentine. _____ 146

LIST OF ABBREVIATIONS

%	Per cent
°C	Degrees Celsius
ADT	Agar Diffusion Test
ANOVA	Analysis of variance
BHI	Brain Heart Infusion
BR	BioRoot RCS
BRF	BioRoot Flow
CH	Calcium Hydroxide
CLSM	Confocal laser scanning microscopy
cm	Centimetre
cm ²	Square centimetre
CO ₃	carbonate
COD	Crystallography Open Database
CSH	Calcium silicate hydrate
CT	Calcium Tungstate
DCS	Dicalcium Silicate
DCT	Direct Contact Test

DW	Distilled water
EDS	Energy-dispersive X-ray spectroscopy
EDTA	Ethylenediaminetetraacetic acid
EU	Ethanol + Ultraviolet
FT-IR	Fourier transform infrared
g	Gram
g/l	Grams per litre
GIC	Glass ionomer cement
GP	Gutta-percha
HBSS	Hank's balanced salt solution
HEDP	1-hydroxyethylidene-1,1-diphosphonic acid/Etidronic acid
hr	Hour
ICP-OES	Inductively coupled plasma-optical emission spectrometry
kV	Kilovolt
l	Litre
mA	Milliampere
mg	Milligram
mg/l	Milligrams per litre
mg/ml	Milligrams per millilitre
MIC	Minimum Inhibitory Concentration
min	Minutes

ml	Millilitre
mm	Millimetre
mm ²	Square millimetre
MTA	Mineral trioxide aggregate
N	Newton
NaOCl	Hypochlorite
OD	Optical density
PBS	Phosphate Buffered Saline
PI	Propidium iodide
PO ₄	Phosphate
rpm	Revolutions per minute
s	Second
SEM	Scanning electron microscopy
TCS	Tricalcium silicate
TCS	Tricalcium silicate
TF	Total Fill
UV	Ultraviolet
wt. %	Per cent by weight
XRD	X-ray diffraction
ZO	Zirconium oxide
µg	Microgram
µg/g	Micrograms per gram
µm	Micrometre

CHAPTER 1: REVIEW OF LITERATURE

1.1 Background to the study

1.1.1 Human Tooth

Human tooth consists of crown and a root portion. Highly mineralised enamel of the crown covers the dentin, while root dentin is capped by cementum, responsible for the attachment of the tooth to bony socket as seen in figure 1.1.

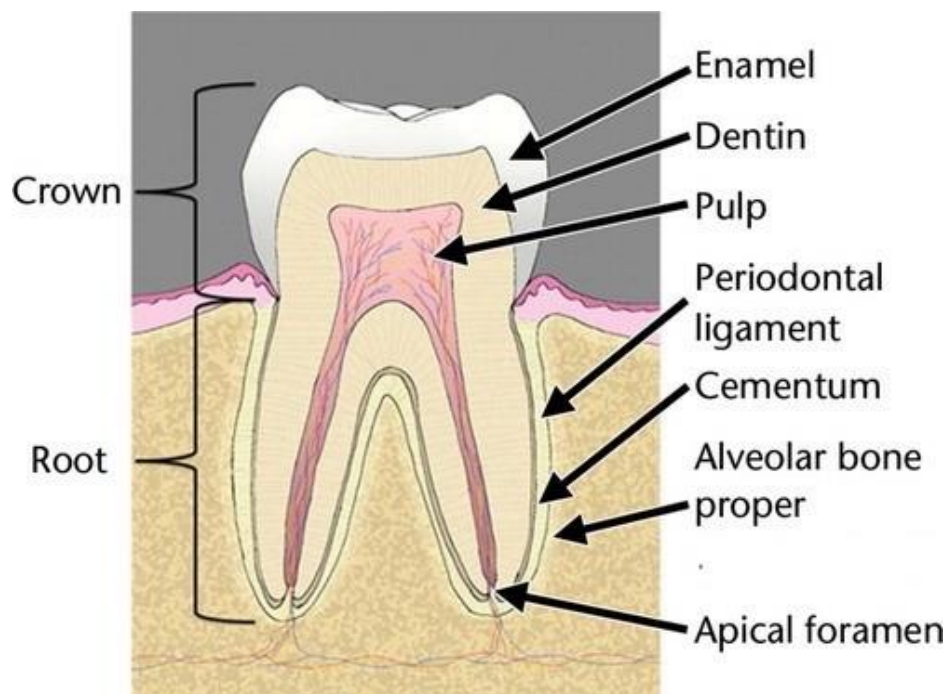


Figure 1.1: Structure of Tooth: Tooth is divided into (a) Crown - enamel, dentine, pulp and (b) Root- cementum, periodontal ligament, alveolar bone proper, pulp root canal, apical foramen.

The cementum along with alveolar bone and periodontal ligament comprises of peri radicular tissue. In the centre lies the pulp which is made of nerves, connective tissue, and blood vessels (1). The blood vessels & nerves of the pulp enter and exit the pulp cavity through an aperture at or near the apex of a tooth known as apical foramen. Coronally, the pulp resides in the pulp chamber and the root portion forms the root canal; with both portions known as the root canal system. Teeth may have one or more main root canals with anastomosis between them along with lateral & accessory canals, making root canal systems complex in nature (2). Dentin constitutes the bulk of the human tooth.

1.1.1.1 Dentin

Dentin is a permeable tubular structure representing most of the dental tissue. It is composed of the organic matrix embedded in a crystalline apatite, having about 70 weights % inorganic (mineral), 20 weight % organic, and 10 weight % water (3). The main inorganic component of dentin is the crystalline hydroxyapatite with some calcium carbonate while, type I collagen fibrils are majorly the organic constituent. Collagen fibres are triple helical structures of polypeptide chains, encapsulated within hydroxyapatite crystals (4). Carbonated apatite in dentin, like bone tissue, provides strong buffering action against acids and this inorganic component renders the dentin its antimicrobial property (5) of water in the dentine keeps the tissue constantly moist maintaining the viscoelasticity of dentine (6).

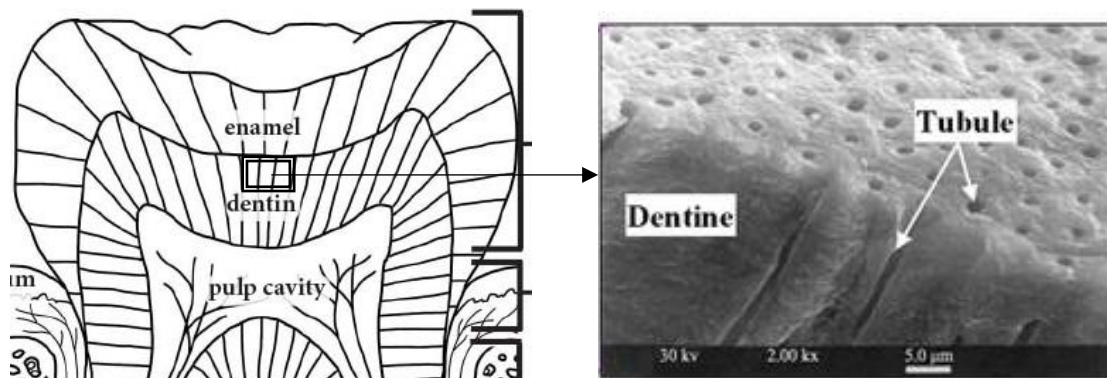


Figure 1.2: Structure of Dentine : Dentine near the DEJ showing tubule lumen (294).

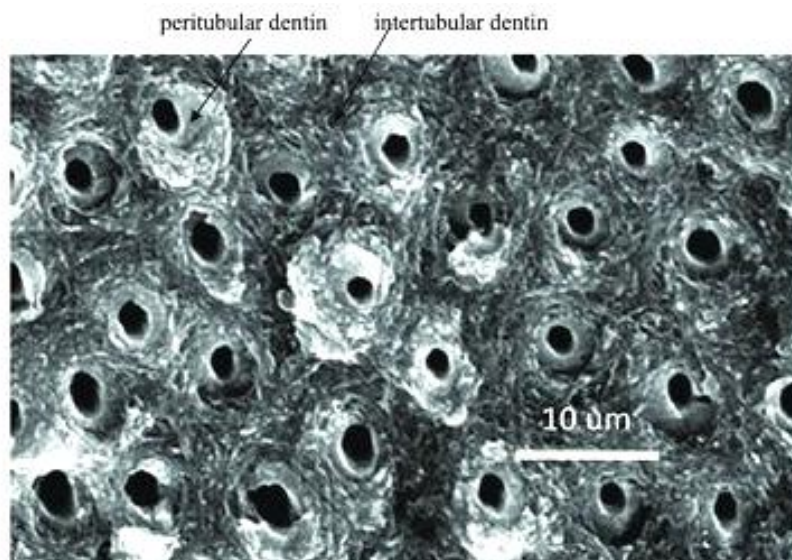


Figure 1.3: Scanning electron micrographic image of dentin tubule showing peritubular dentin and intertubular dentine (293).

The salient feature of dentin is the regular pattern of microscopic dentinal tubules which extend from the pulpal surface to the enamel–dentine junction depicted in Figure 1.2. These dentinal tubules are filled with dentinal fluid and surrounded by peritubular and intertubular dentine

(Figure 1.3). Peritubular dentine is strongly mineralized (3) and therefore harder than intertubular dentin and thus provide added structural support to the latter one. In comparison to intertubular dentine, peritubular dentin dissolves more quickly in acid because to its lower collagen concentration.

1.1.1.2 Dentin Pulp complex

Directly beneath the layer of dentin lies the pulp which is a mass of connective tissue. These two tissues are closely interlinked and dependent on each other's development and survival referred to as the “dentin-pulp” complex. Damage to the dentin via caries/trauma may expose the pulp to oral bacteria, resulting in inflammation and necrosis of the pulp. This may further lead to apical periodontitis (7).

1.1.2 Microbiology of Root canal

The primary etiological factor of pulpal necrosis and apical periodontitis is bacteria and their by-products (8). Apical periodontitis is a biofilm-related disease (9). The biofilm of infected root canals contains a diverse microbiota including Enterococci species (sp), *Fusobacterium* sp, *Prevotella* sp, *Porphyromonas* sp and *Treponema* sp. (10–13). As a result of the ecologic pressure, the flora of untreated necrotic root canals varies significantly to the treated ones. Necrotic root canals are polymicrobial, with both gram-positive and gram-negative bacteria dominated by anaerobic microflora whilst the ecology changes immensely after the root canal treatment. The

limited availability of oxygen & nutrients as well as introduction of antimicrobial substances in the root canal may further contribute to the development of monoinfections, more resistant, gram-positive facultative anaerobic microbiota (5,8).

Enterococcus faecalis (*E. faecalis*), is the most used microorganism utilised in laboratory research to simulate persistent endodontic infections, despite the fact that endodontic infections are caused by many species of biofilms (8,13). *E. faecalis* is a Gram-positive facultative anaerobe which can survive in an environment with scarce nutrients (14). The smear layer formed during instrumentation enhances *E. faecalis* adhesion, invade the dentinal tubules and create biofilms (15) A biofilm is an aggregation of microorganisms in an extracellular polysaccharide matrix associated with a surface like root canal wall (16). *E. faecalis* biofilm also has strong viability and drug resistance (17) property of *E. faecalis* to create biofilm, can hinder its complete elimination by root canal irrigants and intracanal medications. Due to these properties this bacterial species has long been considered the main bacterial species in labs to test the antimicrobial activity of root canal sealers (18) An alkaline pH promotes the elimination of *E. faecalis* and it cannot survive in pH around 11 (19).

However, newer developments in the field of endodontics have revealed that root canal infections are actually polymicrobial in nature, meaning that they involve the presence of multiple bacterial species in addition to *E. faecalis*. In fact, recent research has shown that there are several other bacteria that play important roles in endodontic infections, including *Streptococcus* species, *Porphyromonas* species, *Prevotella* species, and *Fusobacterium* species (19).

As there is a microbial cause of apical periodontitis, the elimination or reduction of microorganisms from the root canal system and subsequent closure will result in healing and prevention of re-infection. This can be accomplished by root canal treatment.

1.2 Root Canal Treatment

Root canal treatment is the procedure of repair & treatment of injured pulp, either by trauma or infection, as well as the periapical tissues, to preserve the function of natural tooth. Root canal treatment has four objectives: 1) to provide a clean root canal keeping the microbial load to minimum, 2) Seal the root at apex to prevent fluid penetration which may act as food for bacteria inside root canal, 3) Provide coronal seal to prevent any reinfection from oral cavity and 4) healing of periapical lesion (20).

Thus, root canal treatment includes 4 major steps:

- 1) Mechanical instrumentation $\left. \vphantom{\begin{array}{l} 1) \\ 2) \end{array}} \right\} \text{Cleaning \& Shaping / Chemo mechanical debridement}$
- 2) Irrigation
- 3) Obturation/ Placement of root canal filling
- 4) Coronal restoration

The flow chart 1.4 below demonstrates the topics to be discussed in each root canal step, further in the literature -

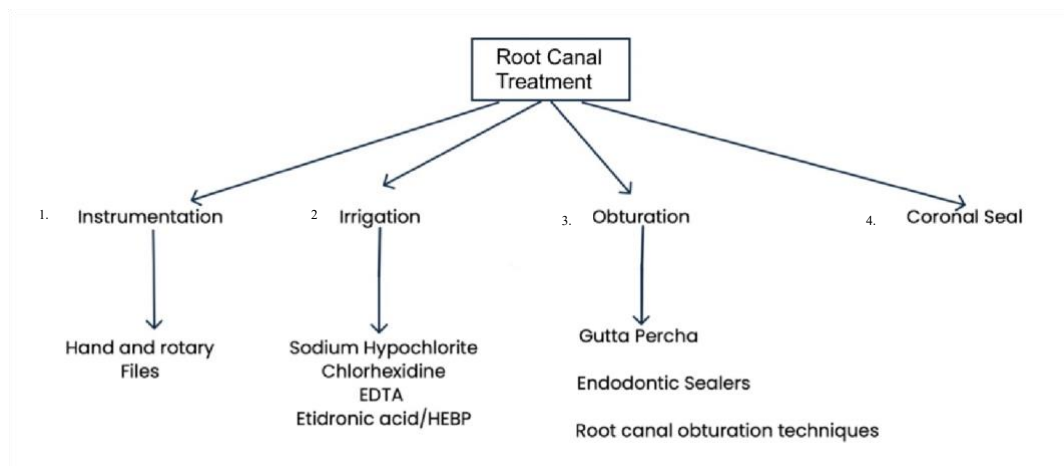


Figure 1.4: Flow chart demonstrates the root canal treatment steps and topics to be discussed in each step.

1.2.1 Mechanical instrumentation

The debridement and shaping of the canal space facilitates the placement of root canal filling. This is done with the help of rotary nickel titanium (NiTi), reciprocating files or traditional stainless-steel hand instruments/files (21).

Although instrumentation helps to remove a lot of debris, the complexities within the root canal system remain mostly untouched (22). These irregularities may harbour infected necrotic tissue and may result in persistent infection (23). Also, a smear layer is produced through mechanical instrumentation (24). A smear layer is an amorphous layer which is made up of the organic component (pulp tissue, denatured collagen, tissue fluid, bacteria, and their by-products) and inorganic component (dentin chips containing calcium hydroxyapatite), created on hard tissues whenever they are cut with hand or rotary instruments (25). This layer covers the radicular dentinal surfaces and obliterates the dentinal tubules, interfering with the adaptation of filling material to dentinal walls and promoting microleakage (26). It may also provide protection to

bacterial biofilms adhering to root canal walls (27). Thus, chemical disinfection with irrigating solutions remains a critical component of root canal therapy.

1.2.2 Irrigation

To predictably remove all microbiologic aetiology from the root canal system and dentinal tubules, mechanical debridement should be accompanied with chemical disinfection through copious antimicrobial irrigants solutions (21). Ideally root canal irrigants should: 1) Flush any loose debris, 2) Dissolve necrotic pulp tissue remnants, 3) provide lubrication, 4) Prevent the formation of a smear layer during or after instrumentation, 5) be antimicrobial, and 6) have low tissue toxicity (28).

Irrigating solutions can be classified as (29) -

Chemical agents

- Tissue dissolving agents- NaOCl
- Antibacterial – Chlorhexidine, Quaternary ammonium compounds, MTAD (tetracycline antibiotics),
- Chelating agents - mild pH- Etidronic acid
- strong pH -EDTA, citric acid

Natural agents

- Antibacterial agents - Green tea, Triphala

1.2.2.1 Sodium hypochlorite (NaOCl)

It is currently the irrigant of choice for chemical debridement of the root canal system as it covers more of the requirements for endodontic irrigant than any other known compound (28). It has the property to dissolve necrotic pulp tissue and organic component of smear layer. In addition to this, it is also antimicrobial in nature (29,30) as it kills pathogens in biofilms and in dentinal tubules along with which it also inactivates the endotoxin produced by bacteria to some extent (31,32) is used clinically in concentrations ranging from 0.5% to 6% (30). Although NaOCl appears to be the most ideal irrigant, it cannot dissolve inorganic component of smear layer formed during instrumentation (28).

1.2.2.2 Chlorhexidine

It is available between 0.12-2%, has antimicrobial properties and is nontoxic but does not dissolve organic or inorganic matter (29). Chlorhexidine has the ability to kill the bacteria by permeating its cell wall and damaging it. It has the capacity to inhibit certain bacterial adherence to dentine (33). It has the ability to bind to phosphate present in the structure of hydroxyapatite of dentine and can be gradually released in small amounts contributing to prolonged antimicrobial effect; the property which is known as substantivity (29).

1.2.2.3 Chelating Agents

Chelants are chemical compounds that form complex soluble molecules with certain metallic ions and inactivate them. Chelating agents were introduced to endodontics by Nygaard-Østby in 1957 as an aid for the preparation of narrow and calcified root canals (34). These agents as an endodontic irrigant, bind with calcium ion present in the hydroxyapatite ($\text{Ca}_5(\text{PO}_4)_3\text{OH}$) crystals of the inorganic component of smear layer, forms soluble calcium chelates favouring its removal. The elimination of smear layer is essential to allow sealer penetration into dentinal tubules producing a better adaption of sealer to the dentinal wall, moreover, it also creates a demineralising effect on the inorganic component of dentine leading to enlargement of the dentinal tubules exposing the bacteria to the disinfecting irrigants (35,36).

1.2.2.3.1 Ethylenediaminetetraacetic acid (EDTA)

EDTA is a strong chelating agent is often used as an adjunct irrigant to remove the smear layer produced during root canal therapy (28). It has slightly alkaline to neutral pH and does not possess the tissue dissolving property. It has limited antimicrobial property (17,37) which can be due to chemical chelation of outer membrane of bacteria and detaching biofilms adhered to root canal walls (30,38). Furthermore, the demineralising effect of strong chelating agents can lead to changes in the structural and biomechanical properties of the dentin (39) resulting in its erosion and weakening. It is used clinically in concentration of 15% to 17%.

As none of these irrigants can be considered as optimum, they are used in combination for a better cleaning and disinfection of root canal, better preparation of dentin for interaction with the sealer and finally leading to a better outcome of treatment. To date, the recommended protocol for irrigation includes the use of NaOCl due to its effective antibacterial properties and ability to dissolve organic tissue. Strong chelating agents like EDTA come next, aiding in the removal of the smear layer's inorganic components (28). However, once the debris created, only 50% could be removed using this protocol. Also, these decalcifying agents like EDTA are incompatible with NaOCl, as they cannot keep the chlorine active in solution, reducing the effectiveness of hypochlorite (40).

1.2.2.3.2 Etidronic acid (HEBP) (1-hydroxyethylidene-1,1-bis-phosphonate)

HEBP is a biocompatible decalcifying agent with weak chelating capacity in contrast to EDTA. Since HEBP is a poor chelator, it cannot be utilized as a last rinse; instead, it can be employed as a more comprehensive root canal irrigant in combination with NaOCl. HEBP does not interact with NaOCl and therefore this mixture maintains the antimicrobial properties and dissolution activity of NaOCl for a brief period (40–43). It can be used as a sole irrigating solution during biomechanical preparation and as a final flush, preventing the smear layer formation throughout (43–46), hence the term “continuous chelation”(47,48).

HEBP also has less effects on the microhardness of dentin when used as a final flush as compared to EDTA (47,49). In addition, it provides larger intratubular dentin area for hybridization of resin-based sealers (like AH Plus), increasing the bond strength (49,50). However, this solution needs 300 seconds to remove the smear layer completely (47,50). It is used in concentrations of 9% or 18%.

Once the mechanical instrumentation and irrigation commences, it leaves the root canal space with minimum microbial load. This space then needs to be obturated and filled with a dense filling material for appropriate sealing of the root canal space to prevent any nutrient source to bacteria and therefore reducing the chances of reinfection.

1.2.3 Obturation/ Placement of root canal filling

A successful obturated system should: prevent leakage of microorganisms, entomb any residual bacteria, and prevent reinfection (51). Root canal obturation is filling and sealing of the entire root canal space with the help of a solid core (most common is gutta-percha) and a cement (endodontic sealer) to provide a fluid tight seal from coronal orifice of the canal to the apical foramen of the root (52). The core material occupies space while role of a sealer is to create a bond between the core material and the prepared dentinal walls (53), filling the irregularities and eliminating any voids in the root canal where core material cannot reach.

Recently a sealer-based obturation technique has become popular which uses calcium silicate root canal sealers (54). Advantages of this obturation technique includes less armamentarium, easy handling and less technique sensitivity (55,56).

Numerous materials have been researched to be used as a core for obturation purpose, which includes solid or metal cores like silver cones that is no longer used in modern endodontics as they corrode when exposed to saliva or tissue fluid, plastics like Gutta-percha (GP), impregnated gutta-percha cones (57), bioceramics, in particular MTA which has extended setting time, good fracture resistance, relatively low compressive strength and antibacterial characteristics (58). Currently, out of all these materials gutta-percha may be regarded as a suitable alternative and

most used core material in modern endodontics owing to its physical and chemical properties (59).

1.2.3.1 Gutta-percha (GP)

Gutta-percha is a trans-isomer of polyisoprene, most used solid core for obturation and is made of natural rubber. Major component of dental gutta-percha is zinc oxide (50-79%), heavy metal salts (1-17%), wax or resin (1-4%) and only 19-22% actual gutta-percha (60).

Gutta-percha exists in two distinctly different crystalline forms (α and β), that can be converted into each other. α is a natural form and β form is commercially available product.

α form - Brittle at room temperature

- Becomes gluey and highly flowable when heated
- Used for warm condensation obturation techniques

β form - Stable and flexible at room temperature

- Becomes less adhesive and flowable when heated
- Used for cold condensation obturation techniques

Gutta-percha is a temperature-sensitive thermoplastic and viscoelastic substance. It exists at room temperature in a rigid and solid state. Over time, exposure to air and light makes it fragile. It is excellent non-conductor. In some solvents, it is soluble. When gutta-percha is manipulated with heat or a solvent, the material shrinks (by 1-2%) (60).

To obtain optimal therapeutic effects and maximum obturation seal, several innovations on gutta-percha have been undertaken (61). These include the following

- Surface modified.

Resin coated.

Glass Ionomer coated

Bioceramic coated

Nonthermal plasma-Argon and oxygen plasma sprayed to GP

- Medicated

Iodoform

Calcium hydroxide (CHG)

Cetylpyridinium chloride (CPC)

Tetracycline

Nanoparticles enriched gutta-percha

Gutta-percha, due to its distinctive features of chemical inertness, ease of manipulation, cost effectiveness, the availability, and the ability to do retreatment in case of failure, makes it crucial obturating material and wherefore considered as a gold standard material for obturation (61,62).

Although, gutta-percha has satisfied most of the criteria for an ideal root filling material, it may cause minimal toxicity and allergy in patients with sensitivity to latex and lack adhesiveness to root canal wall (62). Therefore, to procure a fluid tight seal between the dentine and obturating material the role of cement/sealer remains vital.

1.2.3.2 Endodontic Sealers

To provide a barrier between the core material and the root canal walls, sealants are necessary. Their ability to flow into areas that the core filling materials cannot reach can help create a satisfactory seal. Louis Grossman was the first to provide with the criteria of an ideal root canal sealer in 1982. Although many materials have changed since then but these requirements are still applicable. Following is the “Grossman’s Criteria” for ideal root canal filling material (63) ideal filling material should be-

- easy to introduce into the canal
- able to seal the canal peri apically and laterally
- resistant to shrinkage inside the canal
- impervious to moisture
- bactericidal or bacteriostatic
- radiopaque
- non staining
- non irritating to the periapical tissues
- sterile or easily sterilised

- easily removable from the canal if necessary

Occasionally sealer may extrude from the root canal peri apically, touching the soft and hard tissues leading to periapical biotoxicity. Biotoxicity/cytotoxicity is the inflammatory response in the periapical area due to leaching of sealer itself or its degradable products interfering with the healing process of an apical periodontitis (64), the sealer should also possess the critical property of biocompatibility when in direct contact with the vital tissue at the apical and lateral foramina of the root. Biocompatibility is defined as the ability of a material to aid or stimulate the reorganization of injured structures (65) and not trigger any adverse reaction, such as inflammation of periapical tissues or cytotoxicity. To date various sealers with different chemical composition have been developed keeping these properties in mind, such as: zinc-oxide eugenol, calcium hydroxide, epoxy resin, glass ionomer, silicones, and hydraulic cement-based sealers (66,67).

1.2.3.2.1 Epoxy resin-based sealers

These sealers comprise of low molecular weight epoxy resins and amines. AH 26 and AH Plus (Dentsply Sirona, Konstanz, Germany) are both examples of epoxy resin-based sealers wherein AH Plus is a modified version of AH 26, which does not release formaldehyde (cytotoxic and mutagenic) (64). AH Plus has been considered a gold standard and used as a control material in most studies on endodontic sealers. This can be attributed due to its property of better adaptability and high bonding between canal wall and the core material forming sealer tags & creating a “Monoblock”(68) beneficial properties include, adequate long-term dimensional stability, radio opacity, uniform sealing ability and excellent physicochemical properties (69,70). Fresh AH plus can exhibit antimicrobial activity due to the toxic effects of amines and epoxy

resin present in its components along with the minimum release of formaldehyde during the polymerization process (71). However, it shows some degree of cytotoxicity when extruded periapically causing inflammation of tissues around the apex and does not present any mineralization potential (72).

1.2.3.2.2 Hydraulic cement-based sealers

Hydraulic cements are a group of materials, which set by reaction with water (i.e., a hydration reaction); and further react with environmental fluids like blood, tissue fluids, dentine, and irrigating solutions (73). This hydraulic calcium silicate cements have gained popularity since their introduction, due to this hydraulic nature. They reach their maximum physical and mechanical characteristics once set and do not deteriorate when wet (74). Hydraulic calcium silicate cements have been introduced in dentistry with Portland cement which is a material used in the construction industry for over a century.

Due to its characteristics, it was proposed that it may be applied to persistently wet dental situations, such as root-end surgery and perforation repair (75,76). It was not until Torabinejad patented it as "mineral trioxide aggregate" ("MTA") (77) "tooth filling" that it was widely used in therapeutic settings. In 2005, it was determined to be a hydraulic calcium silicate (78). Since then, several related materials—mostly those utilised in endodontic procedures—have been created and used in a variety of clinical dental applications.

Despite being the primary phase for these forms of cement, tricalcium silicate phase is not usually the only phase. In addition, other phases that affect the material's hydration include tricalcium aluminate and dicalcium silicate (74). Most of the more recent materials have

additives that can change the physical and mechanical qualities as well as the chemical response.

Water can be added to the materials, or the powders can be disseminated in a non-aqueous medium, and the hydration happens afterwards when the materials interact with the fluids in the environment.

These hydraulic cements demonstrated adequate setting time, excellent flowability, good dimensional stability and slight volume expansion during setting; in addition to high pH and sealing ability with sufficient radiopacity (79,80). The property of biocompatibility and antibacterial property of tricalcium silicate sealers prevented reinfection and assisted in the process of healing. These sealers alike MTA, induced apatite deposition on material surface (73) when encountered with the environmental fluids.

The hydraulic calcium silicate cements can be classified according to their chemistry, the modifiers used and whether the material is mixed with water or not (81) as described in the Table 1.1 below.

Table 1.1: Classification of clinically available hydraulic calcium silicate cements (81)

Type	Cement	Additives	Water	Commercial examples
1	Portland Cement	No	Yes	ProRoot MTA
2	Portland Cement	Yes	Yes	MTA Angelus
3	Portland Cement	Yes	No	Bio C
4	Tricalcium/dicalcium silica	Yes	Yes	Biodentine, BioRoot RCS
5	Tricalcium/dicalcium silica	Yes	No	TotalFill

The tricalcium silicate-based sealers could be type 4 materials as shown in Table 1, which are mixed with water, like BioRoot RCS or Type 3 /5 materials which are provided in single phase syringes like TotalFill or BioRoot Flow. The heterogeneous consistency during powder liquid mixing of type 4 materials could influence the physicochemical properties of these cements (82) is then eliminated by the introduction of premixed tricalcium silicate-based sealer. These sealers are premixed with non-aqueous/water-free carrier which does not allow it to set in the syringe in which it is stored. The setting reaction of premixed cements is triggered only when it encounters the moisture present in the dentinal tubules or the environmental fluids (83).

1.2.3.2.2.1 Chemical composition and hydration reaction

Hydraulic calcium silicate-based sealers are composed of tricalcium silicate, radiopacifier and additives. When calcium silicates react with water, which can be added or be absorbed from the environment as in single syringe systems, it forms calcium silicate hydrate (which forms the cement matrix) and calcium hydroxide which leaches out and interacts with environment (84).

The hydration reactions (A and B) are described below (81) -



tricalcium silicate water calcium silicate hydrate calcium hydroxide



dicalcium silicate water calcium silicate hydrate calcium hydroxide

Hydration pattern is different for different types of hydraulic calcium silicate sealers (85–87) Type 4 water based HCSC are mixed with water and so do not rely on OH-ions derived from tissue fluids to form calcium hydroxide. The vehicles used in type 5, a premixed ready-to-use injectable sealer have an influence on rates of ionic dissociation and diffusion of calcium hydroxide and depend entirely on an aqueous environment to hydrate and set (88).

1.2.3.2.2.2 Interaction of the material with the environment

Interaction of hydraulic calcium silicate cement with the root dentine happen through chemical bonding via formation of “mineral infiltration zone” (MIZ) at the dentine sealer interface (89–91). This distinguished property of HCSC sealers is known as “biomineralization” by which minerals are deposited in the matrix of biological tissues (92). Hydration of tricalcium silicate cement releases calcium hydroxide and minerals like silicon. The calcium hydroxide released during the setting reaction causes the alkaline caustic etching of the collagenous component of interfacial dentine. This is followed by diffusion of minerals from tricalcium silicate cement in the porous structure of intertubular dentin leads to the formation of a high mineralised zone improving bonding with the dentine (68,93).

This chemical and physical interaction with the dentin helps in improving the sealing ability of the material and prevent microleakage. The retention of the material inside the dentinal tubules also may entomb the residual bacteria and help in exerting prolonged antimicrobial effect.

Interaction of HCSC sealers with the phosphates of the surrounding blood or tissue fluids releases calcium ions when the setting reaction of these cements occurs, which in turn induces apatite deposition (94–98). This interfibrillar precipitation of hydroxy apatite crystals on dentine surface,

helps in forming micromechanical tags at the dentine sealer interface improving the seal and stimulate tissue repair (73,99).

When immersed in phosphate solutions like Hanks' Balanced Salt Solution (HBSS), calcium silicate cements release high amount of calcium and hydroxyl ion due to hydration reaction (84), as compared to epoxy resin-based sealers (79,100). Earlier studies have shown the significant quantity of calcium release by the BioRoot RCS (73). Prolonged release of calcium and hydroxyl ions characterises these cements (74) but the leaching of ion decreases with time as hydration rate diminishes and cement becomes porous as mentioned in previous studies (101–103). This reduction in ion release may seem to reduce the cement performance and its biological property within initial days of application (104). Interaction with irrigating solutions also changes the chemistry of the sealers and their reaction to dentine which is discussed later in detail.

1.2.3.2.2.3 Antimicrobial property

Although mechanical debridement, irrigation and filling with a sealer reduces the bacterial load, complete elimination of microorganisms from dentinal tubules, lateral canals and apical ramification is difficult (105,106). Furthermore, bacteria have an affinity for sealers and filling materials which results in biofilm formation leading to continuation of microbial growth. Long term percolation can dissolve the sealer and result in apical leakage. Thus, antimicrobial properties of sealing materials play a key role in preventing the growth and number of residual microorganisms (18,107) *in vitro* and *ex vivo* studies have shown the antimicrobial action of tricalcium silicate sealers against root canal pathogens (106,108). TCS based sealers have the advantage of prolonged antimicrobial activity as compared to the epoxy resin-based sealers like

AH Plus, which loses antimicrobial activity after setting (105). The antibacterial effect of tricalcium silicate based cements is attributed to release of hydroxyl ions and raised pH values. Water based sealers like BioRoot RCS exerted superior long-term antimicrobial effects as compared to single syringe sealers which have an alternative vehicle to water such as Total Fill sealer (71). This may be correlated to the difference in ion leaching from the sealer as reported in the literature (74), Camilleri, 2022, wherein BioRoot RCS exhibited higher calcium ion leaching as opposed to premixed TCS sealers for the same time periods under the same conditions. Another study showed leachate analyses before and after exposure to the bacteria found BioRoot RCS fully antimicrobial as compared to AH Plus which had limited effect on the biofilm (109).

The common tests for evaluating antimicrobial effect of root canal sealers are agar diffusion test (ADT), direct contact test (DCT) and intratubular infection test/live and dead assay (110–112). The antimicrobial potential of tricalcium silicate sealers has been tested against a plethora of caries-associated bacteria, such as *Streptococci* and *Staphylococci*, as well as fungi and species associated with refractory endodontic cases, namely *E.faecalis*.

Once the appropriate sealer is selected a technique is required to place this sealer along with the core gutta-percha in the root canal space created, to achieve a void-free, homogeneous filling. Several techniques have been developed to achieve that hermetic seal.

1.2.3.3 Root Canal Obturation Techniques

Post chemo-mechanical debridement of root canal system, it needs to be filled and sealed with gutta-percha and an endodontic sealer to get a proper seal. Albeit several techniques have been developed, there have been significant changes to the root canal obturation procedures.

1.2.3.3.1 Single cone technique

The single cone obturation technique was the first developed in clinical practice. After the completion of cleaning and shaping, a single gutta-percha point of matching root canal taper was used for obturation in this technique. Most canals have an oval shape, whereas gutta-percha has a rounded cross section which left a lot of space that needed to be filled with sealer for a tight seal. This technique was simple to use but majority of sealers were soluble and resulted in high amount of shrinkage. Eventually, tissue fluids leached and washed the sealer out making space for microbial growth which ultimately led to treatment failure. Also, using a single cone technique did not fill all the irregularities of the tooth. Thus, single cone technique was associated with lower quality obturation, so the profession moved to look for techniques to fill root canals hermetically.

1.2.3.3.2 Cold Lateral Compaction

To overcome the problems caused by using large amount of sealer, importance was given to minimise sealer and fill majority of root canal with gutta-percha. Thus, many accessory gutta-percha cones were compacted along with the single master cone and using sealer just as a coating to form a bond between core and the dentin and this technique was known as cold lateral compaction technique. It is the classic obturation technique and most widely taught (113). Heat is not applied to the gutta-percha during obturation.

This technique involves selection of a master point slightly short of the working lengths, placed with sealer in the prepared root canal. Accessory gutta-percha points are then compacted laterally against the sides of the canal walls with a spreader. Remove excess gutta-percha from the canal orifice with a heated plugger, and firmly compact the remaining gutta-percha to seal the coronal access to the canal.

The advantages of using this method include the low cost and simplicity (114). Also, no specific expensive instruments are required, and placement of gutta-percha can be controlled in this technique (52). The main problem is that it is a time-consuming procedure with risk of void formation. Adaptation of gutta-percha to the root canal walls is also inadequate, especially in teeth with complex root canal morphology (60). The resulting fill would lack homogeneity and must rely on sealer to fill the voids, and thus would have a poorer prognosis (115). It could also lead to vertical root fractures due to compaction force.

1.2.3.3.3 Warm vertical condensation

Schilder first proposed the idea of heating gutta-percha to reduce the voids in the root canal in the 1960s. His rationale was to allow the homogeneous plasticised gutta-percha to adapt to the intricate architecture of the root canal system (116) and provide high density filling.

This technique involves fitting a master cone short of the prepared working length with sealer already in place. The coronal portion of the cone is removed in successive increments with a heated instrument, softening the remaining material in the canal. It is followed by vertical compaction with a plugger. The process is repeated until the apical portion has been reached. The coronal space formed is back- filled by thermoplasticised gutta-percha.

Significantly fewer voids are present in the obturation due to movement of plasticised gutta-percha into irregularities of root canal system than cold lateral condensation technique (117) The technique is time consuming and there is potential for vertical root fracture (118) and overextension of obturation materials into the peri radicular tissues.

Since the introduction of Schilder 's technique, other procedures that use heated gutta-percha cones evolved: the continuous wave obturation, injectable gutta-percha, and carrier-based techniques.

1.2.3.3.4 The Continuous Wave of Condensation

A variation of warm vertical compaction is the continuous wave compaction technique. A plugger is inserted into the canal orifice and activated to remove excess coronal material, until the plugger is moved within 3 mm of the binding point of master cone. The apical portion of the gutta-percha remains a single-cone technique as the heat transfer does not take place in the apical

2 to 5 mm of the gutta-percha. The continuous wave technique causes significantly higher rise in temperatures (119).

1.2.3.3.5 Thermoplasticised obturation / Injectable gutta-percha obturation

Injection moulded thermoplasticised gutta-percha was introduced to the dental practice by Yee *et al.* in 1977 where gutta-percha was heated outside the tooth. In this technique pellets of alpha phase gutta-percha were inserted into a delivery system gun softened at about 150- 200°C and extruded through a heated silver needle. As the apical portion is filled, the needle backs out of the canal. Pluggers are used to compact the gutta-percha. The main advantage is the higher adaptability of gutta-percha to the root canal walls. However, there is lack of control over the gutta-percha which results in increased tendency for the extrusion of softened gutta-percha from the apex (59,60).

1.2.3.3.6 Carrier-based gutta-percha obturation

Carrier-based gutta-percha technique involves a solid core (recently plastic core being used) coated with α -phase gutta-percha and a heating device (obturators) that controls the temperature. The carrier is softened in the heating device and swiftly inserted into the root canals. The excess material is removed using the high-speed bur and a plugger is used to compact the coronal gutta-percha. The carrier remains in the canal as a central core.

Advantages include the ease of placement and particularly good three-dimensional obturation as the softened gutta-percha flows well into the lateral and accessory canals (120). The plastic carrier has a groove to provide an access point which makes it easy for retreatment. However, there is extrusion of the material from the apex and additionally there is a tendency of for denuding of the obturator with the gutta-percha coming away leaving back only the plastic carrier in the canal (121,122).

1.2.3.3.7 Hydraulic Condensation/ Sealer-based obturation

All the above obturation techniques were gutta-percha based, relying on compaction of gutta-percha with limited use of sealer to produce a hermetic seal. The minimal use of sealer was due to its shrinkage and degradation over time. With minimal sealer, gutta-percha needed to be heated and compacted to establish a complete root canal filling which could be time consuming, technique sensitive and leading to apical extrusion or vertical root fracture.

With the introduction of hydraulic calcium silicate sealers, there was a shift seen in obturation techniques from traditional gutta-percha-based obturation to a more sealer-based approach as gutta-percha unlike hydraulic sealers can shrink, is not biocompatible, does not form bond with dentin, has reduced flow property and is not hydrophilic allowing flow into dentinal tubules.

Hydraulic calcium silicate sealers made clinicians to revisit traditional single cone obturation technique (123) wherein a single cone can be used with sealer, although more than one cone can be used if required and cone matching for the root canal taper is done. The sealer plays a major part of obturation and a three-dimensional filling in this technique whereas gutta-percha is required to serve as a carrier and condenser for the sealer as well as a path for retreatment. This

technique involves filling of the middle and coronal third of root canal with the sealer after which a lentiulo spiral is used to push the sealer to the working length. Pre-fitted gutta-percha is then coated with sealer, pushed inside the canal, and compacted, with any extra sealer being smeared off coronally. Gutta-percha which is used for this technique could be normal solid cones or bioceramic coated (enhance the bond strength of the sealer to the cone).

The main feature of using a hydraulic cement is its hydraulic nature, which allows it to set in moist environment of the root canal (68). Gutta-percha generates hydraulic pressure when inserted in the sealer coated root canal driving the sealer three-dimensionally throughout the canal system to achieve a seal. Slight volume expansion of sealers during setting allows use of large volumes of sealer without void formation. Properties of biocompatibility and bioactivity of these sealers changed the concept of traditional hermetic seal for obturation to biological bonding (68,73). Advantage of this technique is its simplicity, better bond strength, lesser voids, time and cost efficient, preserving more tooth structure, and avoiding the risk of vertical root fracture. It does not require a long learning curve, is less operator dependent and no thermal damage to periodontal membrane is expected.

1.2.4 Coronal restoration

Root canals obturated with best of techniques if not given a proper coronal seal can develop coronal microleakage (124). This may lead to secondary infection of the root canal and potential endodontic failure. According to one of the studies *E. faecalis* strains recovered from the three sites of saliva, pulp chamber and root canal was identical, suggesting that coronal microleakage is a cause of root canal failure (125). Hence, proper coronal restoration plays a vital role in

treatment success and forms an essential part of root canal treatment. A definitive restoration should be placed as soon as the endodontic treatment commences. The options for permanent restoration include amalgam, composites, metal/fibre posts and crowns (126) well compacted final restoration helps to prevent re-infection and long-term seal of the root canal system coronally (21).

Once the good coronal restoration is done, the root canal treatment completes. Most of the studies in the literature investigates different steps of root canal treatment separately (71,105,127,128) but they are related to each other (87,129,130) it is important to study the correlation of one step with the other and the interactions of varied materials used in each step with each other; along with their interaction with the dentine.

1.3 Interaction of irrigation solutions with dentine and sealers

Irrigating solutions are required for elimination of microbial load and dentin preparation during root canal treatment. It is suggested that these irrigating solutions may cause structural and chemical alterations of root dentin (131) such as changes in wettability, solubility, surface roughness and permeability of dentin (132). These modifications of the dentin surface might affect the mechanical and chemical adhesion of sealers to dentin as well as, it may affect the antimicrobial properties of both dentin and sealer (43). Therefore, consideration should be given to appropriate selection of irrigation protocols for dentine preparation and to match the sealer type (129,132).

NaOCl being antimicrobial, eliminates the microbial load and its proteolytic action dissolves the collagen fibres but, the continued deterioration may lead to detrimental effects on the collagen resulting in dentin degeneration which significantly decreases the elastic modulus,

microhardness, and flexure strength of human dentin (4,133). Some studies show these significant changes in the collagen structure of dentin by NaOCl do not favour the AH Plus monomer penetration into the radicular dentin (134), consequently, the bond strength of the bondable root canal sealers becomes weak (135). However, when the final rinse is with chlorhexidine, it improves the dentin wettability of AH Plus sealer (131,136).

Mechanical instrumentation creates a smear layer blocking the dentinal tubules which can be cleared by the chelating agents. EDTA the most employed calcium chelator, dissolve this inorganic debris, affects the calcium ratio of dentin, altering the proportion of organic to inorganic components in dentin. This may modify dentin properties of permeability & solubility, and cause enlargement of dentinal tubules. The exposure of underlying type I collagen fibril meshwork of the dentin matrix allows easy sealer penetration into demineralised dentinal tubules improving the sealing ability of most of the sealers (137–139) enhanced bond between the adhesive sealing agents and the collagen matrix of dentin can also minimise the chances of apical leakage (140,141).

However, this may not be true in case of hydraulic calcium silicate sealers wherein, HCSC sealers unlike the previously used sealers, interact with the dentine. This interaction with dentin is more of chemical in nature and thus these sealers do not benefit from property of calcium chelation and smear layer removal (129). Calcium released from tricalcium complex during hydration reaction may be disrupted by the chelating agents resulting in weaker cell adhesion to materials (142). Contrary to that, when utilising epoxy-resin-based sealers like AH Plus, calcium chelation of the dentin by EDTA is required for the sealer to attach to the exposed collagen with the help of resin tags and penetrate the dentinal tubules. Whereas antimicrobial activity of BioRoot was shown to be enhanced by using EDTA as irrigation solution (93).

Chelation decreases the apatite/collagen ratio due to demineralization of the hydroxyapatite components of dentin, reducing the root dentine microhardness and post treatment root fracture (36,143). NaOCl and EDTA used in sequence may also cause peritubular and intertubular dentinal erosion (144). Final rinse with acidic irrigants like citric acid, after NaOCl, may increase in porosity with deterioration of dentin and impairment of the microstructure. HEDP is a weak chelating agent and need longer time to remove the smear layer, but it has minimal effects on dentinal wall (47).

The characteristics and morphology of the dentin after being exposed to the irrigating solutions used during root canal therapy must be determined because HCSC sealers interact with it and therefore the irrigation technique is important for the choice of the sealer.

1.4 Interaction of dentin with hydraulic calcium silicate cements

The chemical and physical interaction of HCSC sealers with the dentin in regards of biomineralization and bioactivity helps in improving the sealing ability of the material, prevent microleakage and reinforce the mechanical properties of dentin (129). The interaction and retention of the material inside the dentinal tubules may entomb the residual bacteria and help in exerting prolonged antimicrobial effect of the sealer.

The interaction of HCSC sealers with dentine was influenced by the irrigation protocol but the sealers also affected the dentine chemistry further enforcing the mineral exchange at the interface along with the changes to the dentine structure/chemistry after the sealer placement (99). Hence, it becomes necessary to substantiate changes in the dentine microstructure and its properties following exposure to irrigating solutions. It also becomes important to match the best irrigation protocol to the sealer for a better bonding at dentine sealer interface.

Several studies have addressed the effect of irrigation solutions on sealers 'properties and the effect of disinfection on dentine (132,142,143,145–148). However, there is scant information regarding the effect of the irrigation and smear layer removal protocols on the chemical and antimicrobial properties of HCSC sealers and the best irrigation protocol that prepares the dentine substrate for interaction with HCSC sealers. Till date only one study has investigated the effect of 2% NaOCl and 2% NaOCl followed by 17% EDTA, on the chemical and microstructural characteristics of dentine and correlated this with the antimicrobial properties of HCSC sealers ((87). This study also found that even changing the delivery system affected the properties of sealer. But it did not study continuous chelation effect on dentin. However, one study evaluated the effect of continuous chelation on root canal dentine and push out bond strength of HCSC sealer (149) but hitherto, no studies have evaluated the effect of continuous chelation on dentine microstructure and antimicrobial effect of HCSC sealer on interaction with that dentine, as well as choose a best sealer to match irrigation protocol.

Therefore, the hypothesis of this research is that continuous chelation irrigation results in superior dentin surface characteristics while maintaining the antimicrobial properties of HCSC sealers, leading to improved adhesion and interfacial integrity with these sealers.'

1.5 Aims of the study

The goal of this research was:

- To investigate the best substrate for HCSC sealer interaction at the dentine sealer interface by examining the effects of various irrigation approaches, such as continuous chelation, on dentine microstructure.

- To explore the changes in the antimicrobial properties of HCSC sealers when interacting with dentine after irrigation with different chemical solutions.
- To choose the best irrigation protocol for endodontic treatment using HCSC sealers for obturation.
- To test the properties of BioRoot Flow, a new hydraulic calcium silicate premixed sealer formulation

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

The following commercial materials were investigated to be used as a root canal sealer:

- AH Plus Jet (AH; Dentsply DeTrey GmbH, Konstanz, Germany) (batch no. 1904000728)
- BioRoot RCS (BR; Septodont, Saint-Maur-des-Fossés, France) (batch no. B27015)
- BioRoot Flow (BRF; Septodont, Saint-Maur-des-Fossés, France) (batch no. B24447BB)
- TotalFill BC Sealer (TF; FKG Dentaire, La Chaux-de-Fonds, Switzerland) (batch no. 21001SP)

AH Plus Jet was an auto-mix syringe delivery system. BioRoot RCS powder and liquid were mixed and employed according to the manufacturer's instructions. TotalFill and BioRoot Flow were supplied in a single syringe system.

The following irrigating solutions were used:

- 5.25% NaOCl (Chloraxid; Cerkamed, Stalowa Wola, Poland).
- 17%, EDTA (Endo Solution, Cerkamed, Stalowa Wola, Poland).
- 9% 1-hydroxyethylidene-1,1-diphosphonic acid (HEDP, Dual Rinse HEDP, MedCem gmbH, Weinfelden, Switzerland).
- Distilled water

The 5.25% NaOCl and 17% EDTA used in this study were used as supplied by the manufacturer. The 9% HEDP was freshly prepared by mixing 0.9 g of Dual Rinse® HEDP powder (mean content per capsule) as recommended by the manufacturer with 10 mL of distilled water or 10 mL of 5.25% NaOCl before each experiment.

2.1.1 Condition of materials prior to testing

For the materials used for assessment of antibacterial properties of their leachates in 7- and 90-day periods, immersion of the test sealers in the extraction medium was done once set, following an incubation period, as reported in the literature in several studies. A Gillmore-needle model was used to check material setting, according to ASTM C266-18 specifications as this standard method is used to determine both initial and final setting time periods for hydraulic cements (150).

Hank's balanced salt solution (HBSS) was used as an immersing solution since its inorganic composition of ions is similar as human plasma and it has been vastly used in many biological studies (84,151). The chemical characterization and the antibacterial experiments both employed the same extraction vehicle because different media have varied leaching abilities (85).

2.2 Sample Preparation

2.2.1. Characterization of Materials

Sealer specimens were prepared in 10 mm diameter and 1 mm thick rubber moulds in triplicates. All sealers were allowed to be set at 37°C, 100% humidity in the incubator (Mettler GmbH, Schwabach, Germany). BioRoot Flow and TotalFill were covered with a moist gauze for setting. These samples were used for characterisation of materials/sealers.

2.2.2. Microbiological Analysis

Microbiological analysis comprised of testing of sterility of sealers, assessment of antimicrobial properties of those sealers alone as well as in contact with dentine at dentine sealer interface. All samples for microbiological assays were prepared in the laminar flow cabinet (Guardian MSC T1200, Monmouth Scientific, Bridgwater, UK) under aseptic conditions with autoclaved (121°C, 15 psi for 15mins) instruments (tweezer, mixing spatula) and materials (glass slab, rubber moulds). Sealer discs of dimension 10 x1 mm were prepared in rubber moulds in triplicates for four sealer groups. 1 set of sealer discs was prepared in unsterile conditions in open at the lab bench to be used as control. For BioRoot RCS, 5 droplets of the liquid were transferred inside the powder and the material was manipulated according to the manufacturer's instructions with a sterile metal spatula upon a sterile glass slab. The AH Plus Jet two pastes were also mixed on the sterile glass slab with the help of a sterile metal spatula to avoid using the tips provided that

could not be sterilised. TotalFill and BioRoot Flow are premixed pastes, and these sealers were therefore directly applied in the sterile rubber moulds with the help of a sterile metal spatula. The plastic tips provided by the manufacturers were not used as they could not be autoclaved. These specimens were placed in closed Petri dishes wrapped with cling film inside the incubator (Thermo Scientific, Langenselbold, Germany) at 37°C and 100% relative humidity to set. These samples were used for testing the sterilisation requirement of sealers and for all microbiological assays done in this study.

2.3 Characterisation of Set Materials

The sealers were characterised using scanning electron microscopy (SEM), energy-dispersive spectroscopy (EDS), Fourier transform infrared spectroscopy (FT-IR) and X-ray diffraction (XRD) analysis.

2.3.1 Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray Spectroscopy (EDS)

After setting, materials were either desiccated in the vacuum desiccator overnight for a 0-day characterisation or were immersed in 5 ml of sterile Hank's balanced salt solution (HBSS, Sigma Aldrich, H6648) in sterile 7 ml polystyrene bijoux tubes (no label, sterile) in an incubator at 37°C and 100% humidity for either 7 days or 90 days. After aging, the material cylinders were retrieved from the HBSS solutions and dried in a desiccator overnight. After desiccation, the materials were embedded in a cold cure resin overnight (Epoxy-fix, Struers, Ballerup, Denmark). The resin

blocks with the impregnated specimens were then ground and subsequently polished with an automatic polishing machine (Tegramin 20, Struers, Ballerup, Denmark). The grinding was accomplished with the use of 250, 500, and 1200 grit diamond discs (MD Piano, Struers, Ballerup, Denmark) under water coolant, while polishing was carried out with 3 progressively finer cloths (MD-Largo, MD-Dac, and MD-Nap respectively, Struers, Ballerup, Denmark) and diamond impregnated lubricants (DiaProDac 3 μm and OP-S, 0.04 μm , Struers, Ballerup, Denmark). Discs and cloths were set to rotate at approximately 300 revolutions per minute (rpm). The grinding and polishing period was approximately 2 and 4 min respectively for each disc/cloth. Consequently, specimens were cleaned from debris under water and were subsequently mounted on aluminium stubs, held in place with carbon tape, and sputter coated with gold (K550X Sputter Coater, Quorum Technologies Ltd., Kent, UK). Finally, the sealers were viewed under the SEM (EVO MA10, Carl Zeiss Ltd., Cambridge, UK) in backscatter mode to obtain elemental contrast. Images at different magnifications of 500x, 1,000x, and 2,000x were obtained. Energy dispersive spectroscopy of selected areas was performed to assess the elemental distribution.

2.3.2 X-ray diffraction (XRD) analysis

For the XRD analyses, the aged specimens were retrieved from the solution and dried in a vacuum desiccator overnight at 37°C. For all ages, the sealers discs were then ground to a fine powder using an agate mortar and pestle. XRD was performed with a diffractometer (Bruker D8 Advance, Bruker Corp., Billerica, MA, USA) with the CuK α radiation at 30 mA and 40 kV was set to rotate between 10° and 60° with a 0.02° 2 θ , and step time of 0.6 s. Phase identification was undertaken

using a search-match software using the COD database (Crystallography Open Database <http://www.crystallography.net>).

2.3.3 Fourier transform infrared (FT-IR) spectroscopy

To obtain the FT-IR spectra, aged specimens were retrieved from 5 ml Hank's Balanced salt solution (HBSS) and dried in a desiccator overnight. For all the ages of 0 days (immediately after setting and drying), 7 days, and 90 days, the sealers discs were ground using an agate mortar and pestle to a fine powder. Five mg of each crushed sample of the sealers were mixed with 500 mg potassium bromide in an agate mortar and pestle, pressed into a pellet (13 mm in diameter) with the help of a pellet die (Specac, Orpington, UK) and a manual laboratory compactor (Clarke CSA10bb 10 Tonne Hydraulic Bench Press, UK). This pellet was then analysed in the infrared spectrometer (Thermo Nicolet Nexus 4700 FT-IR Spectrometer) using transmitted infrared spectroscopy. All spectra were collected within the spectral range of 4,000–400 cm^{-1} . Background spectra of KBR were also collected. Each spectrum was scanned several times to improve the signal-to-noise ratio.

2.3.4 Leachate analysis

Analysis of elemental leaching was performed using inductively coupled plasma optical emission spectrometry (ICP-OES; Optima 8000 ICP-OES, Perkin Elmer, Waltham, MA, USA). Materials were prepared as described above, weighed (0.0001 g precision), and immersed in vials containing 5 ml

HBSS at 37°C and 100% humidity. After 7 days and 90 days pellets were retrieved from the vials and leachates were then sampled and diluted in 2% nitric acid. This process was performed to ensure that elements in the leachates remained stable during the assay period. Consequently, solutions were analysed for the presence of calcium, silicon, zirconium, and tungsten using HBSS acidified to 2% HNO₃ as the blank. A standard curve was provided by these standards, the equation of which was used to determine the concentrations of the target ions of the test leachates. Results were expressed with respect to the materials' weight and the volume of the sample according to the following equation:

Amount of element released per weight of disc (mg/g) = {(Amount of leachate per litre (mg/L) - blank (mg/L)} × volume of solution (L) / weight of disc (g).

2.4 Sterilisation Requirement of Sealer Discs

Once set, each disc was sterilised using one of five sterilisation methods and one set of discs was used as control with no sterilisation. The sterilisation methods are illustrated in Table 2.1.

Table 2.1: Table showing different sterilisation methods used for sterilisation of sealer discs.

1. Discs prepared in microbiology hood	
Group A	70 % ethanol 10 mins
Group B	UV Light 1 hour (each side ½ hour)
Group C	70 % ethanol 10 mins + UV light 1 hour
Group D	Autoclave
Group E	Control (No sterilisation)
2. Discs prepared outside microbiology hood (in open lab)	

2.4.1 Characterization of set materials before and after sterilization

Materials (n = 3 per group) were prepared and after setting, the four materials were sterilized using ethanol, UV light, a combination of ethanol and UV light, and autoclaving as described in Table 2.1 of section 2.4, Sterilisation requirement of sealer discs. Characterization was then undertaken by scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS). Fourier transform infrared spectroscopic analysis was also performed. Unsterilized samples were used as a control.

2.4.1.1 Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray Spectroscopy (EDS)

One disc from each of the five groups was mounted on aluminium stubs, held in place with carbon tape, and sputter coated with gold (K550X Sputter Coater, Quorum Technologies Ltd., Kent, UK). The specimen was then viewed under the scanning electron microscope (EVO MA 10, Carl Zeiss Ltd., Cambridge, UK). The sealer disc was assessed in secondary electron mode to obtain elemental contrast at different magnifications. Energy dispersive spectroscopy of selected areas was performed to assess the elemental distribution.

2.4.1.2 Fourier transform infrared spectroscopy

The discs from all 5 groups were crushed into powder and the same technique was followed as in section 2.3.3 to form KBr pellets and the spectra were collected using transmitted infrared spectroscopy within the spectral range of 4,000–400 cm^{-1} .

2.4.2 Macroscopic imaging of sealer discs

After sterilisation, photographs of the discs were taken using a Huawei phone camera to assess any visible changes.

2.4.3 Sterility Test

Sealer discs were prepared in sterile conditions and further sterilized using the four sterilization methods outlined in Table 2.1 and the unsterilized control was compared to sealer discs prepared in an open lab. Each disc was placed in 5 ml of Brain Heart Infusion Broth (BHI) overnight in a universal tube in a shaking incubator (N-Biotek, INC, 110 rpm) at 37°C. After 24 hours, 100 µL of this BHI suspension was spread with a spreader on an agar plate. The streaked agar plates were placed in an incubator (Thermos Scientific, Langenselbold, Germany) overnight at 37°C. The agar plates were checked for bacterial growth after 24 hours.

2.5 Assessment of antimicrobial properties of endodontic sealers

2.5.1 Sample Preparation

All samples were prepared as presented in section 2.2.2 for microbiological analysis. They were aged in 5 ml of sterile HBSS in sterile 7 ml polystyrene bijoux containers in an incubator at 37°C for 7 days and 90 days. After aging, the samples were rinsed with sterile water, dried on sterile filter paper, and subjected to antimicrobial testing.

2.5.2 Microbiological assays

Microbiological assays used to assess the antimicrobial effect of the endodontic sealers in this experiment were direct contact test and minimum inhibitory concentration assay.

2.5.2.1 Attachment Assay/Direct Contact Test for Sealers

2.5.2.1.1 Bacteria and media

The antimicrobial activity tests were conducted under aseptic conditions in a laminar flow chamber. *E. faecalis*, American type Cell Culture Collection number (ATCC) 29212, was grown on Brain Heart Infusion Agar (BHI agar) plates overnight in an incubator at 37°C, (Thermo Scientific, Langenselbold, Germany) from frozen stock cultures stored at -80°C; subsequently, three to four colonies from the agar plate were inoculated in 5ml Brain Heart Infusion broth (BHI) and incubated in a shaking incubator (N-Biotek, INC, 110 rpm) at 37°C. The overnight bacterial culture was suspended again in BHI and adjusted spectrophotometrically (Jenway, 7315, Staffordshire, UK) to an optical density at 600 nanometres (OD₆₀₀) to 1.6, corresponding to approximately 2×10^6 Colony Forming Units (CFUs)/ml. The antimicrobial activity of each sealer was evaluated against planktonic bacteria.

2.5.2.1.2 Methodology of Direct Contact test

The attachment assay performed to assess the antimicrobial effect of the endodontic sealers used in this experiment has been described in previous papers in detail (152). Pictorial representation of the Attachment assay for sealers could be seen in Figure 2.1. Inside a 9 cm diameter sterile petri dish a round sterile filter paper was placed which was saturated with 900 μ L of Phosphate buffered saline (PBS) to increase humidity and a sterile glass slide was set on top of the filter paper. The sterile sealer disc was placed on top of a microscopic slide which was then inoculated with 20 μ L of the diluted *E. faecalis* culture. A 22 mm diameter sterile glass coverslip (Chance Propper Ltd., Warley, England) was gently placed on the droplet to spread out the solution evenly and for better control of the contact between the disk surface and solution. The petri dish was then closed and sealed with cling film to decrease evaporation during the long incubation times. All the petri dishes were incubated at 37 °C in the dark without shaking. Bacterial suspension placed on the sterile glass coverslip, covered with another sterile glass cover slip on top, with no sealer disc, was used as a control. Testing was carried out for 0, 7, and 90 days.

To assess the survival of bacteria, the complete disc with the cover slip assembly was immersed in 10ml of BHI in a falcon tube and vortexed for 30 secs to allow for dispersion of the viable and non-viable bacteria from the surface of the disc. To enumerate the microbial counts, a 7-fold serial dilution in BHI (Miles and Misra) was performed and 20- μ L aliquots were plated in triplicate onto BHI agar plates and incubated overnight at 37°C. After the incubation period, the number of colonies was counted, and the Colony Forming Units/ml (CFU/mL) were calculated. The number of colonies formed on the agar represents the number of viable bacteria that survived the contact with the sealer surface enabling us to know the anti-bacterial efficacy. All experiments were performed in triplicate.

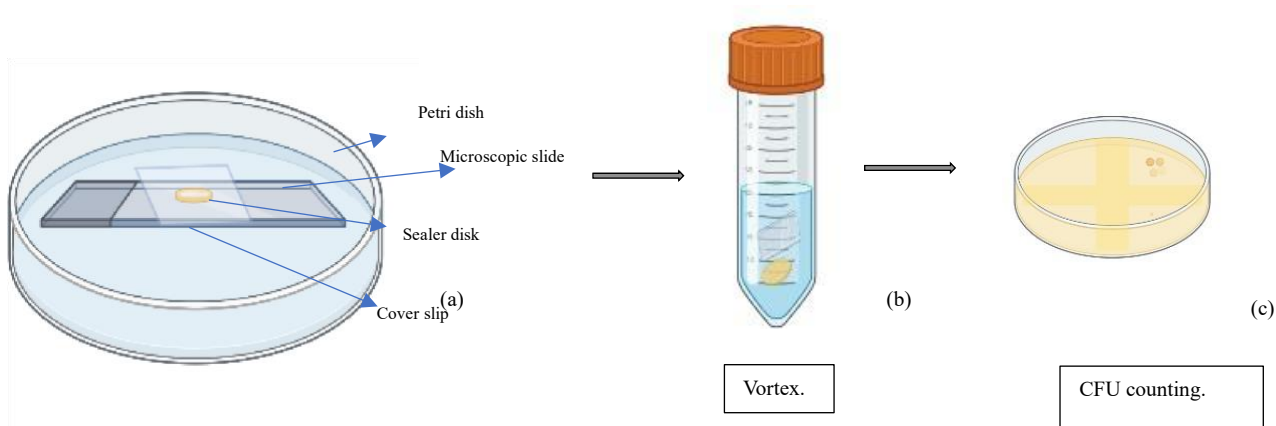


Figure 2.1: Schematic representation of the assembly for Direct Contact Test of different sealer groups : (a) A sterile petri dish was placed on top of the slide which was then inoculated with 20 μ l of the diluted *E. faecalis* culture and a coverslip was gently placed on it. (b) After overnight incubation, complete disc with the cover slip assembly was immersed in 10ml of BHI in a falcon tube and vortexed for 30 secs. (c) Miles and Misra was performed and Colony Forming Units/ml were calculated.

2.5.2.2 Minimum inhibitory concentration (MIC) assay

For MIC (67–75), discs measuring 10 mm in diameter and 1 mm high were prepared as mentioned above and immersed in sterile HBSS for 7 days and 90 days. After aging, the discs were discarded and the potential of leachates to inhibit bacterial growth was tested using the minimum inhibitory concentration (MIC) assay using *E. faecalis*. All procedures were carried out in a laminar flow hood.

E. faecalis was cultured in BHI as mentioned in ‘Bacteria and Media’ section 2.5.2.1 of Microbiological assays. The OD of the bacterial cultures was standardised to 0.1 at 600 nm using a spectrophotometer (Jenway 7315, Staffordshire, UK) and then further diluted in fresh medium to reach a final OD of 0.001 at 600 nm.

Experiments were performed in 96-well plates. One hundred microliters of the final bacterial inoculum were added to each well and an equal amount of the test leachate was then transferred.

The test leachates were analysed either undiluted or following 4 two-fold serial dilutions in sterile water (1/2, 1/4, 1/8, 1/16). Sterile water served as a negative control in the experiments; independent negative controls were carried out for each group, containing sterile broth and the respective test leachate. Culture plates were incubated overnight for 20 - 24 h at 37°C; subsequently, the OD of each well was determined using a microplate reader (ELx800, Bio Tek Instruments, Swindon, UK) at 600 nm. Experiments were performed in triplicate and three independent wells for each group. Results were normalised by subtracting the values of the negative control (leachate + BHI) from each group, to eliminate false positive results from increased OD values obtained due to potential sedimentation effects from the leachates. The MIC is equivalent to the lowest concentration of the eluent where no growth was seen.

2.6 Characterisation of dentine

2.6.1 Sample preparation and Instrumentation

Extracted human premolars with single straight or curved canals less than 10 degrees with fully formed roots were obtained from the Birmingham Dental School Tissue Bank, School of Dentistry, University of Birmingham, Birmingham, UK (ethical approval number: BCHC Dent 005. 2024). All methods were carried out following relevant guidelines and regulations. The split-tooth model was used in this study for the characterisation of dentin which was modified from that of Schmidt et al (153). Each tooth was stuck to an acrylic block with the help of a green stick and the crowns of the teeth were sectioned at the cemento-enamel junction to produce specimens with a standard root length of 14 mm using hard tissue microtome (Isomet, Buhler, Lake Buff, USA) as

shown in Figure 2.2. The roots attached to the acrylic block were then split in the buccolingual direction to obtain 2 root halves. The cleaved roots were reassembled using a light-curing resin barrier (Epoxy fix; Struers, Ballerup, Denmark), covering the root apex with sticky wax (Tenatex red; Kemdent, Swindon, U.K) and placed in an alginate (Zetalgin; Zhermack, Badia Polesine RO, Italy) casing to simulate a closed apical system *in vivo*, increasing stability and preventing leakage of the solutions.

Root canals were prepared with Wave One Gold rotary instruments (Dentsply Sirona, North Carolina, USA) to an apical size of 35 and 0.06 taper. Irrigation using groups A, B, C, D, E, and F was performed with a disposable 3 mL plastic syringe coupled to a 27-G monoject endodontic irrigation needle (Covidien LLC, Mansfield, MA, USA) with a 5 mL monoject syringe (Covidien LLC, Mansfield, MA, USA) placed passively to about 2 mm from the apical foramen without binding. Apical patency was maintained with the #10 K-file.

Afterward, the canals were dried with paper points and separated into their two halves along the cleavage created earlier. Characterisation of dentin was done by SEM, EDS, and FT-IR after the application of different irrigation protocols.

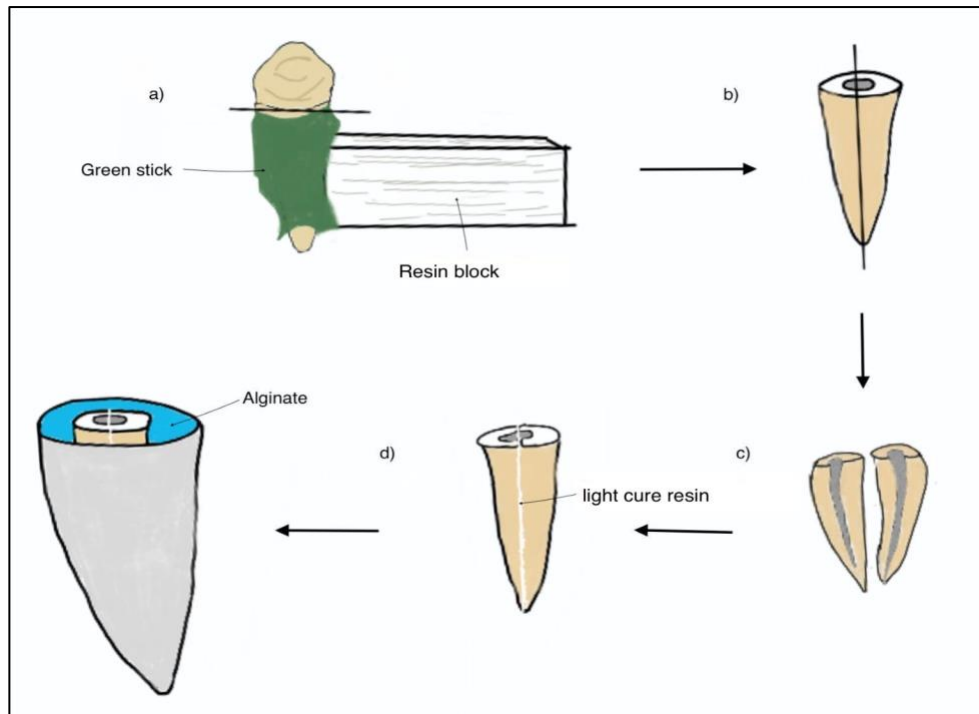


Figure 2.2: Schematic representation of specimen preparation for dentine characterisation. (a) the tooth sample was stuck to an acrylic block with the help of green stick and were decoronated to produce specimens with a root length of 14 mm using hard tissue microtome. (b & c) The roots were then split longitudinally to obtain 2 root halves. (d) The cleaved roots were reassembled and joined into one unit using a light curing resin. (e) This tooth assembly was then placed in an alginate casing to simulate a closed apical system.

2.6.2 Irrigation protocols

Irrigation protocols utilised for characterisation of dentine are described in Table 2.2.

Table 2 2: Irrigants were tested in different sequences. NaOCl (sodium hypochlorite), EDTA (ethylenediaminetetraacetic acid), HEDP (1-hydroxyethylidene-1,1-diphosphonic acid) (mins= minutes)

Groups	Main Irrigants (During instrumentation)	Time	Final Irrigants (After instrumentation)	Time
Group A	Distilled water (control)	5 mins	Distilled water (control)	5 mins
Group B	NaOCl 5.25%	5 mins	NaOCl 5.25%	5 mins
Group C	EDTA 17%	5 mins	EDTA 17%	5 mins
Group D	NaOCl 5.25%	5 mins	EDTA 17%	5 mins
Group E	HEDP 9% + NaOCl 5.25%	5 mins	HEDP 9% + NaOCl 5.25%	5 mins
Group F	HEDP 9% + Distilled water	5 mins	HEDP 9% + Distilled water	5 mins

The solutions were tested in sequences constituting an irrigation protocol that included an antimicrobial (NaOCl) and a calcium chelator (EDTA/HEDP). Distilled water was used as a control. The total volume of solutions and total time was kept constant. For the irrigation sequence the first solution was irrigated for 5ml/5min during instrumentation and the second solution for 5ml/5min after instrumentation was used. For individual solutions, the total volume/time used was 10 ml /10min during and after instrumentation.

2.6.3. Scanning electron microscopy (SEM) and Energy Dispersive Spectroscopy (EDS) analyses

The root halves were dried in a vacuum desiccator for 24 hours after which they were retrieved, attached to aluminium stubs, canal side facing upwards, and sputter coated with a conductive layer of gold using a K550X Gold Target (Emitech Limited, Ashford, UK). The dentine ultrastructure of one-half of each tooth and the irrigation protocol from apical, middle, and coronal points were assessed by scanning electron microscopy (EVO MA10, Carl Zeiss Ltd., Cambridge, UK). In terms of elemental content and elemental distribution under 1K magnification the following parameters were used, EHT = 20 kV, I probe = 1000 PA, and WD = 8.5 mm for a 35° take-off (elevation angle). Data on oxygen (O), carbon (C), calcium (Ca), phosphorus (P), chlorine (Cl), and sodium (Na) were collected from the EDS analysis.

2.6.4. Fourier transform infrared spectroscopy (FT-IR)

FT-IR was used to determine compositional changes in human dentine after each irrigation protocol. The mean of two separate acquisitions of spectra data was obtained for each sample using a Nicolet 6700 FT-IR machine (Thermo Scientific Instruments Corp., Madison, WI, USA) and Omnic 8 software suite (Thermo Scientific Instruments Corp.) within the mid-IR spectrum (range: 2000–700 cm^{-1}) at a resolution of 0.482 cm^{-1} and 32 scans.

2.7 Preliminary investigation of the dentine sealer interface using fluorescent dyes

2.7.1 Sample preparation

Root segments of 4 mm length were obtained by sectioning the decoronated tooth segment horizontally, 1 mm below the cemento-enamel junction, using a microtome (Accuton 50, Struers, Ballerup, Denmark) as explained in Figure 2.3. These root blocks were further sectioned longitudinally to get two halves. The outer cementum of each half was then removed and polished on all sides with 220–800-grit silicon carbide paper to obtain a flat 4x4x2 mm specimen. These samples were then washed with distilled water. A mould was created by silicon putty (Affinis Putty Soft, Coltene) by embedding a tooth sample (which was removed later) as shown in Figure 2.4. Irrigation was performed with 5.25% hypochlorite for 5 minutes followed by 17% EDTA for 5 minutes on each sample and was air dried. BioRoot RCS sealer was mixed according to manufacture instructions and Orange G dye (16230, Sigma-Aldrich, MO, USA) or Rhodamine B dye (Sigma-Aldrich, MO, USA), used in 0.1% concentration was added to the sealer. Sealer on its own with no dye was used as a control. The groups used are shown in Table 2.3. The sealer was then placed on top of the dentine sample with the help of a thin plastic instrument with slight pressure. The assembly was incubated overnight. After 24 hours, the dentine sealer assembly was embedded in cold cure resin (Epofix, Struers, Ballerup, Denmark). Once the resin was set, the block was sectioned longitudinally into two halves with a microtome (Accuton 50, Struers, Ballerup, Denmark).

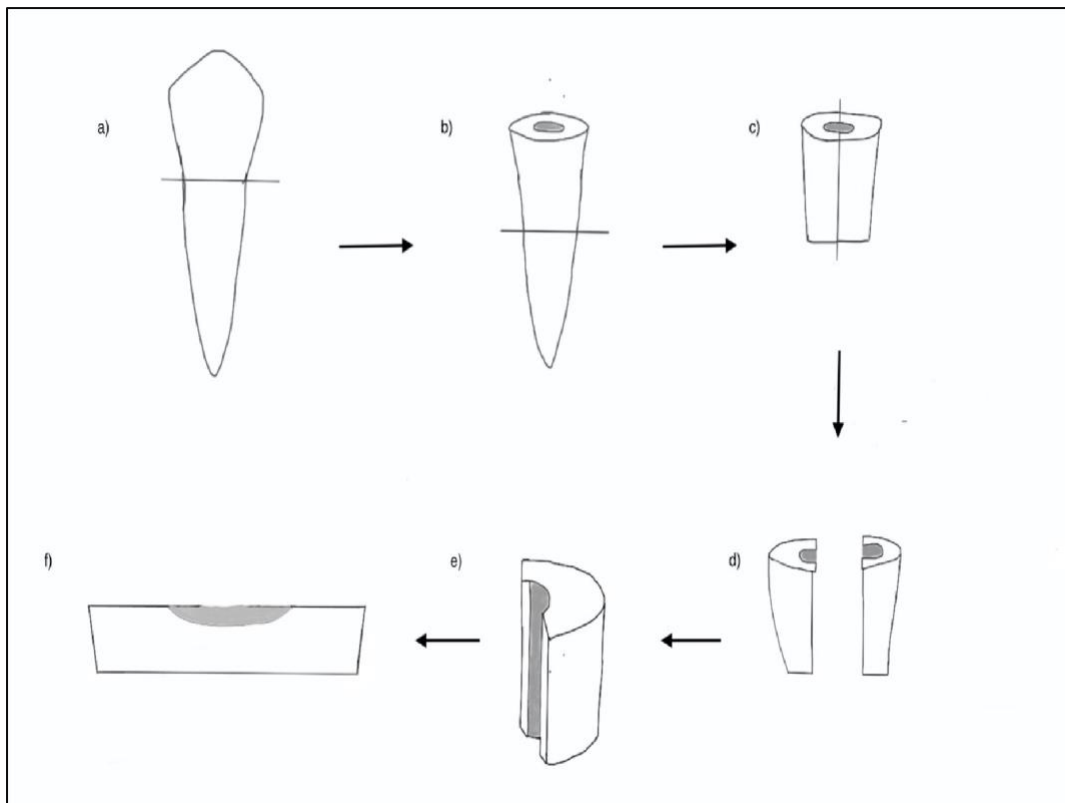


Figure 2.3: Schematic representation of teeth preparation. a) All teeth were decoronated (b) and their roots were horizontally sectioned to form root blocks with a standardized length of 4 mm, using a microtome (c & d). The root blocks were then sectioned longitudinally to get two halves (e & f) one of the halves was polished from all sides to get a flat dentine specimen of 4x2x2 mm.

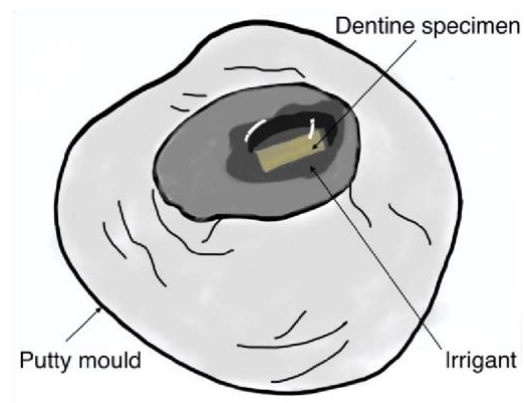


Figure 2.4: A silicon putty mould was created; dentine specimen was then placed in it and irrigation was performed.

Table 2.3: The table depicts three groups of dyes mixed with BioRoot RCS sealer mixed with Rhodamine B, Orange G, and no dye as control. Irrigation of samples was done by 5.25% NaOCl for 5 minutes followed by 17% EDTA (ethylenediaminetetraacetic acid)

Irrigation	BioRoot RCS sealer
5.25% NaOCl followed by 17% EDTA (5 mins)	No dye (control)
	Rhodamine B
	Orange G dye

2.7.2. Confocal laser scanning microscopy (CLSM)

To allow analysis under the CLSM (208–210), after tooth preparation, the samples were secured (to prevent floating of the sample) with the help of dual-cure resin (LuxaCore Z-Dual, DMG, Hamburg, Germany) in a 35 mm cell culture dish, covered with water and examined with an inverted Leica TCS-SP8 confocal system equipped with a DM6 upright microscope and a 40x/0.80 HCX water immersion lens (Leica Microsystems GmbH, Mannheim, Baden-Württemberg, Germany) with an excitation/emission wavelength of 494/521 nm.

2.7.3. Scanning electron microscopy (SEM) and Energy dispersive spectroscopy (EDS)

The specimens to be assessed under the SEM followed the protocol as in section 2.3.1. Images were obtained at 1,000 x magnification in backscatter mode for SEM and four different Energy dispersive spectroscopy (EDS) point scans for Silicon (Si), Calcium (Ca), and Zirconium (Zr) were taken on material, interface, and dentine as described in Figure 2.5. The ratios of Ca/Si and Ca/Zr were obtained.

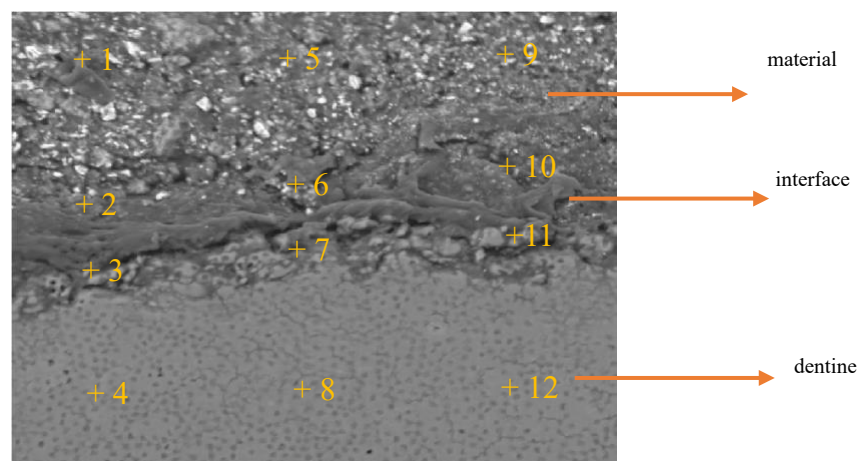


Figure 2.5: Schematic representation of four EDS point scans for dentine sealer interface using fluorescent dyes (3 points in material, 3 points in dentine, 3 points in material towards dentine sealer interface, and 3 points in dentine towards dentine sealer interface).

2.7.4. Fourier transform infrared spectroscopy (FT-IR)

To obtain the FT-IR spectra, 7 days aged specimens were retrieved from 5 ml HBSS, polished to a flat surface using 400-600 μm grit waterproof silicon carbide grinding paper (Struers), and then

air dried. This sample was then analysed using an FT-IR microscope (Nicolet™ iN10 Infrared Microscope, Thermo Scientific) in reflection mode. 7 equidistant points were selected in a vertical direction, three in material, three in dentine, and one on the interface as shown in Figure 2.6. All spectra were obtained within the spectral range of 2,000-700 cm^{-1} . Background spectra of the gold disc were also collected.

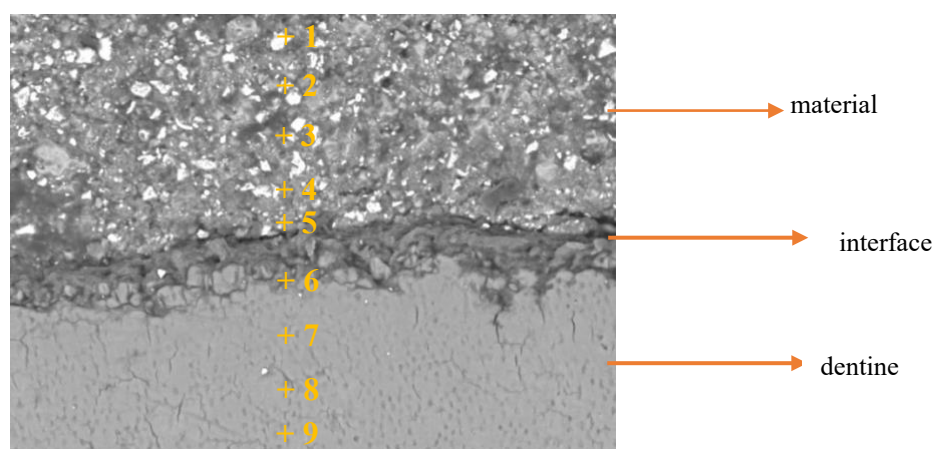


Figure 2.6: Schematic representation of points selected for FT-IR analysis for dentine sealer interface : 1 to 4 points in material, 5 at interface, 6 to 9 in dentine.

2.8 Investigation of the dentine sealer interface using an alternative method

2.8.1 Sample preparation

Samples were prepared as described in section 2.7.1. Irrigation on all the sealers was performed with three different irrigant groups mentioned in Table 2.4 on separate samples. Distilled water was used as a control. Once the resin was set, samples were incubated in 5ml Hank's Balanced salt solution (HBSS) in a bijoux tube to age for 7 days). After aging the dentine sealer interface of the samples was analysed using scanning electron microscopy (SEM), energy-dispersive spectroscopy (EDS) and Fourier transform infrared spectroscopy (FT-IR). For the microbiological investigation of the dentine-sealer interface, an attachment assay/direct contact test was performed.

Table 2.4: Three irrigation groups; Group A: Distilled Water, Group B: Etidronic acid mixed with hypochlorite and Group C: Hypochlorite followed by EDTA; for investigating dentine sealer interface with four sealers AH Plus, BioRoot RCS, BioRoot Flow and TotalFill (NaOCl: sodium hypochlorite, EDTA: Ethylenediaminetetraacetic acid, HEDP: 1-hydroxyethylidene-1,1-diphosphonic acid)

Group A	Distilled water (10 mins)	AH Plus
		BioRoot RCS
		BioRoot Flow
		TotalFill
Group B	HEDP+5.25% NaOCl (10 mins)	AH Plus
		BioRoot RCS
		BioRoot Flow
		TotalFill
Group C	5.25 % NaOCL (5mins) - 17% EDTA (5 mins)	AH Plus
		BioRoot RCS
		BioRoot Flow
		TotalFill

2.8.2. Scanning electron microscopy (SEM) and Energy dispersive spectroscopy (EDS)

The specimens were assessed under the SEM following the protocol as in section 2.3.1. Images were obtained at 2,000 x magnification in backscatter mode and four different Energy dispersive spectroscopy (EDS) point scans followed the approach as in section 2.7.3 and as depicted in figure 2.7 below.

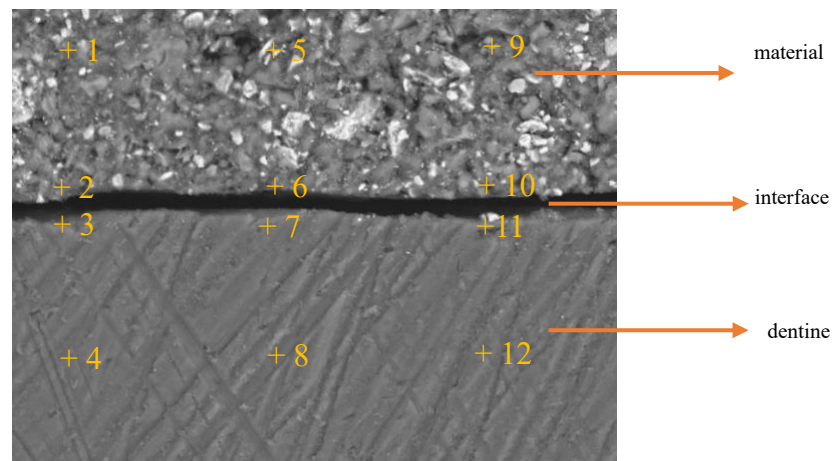


Figure 2.7: Schematic representation of EDS point scan for dentine sealer interface (3 points in material, 3 points in dentine, 3 points in material towards dentine sealer interface, and 3 points in dentine towards dentine sealer interface).

2.8.3. Fourier transform infrared spectroscopy (FT-IR)

The specimens to be assessed by Fourier transform infrared spectroscopy followed the protocol as in section 2.7.4.

2.8.4 Intratubular infection test - pilot study

For the intratubular infection test a previously described methodology was used (112,154) with slight modifications.

2.8.4.1 Sample preparation

Sample preparation for intratubular infection test is described in Figure 2.8. Cylindrical root segments of 4mm length were obtained by sectioning the root horizontally 1mm below the cemento-enamel junction using an Accuton 50 machine (Struers, Ballerup, Denmark). Each root canal was enlarged to the size of a Gates-Glidden bur #4. The root segments were then sectioned longitudinally into halves using a microtome.

The outer cementum of each half (from three sides) was then removed and polished with 220–800-grit silicon carbide paper on three sides maintaining the curve of the root canal on one side, to obtain 4x4x2mm specimens. The smear layer, formed during the preparation of the above dentine specimens, was removed by treating the surface with 17% EDTA for 5min. Then, samples were washed with distilled water for 5min and sterilized by autoclaving for 20min at 121°C. After sterilization, the dentine blocks were kept in a sterile saline solution until used.

Each prepared dentine specimen was placed in the upper chamber of a centrifugal filter tube (VWR, part of Avantor, Radnor, Pennsylvania, U.S.A) with the canal side up, and gaps with the inner wall of the tube were sealed with flowable composite resin (Admira Flow, Voco, Cuxhaven, Germany) and light-cured for the 20s. Sterile tweezers and a probe were used for the specimen's manipulation.

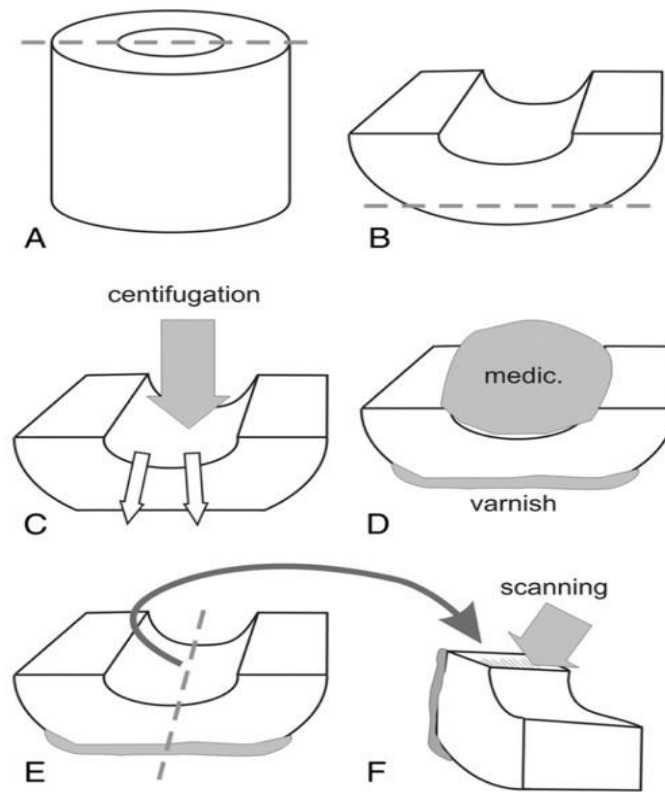


Figure 2.8: Schematic diagram of sample preparation for intratubular infection test. 3 The cylindrical root segments were sectioned longitudinally into halves. Each of these dentine specimen was placed in centrifugal filter with the canal side up for dentinal tubule infection. After centrifugation, the dentine specimens were removed, outer surfaces of the specimens were closed with nail varnish; irrigation followed by different sealer groups (medic) were applied on the canal side. After incubation, samples were vertically cut into two halves, stained with live and dead stain, and viewed under confocal microscope.

2.8.4.2 Dentine infection with *Enterococcus faecalis*

E. faecalis was cultured in BHI as mentioned in ‘Bacteria and Media’ section 2.5.2.1 of Microbiological assays. A previously described protocol (112) used for dentinal tubule infection. 500mL of an *E. faecalis* inoculum was added to the upper chamber of each filter tube with the

dentine specimen inside. The tubes were then submitted to a sequence of eight centrifugation cycles at 1400, 2000, 3600, and 5600 g (Eppendorf 5417R centrifuge, Eppendorf, Hamburg, Germany), at 25 °C, in two cycles of 5 min for each speed. Between the centrifugations, the old suspension was discarded and replaced by fresh 500ml suspension. The same procedure was repeated after two days and five days. In between centrifugations, the samples were placed in an incubator at 37°C and 100% relative humidity in fresh sterile plain broth. The incubation lasted for 5 days in total. On the sixth day, the samples were removed from the microtubes and prepared for treatment with various irrigating solutions.

2.8.4.3 Irrigation

After centrifugation, the dentine specimens were removed from each tube, followed by rinsing in sterile water for 1 min and air drying. The outer surfaces (cemental sides) of the specimens were closed with clear nail varnish.

To check the efficacy of the test, three groups were formed as mentioned in Table 2.5. The first group with no bacterial infection was utilised as control to check dentine autofluorescence , second group was a sample with only bacteria and the third group was bacterial infected sample treated with ethanol. These samples were irrigated with three types of irrigants which was followed by the placement of the most widely used tricalcium silicate sealer, BioRoot RCS on the canal side.

Table 2.5: Three groups for intratubular infection test using different irrigation protocols followed by BioRoot RCS as sealer (NaOCl: sodium hypochlorite; HEDP: etidronic acid; EDTA: Ethylenediaminetetraacetic acid).

Group		Irrigation protocols	Sealer
Group A	No bacteria	Distilled water (10 mins)	BioRoot RCS
	With bacteria		
	With bacteria followed by ethanol		
Group B	No bacteria	5.25 % NaOCl+ 9% HEDP (10mins)	BioRoot RCS
	With bacteria		
	With bacteria followed by ethanol		
Group C	No bacteria	5.25 % NaOCl (5mins) - 17% EDTA (5 mins)	BioRoot RCS
	With bacteria		
	With bacteria followed by ethanol		

The root canal lumen of each sample was irrigated with 5ml of the irrigating solutions for 5 min. The specimens were then dried by placing them on sterile filter papers and each freshly prepared sealer was placed on the surface of the root canal wall using a cavity liner applicator to achieve an approximate thickness of 0.5 mm measured by digital pachymeter [tip of sterile probe] (155). The samples were incubated for 7 days at 37°C at 100% humidity for aging.

2.8.4.4 Confocal laser scanning microscopy analysis

For dentine disinfection analysis, the Syto-9/Propidium iodide (PI) technique (Live/Dead, BacLight, Invitrogen, Eugene, OR, USA) 480/500 nm for SYTO 9 and 490/635 nm for propidium iodide was used as reported earlier (156). The ratio of SYTO 9 /propidium iodide was adjusted to 2.5µl:9 µl as suggested previously (157).

After aging, a scalpel was used to remove the excess material from dentine walls. The specimens were rinsed in sterile water for 1 min to eliminate sealer residues and vertically cut into two halves through the root canal using an Accuton-50 machine. Thereafter, they were washed with sterile water and subsequently stained with 200 µL of the dye, in a light-free environment, for 10 min. All excess stain was removed, samples were rinsed with sterile water and excess water was discarded. This sample was then placed in the fridge until testing under a confocal microscope. In the refrigerator, it was retained in a 96 well plate wrapped with aluminium foil (to avoid light contamination) in a drop of Phosphate buffered saline (PBS) to prevent it from drying.

For confocal imaging, the samples were stuck with dual cure resin in a 20 mm dish, covered with water, and examined with an inverted LeicaTCS-SP8 confocal system equipped with a DM6 upright microscope and a 40x/0.80 HCX water immersion lens (Leica Microsystems GmbH, Mannheim, Baden-Württemberg, Germany). To visualize the true signal from the bacteria

without any interference from autofluorescence coming from the substrate, a sterile tooth sample was stained with the live and dead stain. It showed some fluorescence, so this was considered a baseline, and its gain were deducted from the actual sample set. A low threshold was set for the control sample to create a black background. Another control comprising a sealer filled root canal sample without bacterial contamination was used.

Four confocal 'stacks' of random areas were obtained for each sample at 1 μ m step size. For quantification, ImageJ software (<https://imagej.net/Fiji>) was used to calculate the percentage of green (live cells) and red (dead cells) found.

2.8.5 Attachment Assay/Direct Contact Test for Dentine Sealer Interface

All teeth were decoronated and their roots were further segmented vertically into two halves with the use of a microtome. Before segmenting, the exterior surface of the tooth was wiped with 70% ethanol in the microbiology laminar flow hood and checked for any contamination of the exterior surface of the root sample by rolling on BHI agar plates and incubating at 37C overnight. The next day the agar plate was checked for any contamination. Once satisfied with the sterility the root was segmented and the two segments were repositioned and held tightly by flowable light cure composite (Admira Flow, Flowable light curing ORMOCER based restorative material, Voco, Cuxhaven, Germany) on the sides and wrapped tightly with parafilm (Parafilm M, Curwood, USA), leaving the apex. The roots were instrumented with sterile wave one gold files up to size F3 and irrigants (Irrigation protocol was used as described in Table 2.4) from a fresh bottle were applied from the top of the root blocks with the help of a needle and syringe. Then the canal space was dried with sterile paper points. The sealers were mixed according to the

manufacturer's instructions and placed inside the root canal along with sterile gutta-percha (ethanol for 10 mins and dried) following the lateral condensation technique. It was then coronally sealed with Glass ionomer restoration (GIC) (Ionofil Plus, Voco, Cuxhaven, Germany).

Samples were then aged in 5ml of sterile HBSS [to better mimic the conditions present inside the mouth], in sealed sterile 7 ml polystyrene bijoux containers in an incubator at 37°C for 7 days.

After the incubation period, each root was unwrapped from the Parafilm, the light cure removed, and the root segments were gently detached/debonded with the use of a scalpel that was applied along their contact surfaces. After separating the twin root segments to reveal dentine and sealer, the whole bulk of the sealer adhered to one segment whilst the adjacent dentine segment had fragments of sealer remanent. This sealer remanent was gently detached from the dentine with the help of the scalpel making sure not to scrape the dentine. The sample was then rinsed with sterile water, dried on sterile filter paper, and subjected to the direct contact test. The sealer remnants were gently exposed and retrieved intact from the dentine walls they had been in contact with and tested. The direct contact test was then performed. Dentine in contact with sealer will be named as dentine sample and sealer in contact with dentine will be known as sealer sample.

E. faecalis was cultured in BHI as mentioned in 'Bacteria and Media' section 2.5.2.1 of

Microbiological assays. The antibacterial properties were assessed using planktonic bacteria.

Inside a 9cm diameter petri dish a round sterile filter paper was placed which was saturated with 1000 µL of Phosphate-buffered saline (PBS) to increase humidity and a sterile glass slide was set on top of the filter paper. The dentine and sealer samples were placed in different petri dishes on top of a microscopic slide securing it with dual cure resin to keep it straight. These samples were then inoculated with 20µL of diluted *E. faecalis* culture. A 22 mm diameter glass coverslip (Chance Propper Ltd. Warley, England) was gently placed on the droplet to spread out the solution evenly and for better control of the contact between the disk surface and solution. The petri dish

was then closed and sealed with a cling foil to decrease evaporation during the long incubation times. All the petri dishes were incubated at 37 °C in the dark without shaking. A root sample with distilled water irrigation, without any sealer, and a coronal end sealed with GIC was used as a control. Testing was carried out after 7days of incubation in sterile HBSS.

To assess the survival of bacteria, the dentine/sealer sample with the cover slip assembly was immersed in 5 ml of BHI in a falcon tube and that was vortexed for 30 secs to allow for dispersion of the viable and non-viable bacteria from the surface of the root sample. To enumerate the microbial counts, a 7-fold serial dilution in BHI was performed and 20- μ L aliquots were plated onto BHI agar plates and incubated overnight at 37°C. After the incubation period, the number of colonies was counted, and the Colony Forming Units/ml (CFU/mL) were calculated. The number of colonies formed on the agar represents the number of viable bacteria that survived contact with the sealer surface enabling us to know the anti-bacterial efficacy.

2.9 Statistical analyses

Graph Pad Prism software version 9.3.1 (GraphPad, San Diego, CA, USA) was used for data handling, statistical analyses, and the expression of results in graphical formats, data were assessed for normality with the Shapiro–Wilk test. All data were normally distributed according to Shapiro -Wilk test. Mean values and standard deviations were expressed. The significance level of $p < 0.05$ was used for all analyses.

2.9.1 Studies on antimicrobial properties of eluates

All results were analysed using a one-way ANOVA and Tukey post-hoc tests for multiple comparisons. Additionally, the Spearman correlation coefficient (r) was calculated to evaluate the relationship between the mean values of calcium ion release and the respective antimicrobial performance of the eluates.

2.9.2 Studies on Sterilisation of sealer disks and Dentine Sealer Interface

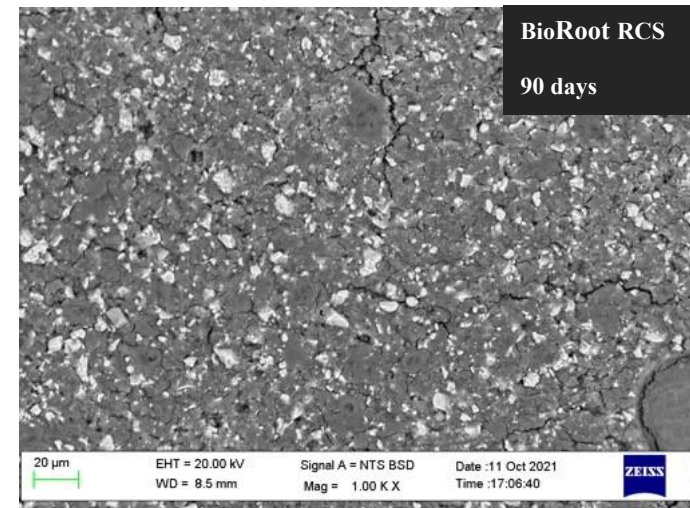
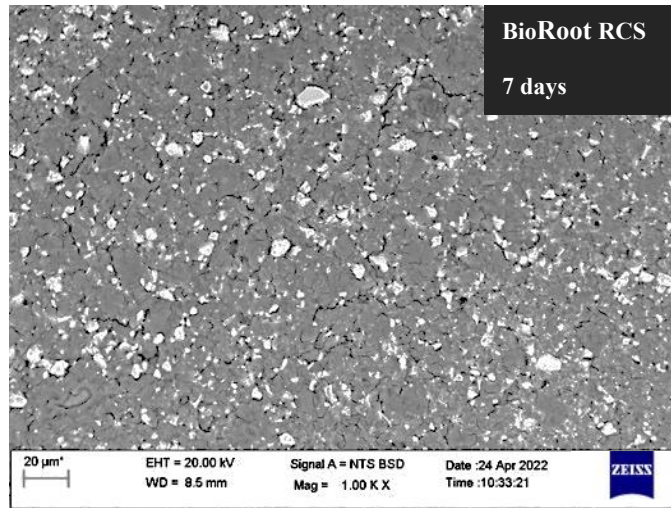
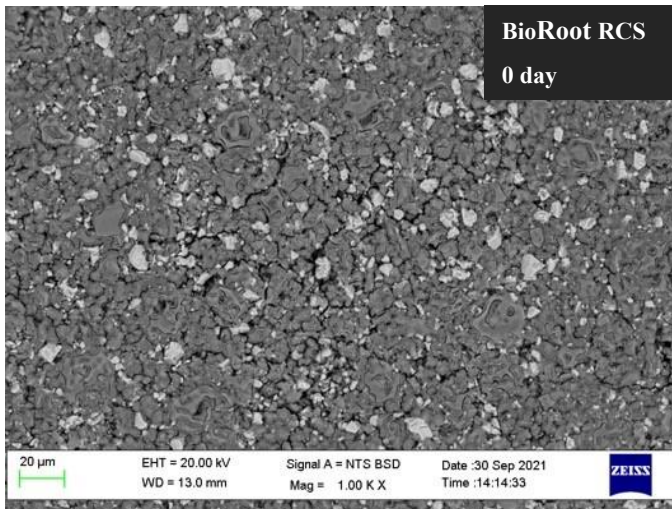
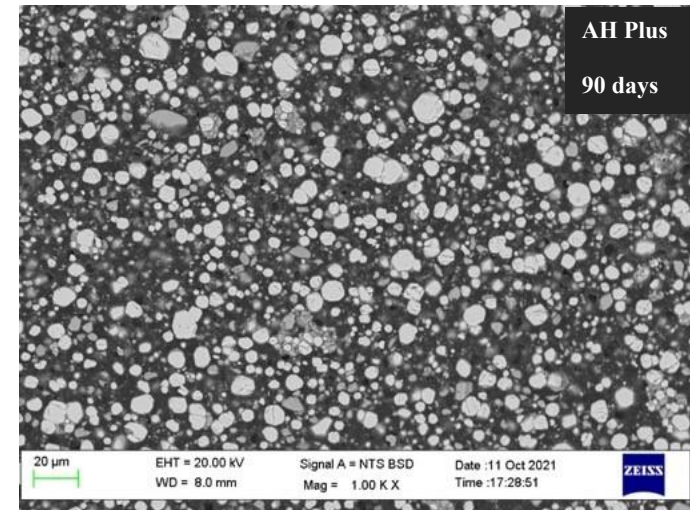
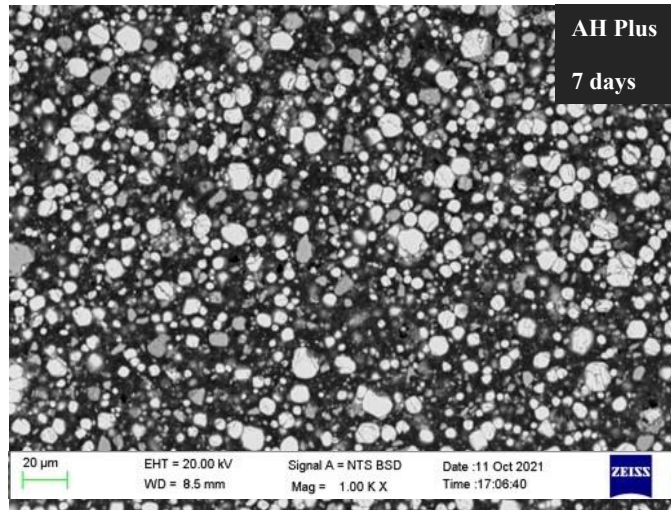
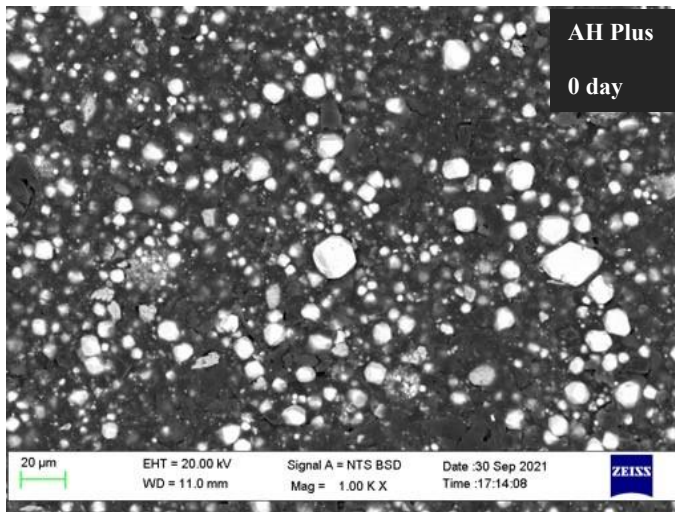
The data were evaluated using Graph Pad Prism software version 9.3.1 (GraphPad, San Diego, CA, USA). All results were analysed using a one-way ANOVA test. Mean values and standard deviations were expressed. The significance level of $p < 0.05$ was used for all analyses. Microbial tests were performed at least in triplicate and CFU values were reported as averaged values. Two-way analysis of variance (ANOVA) and Tukey's multiple comparison tests was performed for the dentine sealer interface attachment assay.

CHAPTER 3: RESULTS

3.1 Characterisation of set materials

3.1.1 Scanning electron microscopy and Energy dispersive spectroscopy

The backscatter scanning electron micrographs (SEM) at 1,000 x magnification and energy dispersive X-ray spectroscopy (EDS) profiles for the randomly selected areas of identical sizes of the tested materials are shown in Figures 3.1 and 3.2 for 0-day, 7 days, and 90 days aging in HBSS, respectively. AH Plus sealer was composed of radio-dense particles rich in calcium and tungsten interspersed in a radiolucent matrix. Smaller particles rich in zirconium were also present. There were no visible changes in microstructure and morphology after immersion in HBSS for 7 days and 90 days. The EDS analysis of the material showed peaks for calcium, zirconium, carbon, oxygen, and tungsten. The TotalFill, BioRoot RCS, and BioRoot Flow were all composed of a cement and radiopacifier phase. The material matrix was dense, and the phases were uniformly distributed, the radiopacifier appeared more radio-dense on the scanning electron micrograph. The EDS analysis of all three sealers revealed peaks of calcium, zirconium, oxygen, silicon, and carbon.



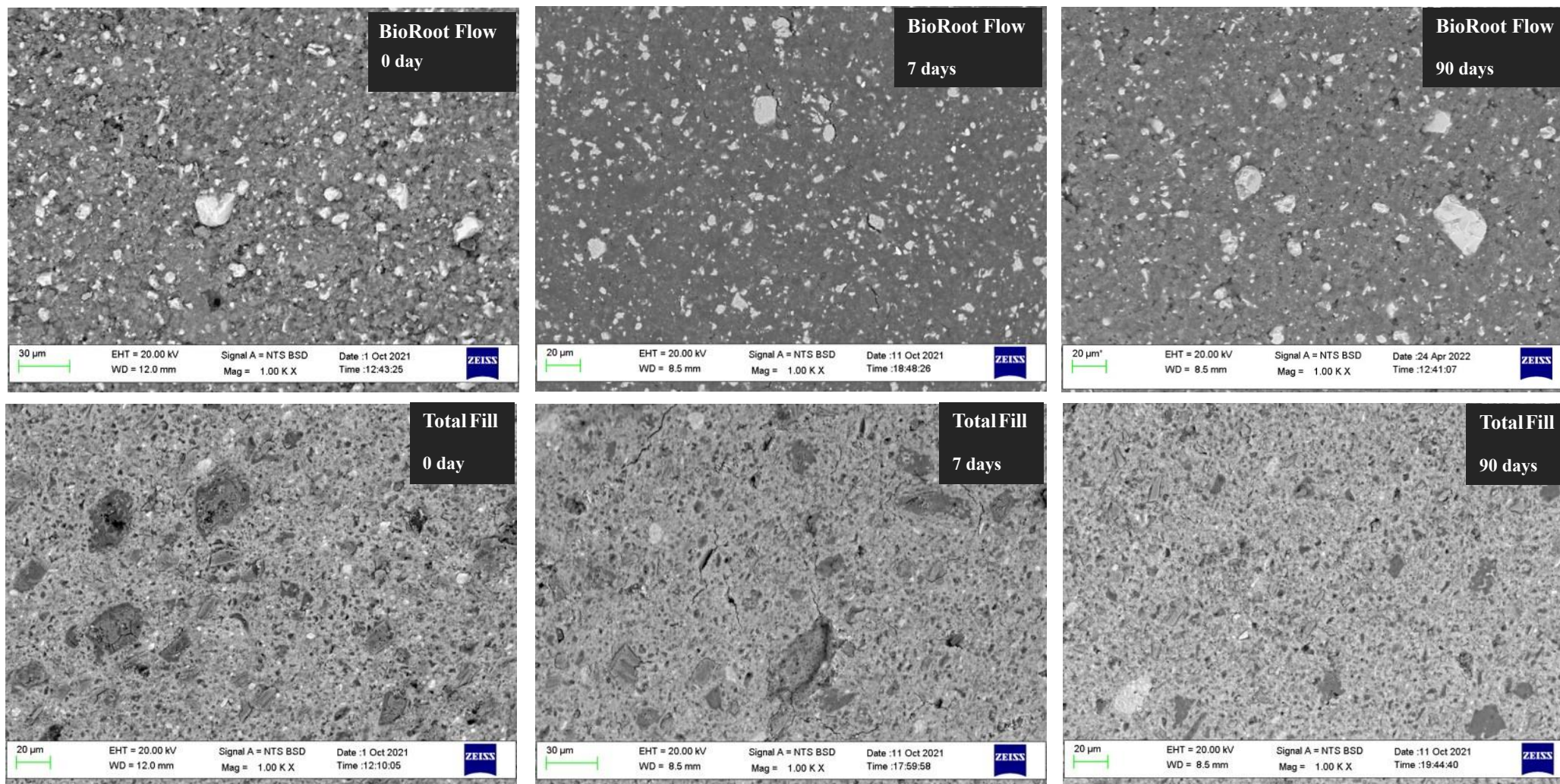
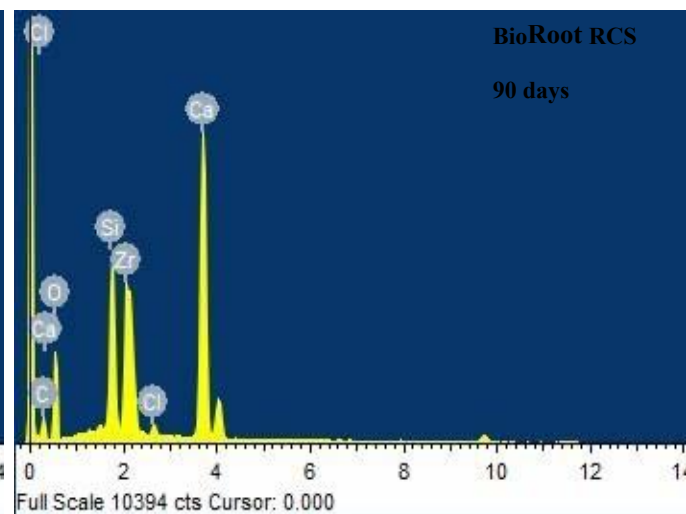
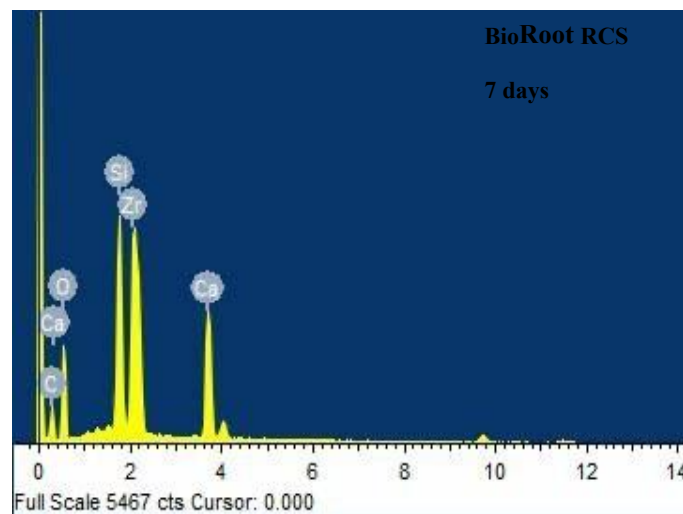
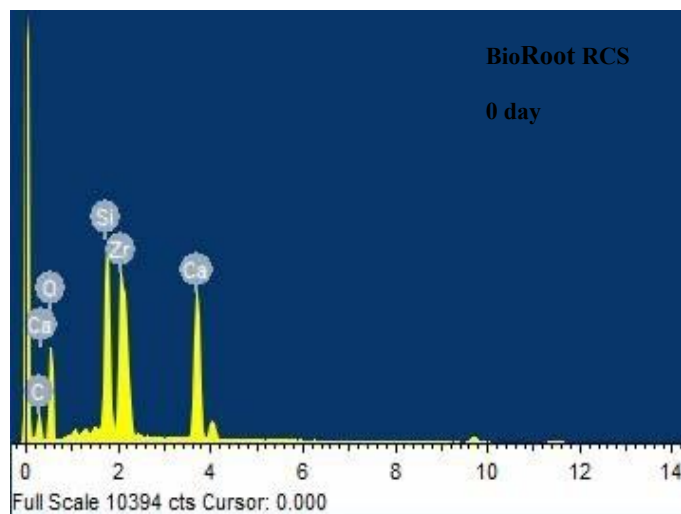
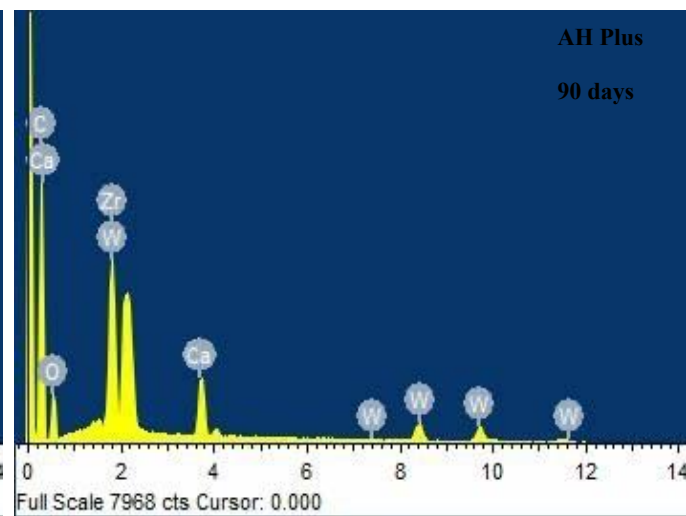
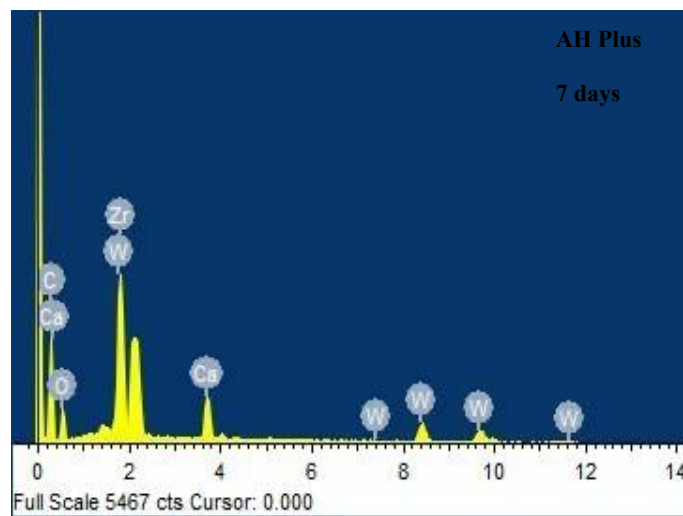
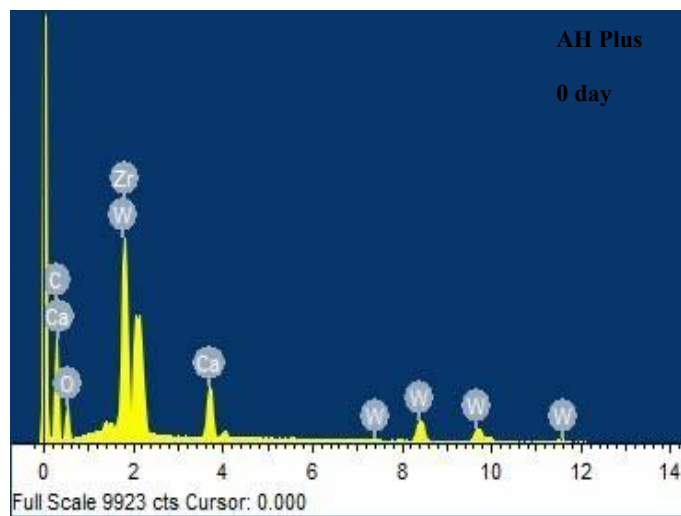


Figure 3.1: Indicative backscatter Scanning Electron Microscopy (SEM) (1,000X magnification) showing microstructural components of AH Plus, BioRoot RCS, BioRoot Flow and TotalFill sealer after immersion in HBSS for 0 day ,7,days and 90 days.



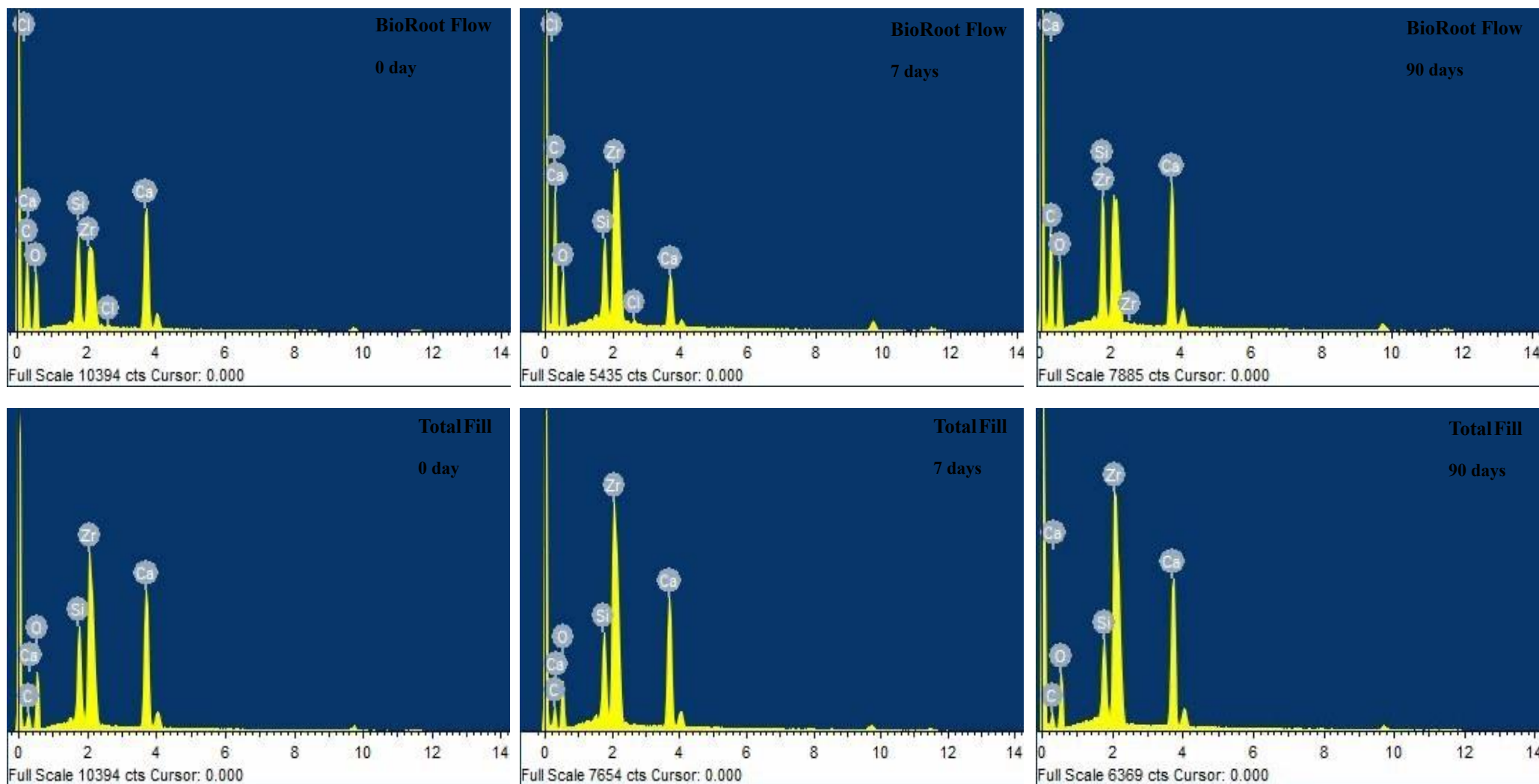


Figure 3.2: Indicative Corresponding Energy-Dispersive X-Ray Spectroscopy (EDS) analyses showing elemental composition of AH Plus, BioRoot RCS, BioRoot Flow and TotalFill sealer after immersion in HBSS for 0 day ,7days and 90 days displaying main peaks of calcium, zirconium, silicon, carbon, and oxygen.

3.1.2 X-ray diffraction analysis

XRD scans of AH Plus, BioRoot RCS, BioRoot Flow, and TotalFill were undertaken immediately after setting and after 7 days and 90 days of immersion in HBSS as presented in Figure 3.3. AH Plus exhibited very definite peaks of calcium tungstate (CT) (COD: 1546034) and zirconium oxide (ZO) (COD:9005833). The XRD plots of BioRoot RCS, BioRoot Flow, and TotalFill showed predominant peaks of zirconium oxide (COD: 9007485), (COD: 9005843) (COD: 9007485) and tricalcium silicate (COD: 9015421) (COD: 9015421) respectively. The BioRoot RCS exhibited a definite peak of calcium hydroxide (COD: 9006832) at 18° 2θ which increased in intensity with aging reaching maximum intensity by 90 days. The TotalFill and BioRoot Flow exhibited a minor peak for calcium hydroxide which was stable throughout the testing phase.

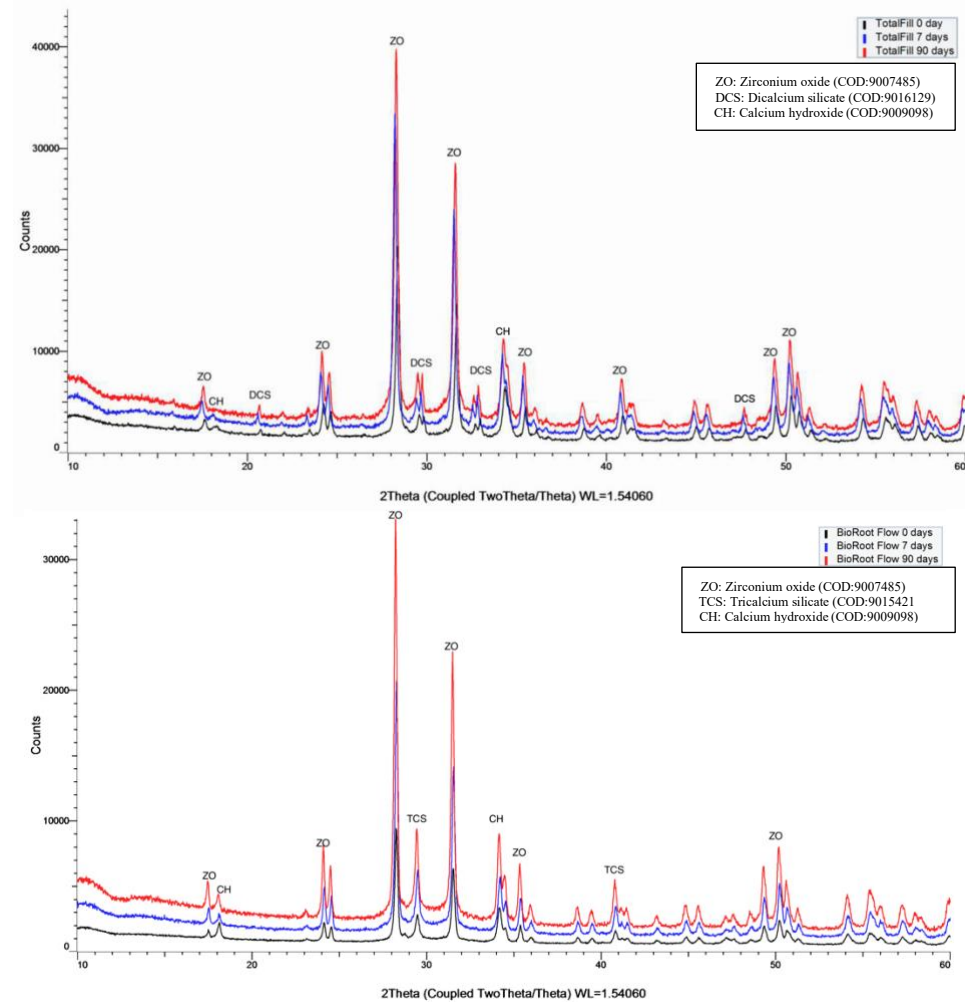
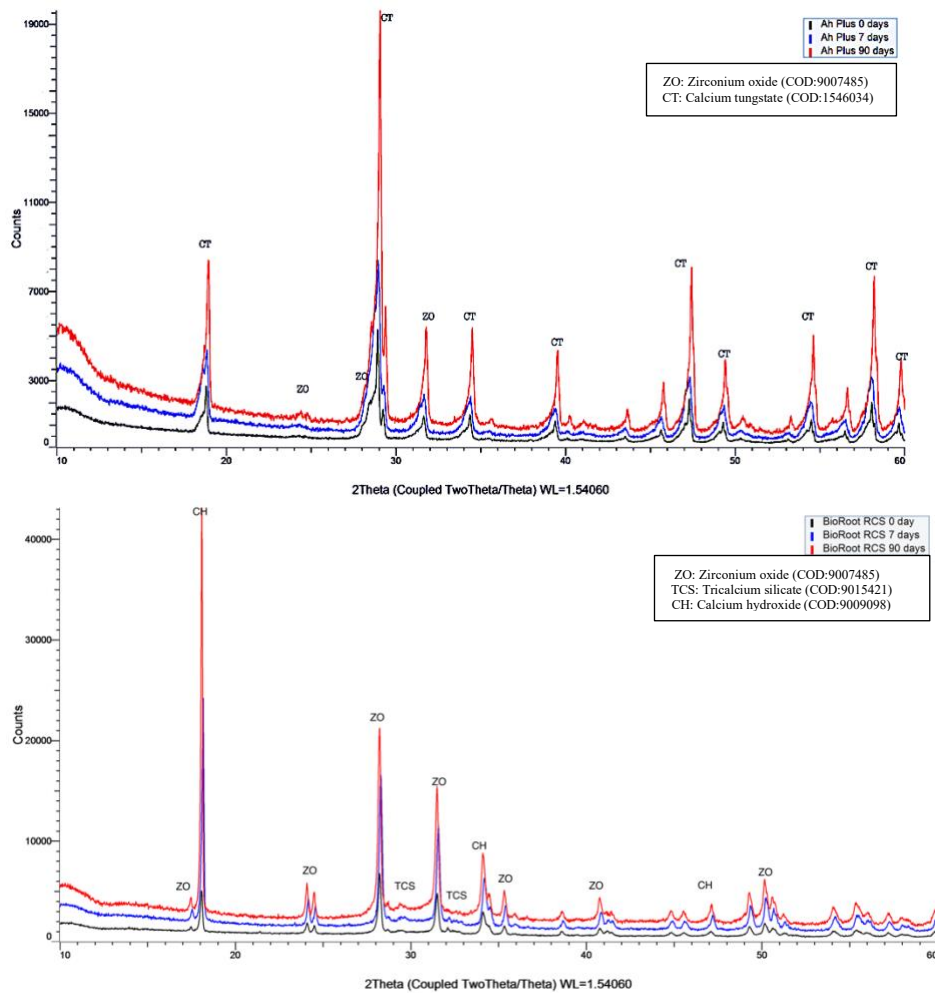


Figure 3.3: X-ray diffraction plots of AH Plus, BioRoot RCS, BioRoot Flow and TotalFill at 0 ,7 and 90 days showing the crystalline phases present after immersion in Hank's balanced salt solution.

3.1.3 Fourier transform infrared spectroscopy

In Figure 3.4 characteristic bands of carbonates (CO_3) were detected from 1400 to 1500 cm^{-1} in all specimens. Absorption intensities from 940 to 980 cm^{-1} were observed in all specimens, indicating the formation of calcium silicate hydrate (CSH). The broad peaks presented at 3350 and 3430 cm^{-1} represent the stretching motions of hydroxyl ions in the crystal network of BioRoot RCS. A broad absorption band at 3400 cm^{-1} was detected in the specimens indicating the presence of water in the specimens. A small peak of calcium hydroxide ($\text{Ca}(\text{OH})_2$) at 3622 cm^{-1} occurred in hydraulic cement samples in this region. For AH Plus in figure 3.4, the peak corresponding to the nitrogen-to-hydrogen bond (N–H) was present at 2880- 2895 cm^{-1} .

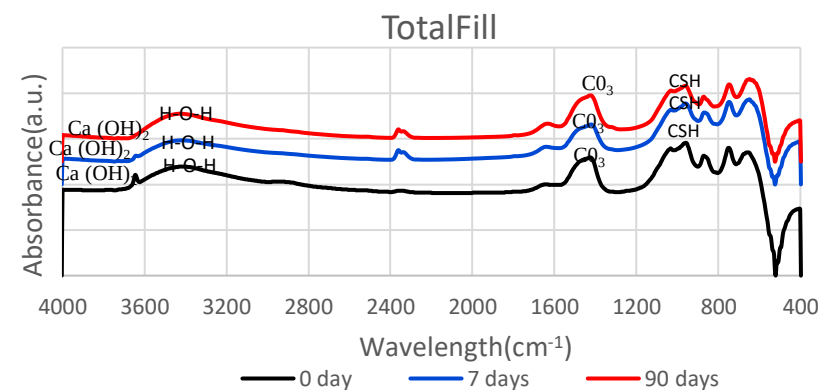
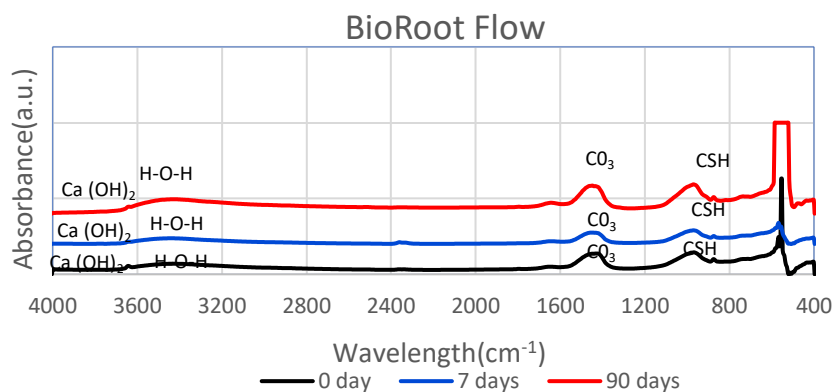
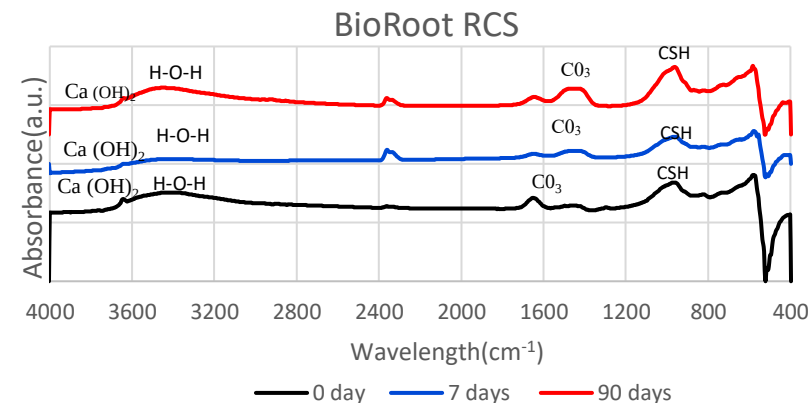
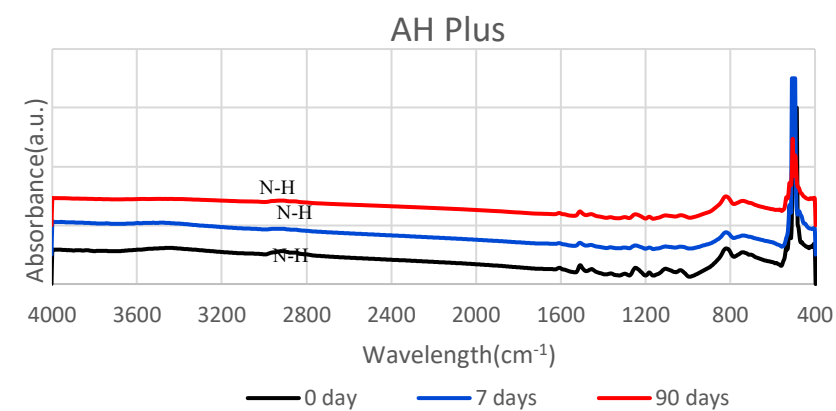


Figure 3.4: Fourier transform infrared spectroscopy plots of the AH Plus, BioRoot RCS, BioRoot Flow and TotalFill at 0-day, 7 days and 90 days. Nitrogen to hydrogen bond (N-H) peaks were detected in AH Plus and Carbonate (CO₃), calcium silicate hydrate (CSH), & calcium hydroxide (Ca (OH)₂) peaks were detected in other tricalcium silicate sealers. Absorbance units (au) on y axis.

3.1.4 Leachate Analysis (calcium release assessment)

Two types of experiments for calcium release assessment were undertaken: one for 7 days and 90 days leachate; the other for leachate changed every 15 days for 90 days duration.

Table 3.1 illustrates calcium release data for 7 days and 90 days. Test eluates of BioRoot RCS exhibited the highest values but were similar for calcium for both 7 days and 90 days ($p=0.023$).

Calcium amounts in the 7-d leachate of BioRoot Flow were not significantly different from TotalFill ($p=0.148$). 90-day leachate of BioRoot Flow observed a significant difference in the calcium level with the other TCS-based materials ($p<0.0001$). Whilst the 90-day leachate of BioRoot RCS and TotalFill did not show much difference ($p=0.0029$). There was a significant rise in calcium ion leaching for TotalFill in 90 days than the 7 days ($p<0.0001$). However, it was the opposite for BioRoot Flow whose calcium leached half as compared to 7 days of immersion in HBSS ($p=0.1495$).

Results for calcium ion leaching in media changed after every 15 days for a total of 90 days duration is shown in Table 3.2. BioRoot Flow and TotalFill showed a similar constant decrease in calcium ion release ($p=0.7242$) for all the changes till 90 days. BioRoot RCS exhibited a comparable decline in the leaching of calcium ions for the first three changes than other TCS sealers ($p=0.356$) but at 60 days there was significant difference in calcium ion leaching ($p=0.034$) with the same reducing pattern further till 90 days. AH Plus being a resin revealed insignificant calcium ion leaching overall.

Table 3.1: Cumulative data of leachate collected after total of 7 days and 90 days: Calcium (Ca) ion concentration in leachate with standard deviation of four sealers: AH Plus, BioRoot RCS, BioRoot Flow and TotalFill. BDL: Below Detection Limit

Sealer	Ca- 7 days	Ca- 90 days
AH Plus	0.03 ± 0.01	BDL
BioRoot RCS	278.33 ± 33.29	300.73 ± 54.15
BioRoot Flow	142.29 ± 52.01	84.08 ± 8.73
TotalFill	123.50 ± 11.68	247.25 ± 37.09

Table 3.2: Data of leachate collected every 15 days for a total of 90 days duration: Mean calcium ion concentration in leachate media of four sealers: AH Plus, BioRoot RCS, BioRoot Flow and TotalFill with standard deviation.

Duration	AH Plus	BioRoot RCS	BioRoot Flow	TotalFill
15 days	0.06 ± 0.10	20.97 ± 1.29	17.34 ± 1.17	17.34 ± 1.17
30 days	0.01 ± 0.01	16.08 ± 0.93	19.36 ± 0.43	19.36 ± 0.43
45 days	0.03 ± 0.03	13.34 ± 0.50	12.62 ± 0.69	12.62 ± 0.69
60 days	0.01 ± 0.01	9.31 ± 0.41	9.27 ± 0.8	9.27 ± 0.8
75 days	0.01 ± 0.01	3.91 ± 0.91	5.72 ± 1.06	5.72 ± 1.06
90 days	0.03 ± 0.03	2.81 ± 1.51	3.59 ± 0.63	3.59 ± 0.63

MICROBIOLOGICAL ANALYSIS

Microbiological analysis included testing of sterility of sealers, assessment of antimicrobial properties of those sealers alone as well as in contact with dentine at dentine sealer interface.

It helped to determine if sterilization processes altered the microbial profile of the disc, which could influence experimental results.

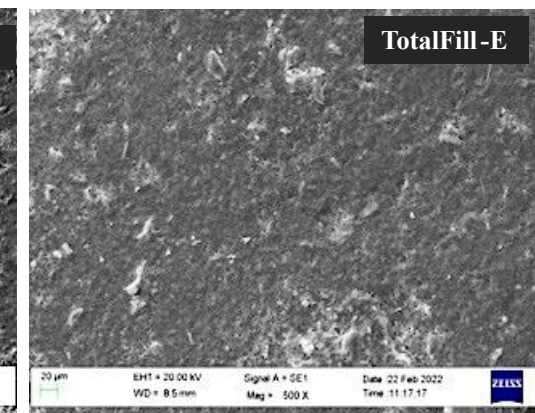
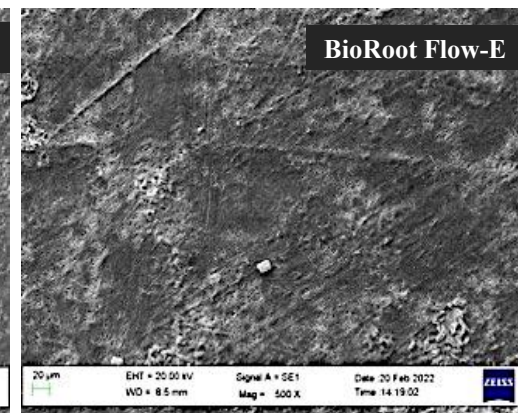
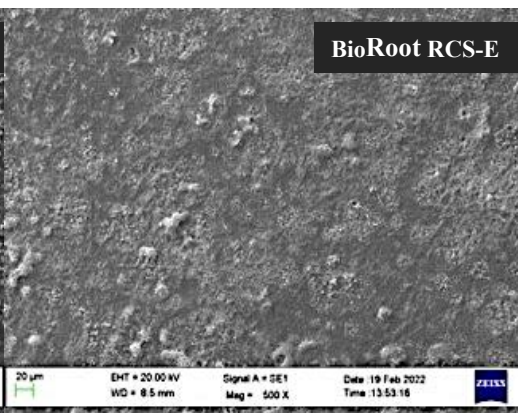
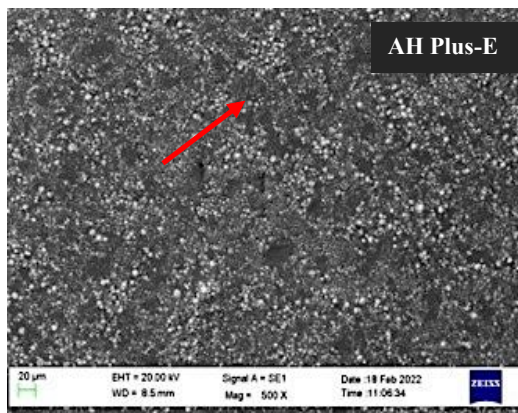
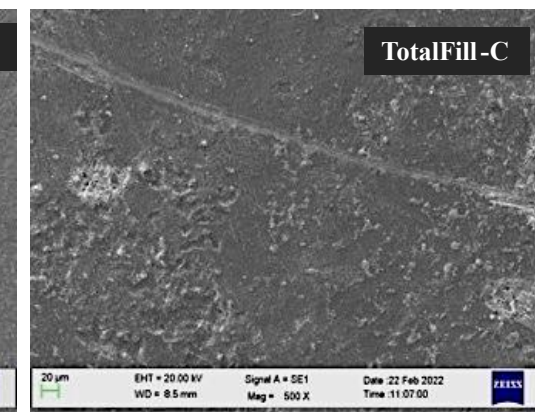
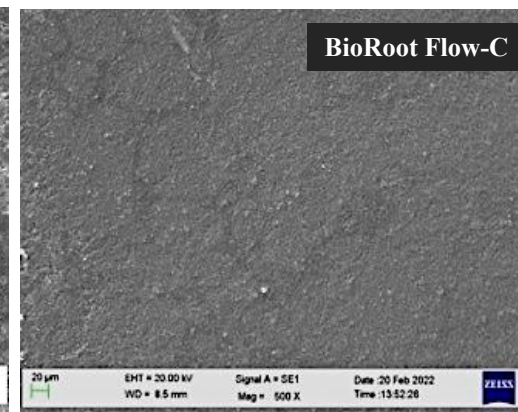
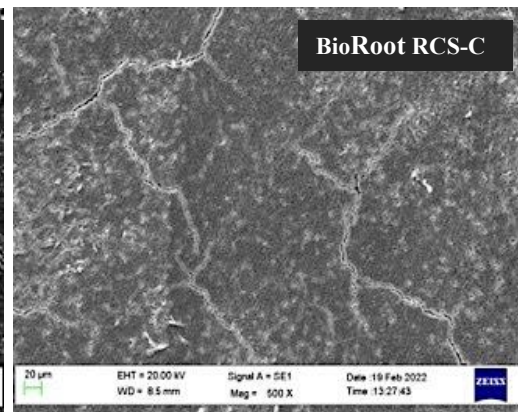
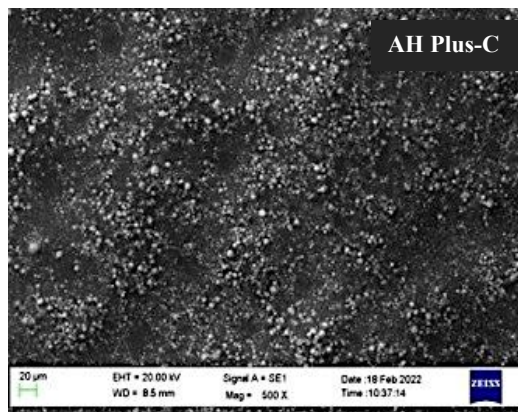
3.2 Sterilization of Sealer Disk

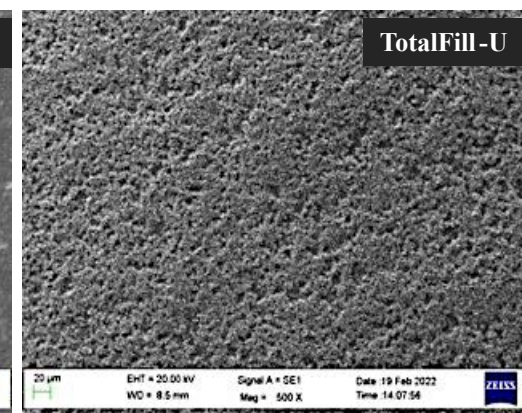
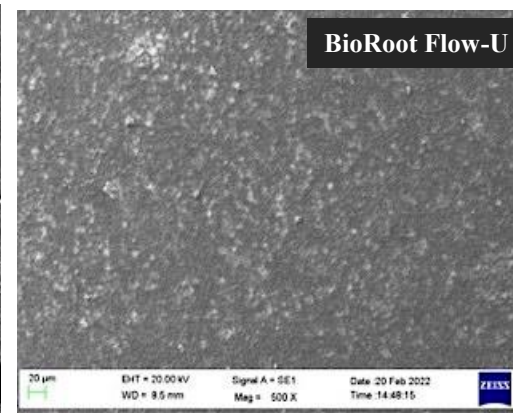
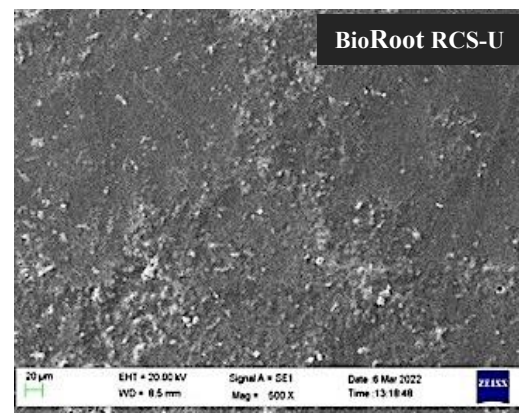
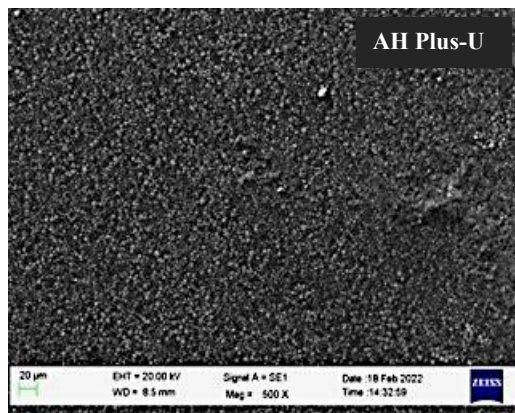
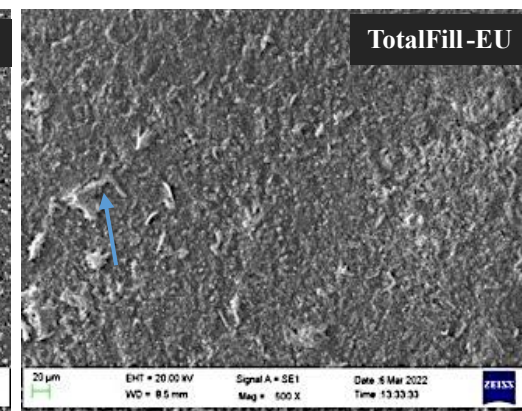
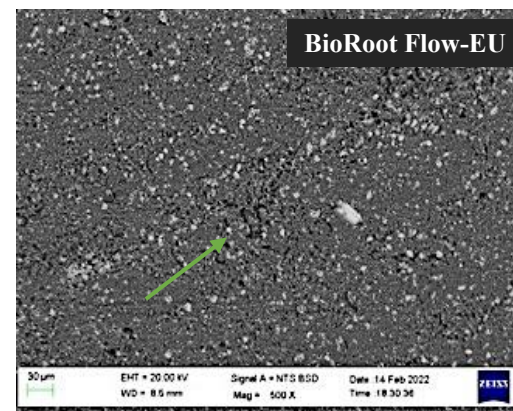
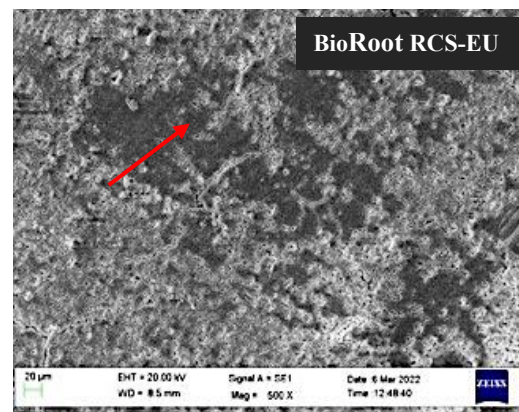
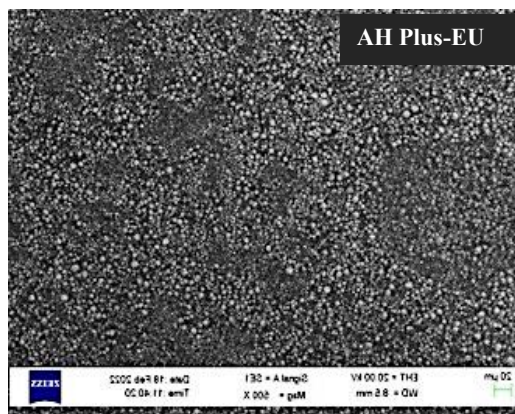
3.2.1 Characterization of set materials before and after sterilization

Discs of set materials were characterised before and after sterilisation using techniques of SEM, EDS and FT-IR.

3.2.1.1 Scanning Electron Microscopy (SEM)

The surface secondary electron micrographs of the materials subjected to different sterilization protocols are shown in Figure 3.5. The autoclave and ethanol caused deterioration to the material surface of AH Plus with channels devoid of radiopacifier present. Some surface deposits were seen when BioRoot RCS was autoclaved. Ultraviolet seemed to have increased the porosity of the material. The ethanol plus ultraviolet was more destructive with areas showing deposits interspersed with areas that are darker and without deposits. In the case of BioRoot Flow, few changes were seen except some cracking and increased porosity in ethanol plus ultraviolet. For TotalFill, no microstructural changes were spotted other than some surface deposits in the ethanol plus ultraviolet ground.





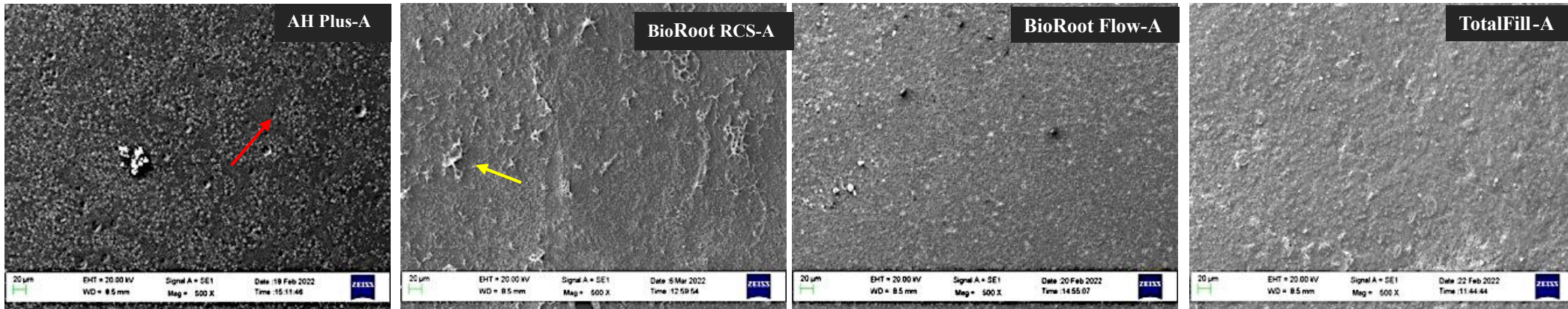


Figure 3.5: Secondary electron Scanning electronic micrographs of AH Plus ,BioRoot RCS ,BioRoot Flow and TotalFill sealers after sterilisation with ethanol (E) ethanol & ultraviolet (EU), ultraviolet alone (U) and autoclave(A) at 500 x magnification; control (C). In ethanol plus ultraviolet group, red arrow indicates areas with deposits interspersed with areas that are darker and without deposits in BioRoot RCS, green arrow indicates cracking and increased porosity in BioRoot Flow, blue arrow indicates some surface deposits in Total Fill. In autoclave group red arrow indicates deterioration to the material surface of AH Plus with channels devoid of radiopacifier. Yellow arrow indicates some surface deposits in BioRoot RCS.

3.2.1.2 Energy-dispersive X-ray spectroscopy (EDS)

EDS analysis of the materials subjected to different sterilization protocols is shown in Table 3.3. The mean Ca/Si ratio and Ca/Zr ratio increased in BioRoot RCS after autoclaving while it was elevated with ultraviolet sterilisation in BioRoot Flow as compared to the control group (no sterilisation). TotalFill depicted opposite effects, where the Ca/Si and Ca/Zr ratios showed a drastic decline in their values with all sterilisation techniques specifically more with Ethanol .

Table 3.3: Energy-dispersive X-ray spectroscopy (EDS) mean values with standard deviation for sterilisation of sealers. Ratio of calcium with silicon and ratio of calcium with zirconium of distinct groups of sterilisation methods on four sealers AH Plus, BioRoot, BioRoot Flow and Total Fill: no sterilisation as control.

Sterilisation Methods	AH Plus (mean ratio)		BioRoot RCS (mean ratio)		BioRoot Flow (mean ratio)		Total Fill (mean ratio)	
	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr
Control	0.47 ± 0.8	0.10± 0.01	5.12 ± 0.1	1.63 ± 0.16	8.1± 0.41	3.11 ± 0.1	57.61 ±5.23	3.18 ± 0.29
Ethanol	1.47 ± 0.49	0.12 ± 0.03	5.43 ± 0.43	1.40 ± 0.11	11.29 ± 0.61	5.52 ± 0.45	13.20 ±1.67	1.50 ± 0.09
Ultraviolet	2.43 ± 0.15	0.15 ± 0.03	4.64 ± 0.14	1.29 ± 0.03	12.68 ± 1.56	6.01 ± 1.46	26.30 ±6.30	1.53 ± 0.11
Ethanol+UV	1.57 ± 0.6	0.09± 0.003	4.27 ± 0.25	1.14 ± 0.1	7.72 ± 0.26	2.81 ± 0.10	9.1 ± 0.29	1.14 ± 0.05
Autoclave	2.24± 0.27	0.11± 0.34	8.45 ± 1.72	3.67 ± 5.94	7.96 ± 0.24	3.03 ± 0.13	14.44 ±1.18	1.25 ± 0.19

3.2.1.3. Fourier transform infrared spectroscopy (FT-IR)

Fourier transform infrared spectroscopy analysis in Figure 3.6 depicted changes in water and alcohol peaks at 3421cm^{-1} for AH Plus and BioRoot RCS, whereas TotalFill and BioRoot Flow seemed unaffected by different sterilisation techniques.

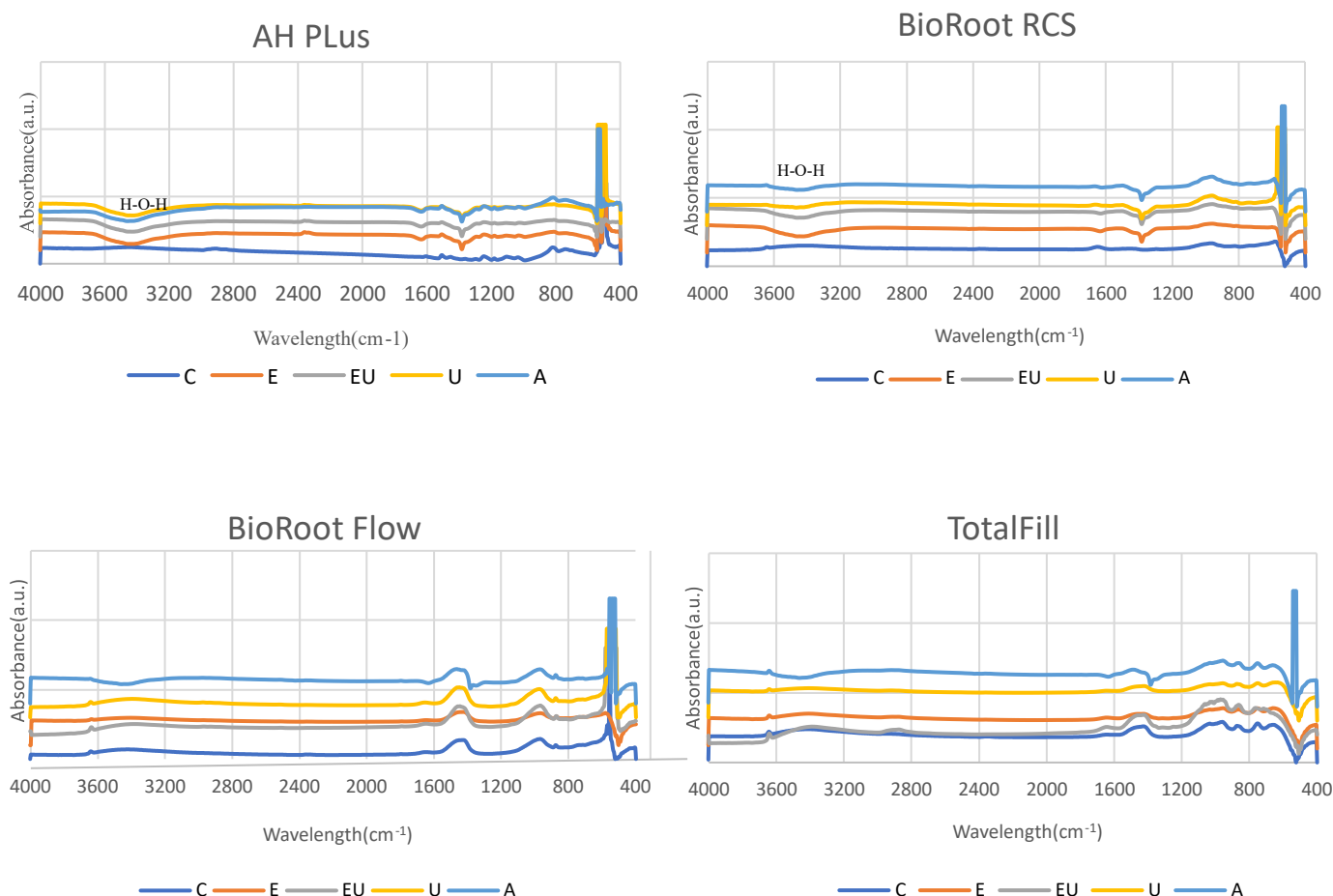


Figure 3.6: Fourier transform infrared spectroscopy of AH Plus, BioRoot RCS, BioRoot Flow (BRF) and Total Fill (TF) after different techniques of sterilisation: No sterilisation as control (C), Ethanol (E), Ethanol + Ultraviolet (EU), Ultraviolet (U) alone and Autoclave (A). Changes in water (H-O-H) and alcohol peaks could be seen at 3421cm^{-1} in AH Plus and BioRoot RCS specimen. No changes could be seen with BRF and TF. Absorbance units (au) on y axis.

3.2.2 Macroscopic imaging of sealer discs

Not much difference could be seen between any of the different sterilisation techniques macroscopically as shown in Figure 3.7.

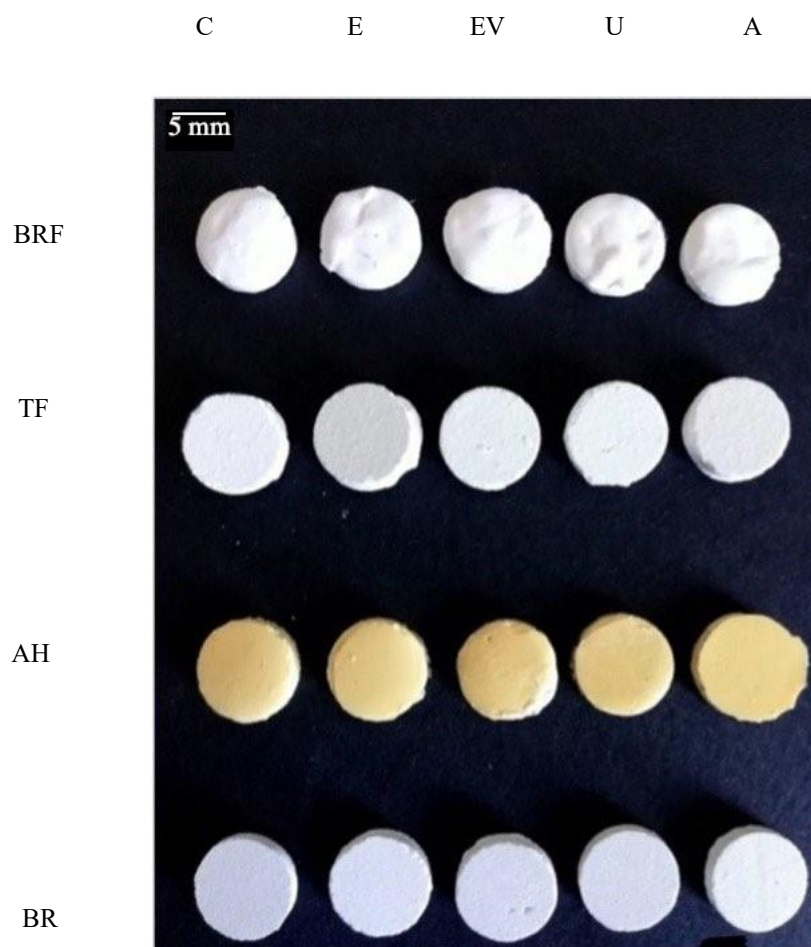


Figure 3.7: Macroscopic view of all four sealer discs (BRF-BioRoot Flow, TF-TotalFill, AH-AH Plus, BioRoot RCS) before and after sterilisation with different techniques (C-control, E-ethanol, EV-ethanol +ultraviolet, U-ultraviolet, A-autoclave).

3.2.3 Sterility Test

All the materials exhibited no contamination when prepared in a laminar flow chamber in sterile conditions as compared to sealers prepared in the open lab as seen in Figure 3.8.

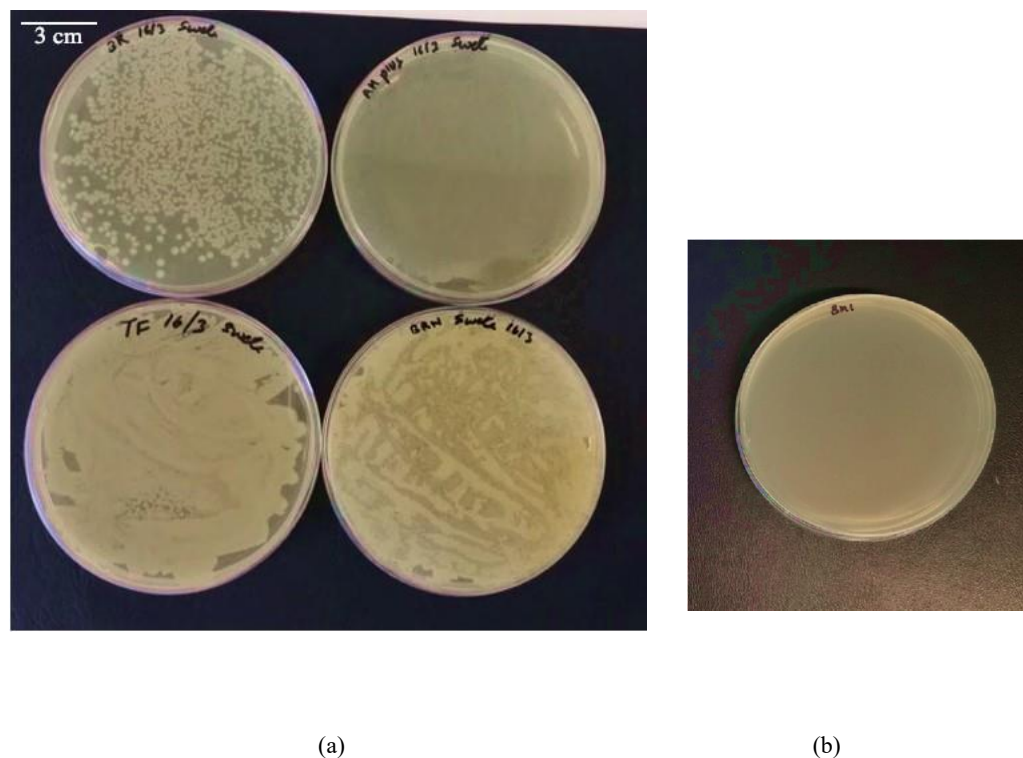


Figure 3.8: (a) Agar plates showing growth when disks of AH Plus, BioRoot RCS, BioRoot Flow and TotalFill were prepared in open lab. (b) Uncontaminated agar plate.

3.3 Assessment of antimicrobial properties of endodontic sealers

A preliminary experiment was conducted to ascertain the association between optical density and CFU/ml, as shown in the table 3.4 below-

Table 3.4: Association between optical density and CFU/ml conducted in a preliminary experiment.

Optical density(nm)	CFU/ml
0.272	1×10^5
1.531	1.2×10^4
1.855	1.6×10^6
2.111	6.3×10^6
3.793	18.6×10^6

3.3.1 Attachment Assay/Direct Contact Test for Sealer

The results of the Direct Contact Test for the antimicrobial activity of all four sealers against *E. faecalis* are shown in Figure 3.9. Groups of BioRoot RCS, BioRoot Flow, and TotalFill specimens presented nondetectable viable counts at all time points suggesting complete inhibition of the microbial activity. The limit of detection was marked at 50 CFU/ml. When 20 μ l of suspension is plated, there are often fewer than 50 CFU/ml colonies present, which are not likely to be visible, therefore the limit of detection. The bacterial concentration that was recovered from the AH Plus control group at 0 days and 7 days was similar with 1×10^4 CFU ml^{-1} . After ninety days of aging, Colony Forming Unit values for the AH Plus sealer disc increased up to 1×10^6 CFU/ml.

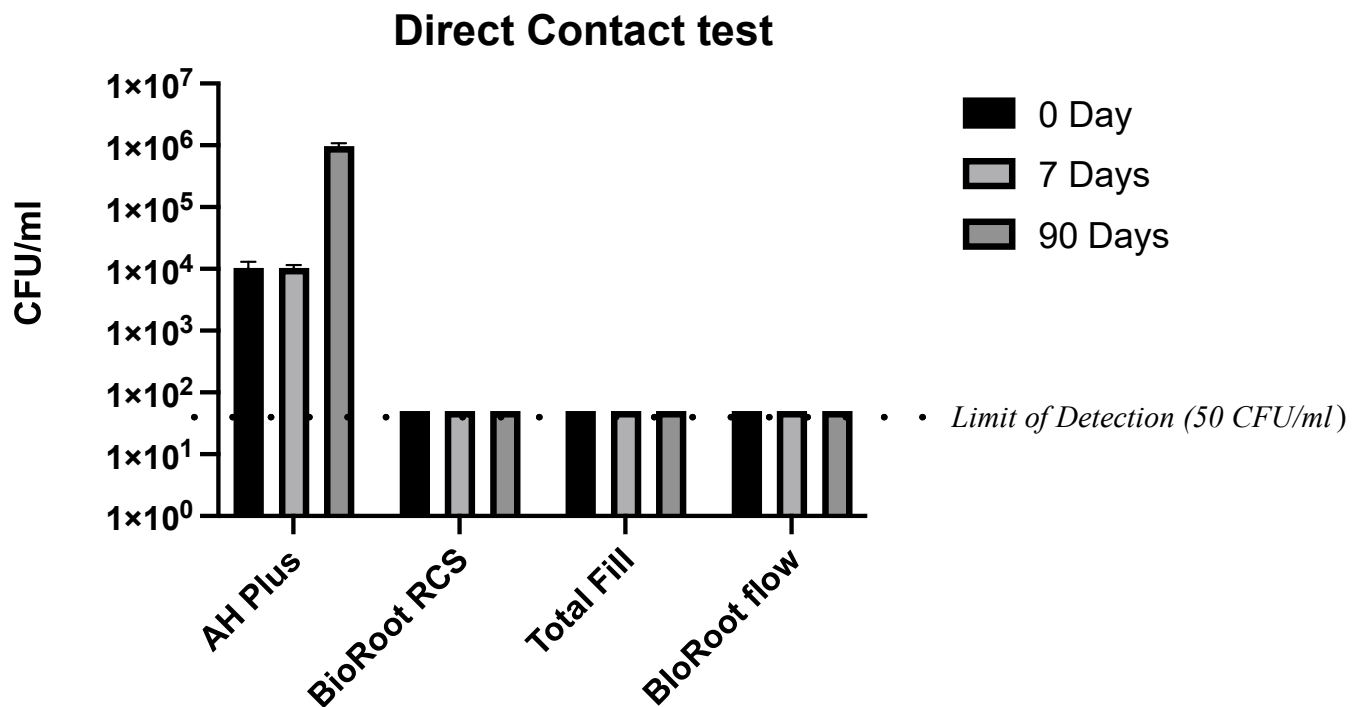


Figure 3.9: Antimicrobial activity in CFU/ml for four different sealers (AH Plus, BioRoot RCS, BioRoot Flow and TotalFill) at 0-day, 7 days and 90 days with the Direct Contact Test. AH plus was the control. Results of two-way ANOVA test with $p < 0.0001$: statistically significant difference. The limit of detection was at 50 CFU/ml. ANOVA: analysis of variance; (n=3).

3.3.2 Minimum Inhibitory Concentration (MIC)

Results from the MIC assays testing 7-day eluates on *E. faecalis* are presented in Figure 3.10

(a). The leachates of BioRoot RCS, BioRoot Flow, and TotalFill resulted in a significant growth reduction of *E. faecalis* ($p < 0.0001$) when compared with the bacterial control. The leachate of BioRoot RCS was more antibacterial compared with all other test leachates.

However, AH Plus leachate demonstrated the least inhibition of microbial growth.

Figure 3.10 (b) shows the results from the 90-day leachates on the inhibition of *E.*

faecalis. BioRoot RCS exhibited a greater inhibitory effect ($p < 0.001$) on bacterial growth as compared to its 7-day eluate and all other test sealers. The 90-day leachate of BioRoot Flow demonstrated a rise in microbial activity as opposed to the 7-day result. However, the eluent of TotalFill exhibited the opposite effect. AH Plus revealed comparable results for both time frames.

The difference in the mean was highly significant when the comparison of means was made between tricalcium silicate sealers and the bacterial control suggesting high antibacterial activity. Whilst comparing the means of AH plus and the control, results were non-significant showing it to be the least antimicrobial.

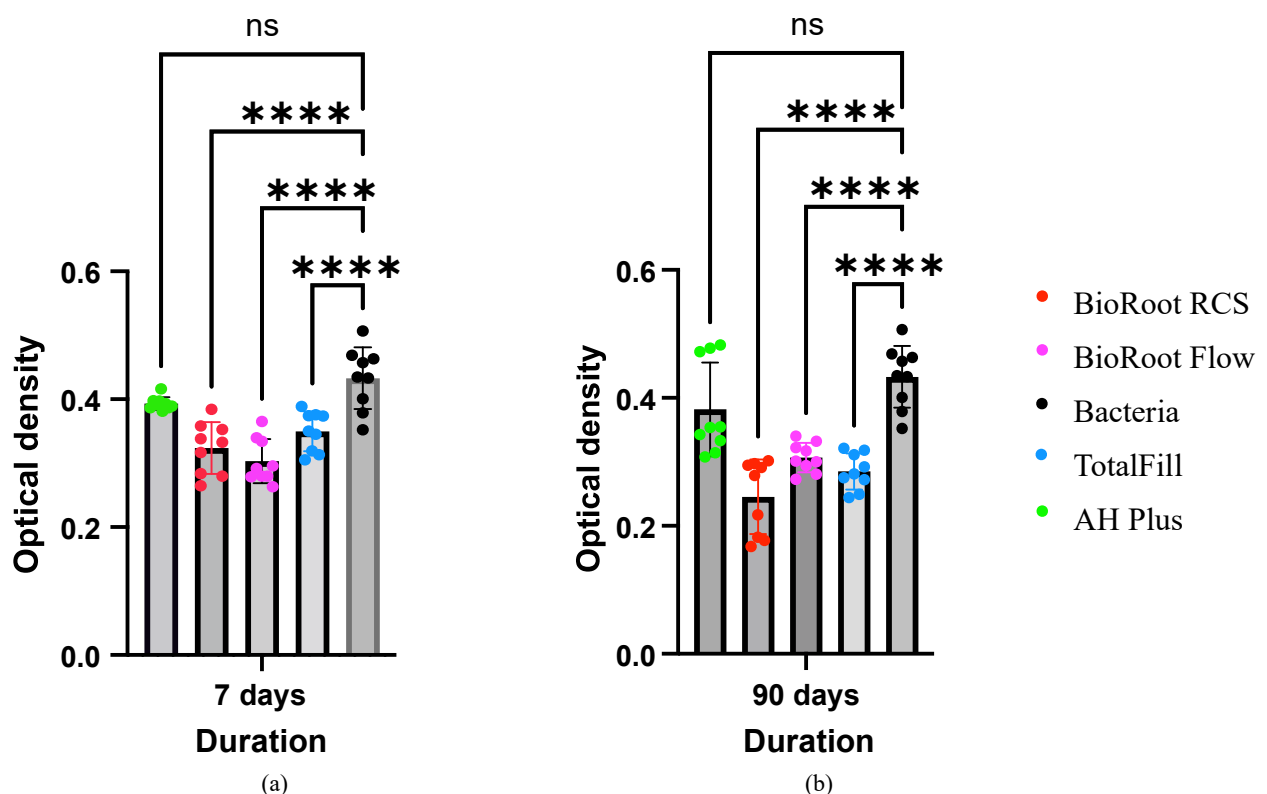


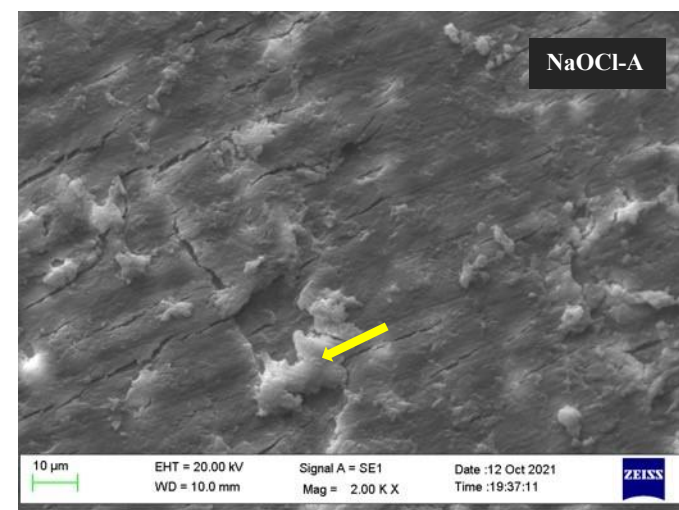
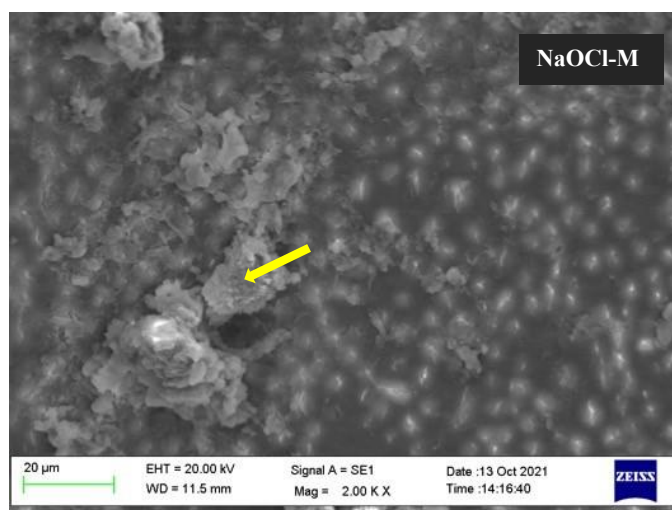
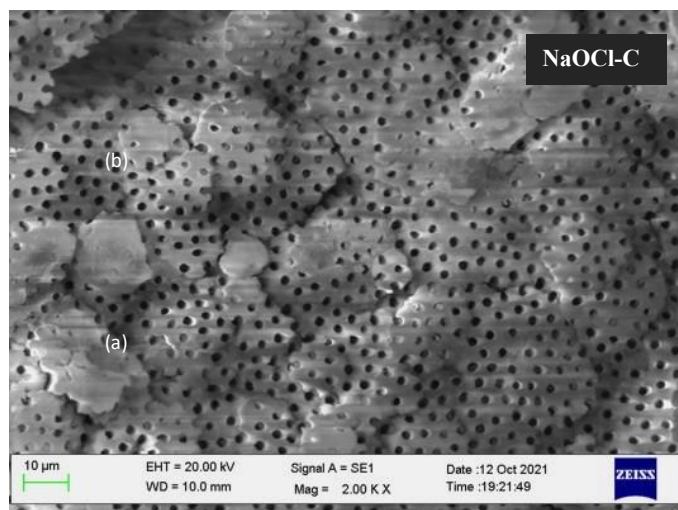
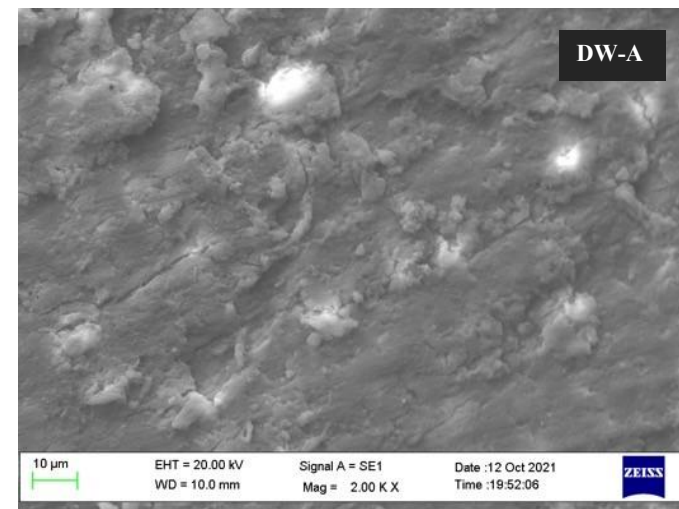
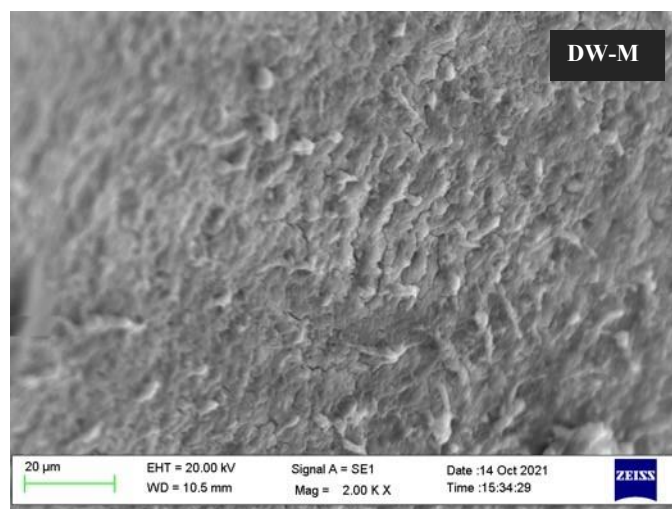
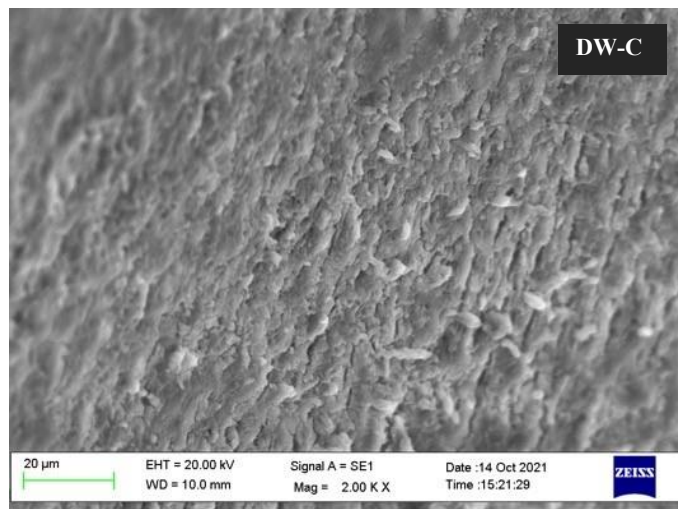
Figure 3.10: This graph represents optical density of various sealer eluents of a time frame of (a) 7 days and (b) 90 days frame. Bacteria only are the control. Results of one-way ANOVA / Dunnett's multiple comparisons test. Bar graph and error bar (mean $\pm$ SD). Differences between means are indicated. ****, $p < 0.0001$. ns, not significant. ANOVA: analysis of variance; (n=9).

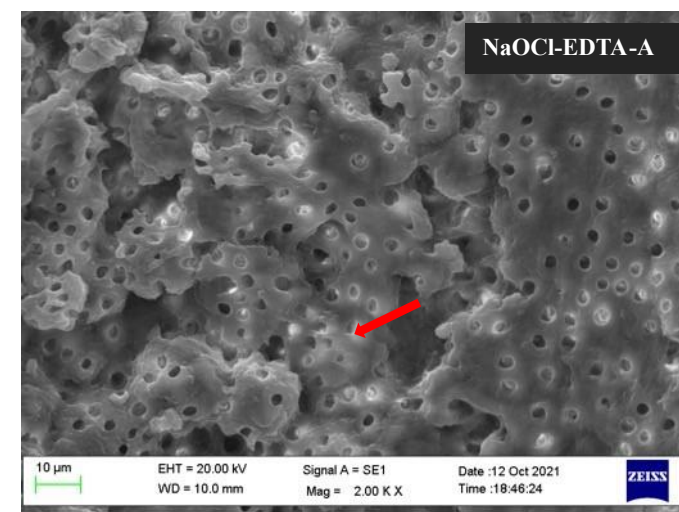
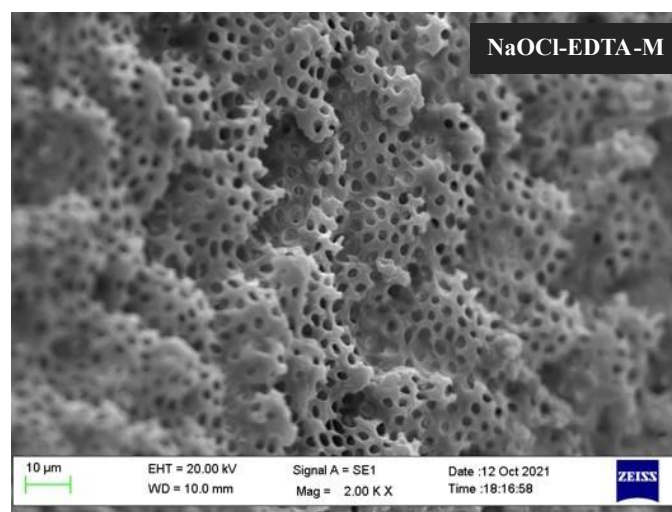
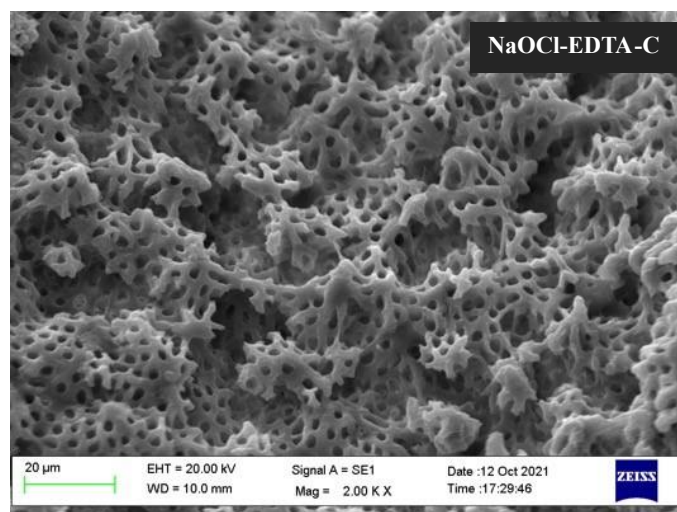
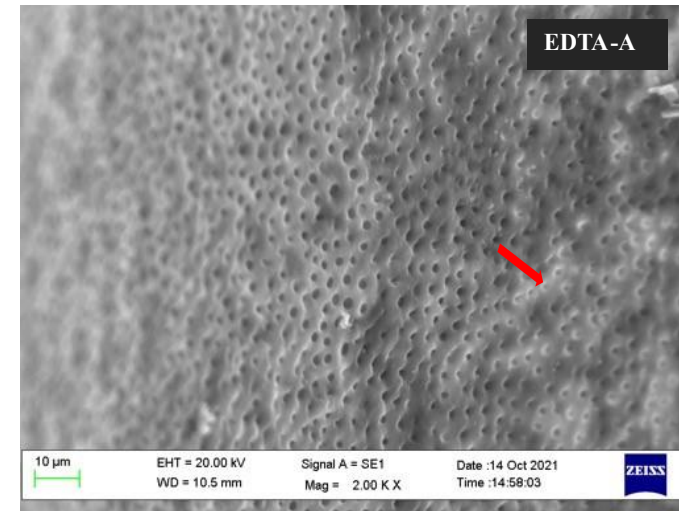
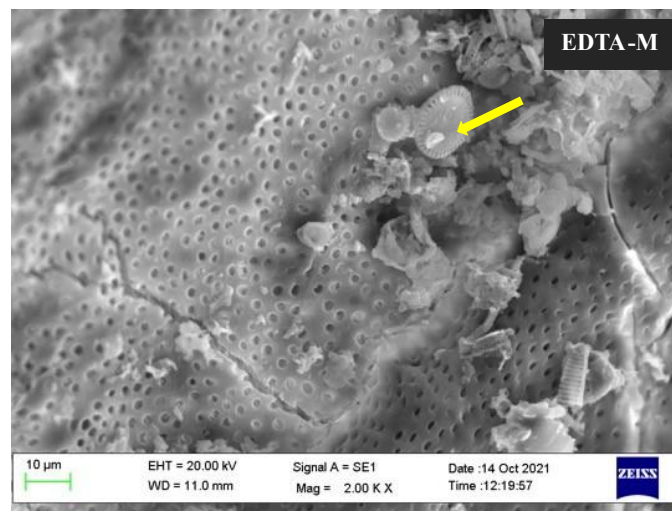
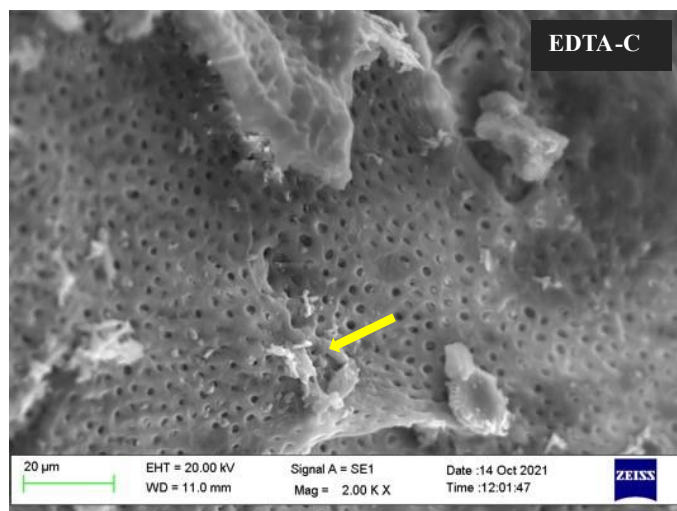
3.4 Characterisation of dentine

3.4.1 Scanning electron microscopy (SEM) and Energy Dispersive Spectroscopy (EDS) analysis

Scanning electronic microscopic images of dentine after being treated with different irrigation protocols are presented in Figure 3.11. When the NaOCl was used as the only irrigant, a smear layer was still observed on the dentin surface. The use of EDTA on its own as irrigating solution and hypochlorite followed by EDTA as in Group C and D (Figure:3.11) resulted in the widening of the dentinal tubules but some smear layer was seen in Group C and erosion was observed (area marked with red arrow in Figure 3.11) with Group D. When HEDP mixed hypochlorite (Group E) was used for irrigation, widening of the dentinal tubules was seen, although tiny amounts of smear layer were present over the dentinal tubules (area marked with yellow arrows in Figure 3.11). Distilled water which was used as a control and HEDP mixed with distilled water which are Groups A and F, revealed a cracked smear layer, which completely covered the dentinal tubules and showed the least cleaning effect.

EDS analysis from Figure 3.12 showed the increased intensity of sodium (Na) and chloride (Cl) with the NaOCl-treated dentin. Carbon (C) was reduced with the use of hypochlorite as compared to the control group. C level increased significantly compared to the NaOCl and control groups in the EDTA alone treated group; a significant decrease in the level of oxygen (O) and calcium (Ca) was seen. Na and Cl levels increased in the HEDP+NaOCl group while the HEDP+DW group displayed similar elemental composition as the control.





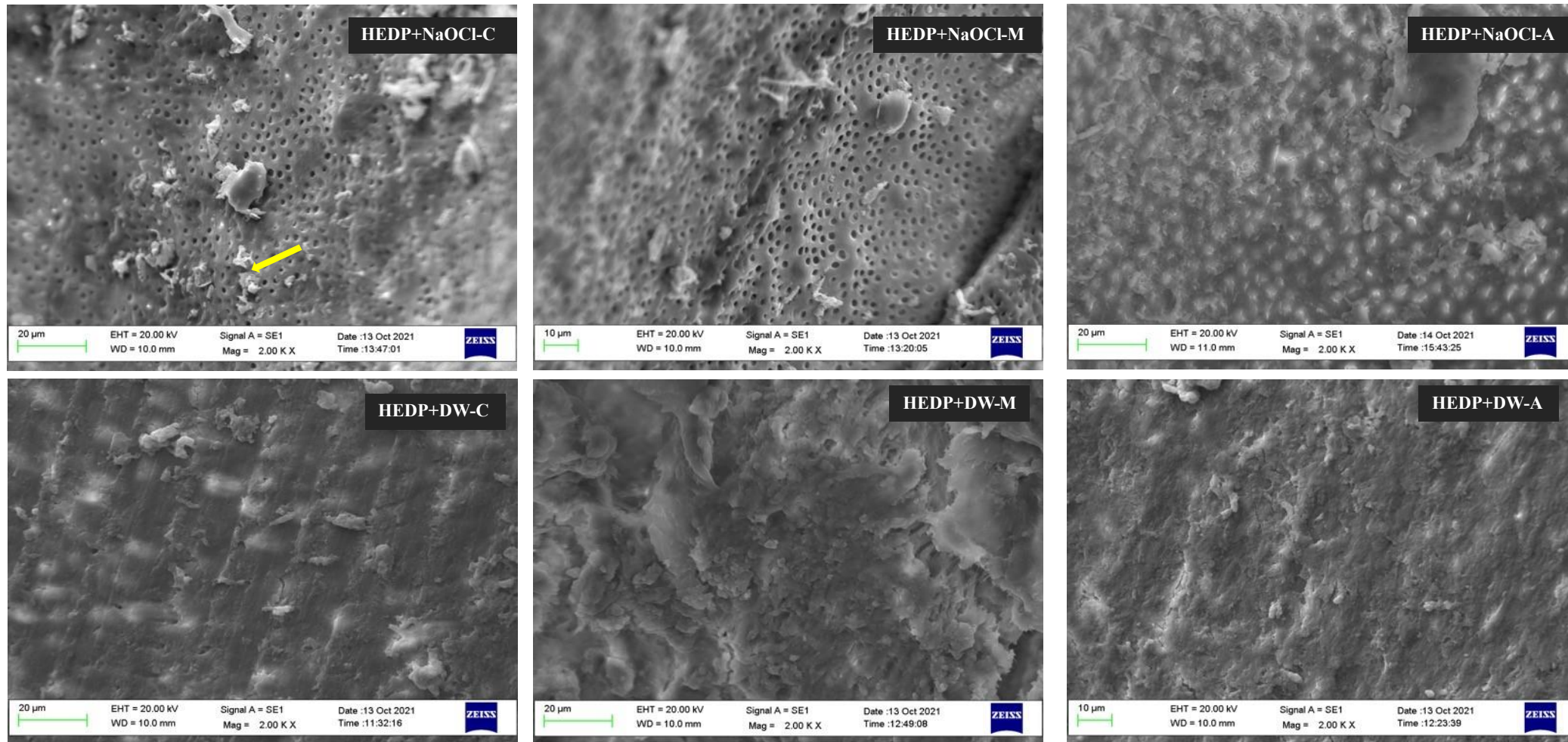
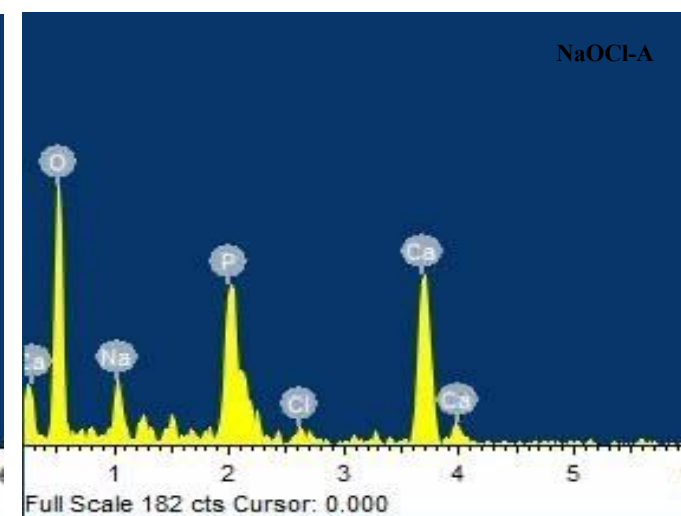
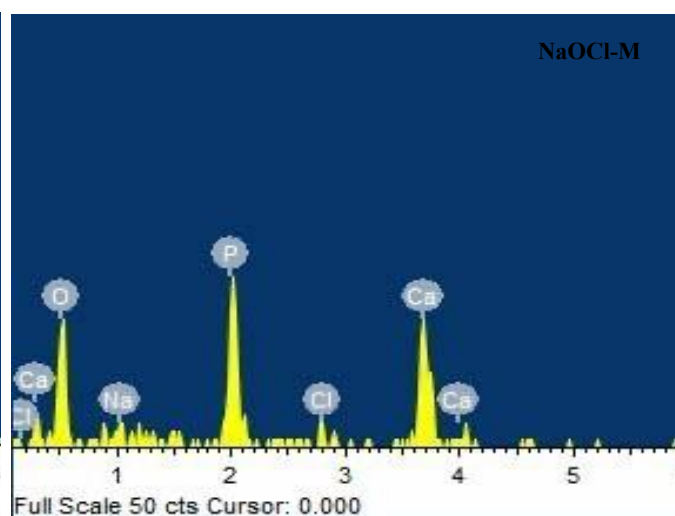
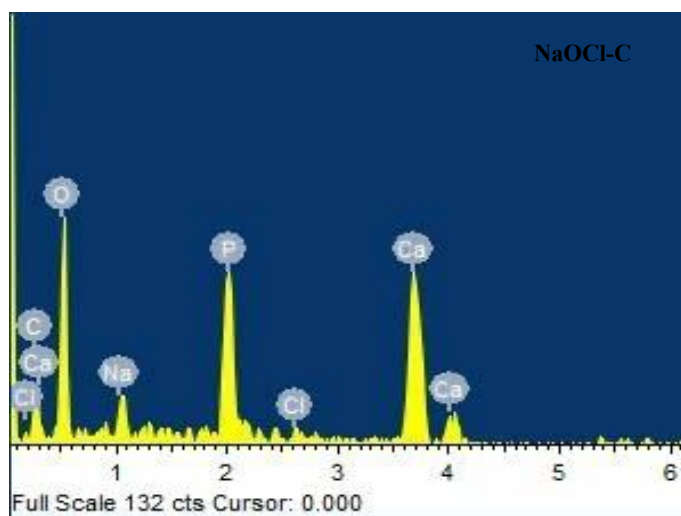
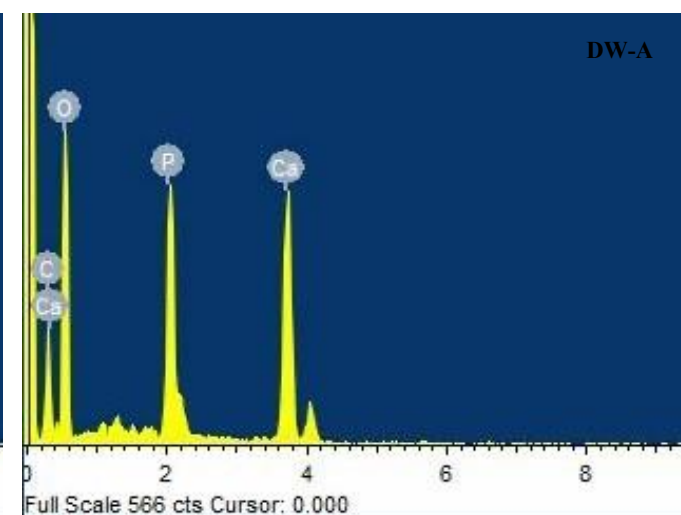
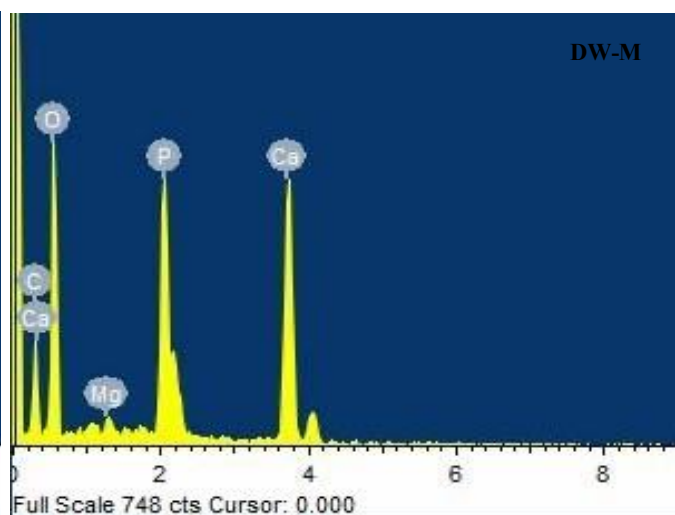
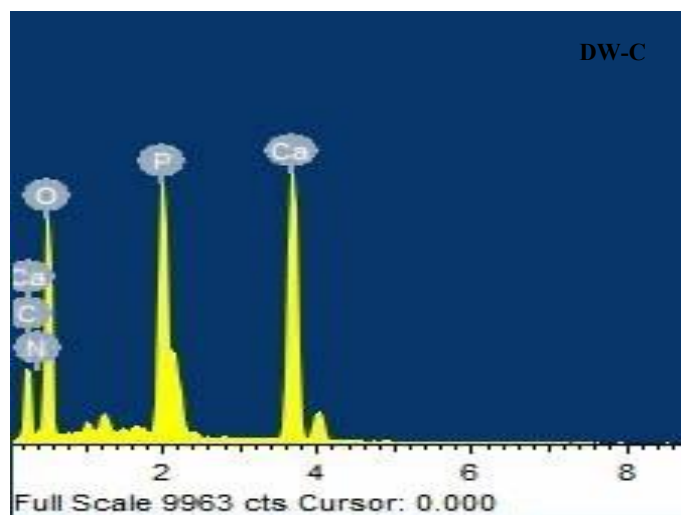
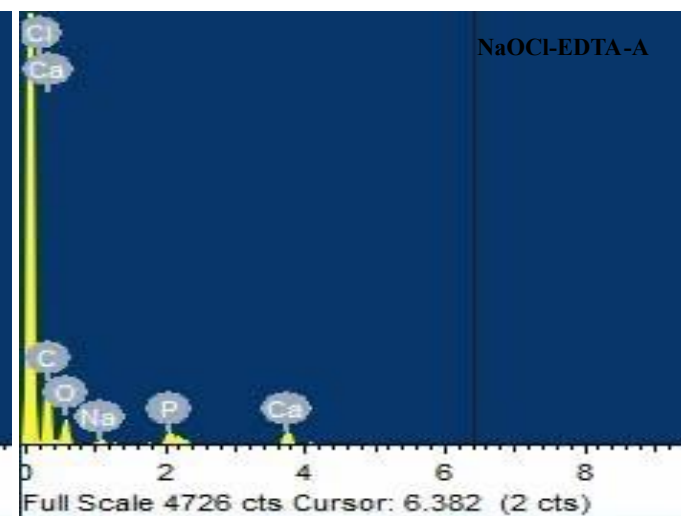
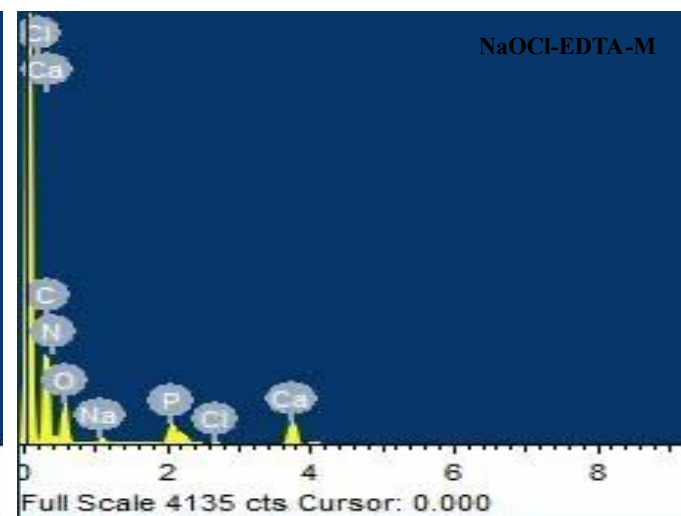
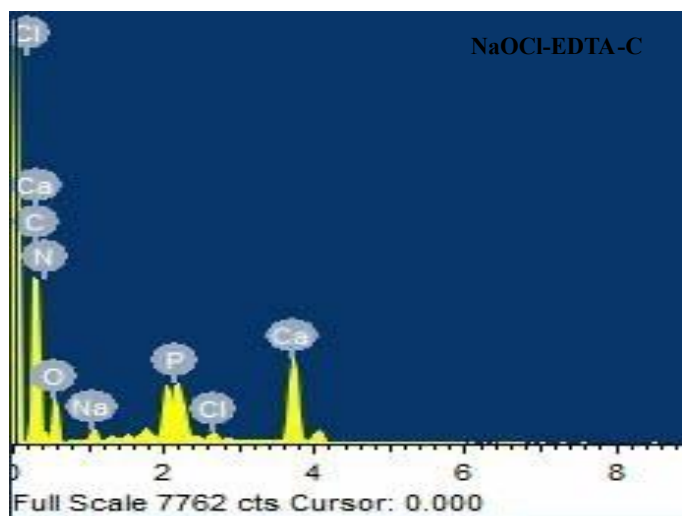
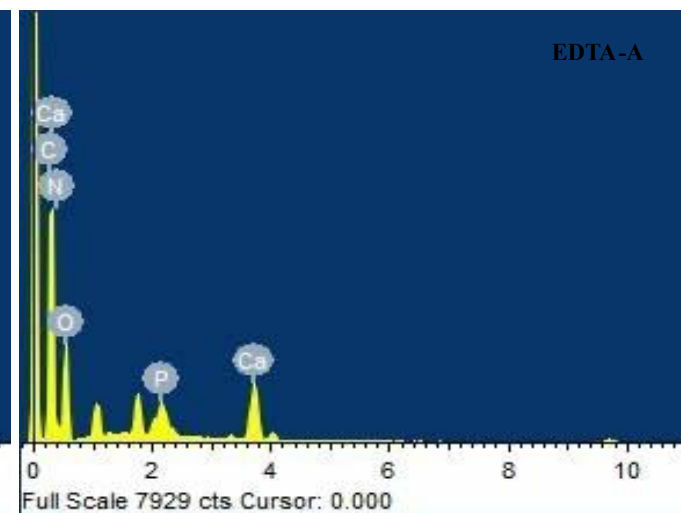
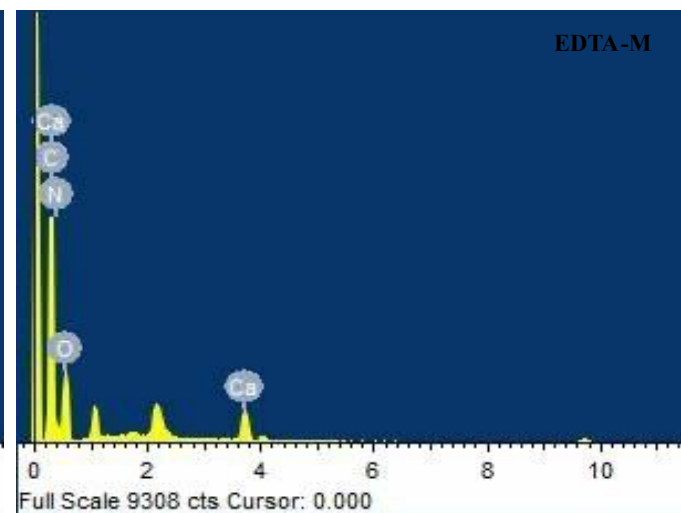
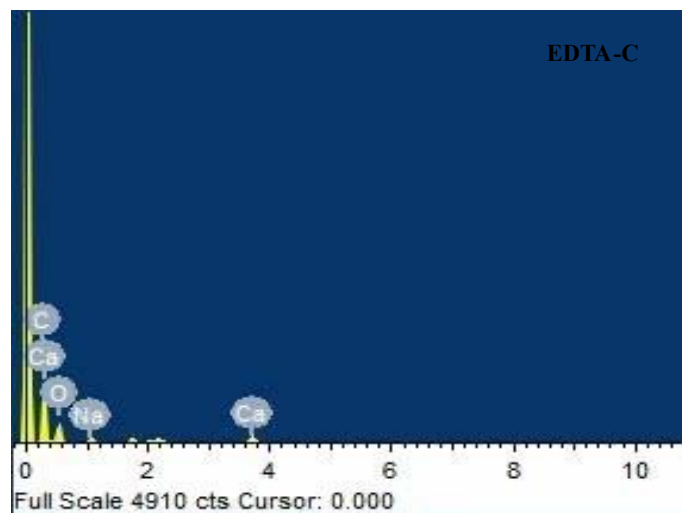


Figure 3.11: Photomicrographs indicative secondary electron Scanning Electron Microscopy (2,000 X magnification) showing microstructural components of human root dentine from the coronal (C), middle(M) and apical (A) thirds after irrigation on surface with Distilled (DW)- Group A, Hypochlorite (NaOCl)-Group B, EDTA - Group C and Hypochlorite followed by EDTA (NaOCl-EDTA)- Group D, Etidronic acid mixed with hypochlorite (HEDP+NaOCl)- Group E and Etidronic acid mixed with Distilled water (HEDP+DW)- Group F. Yellow arrow indicates smear layer, red arrow indicates erosion of dentinal tubule.





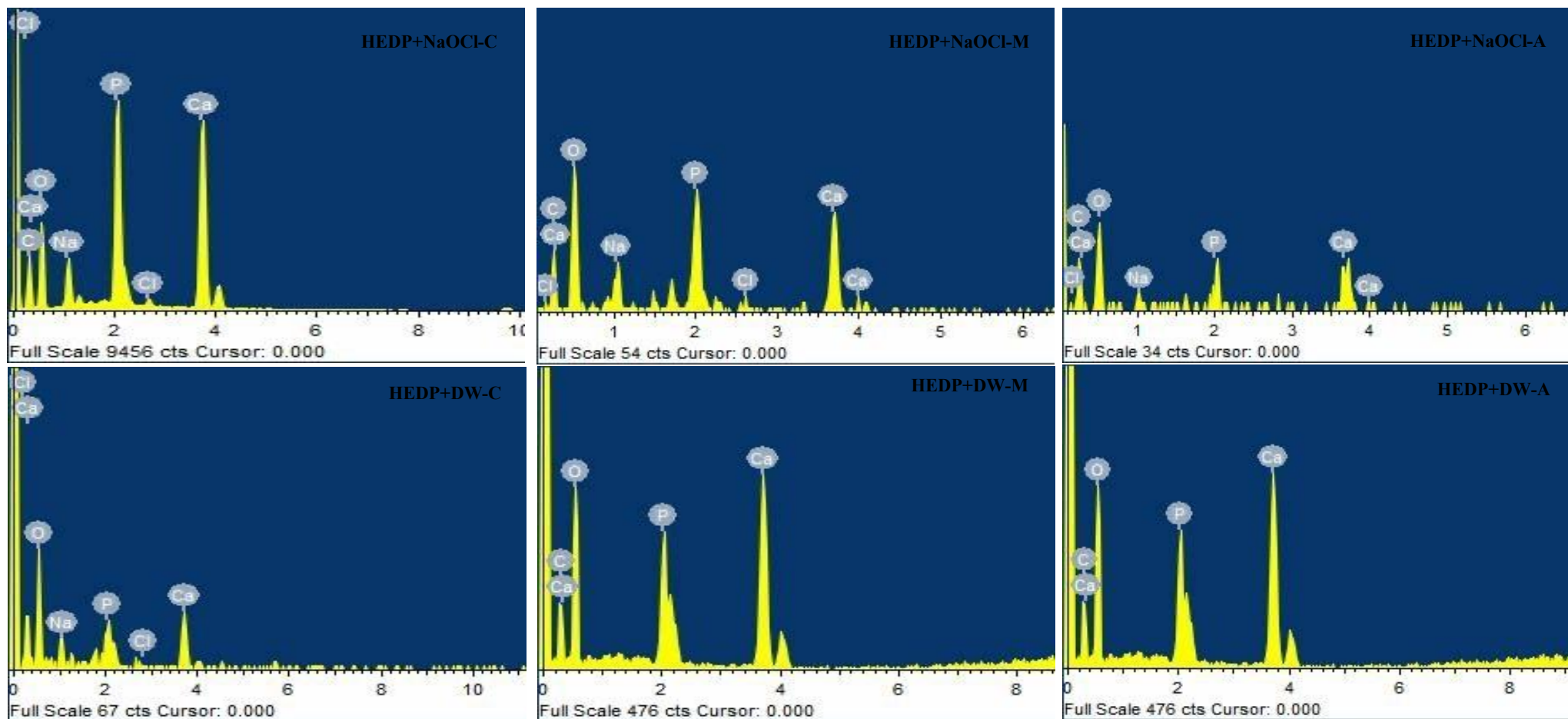


Figure 3.12: Corresponding Energy Dispersive Spectroscopy plots showing elemental components of human root dentine from the coronal (C), middle (M) and apical (A) thirds after irrigation on surface with Distilled Water (DW)- Group A, Hypochlorite (NaOCl)-Group B, EDTA-Group C, Hypochlorite followed by EDTA (NaOCl-EDTA) Group D, Etidronic acid mixed with hypochlorite (HEDP+NaOCl)- Group E and Etidronic acid mixed with Distilled water (HEDP+DW)- Group F. Increased intensity of Sodium (Na) & Chloride (Cl) and reduced Carbon (C) with the NaOCl-treated dentin as compared to control. Increased carbon (C) levels and decrease in the level of oxygen (O) and calcium (Ca) was seen with EDTA groups as compared to controlling and Cl levels increased in HEDP+NaOCl.

3.4.2 Fourier transform infrared spectroscopy

The FT-IR spectra of dentine showed peaks at 1650, 2148, and 1398 cm^{-1} , characteristic of the amide I, amide II, and III bands respectively (Figure 3.13). Bands were also observed at 3350 and 3307 cm^{-1} , representing the stretching of $-\text{OH}$. 860 and 2009 cm^{-1} bands represented carbonate peaks while 980-1000 peaks were of phosphate. The samples of the EDTA group demonstrated a prominent amide peak as compared to phosphate, eliciting demineralisation of dentine in comparison to the HEDP+NaOCl group where amide peaks exhibited weakening and superior phosphate peak indicating denaturation of collagen. The NaOCl-EDTA group displayed a reduction in all phosphate, amide, and carbonate peaks implying both denaturation and demineralisation of dentine.

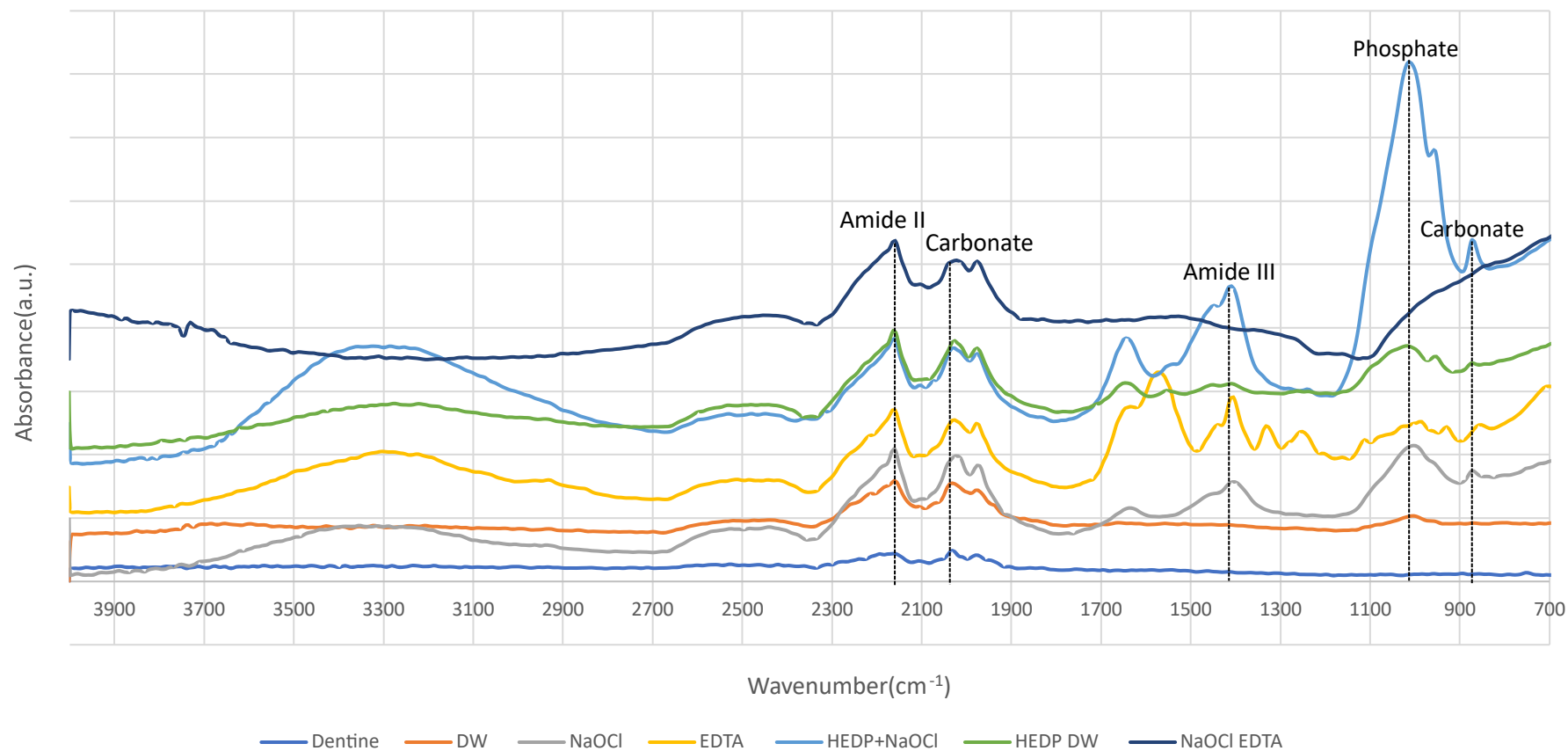


Figure 3.13: Fourier transform infrared spectroscopy (FT-IR) of dentine plain and dentine exposed to different irrigation protocols: Distilled Water (DW)- Group A, Hypochlorite (NaOCl)-Group B, EDTA -Group C, Hypochlorite followed by EDTA (NaOCl-EDTA)- Group D, Etidronic acid mixed with hypochlorite (HEDP+NaOCl)- Group E and Etidronic acid mixed with Distilled water(HEDP+DW)- Group F.

3.5 Preliminary investigation of the dentine sealer Interface using fluorescent dyes

The dentine sealer interface was assessed using confocal laser scanning microscopy using Rhodamine B and Orange G. To ensure that the dyes did not alter the material, the samples used for the confocal microscopy were also assessed using scanning electron microscopy, energy dispersive X-ray analysis and Fourier transform infrared spectroscopy using specimens not exposed to dyes as a control.

3.5.1 Confocal laser scanning microscopy (CLSM)

The resultant images of confocal laser scanning microscopy of dentine sealer interface for BioRoot RCS and AH Plus sealers in Figure 3.14 depicted high infiltration of Rhodamine B and Orange G dyes into the dentinal tubules all along from the interface but were inconclusive of penetration depth of the sealers mixed with those dyes.

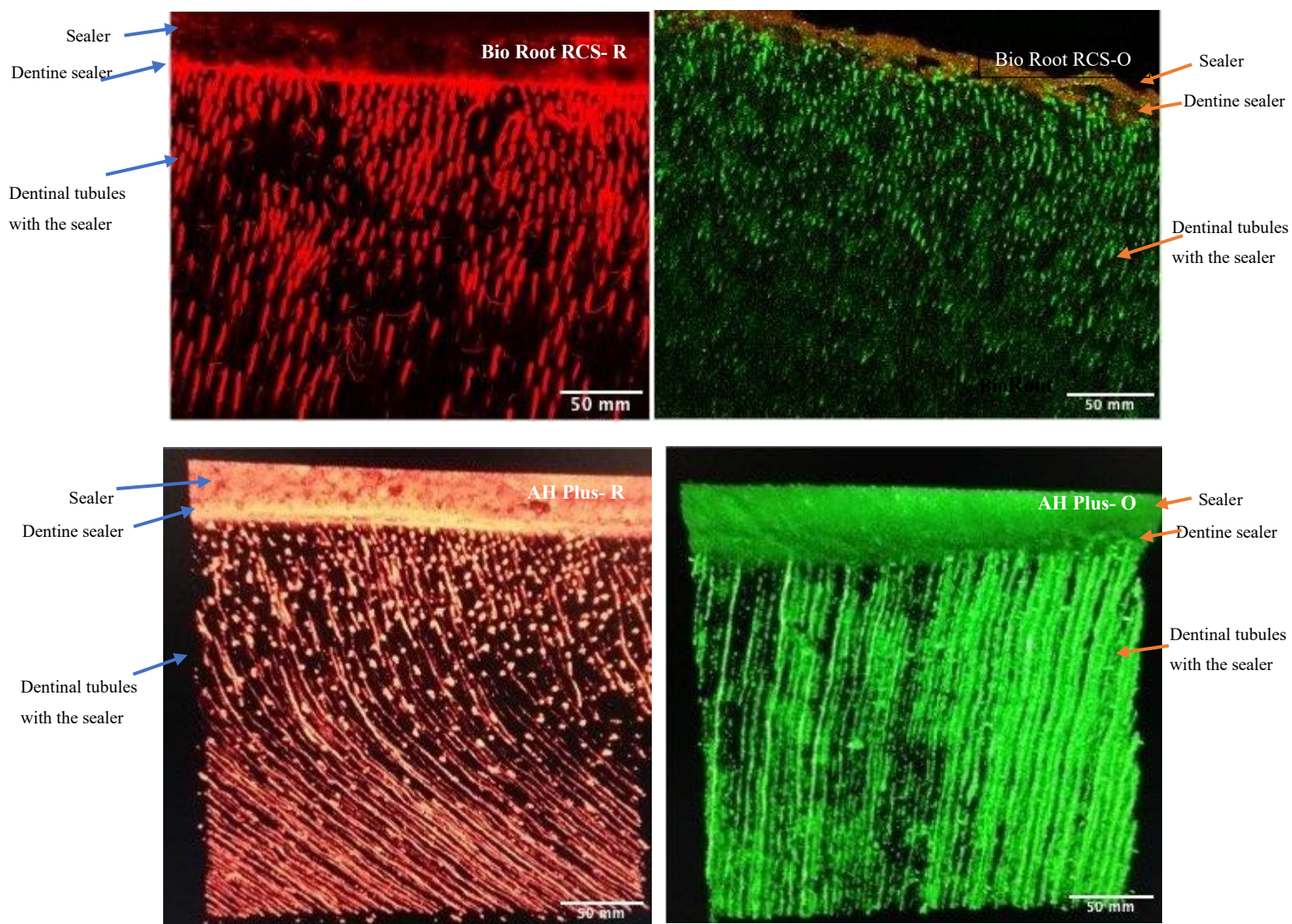


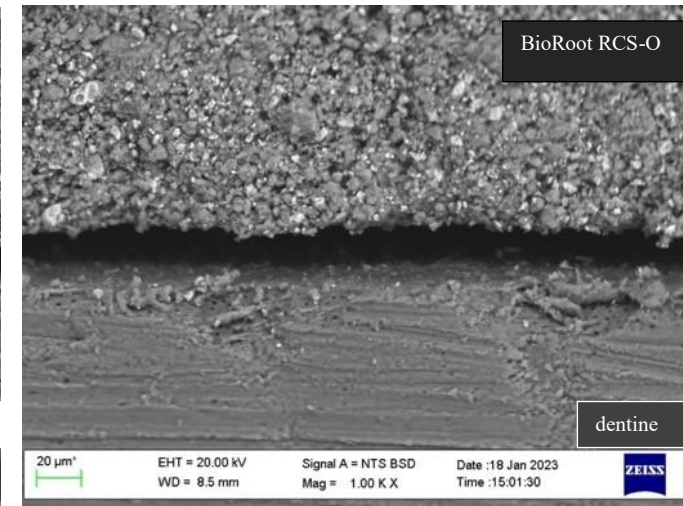
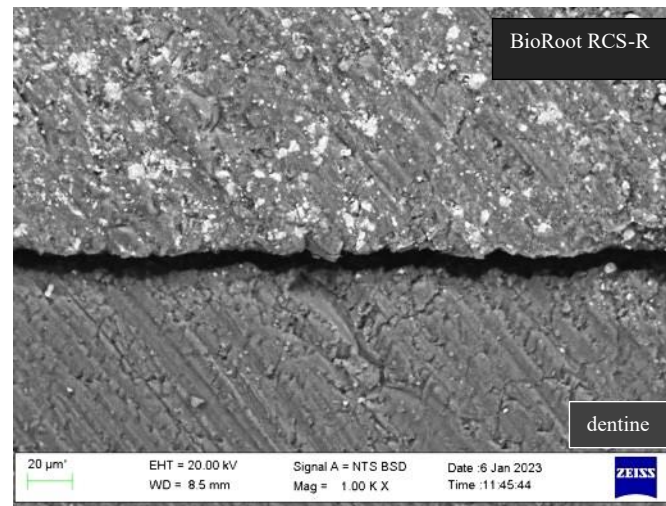
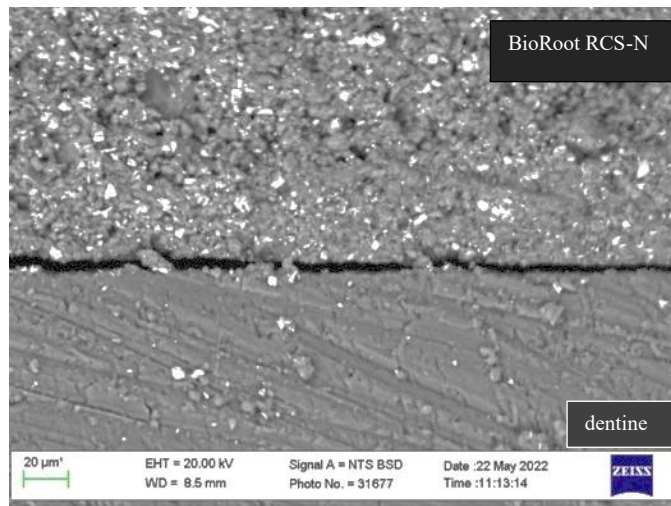
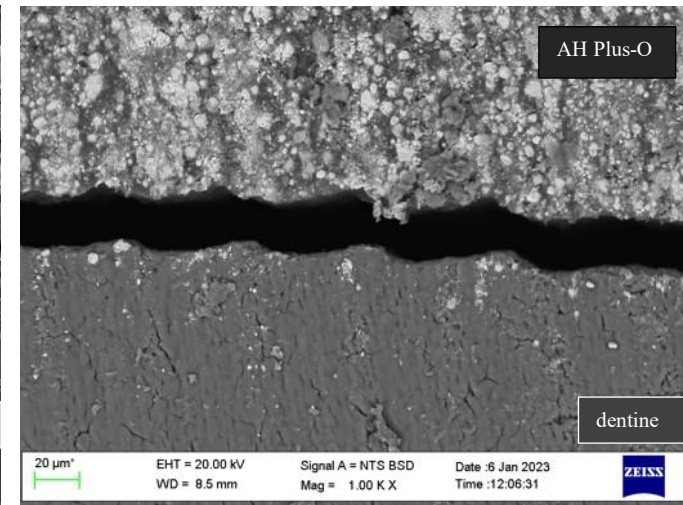
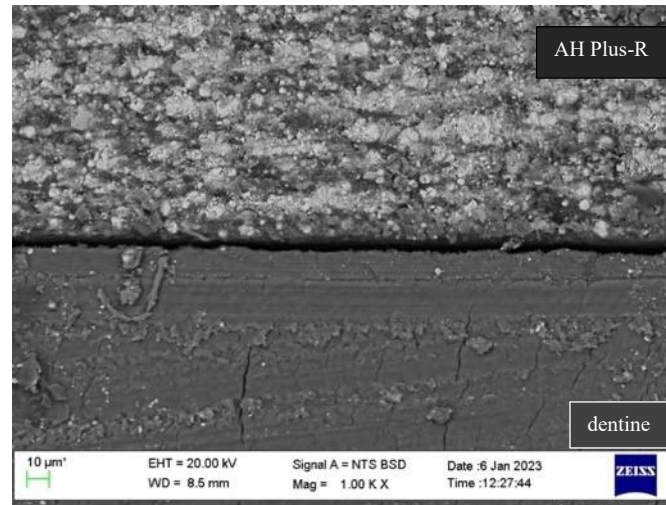
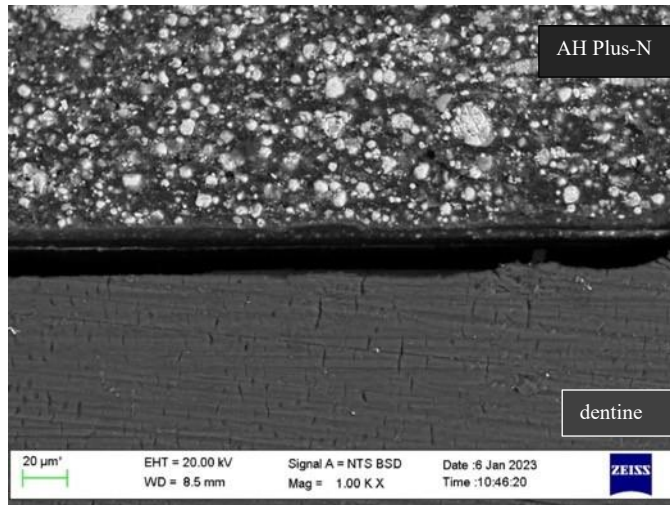
Figure 3.14: Representative fluorescence-mode CLSM images of sealer-dentin interface of BioRoot RCS and AH Plus with Rhodamine B (R) or Orange G (O) added to the sealer; showing a richly dye-infiltrated dentinal tubules.

3.5.2 Scanning Electron Microscopy (SEM)

Representative scanning electron micrographs of sealers with fluorescent dyes at the dentine sealer interface are shown in Figure 3.15. The AH Plus exhibited material microstructural changes when exposed to the dyes whereby the radiopacifier was less evident and presented washout. In the control AH Plus there was a distinct space between the tooth and the material which was not observed in the Rhodamine B group. The Orange G led to some movement of the radiopacifier from the material to the tooth structure. The BioRoot RCS was also affected by the addition of the dyes with an increase in the interfacial distance when the dye was added and microstructural changes in the material. The BioRoot Flow and TotalFill did not show microstructural changes but there was a difference in the size of the gap at the interface.

This can also be attributed to the use of a vacuum for scanning electron microscopy.

material



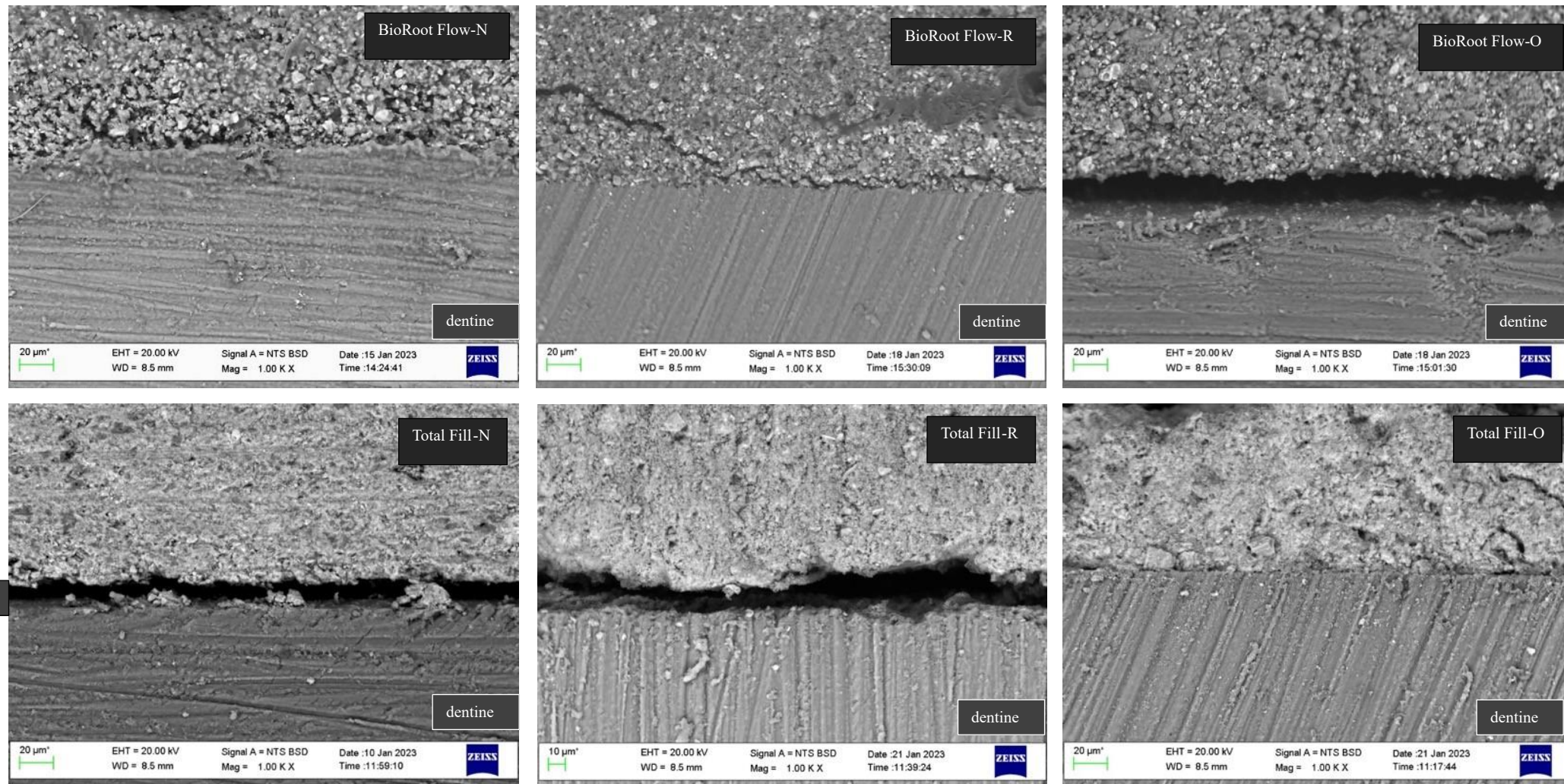


Figure 3.15: Back-scatter scanning electron micrographs of AH Plus, BioRoot RCS, BioRoot Flow and TotalFill at dentine sealer interface (1,000× magnification) showing microstructural components of three groups: Sealer with No dye (N), Sealer mixed with Rhodamine B dye (R) and Sealer mixed with Orange G dye (O).

3.5.3 Energy-dispersive X-ray spectroscopy (EDS)

EDS analyses of the atomic ratios of calcium (Ca) with silicon (Si) and calcium (Ca) with zirconium (Zr), at four different points of BioRoot RCS, BioRoot Flow, and TotalFill sealers with fluorescent dyes are depicted in Table 3.5 to 3.7 respectively. Changes were not anticipated in AH Plus sealer as zirconium is stable in Ah Plus as opposed to zirconium in hydraulic cement sealer; therefore, the ratio was not calculated.

EDS analysis indicated a gradual rise in the Ca/Zr and Ca/Si ratios, moving from material towards dentine, in all the sealer groups with or without dye except for BioRoot Flow where Ca/Si ratio was maximum at the interface for all its groups. Si and Zr content were below the detection limit towards dentine in all the groups. The result suggested the elemental composition of Ca, Si, and Zr did not show much change when Rhodamine B and Orange G dyes were used as opposed to when the dye was not used.

Table 3.5: EDS point scans of mean ratio of calcium with silicon (Ca/Si) and calcium with zirconium (Ca/Zr) of *BioRoot RCS* mixed with Rhodamine B and Orange G dye ,with no dye as control; at four different points, one point in material, two points at interface and one point in dentin. BDL: below detection limit; Inter-M: interface towards material; Inter-D: interface towards dentine.

Dye group	BioRoot RCS (Mean ratio)		Inter-M (Mean ratio)		Inter -D (Mean ratio)		Dentine (Mean ratio)	
	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr
No Dye	3.19	0.68	3.47	16.08	14.80	Zr BDL	Si BDL	Zr BDL
Rhodamine	3.18	0.42	5.80	0.91	28.44	7.89	211.81	6.20
Orange G	4.26	0.68	3.12	16.08	Si BDL	Zr BDL	Si BDL	Zr BDL

Table 3.6: EDS point scans of mean ratio of calcium with silicon (Ca/Si) and calcium with zirconium (Ca/Zr) of *BioRoot Flow* mixed with Rhodamine B and Orange G dye ,with no dye as control; at four different points, one point in material, two points at interface and one point in dentin. BDL: below detection limit; Inter-M: interface towards material; Inter-D: interface towards dentine.

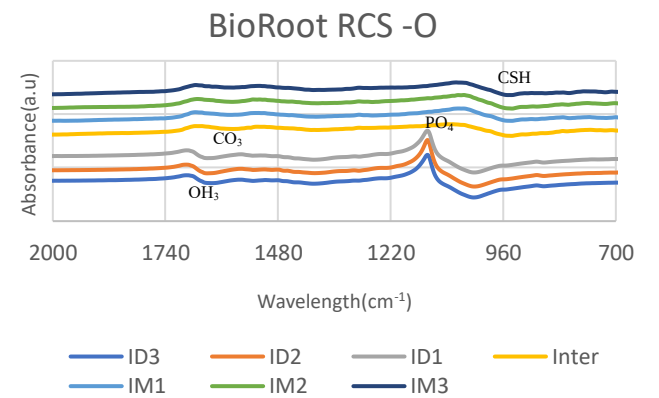
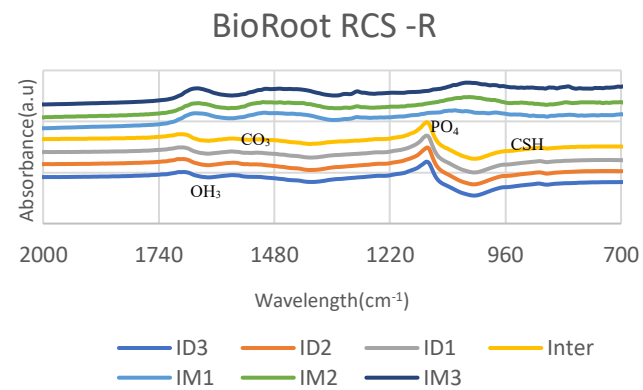
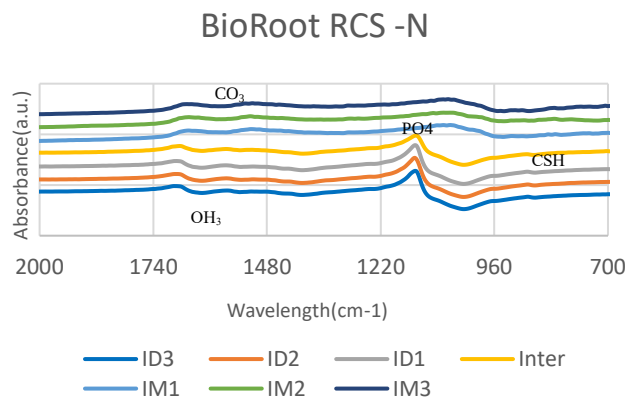
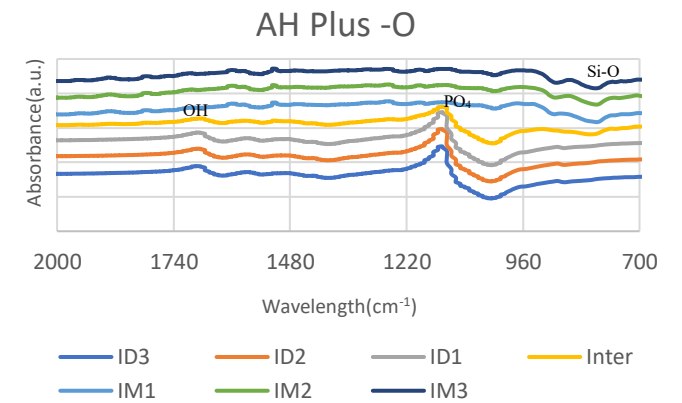
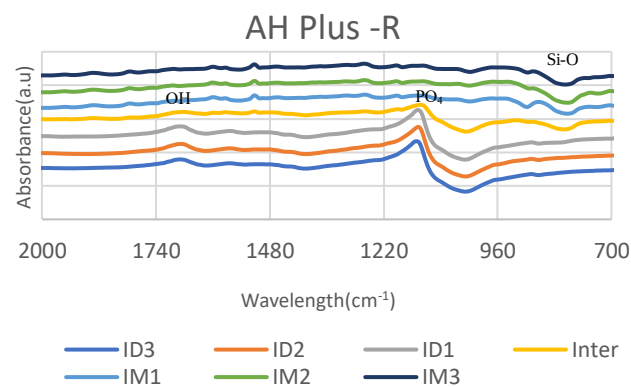
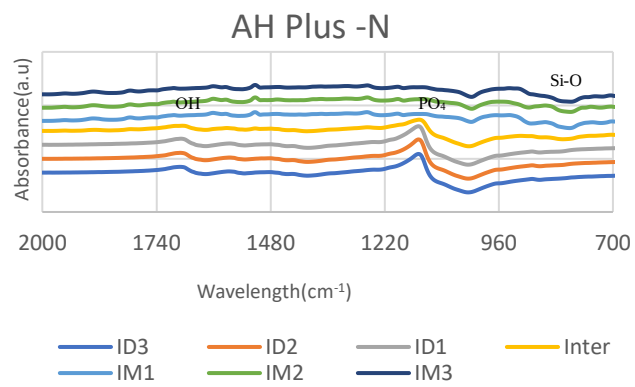
Dye group	BioRoot Flow (Mean ratio)		Inter-M (Mean ratio)		Inter -D (Mean ratio)		Dentine (Mean ratio)	
	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr
No Dye	6.98	0.47	23.21	8.19	28.77	12.30	Si BDL	Zr BDL
Rhodamine	4.63	1.75	78.23	6.72	53.95	14.17	36.96	Zr BDL
Orange G	27.93	3.28	69.61	5.39	59.09	Zr BDL	59.78	Zr BDL

Table 3.7: EDS point scans of mean ratio of calcium with silicon (Ca/Si) and calcium with zirconium (Ca/Zr) of *TotalFill* mixed with Rhodamine B and Orange G dye, with no dye as control; at four different points, one point in material, two points at interface and one point in dentin. BDL: below detection limit; Inter-M: interface towards material; Inter-D: interface towards dentine.

Dye group	TotalFill (Mean ratio)		Inter-M (Mean ratio)		Inter -D (Mean ratio)		Dentine (Mean ratio)	
	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr
No Dye	3.93	0.53	9.21	0.62	23.18	Zr BDL	Si BDL	Zr BDL
Rhodamine	14.19	1.84	4.76	0.70	Si BDL	4.72	Si BDL	15.02
Orange G	5.35	0.98	5.37	0.49	Si BDL	20.22	Si BDL	19.01

3.5.4 Fourier transform infrared spectroscopy

Representative FT-IR spectrums in Figure 3.16 displays phosphate (PO_4) peak at 1100- 1130 in dentin and OH peaks are seen at around 1600 in dentine and sealer both in all the sealer groups. In tricalcium silicate sealer groups a peak at 1400 to 1500 is of carbonate, seen with more intensity towards the material side and 960 peak in the material indicating calcium silicate hydrate (CSH). An increase in the intensity could be seen in the 803 Si-O peaks of the epoxy resin of AH Plus when the dye is incorporated into the resin sealer. Carbonate and CSH peaks intensify with the addition of dyes in the BioRoot RCS, BioRoot Flow, and TotalFill while the phosphate peak fades towards dentin at the interface.



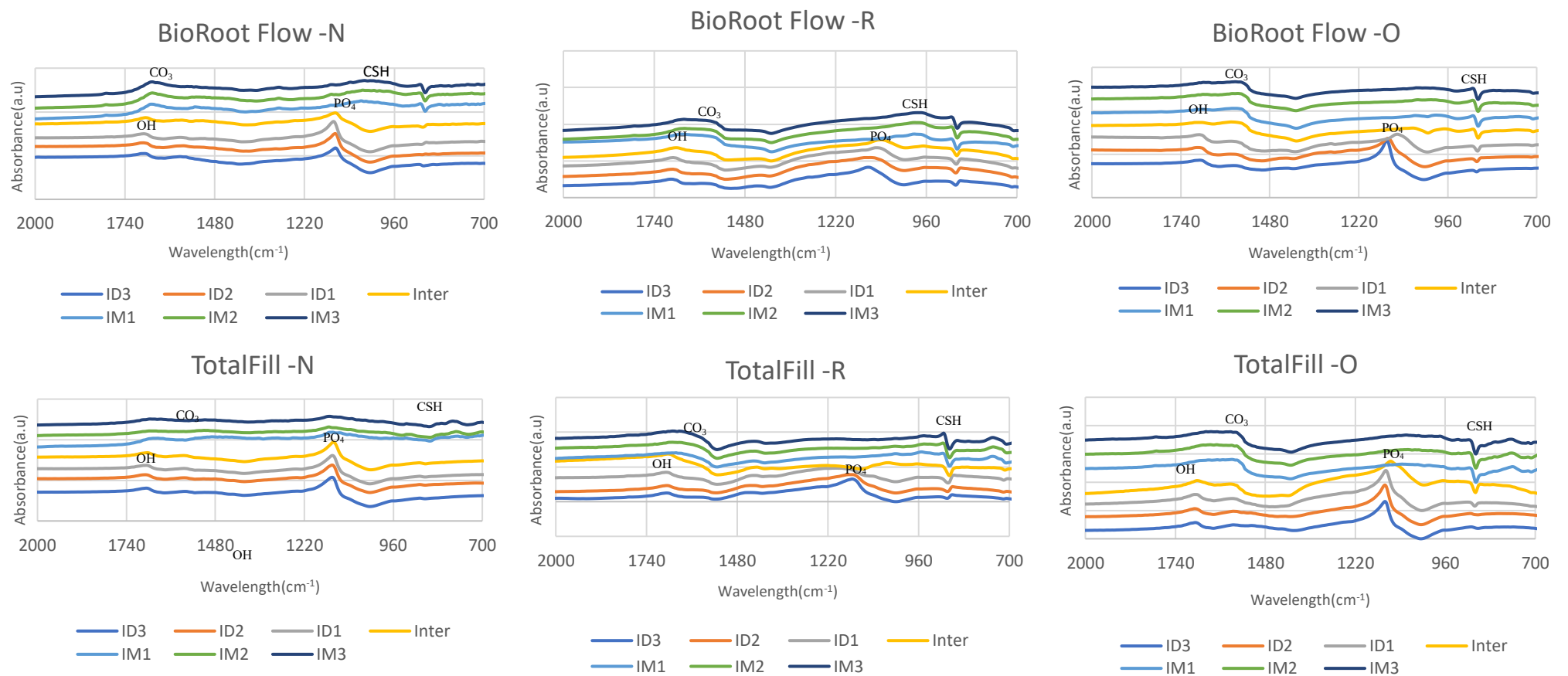
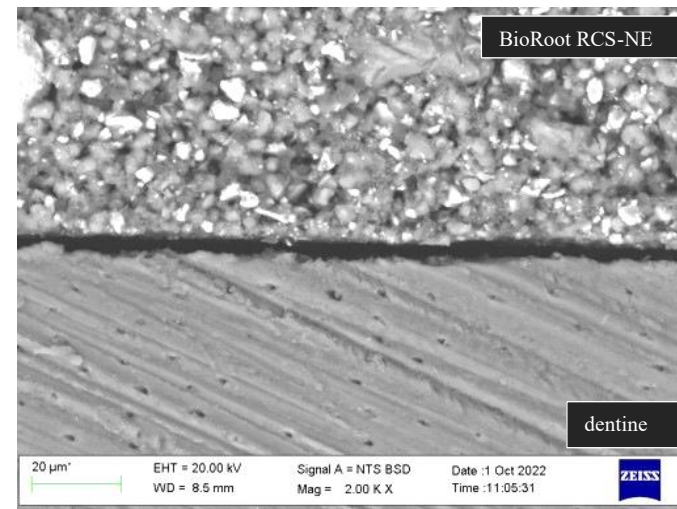
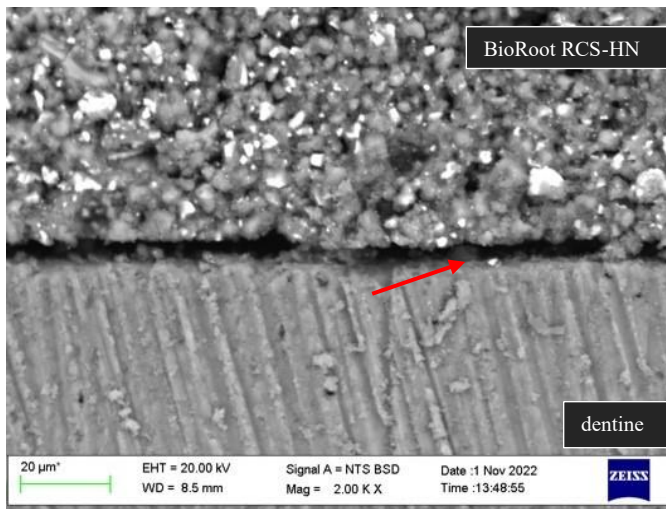
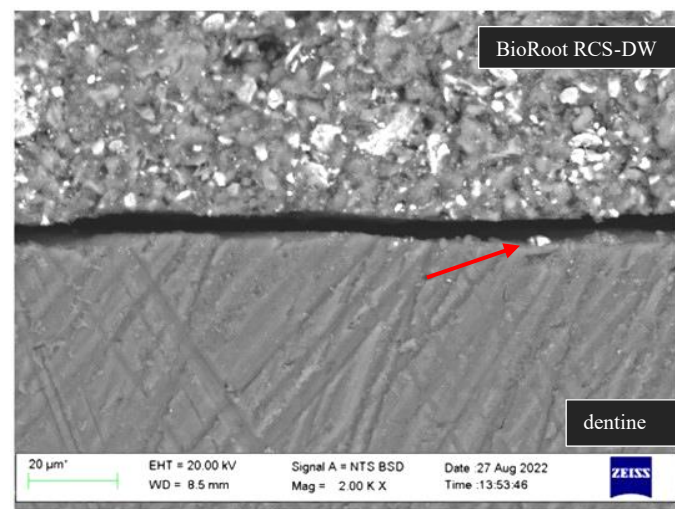
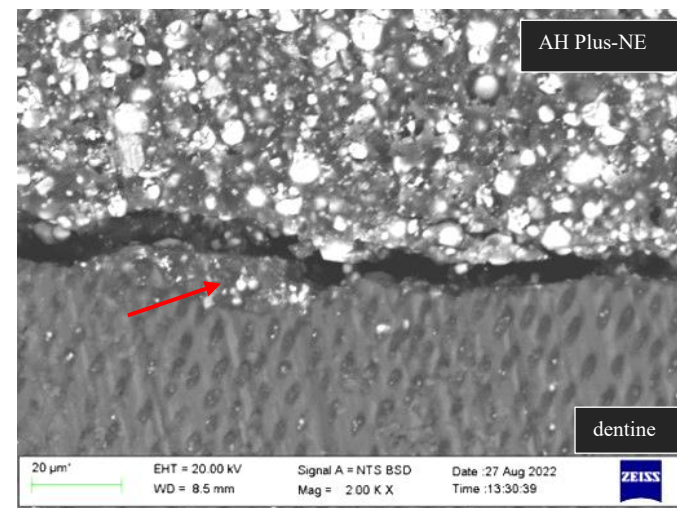
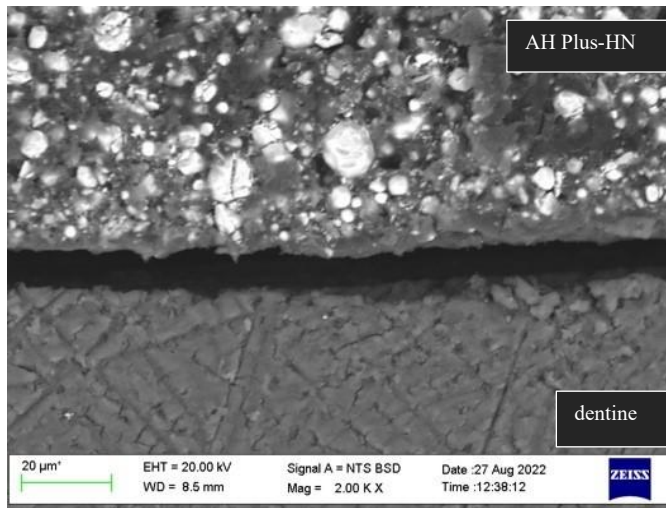
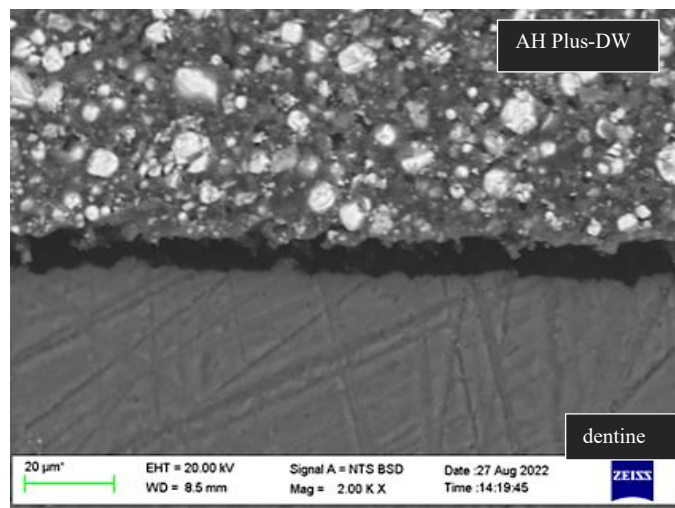


Figure 3.16: Fourier transform infrared spectroscopy point scans of AH Plus, BioRoot RCS, BioRoot Flow and TotalFill at 3 points in dentine(D), 1 point at interface (Inter/I) and 3 points in sealer/material(M) at interface (Inter/I) and 3 points in sealer/material(M) at 300 μ m, 200 μ m and 100 μ m distance from interface. [ID3, ID2, ID1=point at 300,200,100 μ m distance from interface in dentine. [IM3,IM2,IM1= point at 300, 200, 100 μ m distance from interface in material]. Results were assessed within the spectral range of 700 to 2000 of three groups : Sealer with No dye(N) ,Sealer mixed with Rhodamine B dye (R) and Sealer mixed with Orange G dye (O). Increase in intensity in the 803 Si-O peaks of AH Plus when the dye is incorporated into the resin sealer. Carbonate and CSH peaks intensify with the addition of dyes in the BioRoot RCS, BioRoot Flow, and TotalFill while the phosphate peak fades towards dentin at the interface.[Silica(SiO), Phosphate (PO₄),Hydroxyl (OH), Calcium Silicate Hydrate (CSH), and carbonate(CO₃).

3.6 Investigation of dentine sealer interface using a Fourier transform infrared spectroscopy method

3.6.1 Scanning Electron Microscopy (SEM)

The scanning electron micrographs in the backscatter mode of specific microstructural components are presented in Figure 3.17. The samples when viewed under scanning electron microscope revealed cracks, debonding and shrinking from dentine at the interface due to vacuum drying and desiccation during sample preparation. These cracks could be seen depicted by red arrows in most of the samples in Figure 3.17. Therefore, this method of investigation for analysing sealer adaptation to dentine at their interface, could not provide much information, especially when working with hydraulic cements which shrink on final setting.



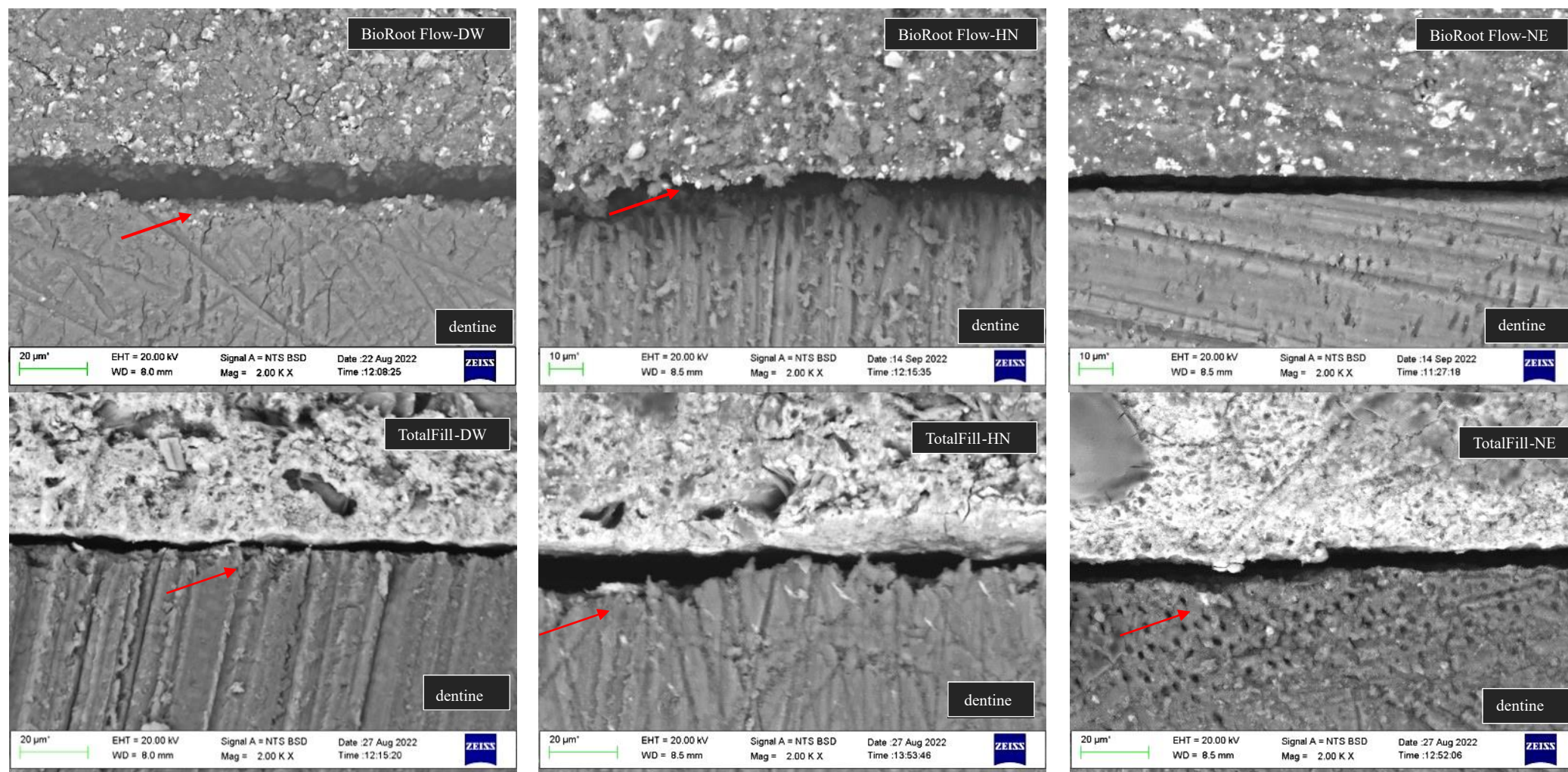


Figure 3.17: Back-scatter scanning electron micrographs of dentine sealer interface of AH Plus, BioRoot RCS, BioRoot Flow and Total Fill sealers (2,000× magnification) showing microstructural components irrigated with three irrigant groups; Distilled water-(DW), HEDP mixed with hypochlorite (NaOCl)-(HN) and Hypochlorite (NaOCl) followed by EDTA-(NE). Red arrow depicts the detached and cracked interface of sealer to dentine.

3.6.2 Energy-dispersive X-ray spectroscopy (EDS)

EDS analyses of the mean atomic ratios (with standard deviation) of calcium (Ca) with silicon (Si) and calcium (Ca) with zirconium (Zr), at four different points, are depicted in Tables 3.8 to 3.10. Since AH Plus is an inert material, the data could not be calculated.

EDS analysis of BioRoot RCS on the dentine surface in Table 3.8 describes that higher concentrations of Si and Zr elements were detected towards the dentine sealer interface in the NaOCl+HEDP irrigant group as compared to the NaOCl-EDTA group and least in DW. This was suggested by the lower Ca/Zr and Ca/Si ratio which was 6.67 and 0.98 respectively in the NaOCl-EDTA group while it was the opposite for BioRoot Flow where Ca/Zr and Ca/Si ratio was 105 and 11.35 respectively for NaOCl-EDTA which was greater than NaOCl+HEDP. . However, in the control group (distilled water) the content of Si and Zr was below the detection limit, towards the dentine, thus ratio could not be calculated. Traces of Zr could be seen in dentine for the other two irrigant groups.

In Table 3.10 of TotalFill, Ca/Si and Ca/Zr ratio was similar for most of the points for NaOCl-EDTA and NaOCl+HEDP groups whereas the ratio for DW was high at the interface. Traces of Zr could be seen in dentine for BioRoot RCS and TotalFill with group A and group B . Overall calcium content increased while progressing from material to dentine.

Table 3.8: EDS point scans of mean ratio of calcium with silicon (Ca/Si) and calcium with zirconium (Ca/Zr) after irrigation with three groups and using sealer as *BioRoot RCS* at three distinct locations, material, interface, and dentin at 4 different points. One point in material, two points at interface and one point in dentin, with standard deviation.

(NaOCl+HEDP= hypochlorite mixed with etidronic acid, NaOCl-EDTA= Hypochlorite followed by EDTA, DW=distilled water), BDL: below detection limit; Inter-M: interface towards material; Inter-D: interface towards dentine.

Irrigants group	BioRoot RCS (Mean ratio)		Inter-M (Mean ratio)		Inter -D (Mean ratio)		Dentine (Mean ratio)	
	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr
DW	4.87 ± 0.84	1.21 ± 0.59	4.36 ± 2.03	4.54 ± 5.52	37.12 ± 46.96	2.09 ± 3.63	Si BDL	Zr BDL
NaOCl+HEDP	5.89 ± 1.94	25.14 ± 21.57	4.11 ± 0.59	3.69 ± 4.05	41.72 ± 28.26	5.92 ± 3.35	51.46 ± 18.37	Zr BDL
NaOCl-EDTA	3.69 ± 0.72	13.62 ± 15.11	7.21 ± 3.24	4.57 ± 3.06	105.44 ± 61.79	11.35 ± 5.49	28.13 ± 36.24	7.29 ± 2.46

Table 3.9: EDS point scans of mean ratio of calcium with silicon (Ca/Si) and calcium with zirconium (Ca/Zr) after irrigation with three groups and using sealer as *BioRoot Flow* at three distinct locations, material, interface, and dentin at 4 different points. One point in material, two points at interface and one point in dentin, with standard deviation. (NaOCl+HEDP= hypochlorite mixed with etidronic acid, NaOCl-EDTA= Hypochlorite followed by EDTA, DW=distilled water, BDL: below detection limit. Inter-M: interface towards material; Inter-D: interface towards dentine.

Irrigants group	BioRoot Flow (Mean ratio)		Inter-M (Mean ratio)		Inter -D (Mean ratio)		Dentine (Mean ratio)	
	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr
NaOCl+HEDP	2.73 ± 0.46	0.88 ± 0.47	3.57 ± 0.71	2.04 ± 0.53	27.30 ± 4.34	13.97 ± 4.35	Si BDL	Zr BDL
NaOCl-EDTA	3.68 ± 0.44	2.38 ± 1.97	27.59 ± 6.95	5.64 ± 5.96	28.03 ± 48.5	Zr BDL	Si BDL	Zr BDL
DW	3.73 ± 1.08	2.489 ± 1.8	5.07 ± 2.75	9.33 ± 10.11	Si BDL	Zr BDL	Si BDL	Zr BDL

Table 3.10: EDS point scans of mean ratio of calcium with silicon (Ca/Si) and calcium with zirconium) after irrigation with three groups using sealer *TotalFill* at three distinct locations, material, interface, and dentin at 4 different points. One point in material, two points at interface and one point in dentin, with standard deviation. (NaOCl+HEDP= hypochlorite mixed with etidronic acid, NaOCl-EDTA= Hypochlorite followed by EDTA, DW=distilled water, BDL: below detection limit; Inter-M: interface towards material; Inter-D: interface towards dentine.

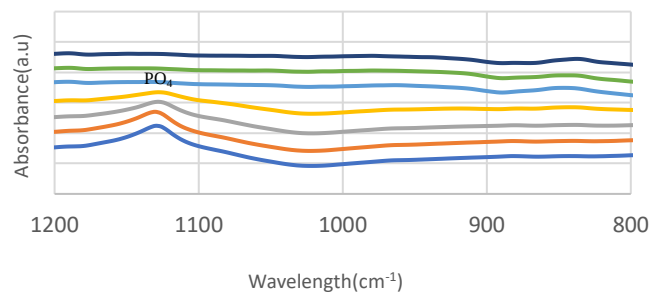
Irrigants group	TotalFill (Mean ratio)		Inter-M (Mean ratio)		Inter -D (Mean ratio)		Dentine (Mean ratio)	
	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr
NaOCl+HEDP	3.61 ± 0.45	0.58 ± 0.44	24.67 ± 19.67	1.80 ± 1.85	Si BDL	Zr BDL	Si BDL	3.81 ± 1.81
NaOCl-EDTA	3.73 ± 0.31	0.29 ± 0.16	4.99 ± 1.53	0.41 ± 0.06	45.14 ± 58.67	4.64 ± 2.99	Si BDL	9.56 ± 0.63
DW	4.23 ± 1.46	0.56 ± 0.1	5.07 ± 1.45	0.35 ± 0.06	31.88 ± 6.72	2.36 ± 0.89	0.63 ± 0.89	7.66 ± 8.13

3.6.3 Fourier transform infrared spectroscopy

The intensity of the dentin characteristic phosphate peak at 1100 cm^{-1} was used to quantify the degree of mineralization across the cement-dentin interface. The peaks at 950 to 1000 which are associated with the presence of calcium silicate hydrate (CSH) were selected as peaks characteristic of hydraulic cement. Representative FT-IR plots of the dentine sealer interface are presented in Figure 3.18.

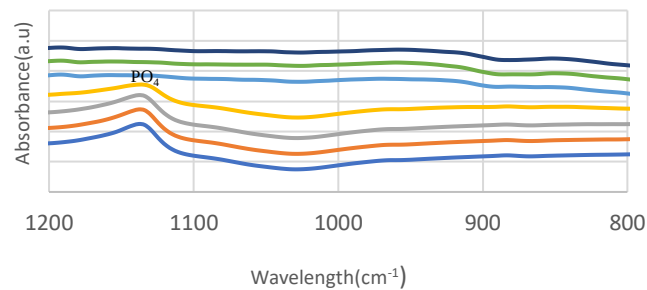
At the transition area of the sealer-dentine interface, the intensity of the phosphate peak was reduced with hydraulic cement as compared to that in the solid dentin area, while the intensities of the CSH peak were reduced gradually towards dentin. Shallowing of the phosphate peak could be seen more when the sealer was used with NaOCl-EDTA irrigation group. AH Plus did not show much difference at the interface with irrigant groups. increased

AH Plus DW



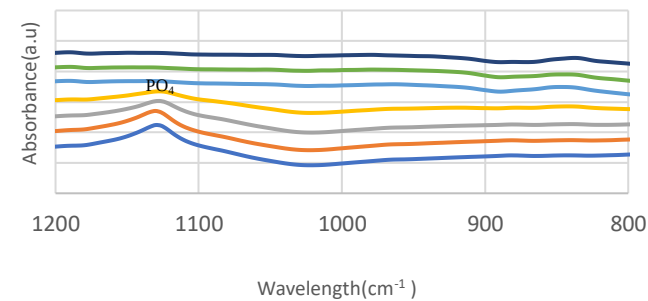
ID3 ID2 ID1 inter
IM1 IM2 IM3

AH Plus NH



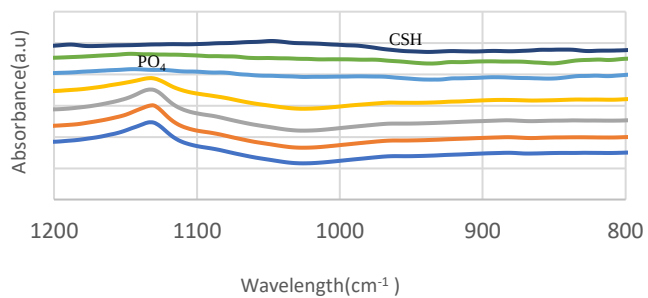
ID3 ID2 ID1 inter
IM1 IM2 IM3

AH Plus NE



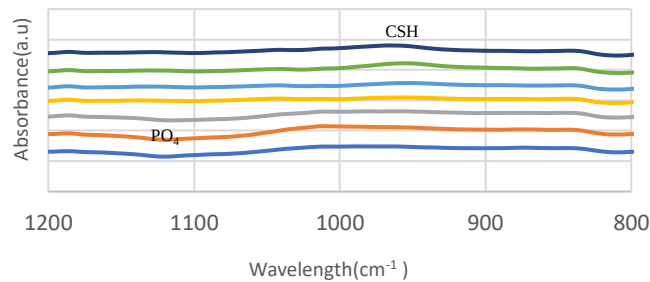
ID3 ID2 ID1 inter
IM1 IM2 IM3

BioRoot RCS DW



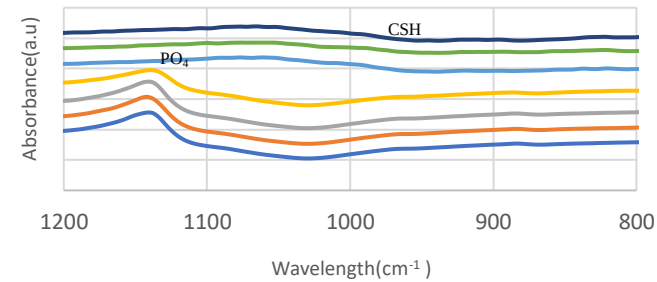
ID3 ID2 ID1 inter
IM1 IM2 IM3

BioRoot RCS NH



ID3 ID2 ID1 inter
IM1 IM2 IM3

BioRoot RCS NE



ID3 ID2 Inter Inter
IM1 IM2 IM3

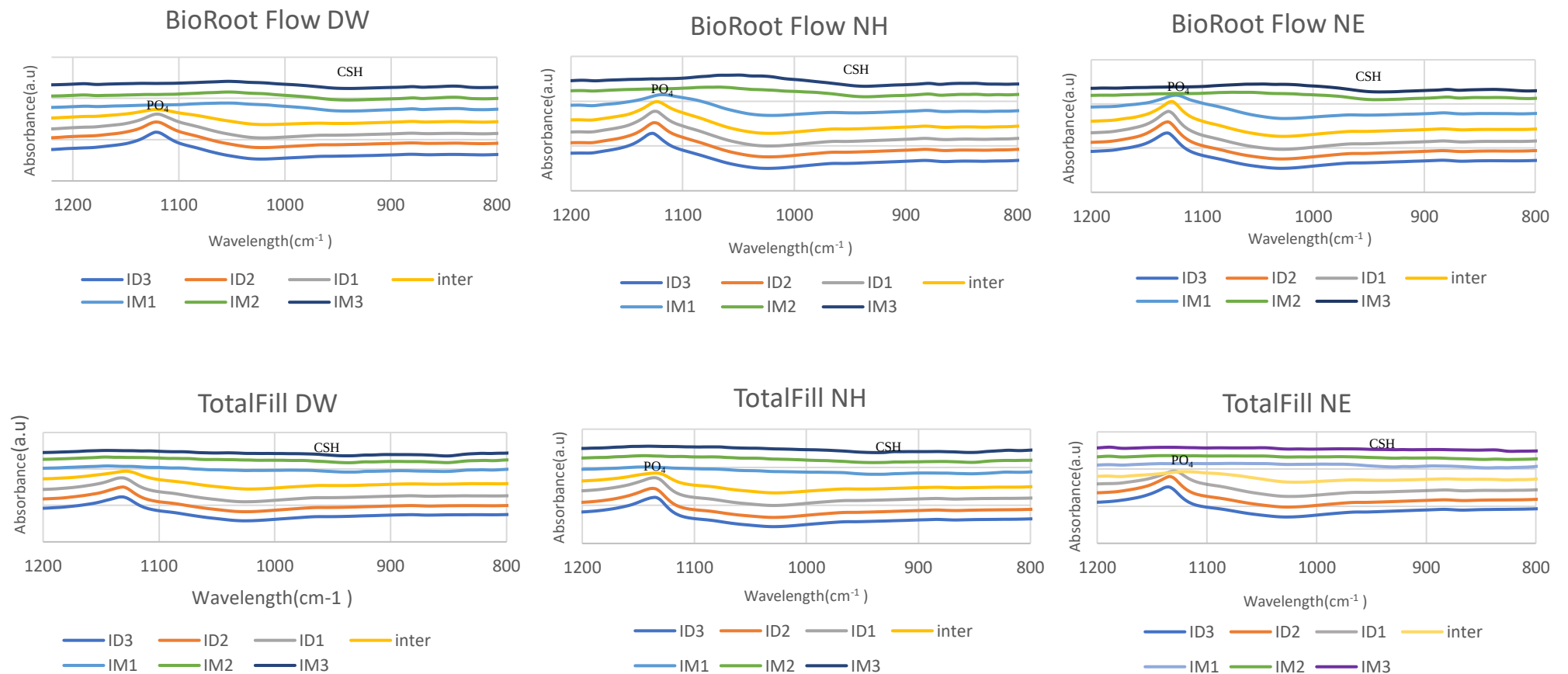


Figure 3.18: Fourier transform infrared spectroscopy point scans of AH Plus, BioRoot RCS, BioRoot Flow and TotalFill at 3 points in dentine(D) ,1 point at interface (Inter/I) and 3 points in sealer/material(M) at 300μm, 200μm and 100μm distance from interface. .[ID3, ID2, ID1=point at 300,200,100 μm distance from interface in dentine. IM3, IM2, IM1= point at 300, 200, 100 μm distance from interface in material]. Results were assessed after irrigation within the spectral range of 800 to 1200 with three irrigant groups: Distilled water-(DW), HEDP mixed with hypochlorite (NH) and Hypochlorite followed by EDTA-(NE). (Phosphate (PO₄) peak in dentine was reduced with hydraulic cement as compared to that dentine area , while the intensities of the Calcium silicate hydrate (CSH) were reduced gradually towards dentine.

3.6.4 Intratubular infection test pilot study

Representative confocal laser scanning microscope images after staining with live and dead stains are shown in Figures 3.19 to 3.21. The green fluorescence indicates live bacteria and red signifies dead ones. Figures 3.20 depicts *E. faecalis* infected dentinal tubules after irrigation with Group A Distilled Water (DW), Group B HEDP+NaOCl (hypochlorite with etidronic acid), Group C NaOCl-EDTA (hypochlorite followed by EDTA), followed by placement of BioRoot RCS sealer. The autofluorescence of dentine is visible so the live and dead cells could not be differentiated. Figure 3.20 depicts a distinct bacterial infiltration in dentinal tubules after modifying the original staining ratio & subtracting the dentine autofluorescence gain. Following the same pattern as Figure 3.20, ethanol was used as a control in Figure 3.21, but despite pursuing the same protocol clear images could not be acquired for counting the dead and live cells. Looking at the results, this methodology was found unsuitable for the research question and thus another approach to determining the antimicrobial activity of sealers after irrigation at the dentine sealer interface was used in this study.

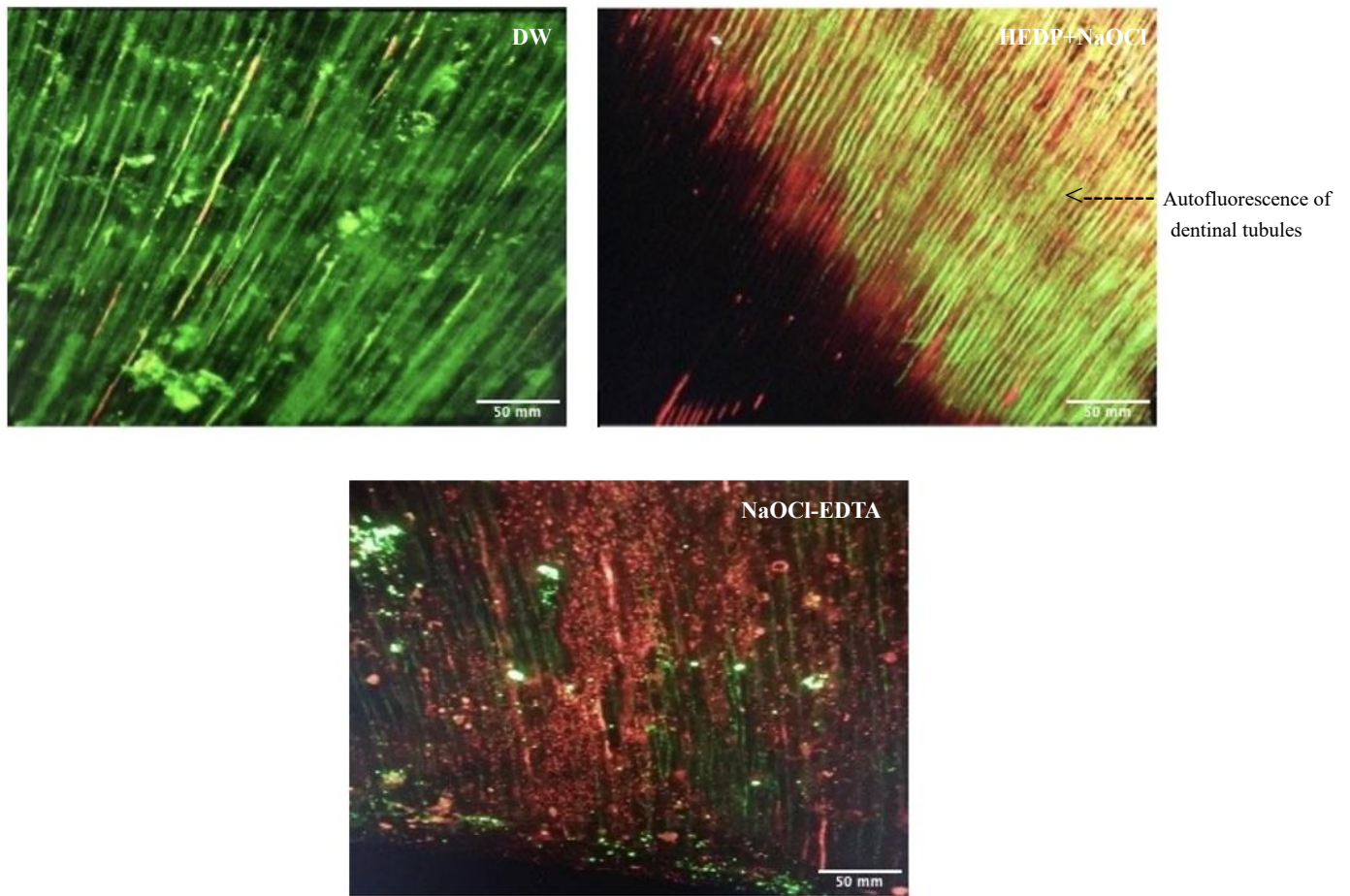


Figure 3.19: Representative confocal laser scanning microscope images of *E. faecalis* infected dentinal tubules after irrigation with distilled water (DW), HEDP+NaOCl (hypochlorite with etidronic acid), NaOCl-EDTA (hypochlorite followed by EDTA), followed by placement of BioRoot RCS sealer. The viability staining indicated live cells in green, and dead cells in red. Scanning was performed using 40xwater lens, 1 μm step size. Dentinal tubules showed autofluorescence.

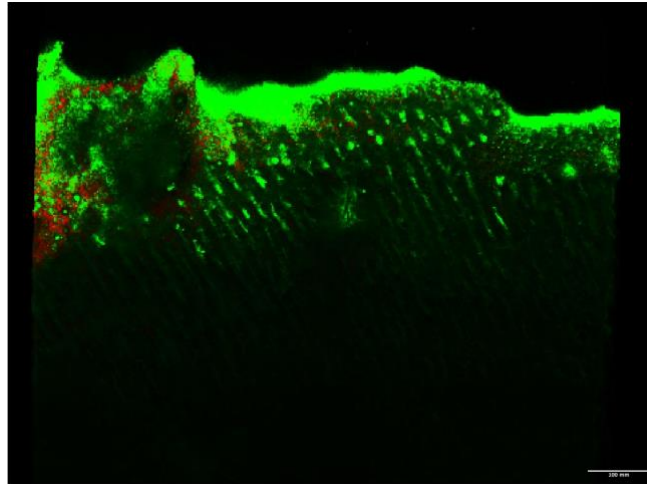


Figure 3.20: Representative confocal laser scanning microscope image of *E. faecalis* infected dentinal tubules after reducing the dentine autofluorescence. The viability staining indicated live cells in green, and dead cells in red. Scanning was performed using 40xwater lens, 1 μ m step size.

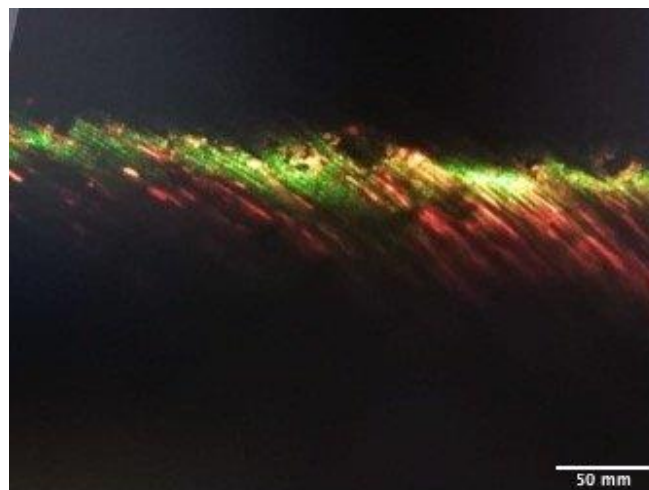


Figure 3.21: Representative confocal laser scanning microscope images of *E. faecalis* infected dentinal tubules after treating with ethanol. The viability staining indicated live cells in green, and dead cells in red. Scanning was performed using 40xwater lens, 1 μ m step size.

3.6.5 Attachment Assay/Direct Contact Test for Dentine Sealer Interface

The results of the DCT after 7 days of aging and 24 hrs of direct contact are shown in Figure 3.22 & Figure 3.23. This test was used to evaluate changes in the antimicrobial effect of each sealer in contact with dentine and vice versa at the dentine sealer interface. In Figure 3.22 all the sealers depicted a reduction in their antimicrobial effect when in contact with dentine as compared to sealers with no contact (control). AH Plus showed the least antibacterial activity of 2×10^4 CFU/ml ($p < 0.05$) with all the irrigants. However, BR RCS sealer showed the highest bactericidal effect as compared to all other sealers. Overall, the sealers with NaOCl- EDTA irrigation exhibited maximum bactericidal effect as opposed to other irrigation groups.

DCT analysis of dentine at the dentine sealer interface is described in Figure 3.23. In general, dentine with or without sealer contact did not show many differences at the dentin sealer interface. However, dentine when irrigated with NaOCl- EDTA, after contact with a sealer, displayed the greatest antibacterial activity at 1×10^5 CFU/ml ($p < 0.05$).

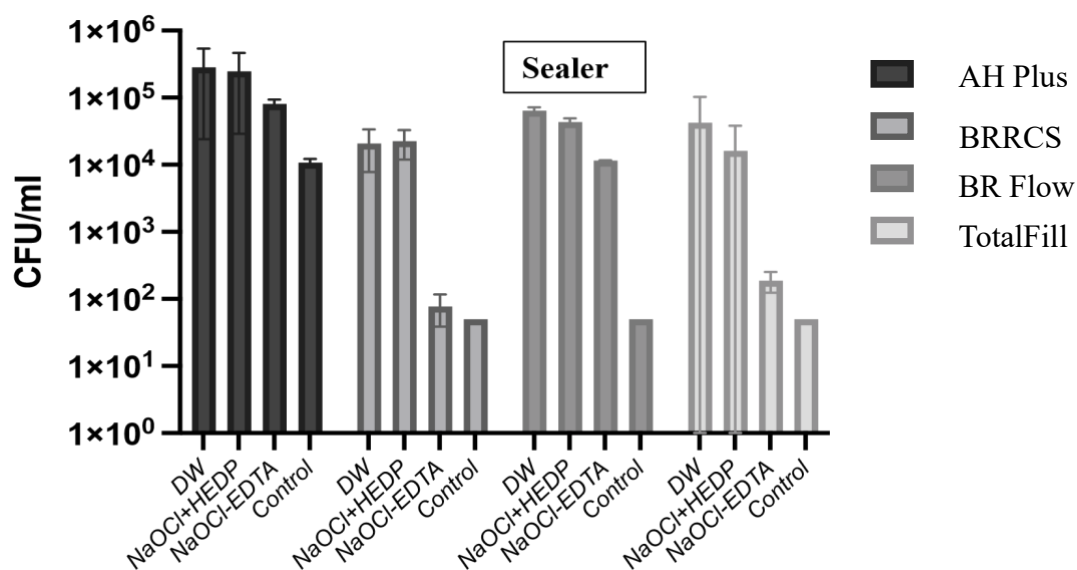


Figure 3.22: Number of CFU (Colony Forming Unit) of *E. faecalis* in the presence of AH Plus, BioRoot RCS, BioRoot Flow, TotalFill sealer in contact with dentine at the dentine sealer interface after 3 irrigation protocols (DW= distilled water; NaOCl+HEDP= hypochlorite mixed with etidronic acid; NaOCl-EDTA= Hypochlorite followed by EDTA), aged for 7 days and 24h of direct contact: ($p < 0.05$). Sealer with no contact with dentine was used as a control. Results of two-way ANOVA and error bar (mean $\pm$ SD). ANOVA: analysis of variance; ($n=3$).

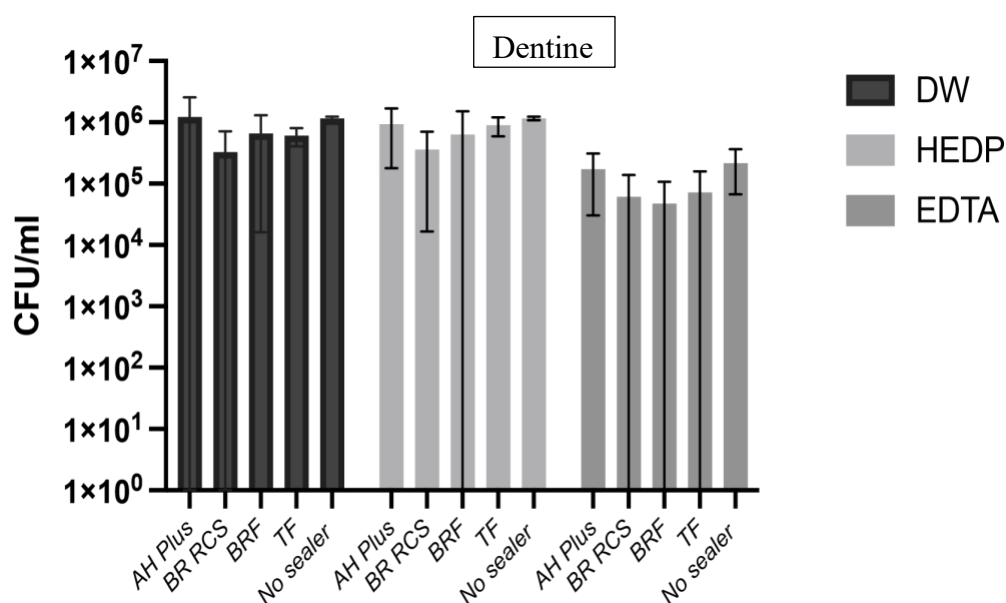


Figure 3.23: Number of CFU (Colony Forming Unit) of *E. faecalis* in Dentine in contact with (AH Plus, BioRoot RCS, BioRoot Flow, Total Fill) sealer at the dentine sealer interface, after 3 irrigation protocols (DW= distilled water; NaOCl+HEDP= hypochlorite mixed with etidronic acid; NaOCl-EDTA= Hypochlorite followed by EDTA), aged for 7 days and 24h of direct contact: ($p < 0.05$). Dentine in no contact with sealer was used as control. Results of two-way ANOVA and error bar (mean $\pm$ SD). ANOVA: analysis of variance; ($n=3$).

Chapter 4: DISCUSSION

4.1 Scope of the project

Prior research has evaluated the interaction of dentine with sealer materials (158–160) and effect of irrigation on dentine properties (143) but there is minimal information on the influence of irrigation on chemical and antibacterial properties of hydraulic cements as well as the optimal irrigation protocol for preparing the dentine to receive the sealer for obturation.

The effect of NaOCl & EDTA on antimicrobial properties of HCSC sealers and on dentine has been evaluated in one of the studies (129) although the effect of continuous chelation has not been studied.

Hence this study aimed at investigating the effect of different irrigation techniques including continuous chelation on dentine microstructure ensuing the best substrate for HCSC sealer interaction at dentine sealer interface.

Furthermore, this research explored the changes in the antimicrobial properties of HCSC sealers on interaction with dentine after irrigation with different chemical solutions and choose the best irrigation protocol for endodontic treatment using HCSC sealers for obturation.

4.2 Selection of materials

Eliminating root canal microorganisms or significantly reducing their load must serve as the primary criterion for selecting a sealer to maximise root canal disinfection and to avoid re-infection through an efficient dentin-sealer interface (161).

4.2.1 Selection of endodontic sealers

The endodontic sealer materials investigated in the current research were mainly based on a tricalcium silicate cement and the control used was an epoxy resin based sealer. From the materials tested, BioRoot RCS was the only formulation presented in a powder and liquid format similarly to MTA. This sealer is water based and the change from cement to sealer is dependent on the addition of a water-soluble polymer. When placed in a storage solution, the sealer leaches high levels of calcium ions when compared to other tricalcium silicate-based sealers, leading to antimicrobial activity. The other two premixed calcium silicate-based endodontic sealers used were TotalFill BC and BioRoot Flow.

These premixed sealers utilize the moisture naturally present in the dentinal tubules to initiate its setting reaction. They have notable antibacterial effects and have also shown high cytocompatibility (162).

Tricalcium silicate sealers have the property of biomineralization and the deposition of phosphates over the material when in contact with the dentine and tissue fluids. This bonding of the sealer to the root canal dentine results in reduction of microleakage.

The fourth sealer used in the study was AH Plus jet. Being an epoxy resin, it sets by an addition reaction between epoxide groups and amines to form polymers. It has an excellent physicochemical property, high dimensional stability, radiopacity and demonstrates high and effective bond to dentine wall by forming sealer tags (68). It also exhibits low solubility, disintegration and antimicrobial property (163). Due to all these properties, AH Plus is commonly used in endodontics and as a reference material for comparison (164). They have limited biocompatibility or osteogenic potential (165).

The choice of sealer should be taken into consideration when selecting the obturation technique. Single cone cold obturation technique was used in the current study. The manufacturer recommends the use of tricalcium silicate sealers with cold obturation techniques only particularly with gutta-percha in a single cone obturation technique or lateral condensation technique. It is shown that on application of heat used in warm vertical compaction obturation techniques to these sealers tend to affect their setting time (166,167) .

4.2.2 Selection of Irrigants

A combination of solutions may be used to effectively irrigate a root canal rather than a single solution (28,30). In the present study, three frequently used endodontic irrigating solutions were used.

The first one selected was NaOCl due to its special ability to disintegrate organic and necrotic tissues like pulp, bacteria, and biofilm remnants (112). Higher concentration (5%) as used in the current study are related to higher chlorine content it releases, which is positively correlated to its tissue-dissolving ability (168,169). Advocated as a supplement irrigant to NaOCl, the use of chelating agent (EDTA & HEDP) is important as it improves the penetration of sealer into dentinal tubules and therefore increases the range of its antimicrobial action and bond to dentine. EDTA was used as one of the chelating agent which dissolves inorganic matter of the smear layer formed during instrumentation to prevent blocking of dentinal tubule, however this action of EDTA can alter the mineral content of root dentine causing peritubular and intertubular dentinal erosion.

Another chelating agent, Etidronate/etidronic acid (HEBP), was used as an alternative to standard chelator, EDTA. Etidronic acid and EDTA have different effects on the structure of dentin, according to the literature (17). Etidronic acid causes less erosion of dentine (18). NaOCl's capacity to dissolve organic tissues was shown to be inhibited by EDTA, while HEDP had a negligible impact on this NaOCl feature (171).

4.2.3 Testing samples for experimental studies

Since the dentine microstructure cannot be intentionally replicated by any method, human teeth were used to enable adequate clinical translation of the results obtained (172–174). Therefore, human teeth were used in the current study to perform all the experiments.

In this work, a split tooth model was used to investigate the antibacterial efficacy at the dentine sealer interface. More explicitly, the bactericidal characteristics of dentine and sealers that have come into contact with dentine were evaluated. This method has been previously employed to assess alteration of the antibacterial properties of dentine and three endodontic sealers irrigated with two irrigation protocols including NaOCl(NaOCl) or chlorhexidine (CHX) (175).

4.3 Sterilisation of the endodontic sealers

During the initial stages of devising the experimental plan for the current project, it was necessary to determine how the sample materials could be sterilised. The importance of evaluating the antibacterial capabilities of endodontic materials is rising. Antimicrobial compounds are supposed to prevent the recontamination of root canal system caused by microorganisms. Various techniques have been employed to evaluate the antibacterial qualities of endodontic materials. These include the agar diffusion test (176–178), minimum inhibitory concentration

(179–182), direct contact test (175,180,183–185), intratubular infection essay (110,112,129). Prior to antimicrobial testing, materials should be disinfected or sterilised to prevent contamination by the organisms. Sterilisation methods include ultraviolet (186,187) gamma radiation (180) ethanol (188) and autoclave. However, in achieving this objective, it is important to recognise that different sterilisation techniques do not modify the surface properties which may interfere with the investigations of the microbial activity of dental materials. There has been a publication on the effects of disinfection or sterilisation procedures on resin restorative materials (189). Steam sterilisation and gamma radiation have been claimed to cause degradation of resin material; ethanol has been shown to affect the physical properties of resin; autoclaving appears to be a more suitable technique without resultant degradation of the material. In the light of these effects, it is important to know what effect can incur on the endodontic sealers after different methods of sterilisation. Furthermore, given the early levels of contamination in endodontic materials, it is unclear if sterilizing the sample before to testing is required.

A careful study of the literature reveals that the effect of sterilisation on the chemical properties of the endodontic sealers has not been thoroughly investigated. Furthermore, sterilisation of endodontic sealers may affect the outcomes of experimental studies through structural alterations and the modification of their chemical and mechanical properties (189–193). Therefore, it is becoming increasingly important for researchers to recognise these possible changes when using sealers material as a research substrate, as these will have an impact on the research outcome.

4.4 Methodological design

So far, tubule penetration (194–197) and push out bond strength (70,141,159,198–200) have been the only methods used to study changes at the dentine sealer interface while keeping the sealer or the dentine alone in mind. However, the interaction between the dentine and sealer following the action of irrigation solution has not been studied to date, which makes it extremely important.

Following this adjustment, both commercial and experimental materials were thoroughly characterised and a potential interrelationship between the leaching profile and biological and antimicrobial properties was investigated.

There were some experiments which were tried but could not get appropriate results, therefore, were discarded. Those include the pilot studies mentioned below.

4.4.1 Pilot studies

4.4.1.1 Preliminary investigation of the dentine sealer Interface using fluorescent dyes

Various tests in microscopic studies have been carried out to determine sealer adaptation to the dentine at the dentine sealer interface such as, SEM, EDS (99,129,201,202) and confocal laser scanning microscopy (CLSM) (91,129,203–205).

SEM images informed about the morphology of the sealer penetration into dentinal tubules. EDS investigated elemental composition of the penetration of sealers and determine the migration of ions in between dentine and sealer. The user can calibrate the apparatus to study the surface by picking out certain points, scanning the entire surface, or employing linear scans. The line scans give information regarding the chemical composition at the dentine sealer surface along those lines and chemically characterise it. This method has been used earlier to characterize the dentine-root canal sealer interface (131) or interface of root end filling materials with dentine (207).

However, to be more specific and compare dentine and material alone, the point scans at the interface could provide better information about the changes, as used in previous studies to study joint or interface (147). Due to sealer separation from dentine at the interface, no data could be gathered at a single centre point, therefore two places were chosen at interface: one on the sealer and one on dentine nearest to the dentine sealer interface. Sealer and dentine both have common element, calcium in them thus studying just the elements alone would not give the appropriate data and not be indicative of sealer remanent, hence in the current study the ratio of calcium with silicon and calcium with zirconium was considered as those (Si, Zr) are the unique elements for tricalcium silicate. Changes were not anticipated in AH Plus sealer as zirconium is stable in AH Plus as opposed to zirconium in hydraulic cement sealer; therefore, the ratio was not calculated.

CLSM with the use of fluorescent organic dyes views underneath the surface of the dentine so no surface preparation which could cause artifacts, is required and thus is non-destructive in nature. It is a standard used in numerous studies to study

sealer penetration (204,208–210). Therefore, this method was chosen for a pilot study to analyse the dentine sealer interface using two sealers, BioRoot RCS and AH Plus, after irrigation with NaOCl followed by EDTA.

A requirement of CLSM is that the sealer must be labelled with a fluorescent dye that can emit fluorescence and measure the depth of sealer penetration in the dentinal tubule. The dyes used in the pilot study were Rhodamine B and Orange G which has been used in previous studies to study sealer penetration (5,58,133,136,137). The results of confocal laser scanning microscopy of the dentine sealer interface in figure 3.14 showed that there was an elevated level of sealer infiltrated into the dentinal tubules all along the interface. Although no such results were detected from the other experiments conducted with fluorescent dyes for comparison, which included, SEM, EDS and FT-IR. SEM showed slight infiltration of sealer into dentinal tubule in figure 3.15. EDS line scans suggested reduction in zirconium and silicon (elements specific for hydraulic cement) with increase in the distance from interface in table 3.5, 3.6 and 3.7. FT-IR showed high intensity of tricalcium hydrate specific CSH peak at interface but decreasing when reaching 300 μm into dentine, suggesting that the sealer flow reduces beyond 300 μm in figure 3.16.

These findings were different from CLSM finding, which showed intense diffusion of dye throughout dentinal tubule. Rhodamine B and Orange G both were acidic in nature as tested with the help of pH meter, water-soluble at room temperature, and when diluted can produce fluorescence (138–140). It shows an enhanced affinity for moisture as compared to the calcium in the sealer (141), due to which the dye leaches out from its mix with sealer, in the presence of moisture in dentine (196). The leached dye in dentinal tubules may emit fluorescence even

beyond the flow of sealer giving flawed results (215) which is also shown in our study where the resultant images of dentine sealer interface for BioRoot RCS and AH Plus sealers shows extensive penetration of Orange G and Rhodamine B dyes into the dentinal tubules starting at the interface. The impressive images of penetration of sealer presented in several studies (208,216,217) are influenced by the nature of the dye.

Therefore, this technique for evaluation of sealer penetration depth was not proceeded further with other sealers and irrigant groups.

4.4.1.2 Intratubular infection test pilot study

The antimicrobial properties of endodontic sealers are important especially in long standing root canal infections where the resistant bacteria are confined in the dentinal tubules of root dentine in form of biofilms (110). This test mimics the above-mentioned conditions and has been used in previous studies (87,105,110,112) to measure the effectiveness of disinfecting solutions and obturating materials against biofilms in dentine. This method provides for an artificial method of dentine tubule bacterial centrifugation and gauge the bacterial viability in dentinal tubules with the help of staining with SYTO 9 (live) and propidium iodide (dead) dyes using confocal imaging technique (110,112,218,219). Hence a pilot study with intratubular infection test was conducted to measure the antibacterial activity of endodontic sealers on its own as well as with different irrigation techniques.

The human root canal samples of 4 mm in length were used. Studies have shown that penetration of irrigant solutions in apical dentine is prevented by tubular sclerosis common in apical third of dentine (218, 220) and this limitation led us discard apical portions. Furthermore, the dentinal tubules are wide enough coronally for easy bacterial infection thus a coronal dentine of 4mm length was chosen for this experiment. The dentinal walls were exposed directly in contact with the irrigant group as suggested previously (112). Ethanol was used as one of the controls as it is a known antiseptic and disinfectant because of its fixative and dehydrating activity and is effective against most bacteria and fungi (71,221,222).

The dentine block was irrigated with three different irrigation groups, distilled water, NaOCl followed by EDTA and NaOCl mixed with HEDP, followed by placement of AH Plus, BioRoot RCS, BioRoot Flow and TotalFill sealer adjacent to dentine infected tooth specimens. The positive control was a sample with only bacteria.

Using the dentine infection model under the experimental conditions the bacterial infiltration in the dentinal tubules were visible with adjustment in the original SYTO 9 and propidium iodide ratio used in one of the previous studies (157) and eliminating the dentine autofluorescence gain.

Despite modifications, bacterial viability could not be gauged, and results could not be obtained in confocal images of *E. faecalis* infected dentinal tubules after irrigating with the above-mentioned irrigation groups as well as the control group.

The reason of unclear images was dentine autofluorescence (223), which persisted in interfering with the interpretation. Another limitation of this model was adverse effects on collagen fibres owing to dentine sterilization, which may result in

inferior biofilm adhesion (71). Thus, this methodology was deemed inappropriate to answer the research questions. Therefore, it was not continued with other groups. A modified direct contact test (152,202) was selected instead, to explore the antimicrobial nature of sealers in contact with dentine and dentine in contact with sealers, using planktonic bacteria.

4.5 Chemical characterisation of set materials

In this study, various techniques have been utilised to investigate the material microstructure and the chemical composition of the sealers. Mineral structures in hydraulic cements are crucial for the material's long-term performance and for comprehending the functionality of the material, hence their significance has been stressed. The details about the distribution of hydrated products, non-hydrated particles and radiopacifiers in the bulk of each set material was performed with SEM; elemental makeup of the materials was achieved with EDS & FT-IR and the mineral phases found in materials by XRD analyses. Calcium leaching was assessed with ICP-OES. For the second part of the study, direct contact test, MIC, SEM, EDS, and FT-IR of dentine sealer interface was carried out. The calcium leaching of eluates was monitored for 7 days, 90 days and changing media every 15 days for 90 days. For antimicrobial experiments, leachates were used in MIC assays to correlate their performance with their chemical profile. In the literature, these techniques have been widely employed to evaluate the chemical composition of dental materials (59,67–75).

In the current study, calcium hydroxide was produced as a by-product of hydration of tricalcium silicate cement. The production of calcium hydroxide was evident from the X-ray diffraction analysis in figure 3.3. This calcium hydroxide was leached in solution and reacted with the phosphate ions present in the Hank's balanced salt solution. A crystalline deposit of calcium phosphate was evident on the surfaces of tricalcium silicate cement. The lower Si/Ca ratio seen in EDS in figure 3.2 results was due to a greater abundance of calcium in tricalcium silicate which can result in a greater deposition of hydroxyapatite when the material is in contact with a physiological solution. Since both sealers and tooth structure include calcium, the elemental mapping was unable to track the calcium's migration from the sealer to the tooth. The presence of sealer residues on dentine was therefore identified by use of the sealer specific elements silicon (Si) for hydraulic cements and zirconium (Zr) for hydraulic cements plus AH Plus sealer which could be tracked in respective sealers. The ratio of calcium to silicon and calcium to zirconium were used to track the flow of calcium from the sealer to the dentine, which could not be tracked using elemental mapping alone as both sealers and tooth structure include calcium. Each functional group of the chemical components absorbs a specific wavelength of radiation in the infrared region and these functional groups were characterised by the Fourier transform infrared spectroscopy (FT-IR) by plotting a graph of radiation intensity versus frequency (72). Since FTIR has such high sensitivity, accuracy, and spectrum quality, it is the recommended technique for infrared spectroscopy. Compared to earlier methods, it offers a higher data gathering speed and does not harm the sample (231). FT-IR is to examine the interaction of calcium silicate cements with various liquids thus showing absorption peaks of calcium silicate hydrate and calcium

hydroxide and broad absorption bands in hydraulic cement samples in the present work.

4.6 Leachate Analysis

The calcium content of the material leachate was measured by ICP OES. This technology is one of the most dependable technologies now in use and is substantially more accurate than other widely used techniques (81). It can test components from trace to high concentrations and perform multi-elemental quantitative analysis in as little as 1 minute with a minimal amount of solution (233).

The release of high levels of calcium by the BioRoot is in accordance with previous studies (73) and accounts for the material's antimicrobial properties. The antimicrobial properties of BioRoot have also been shown in previous research and tested using alternative techniques(43). In the current study, calcium ion leaching was same for both 7 and 90 days. Calcium silicate-based cements are bioactive materials that could release hydroxyl and calcium ions thus creating an adequate alkaline environment. There was a significant rise in calcium ion leaching for TotalFill in 90 days than 7 days as seen in previous studies (234). The water-based sealer BioRoot RCS exhibits a higher calcium ion release compared to syringe systems like TotalFill and BioRoot flow, as it depends on the diffusion of ions through a non-aqueous matrix that delays, or restricts, the calcium ion formation and release (235,236). Furthermore, the presence of calcium phosphate monobasic in TotalFill BC also modifies the hydration reaction (85,236). The

leaching of calcium ions was higher in Bioroot Flow at 7 days than 90 days which is consistent with other research comparing the release of calcium from different calcium silicate-based sealers (237). No calcium ions release was observed in AH plus as it permits the release of a great number of ions during the initial setting, reducing ion release over time. AH Plus like other resin cements has shown a low solubility probably related to its resin matrix which would explain the lower calcium release than other tricalcium silicate cements (103).

4.7 Microbiological Analysis of Sealer materials

4.7.1 Characterisation of set materials before and after sterilisation

When conducting biological and antimicrobial testing, material sterility becomes paramount. Contamination can impede cytocompatibility studies by causing cell death and compromising the accuracy of assessments in microbiological assays. The sterilizing techniques may also modify the microstructure and physical properties of dental and biomaterials (189–192). Therefore, we characterised the set materials before and after sterilisation to assess the changes. The procedures utilized in the current work to assess the material changes were comparable to earlier research (189).

The sealers in our study became contaminated during benchtop preparation and required sterilization. While utilizing a laminar flow cabinet for material preparation is recommended, certain equipment constraints may preclude its use

in all cases. Given the use of a non-sterile laboratory bench for material preparation followed by decontamination, performing a sterility test before initiating actual testing is highly advisable.

The primary way to monitor the growth of pure bacterial cultures is by measuring turbidity in liquid cultures, which is done using a spectrophotometer to determine optical density (OD) (238). However, the spectrophotometer might not always be effective at detecting low viable counts. In these cases, an alternative method involving agar plating can be utilized to obtain a qualitative value indicating the presence or absence of the result (238). To ascertain the presence of bacterial growth, this study utilized the agar plating technique as a confirmatory measure.

To verify the effectiveness of sterilization methods, sterilized samples were compared to untreated controls to assess any property changes. The absence of bacterial growth on plates in the first approach was attributed to the high pH induced by sterilization, potentially inhibiting microbial activity. Additionally, without plate-based culture and specific specimen preparation, the antimicrobial effects of four sterilization techniques, namely steam, ethanol, UV, and ethanol plus ultraviolet, were evaluated directly on several hydraulic cement sealers, including BioRoot RCS, BioRoot Flow, and TotalFill which are popular root canal sealers due to their antibacterial and cytocompatibility nature (101,184).

The suitability of steam sterilization for polymers remains unclear, with conflicting reports showcasing both its effectiveness (189,191) and its potential to induce material damage through hydrolysis, softening, melting, and degradation due to the intense heat, pressure, and presence of water (190). In our study, steam sterilization compromised the material surface of AH Plus and BioRoot RCS

sealers due to elevated temperatures aligning with previous findings that also looked at the effects of steam sterilization on resin-based materials (191).

Ethanol exhibits efficacy as an antiseptic agent against a broad spectrum of bacteria, fungi, and viruses due to its fixative and dehydrating properties. However, it possesses limited sporicidal activity, failing to eradicate bacterial endospores (91). The antimicrobial action of ethanol is attributed to its protein coagulating and membrane-denaturing effects on microbial cells (92). In current study, ethanol exhibits no adverse effects on resin-based materials and biomaterials (51,52), it can impact the mechanical properties of glass ionomer components as shown in previous study (189). There were comparable results, with ethanol having no effect on the physical and chemical properties of BioRoot RCS Flow and TotalFill. This is because these materials do not contain available $(OH)^-$ in the initial stages of the reaction, unlike BioRoot RCS. Therefore, ethanol could potentially be used for sterilizing a limited number of sealers.

At specific wavelengths (200-280 nm), UV light disrupts microorganisms, hindering their ability to multiply and eventually leading to their inactivation. This targeted approach makes it a valuable alternative to other sterilization methods of chemical or heat (241,242). However, this study confirmed that UV light, known to impact light-curable materials (189), altered the surface of the BioRoot RCS sealer and modified the Ca/Si and Ca/Zr ratios in BioRoot Flow and TotalFill. Given these detrimental effects, UV sterilization alone is not recommended for sealers.

This study pioneers the use of a combined ethanol and ultraviolet irradiation approach for sterilizing dental materials to make use of the benefits of both

techniques and minimise their detriments. Notably, previous studies have not explored the use of ethanol and ultraviolet irradiation together for sterilizing dental materials. However, when employing this combination, the surface microstructure, elemental composition of Ca/Si and Ca/Zr ratios, as well as the water and alcohol peaks in all the sealers analysed were modified as well.

4.7.2 Assessment of antimicrobial properties of endodontic sealers

4.7.2.1 Attachment assay / Direct Contact Test for Sealer

In the literature, antibacterial efficacy has primarily been evaluated using agar diffusion test (ADT) with planktonic bacteria (176–178). The agar diffusion test is a simple test and requires relatively low budget (110). It is mostly used for assessment of endodontic materials, but it relies on the solubility and diffusion of the materials and thus is not quantitative (243). This test also does not provide the actual antibacterial effect of the sealers in question due to the buffering capacity of the agar plate (87,244). Therefore, the agar diffusion test was replaced by the direct contact test (DCT) in the current study. This attachment assay, although has some limitations, is a more quantitative and reproducible method to quantify the bacterial growth and to imitate their contact with the materials (71,245).

For the experiments reported here, a single species culture of *E. faecalis* was selected. Although root canal infections are polymicrobial (246), *E. faecalis* is the bacterium used in many studies to assess the anti-microbial properties of materials

and eluates in invitro models (112,246–248) and thus chosen for this current study. Furthermore, the results with a single organism are easily quantifiable and reproducible (249). Afore utilising the bacteria for various tests on material and leachates, it was incubated overnight and then quantified.

With the direct contact test, AH plus showed lesser antibacterial activity as opposed to the HCSC sealers as shown in Figure 3.9 in results section. Its antimicrobial action is due to toxic effects of bisphenol Di glycidyl ether, amines, and epoxy resin present in its components (107) and due to formaldehyde released in the polymerization process (251). Since the DCT in our study was performed after 7 days of aging it reduced the antimicrobial property of AH Plus thus it was unable to reduce the CFU mL⁻¹ at the 7-day contact period evaluated. The inefficacy of this material against *E. faecalis* has been previously reported (252,253). This lack of activity may be related to this material's neutral pH and low solubility (254), confirmed by the present study.

During hydration reaction of hydraulic cements, calcium hydroxide dissociates into calcium and hydroxyl ions (87,255). It has been demonstrated that the hydration patterns of various HCSCs vary. BioRoot RCS is a water-based cement which means its hydration is always maintained, releasing calcium hydroxide, regardless of the amount of fluids present in the environment. Thus, viability of bacteria reduces (116). The premixed ready-to-use injectable sealer systems like TotalFill and BioRoot Flow relies on OH-ions derived from tissue fluids to form calcium hydroxide. The vehicles used have an influence on rates of ionic dissociation and diffusion of calcium hydroxide (88).

Tricalcium silicate cements used in the present study were able to significantly reduce the number of CFU mL⁻¹ at all time points as compared to epoxy-based sealers which is consistent with the previous studies that demonstrated prominent antimicrobial activity of hydraulic cements against the planktonic bacteria when evaluating their killing effect using DCT (18,256–259). This can be due to the strong alkalizing activity of calcium silicate sealers due to their ability to form hydroxyl ions and raising the pH (18,71). However, none of the sealers were successful in completely eliminating the bacteria.

4.7.2.2 Minimum Inhibitory Concentration (MIC)

In the current study, antibacterial assays were also conducted on the bacterial eluates of test materials. Endodontic materials interact with the surrounding tissue, fluids, and bacteria (260). Studying the *in vitro* leachates is also of interest for predicting their anti-microbial activity. The amount of calcium hydroxide that leached into the extraction solution could be directly associated to any potential antibacterial behaviour of endodontic materials, without interfering with the outcomes, making the evaluation of the eluates more pertinent to the scopes of the current project (261–264).

For the evaluation of the antimicrobial potential of the leachates against planktonically grown bacteria, the indirect test Minimum Inhibitory Concentrations (MIC) was chosen. MIC was done for 7 day and 90-day leachate. Measurement of results was performed using a plate reader.

Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. MIC is regarded as a relatively low cost, reproducible technique, and most basic laboratory measurement of the activity of an antimicrobial agent against an organism (230). The lower the MIC value, more effective is the antimicrobial agents. Only a few studies have evaluated the antimicrobial effect of leachates so far using MIC (33,37,41,42).

Data obtained from the MIC experiments also revealed that all tricalcium silicate cements are antimicrobial when compared to epoxy resin-based sealers, which may be linked to hydraulic cements' release of calcium hydroxide as depicted in figure 3.10. BioRoot RCS eluate had higher antibacterial activity as compared to other leachates. This is due to increased calcium hydroxide release when the BioRoot RCS powder comes into contact with water during mixing itself. The hydration of the premixed system sealers TotalFill and BioRoot Flow begins only when they come into contact with environmental fluids, delaying the release of calcium hydroxide. As a consequence, the idea that hydraulic cements are antibacterial was confirmed. Also, this result is in congruence with the ICP results which projected highest values of calcium release of BioRoot RCS for both 7 days and 90 days.

4.8 Characterisation of dentine

Testing the effects of irrigation solutions on the dentine tissues and characterising it, has been crucial, since irrigation treatments may result in changes of the dentine substrate which might affect its interaction with the sealers.

When the NaOCl was used as the only irrigant, smear layer was still observed on the dentine surface shown in figure 3.11, due to its action on organic tissue and it does not remove inorganic debris which appears as smear layer. Some smear layer was still observed with HEDP mixed with hypochlorite group. Only EDTA or hypochlorite followed by EDTA did not show any smear layer, although erosion of dentinal tubules was observed with these groups but not with HEDP group. Collagen fibre which provides the mechanical characteristic to dentinal tubule, is covered with hydroxyapatite (5). NaOCl when used in sequence with strong chelator like EDTA may penetrate and rupture the apatite-encapsulated, highly cross-linked collagen matrix, due to the low molecular size of hypochlorite anion (123), resulting in erosion of dentinal tubule as previously reported (147). Earlier literature indicated EDTA is a stronger chelating agent than HEDP(266) and hypochlorite used after a strong chelating agent erodes the dentinal tubule (267). The spectra obtained from EDS analysis demonstrated that the inorganic materials were further exposed as a result of the prior breakdown of the organic portion of the smear layer on the root canal surface by NaOCl, which favoured the removal of Ca^{2+} by the chelators (4,267) and interaction of EDTA with the calcium ions in hydroxyapatite crystals can change the mineral composition of root dentin (268).

Phosphate and calcium are the main mineral components of dentine, and both are soluble in water. When the strong chelating agents like EDTA comes in contact with dentine, calcium ions are removed from the solution resulting in dissolution of deeper hydroxyapatite crystals uncovering the collagen fibres leading to its destruction (269,270) which can be seen in our results showing widening and erosion of the dentinal tubules. This also coincides with FT-IR finding where there is reduction in all phosphate, amide and carbonate peaks implying both denaturation and demineralisation of dentine with NaOCl–EDTA group. HEDP+NaOCl group exhibited weakening of amide peaks and superior phosphate peaks indicating denaturation of collagen due to NaOCl action mixed with the chelator. The amide peak was more pronounced in the only EDTA group than in the phosphate group attributed to dentine demineralisation, which was not seen in the HEDP+NaOCl group since HEDP is a poor chelator.

4.9 Investigation of dentine sealer interface using an alternative method

The current research looked at three hydraulic calcium silicate cements (HCSCs) and an epoxy resin-based sealer to evaluate how well these materials interacted with dentine that had undergone irrigation with different protocols as well as how well they could alter the properties of the dentine after application at the dentine sealer interface.

4.9.1 Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) is frequently used to assess the morphology of the dentine and sealer particles at dentine sealer interface (195,201,271–273).

In the current work some sealer diffusion could be seen in few groups (figure 3.17) which could be the result of the flowable consistency of the cement flowing into open dentinal tubules (30). A limitation of this method is that it requires surface preparation of specimen and vacuum desiccation, due to which sealer properties especially of hydraulic cements could be greatly affected, shrink, and detach from the surface of the dentine (67) and cannot properly represent the adaptation which can also be seen in this study. In the current work it revealed cracks and adhesive mode of failure at the dentine sealer interface particularly more when hydraulic cements like BioRoot RCS, TotalFill and BioRoot Flow were used. While epoxy resin does not shrink during setting (201), it can account for their better adaptation at the dentine sealer interface when tested without dyes, compared to tricalcium silicate cements. Therefore, this research approach could not yield much information regarding the analysis of sealer adaptation to dentine at their interface, particularly when dealing with hydraulic cements that shrink, debonded, and crack from dentine at the interface upon final setting.

4.9.2 Energy-dispersive X-ray spectroscopy (EDS)

EDS point scans of mean ratio of calcium with silicon (Ca/Si) and calcium with zirconium depicted in table 3.8, signified superior sealer penetration at the interface for BioRoot RCS with the NaOCl+HEDP group and BioRoot Flow when irrigating with group A. Traces of Zr could be seen in dentine for BioRoot RCS and TotalFill with group A and group B indicating sealer penetration further from the interface.

4.9.3 Fourier transform infrared spectroscopy (FT-IR)

FT-IR enables to identify the migration of ions between two substrates and to chemically characterise the interface between them. The present study describes for the first time how the chemical interaction between the dentine surface and a tricalcium silicate sealer can be studied by means of FTIR photoacoustic spectroscopy. This method was used to characterize the clinker phases of hydraulic cements in cement industry (73) and has previously been used to study the chemical nature of tricalcium silicate cements (274–276). The samples in this technique can be analysed without any further handling or sample preparation procedures like vacuum storage as in SEM, which could impair the interface (228) which is a major benefit of this technique. Furthermore, the data can be received immediately within few minutes upon analysis by FT-IR without

requiring complex treatment, unlike other procedures which take several hours to prepare and investigate a single sample.

It has been suggested that tricalcium silicate sealers form mineral infiltration zone chemically at dentine sealer interface due to their ability to deposit apatite in the presence of phosphate. According to scientific research, dentine may absorb the minerals like calcium and silicon released by biomaterials, leading to enhanced mineralization in the neighbouring dentine. We can recognise the migration of specific compounds for sealer and dentine, thus can determine whether the test material was able to induce some mineralisation in the adjacent dentine or not.

In the present study Calcium Silicate Hydrate (CSH) of the material with absorption bands at 950 to 1000 and phosphate of the dentine were the main peaks that were observed at dentine sealer interface via FT-IR seen in figure 3.18. Such interface can be demonstrated spectroscopically by shift in the absorption bands attributable to the sealer and dentine substrate, respectively. So, if a sealer is chemically bound to dentine at the interface, a shift in the absorption bands should be recorded at the interface. In contrast if the sealer does not interact with the dentine, there would be no change observed on a dentine spectrum in absorption band position.

Following the results in this study, it is apparent that there is a shift in the spectrum from sealer to dentine in the hydraulic cements as opposed to the epoxy resin. At the interface, AH Plus did not demonstrate a significant difference with irrigant groups which could be related to the penetration of resin matrix forming resin tags and not of the inorganic compounds as shown by previous studies (99). The intensities of the CSH peak were high at interface but steadily decreased on

approaching dentine especially in Bioroot RCS with HEDP mixed with NaOCl group which could be the sealer penetrating the dentine in this group more as compared to other groups. When transitioning from the solid dentine region to the hydraulic cements, the phosphate peak shallowed. Other finding was that the intensity of phosphate peak at interface was raised when the BioRoot RCS and BioRoot Flow sealer was utilised in conjunction with the NaOCl-EDTA group as compared to other irrigation groups. Phosphorous peaks were verified which could be because of immersion in HBSS for 7 days after obturation.

4.9.4 Attachment Assay/Direct Contact Test for Dentine Sealer Interface

In the current study, Direct contact test was modified and instead of testing the root with the sealer together as one unit, sealer was separated from the root dentine and studied individually. The sealer was aged for 7 days inside root canal with 24 hours of direct contact with the bacteria; CFU counts were then done. This helped to reflect the actual antimicrobial activity of just the material without getting affected by dentine buffering action which is one of the disadvantages of DCT in earlier studies (5,277). However, the investigated material's adhesion to dentin may result in a carryover effect (44) or impede the removal of all sealer from the dentine and impact the dentine's antibacterial activity.

One of the limitations of this method is the use of mono species model. Irrigants and root canal sealers should also be tested in multispecies biofilm environment.

Every sealer showed a decrease in antibacterial efficacy upon contact with dentine in comparison to sealers without contact as represented in figure 3.22. In the

present study no inhibitory effect against the *E.faecalis* was observed after 7 days of aging for AH Plus which indicates Ah Plus loses its antimicrobial activity after setting and these findings are in line with the previous studies (175,183,278,279). The material's neutral pH and poor solubility may be the cause of this lack of action (183). BioRoot RCS on its own exerted the highest antibacterial efficacy in comparison with other test sealers. Our result for BioRoot RCS alone without irrigation is in agreement with the literature (93,175,280,281). This finding may be explained by calcium hydroxide principally formed after hydration of calcium silicate cements and this hydration state of HCSC sealers is highly correlated to their antimicrobial properties. Additionally, the various sealers differ in the release of calcium ions (86,282,283). BioRoot RCS is mixed with water, ensuring assured hydration with higher calcium ion release and hydroxyl ion resulting in elevated alkalinity therefore have greater antimicrobial properties than the premixed sealers (87) as seen in the current study. Premixed sealers like BioRoot Flow and TotalFill rely on the presence of environmental fluids which delays calcium ion release (235). The tissue fluid conditions of single syringe sealers cannot be mimicked in laboratory settings.

Although the material's capacity to enter dentinal tubules and reach the bacteria, following the removal of the contaminated smear layer, may determine the sealer's antibacterial activity (183). The irrigation protocols followed before root canal filling and the interaction of sealer with the dentine, both had an impact on the characteristics of HCSC and epoxy resin sealers. Regarding the planktonic bacteria analysed by the DCT in the current investigation, all sealers showed considerable antibacterial action after 7 days in contact with irrigated dentine.

BR RCS had the greatest bactericidal impact with different type of irrigant groups as seen in figure 3.23 The sealers with NaOCl- EDTA irrigation and dentine when irrigated with NaOCl- EDTA and after contact with sealer, displayed the greatest antibacterial activity as opposed to other irrigation groups. EDTA and hypochlorite both have some capacity of killing microorganisms (284) thus these groups resulted in lower bacterial viability. This finding confirms the results obtained by previous studies (17,285). High antibacterial efficacy for BioRoot RCS was shown after final irrigation with EDTA as seen in previous study (286). NaOCl dissolves necrotic pulp tissues and is antibacterial (151) and chelating agents like EDTA & HEDP removes the inorganic portion of smear layer (87,287) augmenting sealer infiltration into dentinal tubules (142,288,289). The reason for enhanced sealer and dentine activity could be multifactorial where chelation affected the dentine rather than the sealer. The greater diffusion of hydraulic cements through the smear layer free dentine escalating the ion exchange and hydration of HCSC resulted in enhanced antimicrobial effect. Contact with the irrigated dentine rendered BioRoot RCS to lose its antibacterial properties compared to control. Sealers with NaOCl EDTA application eliminated greater bacteria thus NaOCl EDTA when applied on the surface of dentine improved the antimicrobial properties of sealers especially BioRoot RCS and TotalFill against planktonic bacteria as compared to other irrigant groups.

When HCSC sealer comes in contact with dentine it loses its antimicrobial property as also seen in current study. One of the reasons could be the ability of bacteria to penetrate dentinal tubules, build biofilms contributing to their survival and thus may be shielded from the direct antibacterial action of sealers (151). It may also be brought about by the interaction of the hydraulic cement with the

surroundings (57,180) resulting in the formation of calcium carbonate by consuming the available calcium hydroxide (181). This lowers the pH, which has a detrimental effect on antibacterial action (106).

The direct contact test of irrigated dentine (after obturation) in contact HCSC sealer overall displayed reduction in bacterial viability as compared to epoxy resin. This can be attributed to interaction of dentine with hydraulic cements through mineral exchange at the interface (99,275). When comparing the conventional HCSC and premixed sealer, BioRoot RCS showed decreased microbial growth with distilled water or HEDP irrigant group. While dentine in contact with the premixed sealers showed better antimicrobial response when irrigated with EDTA. The reason for this effect could be that the smear layer must be removed for single-syringe sealers to function since they need the dentine fluid to hydrate (87,292). Formulations that include powder and liquid, like BioRoot RCS, chemically interact with dentine to produce an antibacterial activity that is not dependent on the smear layer (87,270).

In two of the studies, bovine dentine or human dentine were used as substrates to grow *E. faecalis* biofilms (31, 34). No matter how long the sealers were in touch with the bacteria, none of them completely removed it. Therefore, it may be deemed necessary if an obturating substance has the capacity to aid in the partial and superficial eradication of bacteria that remain following endodontic cleaning and disinfection processes.

CHAPTER 5: CONCLUSIONS

The present project assessed an optimal irrigation technique, potentially including continuous chelation, which could prepare dentin with the ideal microstructure to ensure best interaction with the HCSC sealer at the dentine sealer interface, as well as tested different irrigation protocols that could impact the antimicrobial efficacy of HCSC sealers after interacting with dentin. Given that continuous irrigation results in better dentine surface characteristics, half of the hypothesis was accepted; however, the other part was denied as contact with irrigated dentine can alter the antibacterial properties of tricalcium silicates.

Under the experimental conditions of this investigation following conclusions can be drawn:

1. HCSCs require hydration for setting and achieving their full properties. Scanning electron microscope sample preparation methods involve dehydration steps which can cause shrinkage and debonding of the HCSC from the dentine, leading to inaccurate assessment of the interface. SEM is thus not the appropriate method for viewing dentine sealer interface when using hydraulic cements.
2. There are limitations to using confocal laser scanning microscopy (CLSM) for assessing dentine sealer penetration, particularly regarding dye leaching. Certain dyes used in CLSM can diffuse beyond the sealer into dentinal tubules, masking the true extent of sealer penetration and providing inaccurate results rendering this method unsuitable for analysing dentine sealer interface.

3. The process of sterilizing sealers can result in both physical and chemical changes. It is important to prepare the materials in a controlled environment, such as a laminar flow cabinet, and conduct a sterility test before conducting any biological experiments. In case the materials are not sterile, it is advisable to assess the impact of the sterilization methods.
4. EDTA is the more commonly used chelator due to its effectiveness in smear layer removal, but its potential for excessive dentine demineralization and erosion necessitates use of weak chelating agent like HEDP which lowers the deterioration of dentinal tubules .
5. Irrigation with HEDP results in higher mineral concentration zone for hydraulic cements at dentine sealer interface as compared to EDTA and distilled water.
6. The direct contact test of irrigated dentine (after obturation) in contact with BioRoot RCS revealed lower microbial growth with HEDP irrigant group compared to other groups. BioRoot RCS interacts chemically with dentine to create antibacterial action that is independent of the smear layer.
7. While tricalcium silicate sealers are generally known for their antimicrobial properties, their effectiveness can indeed be affected by contact with irrigated dentine.

Therefore, understanding these interactions and optimizing irrigation protocols is essential for maximizing the benefits of tricalcium silicate cement sealers in root canal treatment.

CHAPTER 6: FUTURE WORK

1. *In vitro* method used in the current study evaluated the antimicrobial effectiveness against single species. The single species biofilm model, although accepted, is not representative of the complex polymicrobial biofilm found within infected root canals. In clinical cases, endodontic pathology is biofilm induced and therefore further studies are indicated to evaluate the antibacterial activity of hydraulic cement sealers against polymicrobial biofilm.
2. More research is needed to fully understand the mechanisms behind the observed effect of HEDP on mineral concentration zone formation.
3. It is proposed that in the process of evaluating the antibacterial properties of dental materials, in particular endodontic sealers, more than one assaying method should be used for better understanding. The direct contact test has limitations which include its failure to assess bacteria in biofilms and its inability to be utilized with freshly mixed sealer. This could lead the sealer to adhere to the substrate. Therefore, further antimicrobial testing techniques could be tried.

APPENDIX

Publication

Bhandari SS, Palin W, Kuehne SA, Camilleri J. Investigating best practice for specimen preparation for biological testing of root canal sealers. *Dental Materials*. 2024 Mar 1; 40(3):387–92.

LIST OF REFERENCES

1. Avery JK, Steele PF. Oral development and histology . 3rd Edition,. Avery JK, Steele PF, editors. New York, USA: Thieme Medical Publishers,; 2002 :72-108,153-190 .
2. Carrotte P. Endodontics: Part 4. Morphology of the root canal system. *Br Dent J*. 2004 Oct 9;197(7):379–83.
3. Kinney JH, Marshall SJ, Marshall GW. The Mechanical Properties of Human Dentin: a Critical Review and Re-evaluation of the Dental Literature. *Critical Reviews in Oral Biology & Medicine*. 2003;14(1):13–29.
4. Oyarzun A, Cordero A, Whittle M. Immunohistochemical Evaluation of the Effects of NaOCl on Dentin Collagen and Glycosaminoglycans. *J Endod*. 2002;28(3):152–6.
5. Haapasalo M, Qian W, Portenier I, Waltimo T. Effects of Dentin on the Antimicrobial Properties of Endodontic Medicaments. *Journal of Endodontics*. 2007;33:917–25.

6. Pashley DH, Carvalho RM. Dentine permeability and dentine adhesion. *J Dent*. 1997 Sep;25(5):355–72.
7. Möller AJ, Fabricius L, Dahlén G, Ohman AE, Heyden G. Influence on periapical tissues of indigenous oral bacteria and necrotic pulp tissue in monkeys. *Scand J Dent Res*. 1981;89:475–84.
8. Hancock HH, Sigurdsson A, Trope M, Moiseiwitsch J. Bacteria isolated after unsuccessful endodontic treatment in a North American population. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2001;91(5):579–86.
9. Nair PNR. On the causes of persistent apical periodontitis: A review. *International Endodontic Journal*. 2006;39:249–81.
10. Pereira RS, Rodrigues VAA, Furtado WT, Gueiros S, Pereira GS, Avila Campos MJ. Microbial analysis of root canal and periradicular lesion associated to teeth with endodontic failure. *Anaerobe*. 2017 Dec 1;48:12–8.
11. Zehnder M, Rechenberg DK, Thurnheer T, L  Uthi-Schaller H, Belibasakis GN. FISHing for gutta-percha-adhered biofilms in purulent post-treatment apical periodontitis. *Molecular Oral Microbiology* . 2017;32:226–35.
12. Siqueira JF, R  as IN. Clinical Implications and Microbiology of Bacterial Persistence after Treatment Procedures. *JOE*. 2008;34:1291–301.
13. Gomes BPFA, Pinheiro ET, Jacinto RC, Zaia AA, Ferraz CCR, Souza-Filho FJ. Microbial Analysis of Canals of Root-filled Teeth with Periapical Lesions Using Polymerase Chain Reaction. *J Endod*. 2008 May;34(5):537–40.
14. Stuart CH, Schwartz SA, Beeson TJ, Owatz CB. *Enterococcus faecalis*: Its role in root canal treatment failure and current concepts in retreatment. *Journal of Endodontics*. 2006;32:93–8.
15. Alghamdi F, Shakir M. The Influence of *Enterococcus faecalis* as a Dental Root Canal Pathogen on Endodontic Treatment: A Systematic Review. *Cureus*. 2020 Mar 13;12(3):e7257.

16. Costerton JW, Lewandowski Z, DeBeer D, Caldwell D, Korber D, James G. Biofilms, the customized microniche. *J Bacteriol.* 1994 Apr;176(8):2137–42.
17. Zhang R, Chen M, Lu Y, Guo X, Qiao F, Wu L. Antibacterial and residual antimicrobial activities against *Enterococcus faecalis* biofilm: A comparison between EDTA, chlorhexidine, cetrimide, MTAD and QMix. *Sci Rep.* 2015 Aug 6;5:12944.
18. Al-Haddad A, Aziz ZACA. Bioceramic-Based Root Canal Sealers: A Review. *International Journal of Biomaterials.* 2016:9753210.
19. Mchugh c, Zhang p, michalek s, Eleazer p. pH Required to Kill *Enterococcus faecalis* in Vitro. *J Endod.* 2004 Apr;30(4):218–9.
20. Nicholson J, Czarnecka B. Materials for root canal filling. In: *Materials for the Direct Restoration of Teeth.* Elsevier; 2016:197–219.
21. Alan H.Gluskin. Shaping, Disinfecting and Obturating Root Canals. *Decisions in Dentistry.* 2016;2(11):26–8.
22. Peters OA, Paqué F. Root canal preparation of maxillary molars with the self-adjusting file: A micro-computed tomography study. *J Endod.* 2011 Jan;37(1):53–7.
23. Karunakaran J V., Senthil Kumar S, Kumar M, Chandrasekhar S, Namitha D. The effects of various irrigating solutions on intra-radicular dentinal surface: An SEM analysis. *J Pharm Bioallied Sci.* 2012 Aug 1;4(suppl 2):125–30.
24. Gwinnett AJ. Smear layer: morphological considerations. *Oper Dent Suppl.* 1984;3:2–12.
25. Pashley DH. Smear layer: overview of structure and function. *Proc Finn Dent Soc.* 1992;88 Suppl 1:215–24.
26. Cobankara FK, Erdogan H, Hamurcu M. Effects of chelating agents on the mineral content of root canal dentin. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology.* 2011 Dec;112(6):e149–54.

27. Hakan B, Safavi KE, Sp&ngberg LSW. Antifungal Effects of NaOCl and Chlorhexidine in Root Canals. 1999;25:235-38.
28. Zehnder M. Root Canal Irrigants. JOE. 2006;32(5):389–98.
29. Kandaswamy D, Venkateshbabu N. Root canal irrigants. Journal of Conservative Dentistry. 2010;13(4):256.
30. Haapasalo M, Shen Y, Wang Z, Gao Y. Irrigation in endodontics. Br Dent J. 2014;216(6):299–303.
31. Sarbinoff JA, O’Leary TJ, Miller CH. The comparative effectiveness of various agents in detoxifying diseased root surfaces. J Periodontol. 1983 Feb;54(2):77–80.
32. Spratt DA, Pratten J, Wilson M, Gulabivala K. An in vitro evaluation of the antimicrobial efficacy of irrigants on biofilms of root canal isolates. Int Endod J. 2001 Jun;34(4):300–7.
33. Kishen A, Sum CP, Mathew S, Lim CT. Influence of irrigation regimens on the adherence of *Enterococcus faecalis* to root canal dentin. J Endod. 2008 Jul;34(7):850–4.
34. Nygaard-Østby B. Chelation in root canal therapy: ethylenediaminetetraacetic acid for cleansing and widening of root canals. Odontologisk Tidskri. 1957;65:3–11.
35. de Souza Matos F, Rosatto CMP de, Cunha TC, Vidigal MTC, Blumenberg C, Paranhos LR, et al. Influence of chelating solutions on tubular dentin sealer penetration: A systematic review with network meta-analysis. Australian Endodontic Journal. 2021;16:516–38.
36. Cruz-Filho AM, Sousa-Neto MD, Savioli RN, Silva RG, Vansan LP, Pécora JD. Effect of Chelating Solutions on the Microhardness of Root Canal Lumen Dentin. J Endod. 2011 Mar;37(3):358–62.
37. Torabinejad M, Khademi A, Babagoli J, Cho Y, Johnson W, Bozhilov K, et al. A New Solution for the Removal of the Smear Layer. J Endod. 2003 Mar;29(3):170–5.

38. Mohammadi Z, Shalavi S, Jafarzadeh H. Ethylenediaminetetraacetic acid in endodontics. *Eur J Dent.* 2013;7(SUPPL 1):s135–42.
39. Torabinejad M, Handysides R, Ali Khademi A, Bakland LK, Linda L. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2002;94(6):658–66.
40. Paqué F, Rechenberg DK, Zehnder M. Reduction of hard-tissue debris accumulation during rotary root canal instrumentation by etidronic acid in a NaOCl irrigant. *J Endod.* 2012 May;38(5):692–5.
41. Zehnder M, Schmidlin P, Sener B, Waltimo T. Chelation in Root Canal Therapy Reconsidered. *JOE.* 2005;31(11):817–20.
42. Tejada S, Baca P, Ferrer-Luque CM, Ruiz-Linares M, Valderrama MJ, Arias-Moliz & MT. Influence of dentine debris and organic tissue on the properties of NaOCl solutions. *Int Endod J.* 2019;52:114–22.
43. Arias-Moliz MT, Camilleri J. The effect of the final irrigant on the antimicrobial activity of root canal sealers. *J Dent.* 2016 Sep;52:30–6.
44. Lottanti S, Gautschi H, Sener B, Zehnder M. Effects of ethylenediaminetetraacetic, etidronic and peracetic acid irrigation on human root dentine and the smear layer. *Int Endod J.* 2009 Apr;42(4):335–43.
45. Tartari T, Guimar~ Aes BM, Amoras LS, Duarte MAH, Silva E Souza PAR, Bramante CM. Etidronate causes minimal changes in the ability of NaOCl to dissolve organic matter. *Int Endod J.* 2015;48:399– 404.
46. Morago A, Ordinola-Zapata R, Mar Ia Ferrer-Luque C, Baca P, Ruiz Linares M, Arias-Moliz T. Influence of Smear Layer on the Antimicrobial Activity of a Sodium Hypochlorite/Etidronic Acid Irrigating Solution in Infected Dentin. 2016;11;1647-50.
47. Tartari T, De Almeida Rodrigues Silva E Souza P, Vila Nova De Almeida B, Carrera Silva Júnior JO, Faciola Pessoa O, Silva E Souza Junior MH. A new weak chelator in endodontics: Effects of different irrigation regimens with etidronate on root dentin microhardness. *Int J Dent.*

- 2013;2013:743018.
48. Neelakantan P, Varughese AA, Sharma S, Subbarao C V, Zehnder M, DeDeus G. Continuous chelation irrigation improves the adhesion of epoxy resin-based root canal sealer to root dentine. *Int Endod J.* 2012;45:1097–102.
 49. Dineshkumar MK, Vinothkumar TS, Arathi G, Shanthisree P, Kandaswamy D. Effect of ethylene diamine tetra-acetic acid, MTAD TM, and HEBP as a final rinse on the microhardness of root dentin. *Journal of Conservative Dentistry.* 2012 Apr;15(2):170–3.
 50. De-Deus G, Zehnder M, dent med, Reis C, Fidel S, Antonio Sergio Fidel R, et al. Longitudinal Co-site Optical Microscopy Study on the Chelating Ability of Etidronate and EDTA Using a Comparative Single-tooth Model. *JOE.* 2008;34:71–5.
 51. Tomson RME, Polycarpou N, Tomson PL. Contemporary obturation of the root canal system. *Br Dent J.* 2014 Mar 21;216(6):315–22.
 52. Bhandi S, Mashyakhy M, Abumelha AS, Alkahtany MF, Jamal M, Chohan H, et al. Complete obturation—cold lateral condensation vs. Thermoplastic techniques: A systematic review of micro-CT studies. *Materials.* 2021;14:4013.
 53. Ingle JI. *Ingle's Endodontics.* 6th ed. Connecticut: Pmph USA Ltd; 2008.
 54. Heran J, Khalid S, Albaaj F, Tomson PL, Camilleri J. The single cone obturation technique with a modified warm filler. *J Dent.* 2019 Oct;89:103181.
 55. Iglecias EF, Freire LG, de Miranda Candeiro GT, dos Santos M, Antoniazzi JH, Gavini G. Presence of Voids after Continuous Wave of Condensation and Single-cone Obturation in Mandibular Molars: A Microcomputed Tomography Analysis. *J Endod.* 2017 Apr;43(4):638–42.

56. Kim J Hyung, Cho SY, Choi Y, Kim D Hyun, Shin SJ, Jung IY. Clinical Efficacy of Sealer-based Obturation Using Calcium Silicate Sealers: A Randomized Clinical Trial. *J Endod.* 2022 Feb;48(2):144–51.
57. Gasner NS, Brizuela M. Endodontic Materials Used To Fill Root Canals. 2023.
58. Parirokh M, Torabinejad M. Mineral Trioxide Aggregate: A Comprehensive Literature Review—Part I: Chemical, Physical, and Antibacterial Properties. *J Endod.* 2010 Jan;36(1):16–27.
59. Aun S, Al-Shimari TBDS, Sc M. TheEffect of Insertion Rate on The Adaptability of Gutta-Percha and The Apical Extrusion of Thermoplasticised Gutta-percha Obturation Techniques. *J Bagh College Dentistry.* 2017;29:33-36.
60. Frantzeska K. Gutta-percha and Updated Obturating Techniques. *J Dent Health Oral Disord Ther.* 2017 Sep 1;8(2):470–4.
61. Vishwanath Vijetha, Murali Rao H. Gutta-percha in endodontics-A comprehensive review of material science. 2019.
62. Li G hua, Niu L na, Zhang W, Olsen M, De-Deus G, Eid AA, et al. Ability of new obturation materials to improve the seal of the root canal system: A review. *Acta Biomater.* 2014 Mar;10(3):1050–63.
63. Grossman L. Obturation of root canal. *Endodontic Practice.* 10th ed. Philadelphia, PA: Lea and Febiger; 1982:297.
64. Kaur A, Shah N, Logani A, Mishra N. Biototoxicity of commonly used root canal sealers: A meta-analysis. Vol. 18, *Journal of Conservative Dentistry.* 2015;2:83–8.
65. Chandra A, Rahman H, Agarwal A, Verma A. Toxicity of root canal sealers in vitro. *Oral Biology and Dentistry.* 2014;2(1):2.
66. Ørstavik D. Endodontic filling materials. *Endod Topics.* 2014 Nov;31(1):53–67.

67. Komabayashi T, Colmenar D, Cvach N, Bhat A, Primus C, Imai Y. Comprehensive review of current endodontic sealers. *Dental Materials Journal. Japanese Society for Dental Materials and Devices*; 2020;39:703–20.
68. Camilleri J. Will bioceramics be the future root canal filling materials? *Current Oral Health Reports*. 2017;4:228–38.
69. Marciano MA, Guimaraes BM, Ordinola-Zapata R, Bramante CM, Cavenago BC, Garcia RB, et al. Physical properties and interfacial adaptation of three epoxy resin-based sealers. *J Endod*. 2011 Oct;37(10):1417–21.
70. Aktemur Türker S, Uzunoğlu E, Purali N. Evaluation of dentinal tubule penetration depth and push-out bond strength of AH 26, BioRoot RCS, and MTA Plus root canal sealers in presence or absence of smear layer. *J Dent Res Dent Clin Dent Prospects*. 2018 Dec 19;12(4):294–8.
71. Alsubait S, Albader S, Alajlan N, Alkhunaini N, Niazy A, Almahdy A. Comparison of the antibacterial activity of calcium silicate- and epoxy resin-based endodontic sealers against *Enterococcus faecalis* biofilms: a confocal laser-scanning microscopy analysis. *Odontology*. 2019 Oct 1;107(4):513–20.
72. Viapiana R, Guerreiro-Tanomaru JM, Hungaro-Duarte MA, Tanomaru Filho M, Camilleri J. Chemical characterization and bioactivity of epoxy resin and Portland cement-based sealers with niobium and zirconium oxide radiopacifiers. *Dental Materials*. 2014;30(9):1005–20.
73. Khalil I, Naaman A, Camilleri J. Properties of Tricalcium Silicate Sealers. *J Endod*. 2016 Oct 1;42(10):1529–35.
74. Camilleri J, Atmeh A, Li X, Meschi N. Present status and future directions: Hydraulic materials for endodontic use. *Int Endod J. John Wiley and Sons Inc*. 2022;55:710–77.

75. Torabinejad M, Watson TF, Pitt Ford TR. Sealing ability of a mineral trioxide aggregate when used as a root end filling material. *J Endod.* 1993 Dec;19(12):591–5.
76. Lee SJ, Monsef M, Torabinejad M. Sealing ability of a mineral trioxide aggregate for repair of lateral root perforations. *J Endod.* 1993 Nov;19(11):541–4.
77. United States Patent (19) Torabinejad et al. (54). Tooth filling material and method of use. 1993.
78. Camilleri J, Montesin F, Brady K, Sweeney R, Curtis R, Ford T. The constitution of mineral trioxide aggregate. *Dental Materials.* 2005 Apr;21(4):297–303.
79. Candeiro GTDM, Correia FC, Duarte MAH, Ribeiro-Siqueira DC, Gavini G. Evaluation of radiopacity, pH, release of calcium ions, and flow of a bioceramic root canal sealer. *J Endod.* 2012 Jun;38(6):842–5.
80. Zhou HM, Shen Y, Zheng W, Li L, Zheng YF, Haapasalo M. Physical properties of 5 root canal sealers. *J Endod.* 2013 Oct;39(10):1281–6.
81. Camilleri J. Classification of Hydraulic Cements Used in Dentistry. *Frontiers in Dental Medicine.* 2020 Sep 8;1.
82. Cavenago BC, Pereira TC, Duarte MAH, Ordinola-Zapata R, Marciano MA, Bramante CM, et al. Influence of powder-to-water ratio on radiopacity, setting time, pH, calcium ion release and a micro-CT volumetric solubility of white mineral trioxide aggregate. *Int Endod J.* 2014;47(2):120–6.
83. Debelian G, Trope M. The use of premixed bioceramic materials in endodontics. Vol. 30, *Giornale Italiano di Endodonzia.* Masson SpA. 2016. :70–80.
84. Gandolfi MG, Taddei P, Tinti A, De Dorigo ES, Rossi PL, Prati C. Kinetics of apatite formation on a calcium-silicate cement for root-end

- filling during ageing in physiological-like phosphate solutions. *Clin Oral Investig*. 2010 Dec;14(6):659–68.
85. Kebudi Benezra M, Schembri Wismayer P, Camilleri J. Influence of environment on testing of hydraulic sealers. *Sci Rep*. 2017 Dec 1;7(1):17927.
 86. Marciano MA, Duarte MAH, Camilleri J. Calcium silicate-based sealers: Assessment of physicochemical properties, porosity and hydration. *Dental Materials*. 2016 Feb 1;32(2):e30–40.
 87. Zancan RF, Maio A Di, Tomson PL, Duarte MAH, Camilleri J. The presence of smear layer affects the antimicrobial action of root canal sealers. *IEJ*. 2021;54(8):1369–82.
 88. Zancan RF, Vivan RR, Milanda Lopes MR, Weckwerth PH, de Andrade FB, Ponce JB, et al. Antimicrobial Activity and Physicochemical Properties of Calcium Hydroxide Pastes Used as Intracanal Medication. *J Endod*. 2016 Dec 1;42(12):1822–8.
 89. Atmeh AR, Chong EZ, Richard G, Festy F, Watson TF. Dentin-cement interfacial interaction: Calcium silicates and polyalkenoates. *J Dent Res*. 2012 May;91(5):454–9.
 90. Viapiana R, Moinzadeh AT, Camilleri L, Wesselink PR, Tanomaru Filho M, Camilleri J. Porosity and sealing ability of root fillings with gutta-percha and BioRoot RCS or AH Plus sealers. Evaluation by three ex vivo methods. *Int Endod J*. 2016 Aug 1;49(8):774–82.
 91. Hadis M, Wang J, Zhang ZJ, Di Maio A, Camilleri J. Interaction of hydraulic calcium silicate and glass ionomer cements with dentine. *Materialia (Oxf)*. 2020 Mar 1;9:100515.
 92. Boskey AL. Biomineralization: An Overview. *Connect Tissue Res*. 2003 Jan 6;44(1):5–9.
 93. Camilleri J. Endodontics Bioroot RCS-is a paradigm shift for root canal obturation possible? 2022. (Available from: www.septodont.co.uk)

94. M Maroto, E Barbería, P Planells. Dentin bridge formation after mineral trioxide aggregate (MTA) pulpotomies in primary teeth. *Am J Dent*. 2005;18(3):151–4.
95. Ricucci D, Grande NM, Plotino G, Tay FR. Histologic Response of Human Pulp and Periapical Tissues to Tricalcium Silicate-based Materials: A Series of Successfully Treated Cases. *J Endod*. 2020 Feb 1;46(2):307–17.
96. Giacomino CM, Wealleans JA, Kuhn N, Diogenes A. Comparative Biocompatibility and Osteogenic Potential of Two Bioceramic Sealers. *J Endod*. 2019 Jan 1;45(1):51–6.
97. Zhang W, Li Z, Peng B. Effects of iRoot SP on mineralization-related genes expression in MG63 Cells. *J Endod*. 2010 Dec;36(12):1978–82.
98. Schmalz G, Hickel R, Price RB, Platt JA. Bioactivity of Dental Restorative Materials: FDI Policy Statement. *Int Dent J*. 2023 Dec;73(1):21–7.
99. Viapiana R, Guerreiro-Tanomaru J, Tanomaru-Filho M, Camilleri J. Interface of dentine to root canal sealers. *J Dent*. 2014 Mar;42(3):336–50.
100. Lee YL, Wang WH, Lin FH, Lin CP. Hydration behaviors of calcium silicate-based biomaterials. *Journal of the Formosan Medical Association*. 2017 Jun 1;116(6):424–31.
101. Koutroulis A, Kuehne SA, Cooper PR, Camilleri J. The role of calcium ion release on biocompatibility and antimicrobial properties of hydraulic cements. *Sci Rep*. 2019 Dec 13;9(1):19019.
102. Atmeh AR. Investigating the effect of bicarbonate ion on the structure and strength of calcium silicate-based dental restorative material—Biodentine. *Clin Oral Investig*. 2020 Dec 26;24(12):4597–606.
103. Gandolfi MG, Siboni F, Botero T, Bossù M, Riccitiello F, Prati C. Calcium silicate and calcium hydroxide materials for pulp capping: bio interactivity, porosity, solubility and bioactivity of current formulations. *J Appl Biomater Funct Mater*. 2015;13(1):43–60.
104. Beaudoin J, Odler I. Hydration, setting and hardening of Portland cement.

- In: Lea's chemistry of cement and concrete. 3rd ed. London,UK: Butterworth- Heinemann; 2019.
105. Wang Z, Shen Y, Haapasalo M. Dentin extends the antibacterial effect of endodontic sealers against *Enterococcus faecalis* biofilms. J Endod. 2014;40(4):505–8.
 106. Morgental RD, Vier-Pelisser F V., Oliveira SD, Antunes FC, Cogo DM, Kopper PMP. Antibacterial activity of two MTA-based root canal sealers. Int Endod J. 2011 Dec;44(12):1128–33.
 107. Du T, Wang Z, Shen Y, Ma J, Cao Y, Haapasalo M. Combined Antibacterial Effect of NaOCl and Root Canal Sealers against *Enterococcus faecalis* Biofilms in Dentin Canals. J Endod. 2015 Aug 1;41(8):1294–8.
 108. Wang Z, Shen Y, Haapasalo M. Dental materials with antibiofilm properties. Dental Materials. 2014;30: e1–16.
 109. Long J, Kreft JU, Camilleri J. Antimicrobial and ultrastructural properties of root canal filling materials exposed to bacterial challenge. J Dent. 2020 Feb 1;93.
 110. Farrugia C, Baca P, Camilleri J, Arias Moliz MT. Antimicrobial activity of ProRoot MTA in contact with blood. Sci Rep. 2017 Jan 27;7:41359.
 111. Kharouf N, Arntz Y, Eid A, Zghal J, Sauro S, Haikel Y, et al. Physicochemical and antibacterial properties of novel, premixed calcium silicate-based sealer compared to powder–liquid bioceramic sealer. J Clin Med. 2020 Oct 1;9(10):1–17.
 112. Ma J, Wang Z, Shen Y, Haapasalo M. A new non-invasive model to study the effectiveness of dentin disinfection by using confocal laser scanning microscopy. J Endod. 2011 Oct;37(10):1380–5.
 113. Cueva-Goig R, Forner-Navarro L, Llena-Puy MC. Microscopic assessment of the sealing ability of three endodontic filling techniques. Vol. 8, J Clin Exp Dent. 2016.

114. Evans JT, Simon JHS. Evaluation of the apical seal produced by injected thermoplasticised gutta-percha in the absence of smear layer and root canal sealer. *J Endod.* 1986 Mar;12(3):101–7.
115. Peters CI, Sonntag D, Peters OA. Homogeneity of root canal fillings performed by undergraduate students with warm vertical and cold lateral techniques. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology.* 2010 Sep;110(3):e41–9.
116. Marlin J, Schilder H. Physical properties of gutta-percha when subjected to heat and vertical condensation. 1973.
117. Wu MK, Kašt'áková A, Wesselink PR. Quality of cold and warm gutta-percha fillings in oval canals in mandibular premolars. *Int Endod J.* 2001 Sep;34(6):485–91.
118. Blum JY, Micallef JP. Analysis of Forces Developed During Obturations. Comparison of Three Gutta-Percha Techniques. 1997 May;23(5):340-5.
119. Donnermeyer D, Schäfer E, Bürklein S. Real-time Intracanal Temperature Measurement During Different Obturation Techniques. *J Endod.* 2018 Dec 1;44(12):1832–6.
120. Wolcott J, Himmel VT, Powell W, Penney J. Effect of Two Obturation Techniques on the Filling of Lateral Canals and the Main Canal. 1997 Oct;23(10):632-5.
121. Alhashimi RA, Foxton R, Romeed S, Deb S. An in vitro assessment of gutta-percha coating of new carrier-based root canal fillings. *ScientificWorldJournal.* 2014;2014:239754.
122. Gambarini G, Piasecki L, Schianchi G, Di Nardo D, Miccoli G, Al Sudani D, et al. In vitro evaluation of carrier based obturation technique: a CBCT study. *Ann Stomatol (Roma).* 2016;7(1–2):11–5.
123. Guivarc'h M, Jeanneau C, Giraud T, Pommel L, About I, Azim AA, et al. An international survey on the use of calcium silicate-based sealers in

- nonsurgical endodontic treatment. Clin Oral Investig. 2020 Jan 1;24(1):417–24.
124. RAY HA, TROPE M. Periapical status of endodontically treated teeth in relation to the technical quality of the root filling and the coronal restoration. Int Endod J. 1995 Jan;28(1):12–8.
 125. Delboni MG, Gomes BPFA, Francisco PA, Teixeira FB, Drake D. Diversity of *Enterococcus faecalis* Genotypes from Multiple Oral Sites Associated with Endodontic Failure Using Repetitive Sequence-based Polymerase Chain Reaction and Arbitrarily Primed Polymerase Chain Reaction. J Endod. 2017 Mar;43(3):377–82.
 126. Eliyas S, Jalili J, Martin N. Restoration of the root canal treated tooth. Br Dent J. 2015 Jan 23;218(2):53–62.
 127. Machado R, Garcia L da FR, da Silva Neto UX, Cruz Filho A de M da, Silva RG, Vansan LP. Evaluation of 17% EDTA and 10% citric acid in smear layer removal and tubular dentin sealer penetration. Microsc Res Tech. 2018 Mar 1;81(3):275–82.
 128. Dotto L, Kalil Rocha Pereira G, Tomm A, Bacchi A, Sarkis-Onofre R, is Bacchi A. Effect of root canal irrigants on push-out bond strength of endodontic sealers: a systematic review. J Adhes Sci Technol. 2020; 35:1701–22.
 129. Fernandes Zancan R, Hadis M, Burgess D, Zhang ZJ, Maio AD, Tomson P, et al. A matched irrigation and obturation strategy for root canal therapy. Sci Rep. 2021;11:4666–4666.
 130. Donnermeyer D, Vahdat-Pajouh N, Schäfer E, Dammaschke T. Influence of the final irrigation solution on the push-out bond strength of calcium silicate-based, epoxy resin-based and silicone-based endodontic sealers. Odontology. 2019 Apr;107(2):231–6.

131. De Assis DF, Prado M Do, Simão RA. Evaluation of the interaction between endodontic sealers and dentin treated with different irrigant solutions. *J Endod.* 2011 Nov;37(11):1550–2.
132. Elbahary S, Haj-yahya S, Khawalid M, Tsisis I, Rosen E, Habashi W, et al. Effects of different irrigation protocols on dentin surfaces as revealed through quantitative 3D surface texture analysis. *Sci Rep.* 2020 Dec 1;10(1).
133. Rath PP, Yiu CKY, Matinlinna JP, Kishen A, Neelakantan P. The effect of root canal irrigants on dentin: a focused review. *Restor Dent Endod.* 2020;45(3).
134. Sayed MAM El. Impact of Irrigation Solutions on the Apical Sealing Potential of Different Endodontic Sealers Used with the Continuous-wave Obturation Technique: An In vitro Study. *European J Gen Dent.* 2020 Sep 1;9(03):134–40.
135. Ozturk B, Ozer F. Effect of NaOCl on Bond Strengths of Bonding Agents to Pulp Chamber Lateral Walls. *J Endod.* 2004 May;30(5):362–5.
136. Carrilho MRO, Geraldeli S, Tay F, de Goes MF, Carvalho RM, Tjäderhane L, et al. In vivo preservation of the Hybrid Layer by Chlorhexidine. *J Dent Res.* 2007 Jun 18;86(6):529–33.
137. Machado R, Garcia L da FR, da Silva Neto UX, Cruz Filho A de M da, Silva RG, Vansan LP. Evaluation of 17% EDTA and 10% citric acid in smear layer removal and tubular dentin sealer penetration. *Microsc Res Tech.* 2018 Mar;81(3):275–82.
138. Moon YM, Shon WJ, Baek SH, Bae KS, Kum KY, Lee W. Effect of Final Irrigation Regimen on Sealer Penetration in Curved Root Canals. *J Endod.* 2010 Apr;36(4):732–6.
139. Kara Tuncer A, Tuncer S. Effect of Different Final Irrigation Solutions on Dentinal Tubule Penetration Depth and Percentage of Root Canal Sealer. *J Endod.* 2012 Jun;38(6):860–3.

140. El Sayed M. Impact of irrigation solutions on the apical sealing potential of different endodontic sealers used with the continuous-wave obturation technique: An in vitro study. *European J Gen Dent*. 2020 Sep 1;9(3):134–40.
141. Fisher M, Berzins D, Bahcall J. An In Vitro Comparison of Bond Strength of Various Obturation Materials to Root Canal Dentin Using a Push-Out Test Design. *J Endod*. 2007 Jul;33(7):856–8.
142. Y.-L. Lee¹, F.-H. Lin², W.-H. Wang², H.H. Ritchie³, W.-H. Lan¹, C.-P. Lin¹. Effects of EDTA on the Hydration Mechanism of Mineral Trioxide Aggregate. 2007.
143. Marending M, Paqué F, Fischer J, Zehnder M. Impact of irrigant sequence on mechanical properties of human root dentine. *JOE*. 2007 Nov;33(11):1325–8.
144. Çalt S, Serper A. Time-Dependent Effects of EDTA on Dentin Structures. *J Endod*. 2002 Jan;28(1):17-9.
145. Chande KP, Manwar NU, Chandak MG, Lokade J, Chandak SR. Effect of Chelating Agents and Irrigants on Mineral Content of Root Canal Dentin: An In Vitro Study. *International Journal of Clinical Preventive Dentistry*. 2014 Sep 30;10(3):135–8.
146. Kumar P, Prasad N, Darawade A, Kumar Bhagat S, Narayana V, Darawade P. The Effect of Four Commonly used Root Canal Irrigants on the Removal of Smear Layer: An In-vitro Scanning Electron Microscope Study. *Journal of International Oral Health*. 2015;7:88-93.
147. Barón M, Morales V, Fuentes MV, Linares M, Escribano N, Ceballos L. The influence of irrigation solutions in the inorganic and organic radicular dentine composition. *Australian Endodontic Journal*. 2020 Aug 1;46(2):217–25.
148. Lima Nogueira BM, Da Costa Pereira TI, Pedrinha VF, de Almeida Rodrigues P. Effects of different irrigation solutions and protocols on

- mineral content and ultrastructure of root canal dentine. Iran Endod J. 2018 Mar 1;13(2):209–15.
149. Ballal NV, Roy A, Zehnder M. Effect of NaOCl concentration in continuous chelation on dislodgement resistance of an epoxy resin and hydraulic calcium silicate sealer. Polymers (Basel). 2021 Oct 1;13(20):3482.
 150. American Society for Testing and Material. ASTM C266-18. Standard test method for time of setting of hydraulic-cement paste by gillmore needles. 2008;
 151. Arias-Moliz MT, Farrugia C, Lung CYK, Schembri Wismayer P, Camilleri J. Antimicrobial and biological activity of leachate from light curable pulp capping materials. Journal of Dentistry. 2017;64:45-51.
 152. Formosa D, Li X, Sammons R, Dong H. Development and characterisation of novel anti-bacterial S-phase based coatings. Thin Solid Films. 2017 Dec 31;644:71–81.
 153. Schmidt TF, Teixeira CS, Felipe MCS, Felipe WT, Pashley DH, Bortoluzzi EA. Effect of Ultrasonic Activation of Irrigants on Smear Layer Removal. J Endod. 2015 Aug 1;41(8):1359–63.
 154. Arias-Moliz MT, Ordinola-Zapata R, Baca P, Ruiz-Linares M, García García E, Hungaro Duarte MA, et al. Antimicrobial activity of Chlorhexidine, Peracetic acid and Sodium hypochlorite/etidronate irrigant solutions against *Enterococcus faecalis* biofilms. Int Endod J. 2015 Dec 1;48(12):1188–93.
 155. Arias-Moliz MT, Camilleri J. The effect of the final irrigant on the antimicrobial activity of root canal sealers. Journal of Dentistry. 2016;52:30-36.
 156. Ordinola-Zapata R, Bramante CM, Cavenago B, Graeff MSZ, Gomes de Moraes I, Marciano M, et al. Antimicrobial effect of endodontic solutions used as final irrigants on a dentine biofilm model. Int Endod J. 2012 Feb;45(2):162–8.

157. Deng Y, Wang L, Chen Y, Long Y. Optimization of staining with SYTO 9/propidium iodide: Interplay, kinetics and impact on *Brevibacillus brevis*. *Biotechniques*. 2020;69(2):89–99.
158. Sajini S, Atmeh AR, Banerjee A, Festy F, Cook RJ, Andiappan M, et al. Glass-ionomer and calcium silicate-based cements interactions with human dentine in health and disease: Two-photon fluorescence microscopy and Raman spectroscopy analysis. *Dental Materials*. 2022 Sep;38(11):1710–20.
159. Reyes-Carmona J, Felipe M, Felipe W. The biomineralization ability of Mineral Trioxide Aggregate and Portland Cement on dentine enhances the push-out strength. *JOE*. 2010 Feb;36(2):286–91.
160. Li X, Pongprueksa P, Van Landuyt K, Chen Z, Pedano M, Van Meerbeek B, et al. Correlative micro-Raman/EPMA analysis of the hydraulic calcium silicate cement interface with dentin. *Clin Oral Investig*. 2016 Sep 1;20(7):1663–73.
161. Nair PNR, Henry S, Cano V, Vera J. Microbial status of apical root canal system of human mandibular first molars with primary apical periodontitis after “one-visit” endodontic treatment. *Oral Surg Oral Med Oral Pathol Endod*. 2005 Feb;99(2):231–52.
162. Radwanski M, Rozpedek-Kaminska W, Galita G, Siwecka N, Sokolowski J, Majsterek I, et al. Cytotoxicity and genotoxicity of bioceramic root canal sealers compared to conventional resin-based sealer. *Sci Rep*. 2024 Dec 1;14(1).
163. Versiani MA, Carvalho-Junior JR, Padilha MIAF, Lacey S, Pascon EA, Sousa-Neto MD. A comparative study of physicochemical properties of AH Plus and Epiphany root canal sealants. *Int Endod J*. 2006 Jun;39(6):464–71.
164. Silva Almeida LH, Moraes RR, Morgental RD, Pappen FG. Are Premixed Calcium Silicate-based Endodontic Sealers Comparable to Conventional Materials? A Systematic Review of In Vitro Studies. *JOE*. 2017;43:527–35.

165. Kim TG, Lee YH, Lee NH, Bhattarai G, Lee IK, Yun BS, et al. The antioxidant property of pachymic acid improves bone disturbance against AH plus-induced inflammation in MC-3T3 E1 cells. *J Endod*. 2013 Apr;39(4):461–6.
166. Hadis M, Camilleri J. Characterization of heat resistant hydraulic sealer for warm vertical obturation. *Dental Materials*. 2020 Sep 1;36(9):1183–9.
167. Drukteinis S. Root canal obturation techniques with hydraulic calcium silicate-based materials. *Clinical Dentistry Reviewed*. 2021 Dec;5(1).
168. Dumitriu D, Dobre T. Effects of Temperature and Hypochlorite Concentration on the Rate of Collagen Dissolution. *J Endod*. 2015 Jun;41(6):903–6.
169. Breschi L, Gobbi P, Chersoni S, Mazzotti G, Prati C. Effects of different acid and NaOCl treatments on dentin collagen: a FEISEM analysis. *Am J Dent*. 2003 Sep;16 Spec No:77A-81A.
170. Ulusoy Öİ, Zeyrek S, Çelik B. Evaluation of smear layer removal and marginal adaptation of root canal sealer after final irrigation using ethylenediaminetetraacetic, peracetic, and etidronic acids with different concentrations. *Microsc Res Tech*. 2017 Jul 1;80(7):687–92.
171. Tartari T, Guimarães BM, Amoras LS, Duarte MAH, Silva e Souza PAR, Bramante CM. Etidronate causes minimal changes in the ability of NaOCl to dissolve organic matter. *Int Endo J*. 2015 Apr 1;48(4):399– 404.
172. Stewart G. Determination of the approximate volumes of medication used in endodontic treatment. *J Dent Res*. 1948 Feb;27(1):24–6.
173. Goldberg F, Gurfinkel J. Analysis of the use of Dycal with gutta-percha points as an endodontic filling technique. *Oral Surgery, Oral Medicine, Oral Pathology*. 1979 Jan;47(1):78–82.
174. De-Deus G, Souza EM, Silva EJNL, Belladonna FG, Simões-Carvalho M, Cavalcante DM, et al. A critical analysis of research methods and

- experimental models to study root canal fillings. *Int Endod J*. John Wiley and Sons Inc; 2022;55:384–445.
175. Kapralos V, Rukke HV, Ørstavik D, Koutroulis A, Camilleri J, Sunde PT. Antimicrobial and physicochemical characterization of endodontic sealers after exposure to chlorhexidine digluconate. *Dental Materials*. 2021 Feb 1;37(2):249–63.
 176. Özyürek T, Demiryürek E. Comparison of the antimicrobial activity of direct pulp-capping materials: Mineral trioxide aggregate-Angelus and Biodentine. *Journal of Conservative Dentistry*. 2016;19(6):569.
 177. Elshamy FM, Elraih H, Gupta I, Idris FA. Antibacterial Effect of New Bioceramic Pulp Capping Material on the Main Cariogenic Bacteria. *J Contemp Dent Pract*. 2016 May;17(5):349–53.
 178. Poggio C, Arciola CR, Beltrami R, Monaco A, Dagna A, Lombardini M, et al. Cytocompatibility and Antibacterial Properties of Capping Materials. *The Scientific World Journal*. 2014;2014:1–10.
 179. Arias-Moliz MT, Farrugia C, Lung CYK, Wismayer PS, Camilleri J. Antimicrobial and biological activity of leachate from light curable pulp capping materials. *J Dent*. 2017 Sep 1;64:45–51.
 180. Coelho dos Santos D, da Silva Barboza A, Rodrigues Schneider L, Enrique Cuevas-Suárez C, Silva Ribeiro J, Feres Damian M, et al. Antimicrobial and physical properties of experimental endodontic sealers containing vegetable extracts. *Sci Rep*. 2021 Aug11;11(1):6450.
 181. Sedigh-Shams M, Gholami A, Abbaszadegan A, Yazdanparast R, Nejad MS, Safari A, et al. Antimicrobial efficacy and cytocompatibility of calcium hypochlorite solution as a root canal irrigant: An in Vitro investigation. *Iran Endod J*. 2016 Jun 1;11(3):169–74.
 182. Álvarez Reyes RI, Rodríguez- Zaragoza DE, Coria-Valdiosera FE. In-vitro comparison of antibacterial effect of epoxic resin and tricalcium silicate based cements. *J Dent Oral Sci*. 2021 May 5;3(2):1–10.

183. Slutzky-Goldberg I, Slutzky H, Solomonov M, Moshonov J, Weiss EI, Matalon S. Antibacterial Properties of Four Endodontic Sealers. *J Endod.* 2008 Jun;34(6):735–8.
184. Kapralos V, Koutroulis A, Ørstavik D, Sunde PT, Rukke HV. Antibacterial Activity of Endodontic Sealers against Planktonic Bacteria and Bacteria in Biofilms. *J Endod.* 2018 Jan 1;44(1):149–54.
185. Farrugia C, Camilleri J. Antimicrobial properties of conventional restorative filling materials and advances in antimicrobial properties of composite resins and glass ionomer cements - A literature review. *Dental Materials.* 2015 Apr 1;31(4):e89–99.
186. Bose R, Ioannidis K, Foschi F, Bakhsh A, Kelly RD, Deb S, et al. Antimicrobial effectiveness of calcium silicate sealers against a nutrient stressed multispecies biofilm. *J Clin Med.* 2020 Sep 1;9(9):1–15.
187. Liu M, He L, Wang H, Su W, Li H. Comparison of in vitro biocompatibility and antibacterial activity of two calcium silicate-based materials. *J Mater Sci: Mater Med.* 2021;32:52.
188. Long J, Ju K, Camilleri J. Antimicrobial and ultrastructural properties of root canal filling materials exposed to bacterial challenge. *J Dent.* 2020;93:103283.
189. Farrugia C, Cassar G, Valdramidis V, Camilleri J. Effect of sterilization techniques prior to antimicrobial testing on physical properties of dental restorative materials. *J Dent.* 2015 Jun 1;43(6):703–14.
190. Ahmed M, Punshon G, Darbyshire A, Seifalian AM. Effects of sterilization treatments on bulk and surface properties of nanocomposite biomaterials. *J Biomed Mater Res B Appl Biomater.* 2013;101(7):1182–90.
191. André CB, dos Santos A, Pfeifer CS, Giannini M, Girotto EM, Ferracane JL. Evaluation of three different decontamination techniques on biofilm formation, and on physical and chemical properties of resin composites. *J Biomed Mater Res B Appl Biomater.* 2018 Apr 1;106(3):945–53.

192. Horakova J, Klicova M, Erben J, Klapstova A, Novotny V, Behalek L, et al. Impact of various sterilization and disinfection techniques on electrospun poly- ϵ -caprolactone. *ACS Omega*. 2020 Apr 21;5(15):8885–92.
193. Bhandari S, Palin W, Kuehne SA, Camilleri J. Investigating best practice for specimen preparation for biological testing of root canal sealers. *Dental Materials*. 2024;40(3):387-92.
194. McMichael GE, Primus CM, Opperman LA. Dentinal Tubule Penetration of Tricalcium Silicate Sealers. *J Endod*. 2016 Apr;42(4):632–6.
195. Balguerie E, van der Sluis L, Vallaey K, Gurgel-Georgelin M, Diemer F. Sealer Penetration and Adaptation in the Dentinal Tubules: A Scanning Electron Microscopic Study. *J Endod*. 2011 Nov;37(11):1576–9.
196. Jeong JW, DeGraft-Johnson A, Dorn SO, Di Fiore PM. Dentinal Tubule Penetration of a Calcium Silicate-based Root Canal Sealer with Different Obturation Methods. *J Endod*. 2017 Apr 1;43(4):633–7.
197. Ordinola-Zapata R, Bramante CM, Graeff MSZ, del Carpio Perochena A, Vivan RR, Camargo EJ, et al. Depth and percentage of penetration of endodontic sealers into dentinal tubules after root canal obturation using a lateral compaction technique: A confocal laser scanning microscopy study. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology*. 2009 Sep;108(3):450–7.
198. Wang JS, Bai W, Wang Y, Liang YH. Effect of different dentin moisture on the push-out strength of bioceramic root canal sealer. *J Dent Sci*. 2023 Jan;18(1):129–34.
199. Tedesco M, Chain MC, Felipe WT, Alves AMH, Garcia LDFR, Bortoluzzi EA, et al. Correlation between bond strength to dentin and sealers penetration by push-out test and CLSM analysis. *Braz Dent J*. 2019 Nov 1;30(6):555–62.
200. Neelakantan P, Sharma S, Shemesh H, Wesselink PR. Influence of Irrigation Sequence on the Adhesion of Root Canal Sealers to Dentin: A

- Fourier Transform Infrared Spectroscopy and Push-out Bond Strength Analysis. *J Endod*. 2015 Jul 1;41(7):1108–11.
201. Mohammadian F, Farahanimastary F, Dibaji F, Kharazifard MJ. Scanning Electron Microscopic Evaluation of the Sealer-Dentine Interface of Three Sealers. *IEJ Iranian Endodontic Journal*. 2017;12(1):38–42.
 202. Kapralos V, Valen H, Koutroulis A, Camilleri J, Ørstavik D, Sunde PT. The dentine-sealer interface: Modulation of antimicrobial effects by irrigation. *Int Endod J*. 2022 May 1;55(5):544–60.
 203. Atmeh AR, Chong EZ, Richard G, Festy F, Watson TF. Dentin-cement interfacial interaction: Calcium silicates and polyalkenoates. *J Dent Res*. 2012 May;91(5):454–9.
 204. Tedesco M, Chain MC, Bortoluzzi EA, Garcia L da FR, Alves AMH, Teixeira CS. Comparison of two observational methods, scanning electron and confocal laser scanning microscopies, in the adhesive interface analysis of endodontic sealers to root dentine. *Clin Oral Investig*. 2018 Jul 1;22(6):2353–61.
 205. Majumdar T, Mukherjee S, Mazumdar P. Microscopic evaluation of sealer penetration and interfacial adaptation of three different endodontic sealers: An in vitro study. *J Conserv Dent*. 2021;24(5):435–9.
 206. Zhang C, Wu M, Pu J, Shan Q, Sun Y, Wang S, et al. Effect of Cu Coating on Microstructure and Properties of Al/Steel Welding–Brazing Joints Obtained by Cold Metal Transfer (CMT). *Coatings*. 2022 Aug 1;12(8):1123.
 207. Camilleri J, Grech L, Galea K, Keir D, Fenech M, Formosa L, et al. Porosity and root dentine to material interface assessment of calcium silicate-based root-end filling materials. *Clin Oral Investig*. 2014;18(5):1437–46.
 208. Russell A, Friedlander L, Chandler N. Sealer penetration and adaptation in root canals with the butterfly effect. *Australian Endodontic Journal*. 2018 Dec 1;44(3):225–34.

209. Kamin R, Vikram R, Meena N, Anitha Kumari R, Adarsha MS, Murthy CS. Effect of final irrigating solutions on penetration depth of resin-based sealers into dentinal tubules. *Journal of Conservative Dentistry*. 2021 Jul 1;24(4):374–8.
210. Ozasir T, Eren B, Gulsahi K, Ungor M. The Effect of Different Final Irrigation Regimens on the Dentinal Tubule Penetration of Three Different Root Canal Sealers: A Confocal Laser Scanning Microscopy Study in Vitro. *Scanning*. 2021 Oct 14;2021:8726388.
211. Kim H, Kim E, Lee SJ, Shin SJ. Comparisons of the Retreatment Efficacy of Calcium Silicate and Epoxy Resin-based Sealers and Residual Sealer in Dentinal Tubules. *J Endod*. 2015 Dec;41(12):2025–30.
212. Chandra SS, Shankar P, Indira R. Depth of penetration of four resin sealers into radicular dentinal tubules: a confocal microscopic study. *J Endod*. 2012 Oct;38(10):1412–6.
213. Camilleri J, Pitt Ford TR. Evaluation of the effect of tracer pH on the sealing ability of glass ionomer cement and mineral trioxide aggregate. *J Mater Sci Mater Med*. 2008 Aug;19(8):2941–8.
214. Kwon SR, Wertz PW, Li Y, Chan DCN. Penetration pattern of rhodamine dyes into enamel and dentin: Confocal laser microscopy observation. *Int J Cosmet Sci*. 2012 Feb;34(1):97–101.
215. Donnermeyer D, Schmidt S, Rohrbach A, Berlandi J, Bürklein S, Schäfer E. Debunking the concept of dentinal tubule penetration of endodontic sealers: Sealer staining with rhodamine b fluorescent dye is an inadequate method. *Materials*. 2021 Jun 2;14(12):3211.
216. Machado MEL, Veintimilla Lozada VN, Carrillo Rengifo KJ, Guillén REG, Caballero-Flores H, Nabeshima CK. Confocal laser scanning microscopic analysis of the penetration of an epoxy resin-based sealer into dentinal tubules after calcium hydroxide dressing. *Australian Endodontic Journal*. 2021;47(3):480–6.

217. El Hachem R, Khalil I, Le Brun G, Pellen F, Le Jeune B, Daou M, et al. Dentinal tubule penetration of AH Plus, BC Sealer and a novel tricalcium silicate sealer: a confocal laser scanning microscopy study. *Clin Oral Investig*. 2019 Apr;23(4):1871–6.
218. Giardino L, Del Fabbro M, Morra M, Pereira T, Bombarda de Andrade F, Savadori P, et al. Dual Rinse® HEDP increases the surface tension of NaOCl but may increase its dentin disinfection efficacy. *Odontology*. 2019 Oct 1;107(4):521–9.
219. Huang X, Haapasalo M, Wang Z, Hancock REW, Wei X, Shen Y. Effect of Long-term Exposure to Peptides on Mono- and Multispecies Biofilms in Dentinal Tubules. *J Endod*. 2019 Dec 1;45(12):1522–8.
220. Generali L, Campolongo E, Consolo U, Bertoldi C, Giardino L, Cavani F. NaOCl penetration into dentinal tubules after manual dynamic agitation and ultrasonic activation: a histochemical evaluation. *Odontology*. 2018 Oct 1;106(4):454–9.
221. Thiruvankadam G, Asokan S, John B, Priya PG. Effect of 95% Ethanol as a Final Irrigant before Root Canal Obturation in Primary Teeth: An in vitro Study. *Int J Clin Pediatr Dent*. 2016;9(1):21–4.
222. Sissons CH, Wong L, Cutress TW. Inhibition by ethanol of the growth of biofilm and dispersed microcosm dental plaques. *Archs oral Biol*. 1996;41:27-34.
223. Matsumoto H, Kitamura S, Araki T. Autofluorescence in human dentine in relation to age, tooth type and temperature measured by nanosecond time resolved fluorescence microscopy. 1999.
224. Craigbaumgartner J, Mader C. A scanning electron microscopic evaluation of four root canal irrigation regimens. *J Endod*. 1987 Apr;13(4):147–57.
225. McComb D, Smith DC, Beagrie GS. The Results of in vivo Endodontic Chemomechanical Instrumentation ? A Scanning Electron Microscopic Study. *Int Endod J*. 1976 Jan;9(1):11–8.

226. Spencer P, Byerley TJ, Eick JD, Witt JD. Chemical characterization of the dentin/adhesive interface by Fourier Transform Infrared Photoacoustic Spectroscopy. *Dent Mater.* 1992;8:10-15.
227. Ylmén R, Jäglid U. Carbonation of Portland Cement Studied by Diffuse Reflection Fourier Transform Infrared Spectroscopy. *Int J Concr Struct Mater.* 2013;7(2):119–25.
228. Horgnies M, Chen JJ, Bouillon C. Overview about the use of fourier transform infrared spectroscopy to study cementitious materials. In: *WIT Transactions on Engineering Sciences.* 2013. p. 251–62.
229. Lopes C de CA, Limirio PHJO, Novais VR, Dechichi P. Fourier transform infrared spectroscopy (FTIR) application chemical characterization of enamel, dentin and bone. *Applied Spectroscopy Reviews.* 2018;53:747–69.
230. Andrews JM. Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy.* 2001 Jul 1;48(suppl_1):5–16.
231. Ouyang Q, Xie Z, Liu J, Gong M, Yu H. Application of Atomic Force Microscopy as Advanced Asphalt Testing Technology: A Comprehensive Review. *Polymers.* 2022;14:1–25.
232. Chojnacka K, Samoraj M, Tuhy Ł, Michalak I, Mironiuk M, Mikulewicz M. Using XRF and ICP-OES in Biosorption Studies. *Molecules.* 2018 Aug 19;23(8):2076.
233. National Research Council. *Forensic Analysis: Weighing Bullet Lead Evidence* . 2004.
234. Raman V, Camilleri J. Characterization and Assessment of Physical Properties of 3 Single Syringe Hydraulic Cement–based Sealers. *J Endod.* 2024 Mar 1;50(3):381–8.
235. Xuereb M, Vella P, Damidot D, Sammut C V, Camilleri J. In Situ Assessment of the Setting of Tricalcium Silicate-based Sealers Using a Dentin Pressure Model. *JOE.* 2015;41(1):111–24.

236. Bonsor SJ, Camilleri J. Hydraulic cements for various applications in endodontics: Part 2. *Dent Update*. 2021 Sep;48(8):662–8.
237. Kim HI, Jang YE, Kim Y, Kim BS. Physicochemical Changes in Root-Canal Sealers under Thermal Challenge: A Comparative Analysis of Calcium Silicate- and Epoxy-Resin-Based Sealers. *Materials*. 2024 Apr 22;17(8):1932.
238. Maia MRG, Marques S, Cabrita ARJ, Wallace RJ, Thompson G, Fonseca AJM, et al. Simple and versatile turbidimetric monitoring of bacterial growth in liquid cultures using a customized 3D printed culture tube holder and a miniaturized spectrophotometer: Application to facultative and strictly anaerobic bacteria. *Front Microbiol*. 2016 Aug 31;7:1381.
239. Nerandzic MM, Sunkesula VCK, Sankar C. T, Setlow P, Donskey CJ. Unlocking the sporicidal potential of ethanol: Induced sporicidal activity of ethanol against *clostridium difficile* and *bacillus* spores under altered physical and chemical conditions. *PLoS One*. 2015 Jul 15;10(7):e0132805.
240. McDonnell G, Russell AD, Operations L, Louis S. Antiseptics and Disinfectants: Activity, Action, and Resistance. *Clinical microbiology reviews*. 1999;12:147-79.
241. Fabíola Galbiatti De Carvalho¹, Suzana Beatriz Portugal De Fucio¹, Fernanda Miori Pascon², Kamila Rosamília Kantovitz², Lourenço CorrerSobrinho¹, Regina Maria Puppim-Rontani. Effect of Gamma Irradiation on Fluoride Release and Antibacterial Activity of Resin Dental Materials. *Braz Dent J*. 2009;20(2):122–6.
242. Gorna K, Gogolewski S. The effect of gamma radiation on molecular stability and mechanical properties of biodegradable polyurethanes for medical applications. *Polymer Degradation and Stability*. 2002;79:465-74.
243. Farrugia C, Camilleri J. Antimicrobial properties of conventional restorative filling materials and advances in antimicrobial properties of

- composite resins and glass ionomer cements-A literature review. *Dent Mater.* 2015 Apr;31(4):e89-99.
244. Samiei M, Aghazadeh M, Lotfi M, Shakoei S, Aghazadeh Z, Vahid Pakdel SM. Antimicrobial Efficacy of Mineral Trioxide Aggregate with and without Silver Nanoparticles. *Iran Endod J.* 2013;8(4):166–70.
 245. Weiss EI et al. Assessment of antibacterial activity of endodontic sealers by a direct contact. *Endod Dent Traumatol.* 1996;12:179–84.
 246. Zhang H, Shen Y, Dorin Ruse N, Haapasalo M. Antibacterial Activity of Endodontic Sealers by Modified Direct Contact Test Against *Enterococcus faecalis*. *JOE.* 2009;35(7):1051–5.
 247. Wang QQ, Zhang CF, Chu CH, Zhu XF. Prevalence of *Enterococcus faecalis* in saliva and filled root canals of teeth associated with apical periodontitis. *Int J Oral Sci.* 2012 Apr;4(1):19–23.
 248. Arias-Moliz MT, Ferrer-Luque CM, González-Rodríguez MP, Valderrama MJ, Baca P. Eradication of *Enterococcus faecalis* Biofilms by Cetrimide and Chlorhexidine. *J Endod.* 2010 Jan;36(1):87–90.
 249. Koutroulis A, Valen H, Ørstavik D, Kapralos V, Camilleri J, Sunde PT. Surface characteristics and bacterial adhesion of endodontic cements. *Clin Oral Investig.* 2022 Dec 1;26(12):6995–7009.
 250. Schweikl H, Schmalz G. The induction of micronuclei in V79 cells by the root canal filling material AH Plus. 2000.
 251. Huang Y, Li X, Mandal P, Wu Y, Liu L, Gui H, et al. The in vitro antimicrobial activities of four endodontic sealers. *BMC Oral Health.* 2019 Jun 18;19(1):118.
 252. Gomes BPF de A, Pedroso JA, Jacinto RC, Vianna ME, Ferraz CCR, Zaia AA, et al. In vitro evaluation of the antimicrobial activity of five root canal sealers. *Braz Dent J.* 2004;15(1):30–5.
 253. Miyagak DC, Carvalho EMOF de, Robazza CRC, Chavasco JK, Levorato GL. In vitro evaluation of the antimicrobial activity of endodontic sealers.

- Braz Oral Res. 2006 Dec;20(4):303–6.
254. Kaplan AE, Picca M, Gonzalez MI, Macchi RL, Molgatini SL. Antimicrobial effect of six endodontic sealers: an in vitro evaluation. Endod Dent Traumatol. 1999 Feb;15(1):42–5.
 255. Trope M, Bunes A, Debelian G. Root filling materials and techniques: bioceramics a new hope? Endod Topics. 2015 May;32(1):86–96.
 256. Munitić MS, Perićić TP, Utrobičić A, Bago I, Puljak L. Antimicrobial efficacy of commercially available endodontic bioceramic root canal sealers: A systematic review. PLoS One. 2019 Oct 1;14(10):e0223575.
 257. Farmakis ETR, Kontakiotis EG, Tseleni-Kotsovili A, Tsatsas VG. Comparative in vitro antibacterial activity of six root canal sealers against *Enterococcus faecalis* and *Proteus vulgaris*. J Investig Clin Dent. 2012;3(4):271–5.
 258. Zhang H, Pappen FG, Haapasalo M. Dentin Enhances the Antibacterial Effect of Mineral Trioxide Aggregate and Bioaggregate. J Endod. 2009 Feb;35(2):221–4.
 259. Komabayashi T, Colmenar D, Cvach N, Bhat A, Primus C, Imai Y. Comprehensive review of current endodontic sealers. Dental Materials Journal. Japanese Society for Dental Materials and Devices; 2020;39:703–20.
 260. Hauman CHJ, Love RM. Biocompatibility of dental materials used in contemporary endodontic therapy: a review. Part 1. Intracanal drugs and substances. Int Endod J. 2003 Feb;36(2):75–85.
 261. Teughels W, Van Assche N, Sliepen I, Quirynen M. Effect of material characteristics and/or surface topography on biofilm development. Clin Oral Implants Res. 2006 Oct;17 Suppl 2:68–81.
 262. Shen Y, Peng B, Yang Y, Ma J, Haapasalo M. What do different tests tell about the mechanical and biological properties of bioceramic materials? Endod Topics. 2015 May;32(1):47–85.

263. Camilleri J, Montesin FE, Di Silvio L, Pitt Ford TR. The chemical constitution and biocompatibility of accelerated Portland cement for endodontic use. *Int Endod J*. 2005 Nov;38(11):834–42.
264. Farrugia C, Lung CYK, Schembri Wismayer P, Arias-Moliz MT, Camilleri J. The Relationship of Surface Characteristics and Antimicrobial Performance of Pulp Capping Materials. *J Endod*. 2018 Jul 1;44(7):1115–20.
265. Xu H, Ye Z, Zhang A, Lin F, Fu J, Fok ASL. Effects of concentration of NaOCl as an endodontic irrigant on the mechanical and structural properties of root dentine: A laboratory study. *Int Endod J*. 2022 Oct 1;55(10):1091–102.
266. Neelakantan P, Hani , Ounsi F, Devaraj S, Gary , Cheung SP, et al. Effectiveness of irrigation strategies on the removal of the smear layer from root canal dentin. *Odontology*. 2019 Jul 27;107:142–9.
267. Doğan H, Qalt S. Effects of chelating agents and NaOCl on mineral content of root dentin. *J Endod*. 2001 Sep;27(9):578–80.
268. Cury JA, Bragotto C, Valdrighi L, Paula S. The demineralizing efficiency of EDTA solutions on dentin I. Influence of pH. *J Endod*. 1988;14:446–48.
269. Wagner MH, da Rosa RA, de Figueiredo JAP, Duarte MAH, Pereira JR, Só MVR. Final irrigation protocols may affect interradicular dentin ultrastructure. *Clin Oral Investig*. 2017 Sep;21(7):2173–82.
270. Tartari T, Bachmann L, Zancan RF, Vivan RR, Duarte MAH, Bramante CM. Analysis of the effects of several decalcifying agents alone and in combination with NaOCl on the chemical composition of dentine. *Int Endod J*. 2018 Jan 1;51:e42–54.
271. Steier L, Poli De Figueiredo JA, Belli S. Comparison of the interface dentin-endodontic sealer using two SEM magnifications Comparação da interface dentina-cimento endodôntico usando dois aumentos de MEV. *Rev. odonto ciênc*. 2010;25:296-99.

272. Chisnoiu RM, Moldovan M, Prodan D, Chisnoiu AM, Hrab D, Delean AG, et al. In-vitro comparative adhesion evaluation of bioceramic and dual-cure resin endodontic sealers using SEM, AFM, push-out and FTIR. *Applied Sciences (Switzerland)*. 2021 May 2;11(10):4454.
273. Reyes-Carmona J, Felipe M, Felipe W. Biomineralization ability and interaction of Mineral Trioxide Aggregate and white Portland Cement with dentine in a phosphate-containing fluid. *JOE*. 2009 May;35(5):731–6.
274. Viapiana R, Baluci CA, Tanomaru-Filho M, Camilleri J. Investigation of chemical changes in sealers during application of the warm vertical compaction technique. *Int Endod J*. 2015 Jan 1;48(1):16–27.
275. Kebudi Benezra M, Schembri Wismayer P, Camilleri J. Interfacial Characteristics and Cytocompatibility of Hydraulic Sealer Cements. *J Endod*. 2018 Jun 1;44(6):1007–17.
276. Donnermeyer D, Ibing M, Bürklein S, Weber I, Reitze MP, Schäfer E. Physico-chemical investigation of endodontic sealers exposed to simulated intracanal heat application: Hydraulic calcium silicate-based sealers. *Materials*. 2021 Feb 2;14(4):1–11.
277. Morgental RD, Singh A, Sappal H, Kopper PMP, Vier-Pelisser FV, Peters OA. Dentin inhibits the antibacterial effect of new and conventional endodontic irrigants. *J Endod*. 2013 Mar;39(3):406–10.
278. Pizzo G, Giammanco GM, Cumbo E, Nicolosi G, Gallina G. In vitro antibacterial activity of endodontic sealers. *J Dent*. 2006 Jan;34(1):35–40.
279. Kayaoglu G, Erten H, Alaçam T, Ørstavik D. Short-term antibacterial activity of root canal sealers towards *Enterococcus faecalis*. *Int Endod J*. 2005 Jul;38(7):483–8.
280. Camilleri J, Ch BD, Phil M. BioRoot™ RCS Is a paradigm shift for root canal obturation possible? *Scientific inSightS 2*. 2017 Oct.
281. Wang Z, Shen Y, Haapasalo M. Antimicrobial and Antibiofilm Properties of Bioceramic Materials in Endodontics. *Materials*. 2021 Dec10;14(24):7594.

282. Camilleri J, Sorrentino F, Damidot D. Investigation of the hydration and bioactivity of radiopacified tricalcium silicate cement, Biodentine and MTA Angelus. *Dental Materials*. 2013 May;29(5):580–93.
283. Schembri-Wismayer P, Camilleri J. Why Biphasic? Assessment of the Effect on Cell Proliferation and Expression. *J Endod*. 2017 May 1;43(5):751–9.
284. de Andrade FB, Arias MPC, Maliza AGA, Duarte MAH, Graeff MSZ, Amoroso-Silva PA, et al. A new improved protocol for in vitro intratubular dentinal bacterial contamination for antimicrobial endodontic tests: Standardization and validation by confocal laser scanning microscopy. *Journal of Applied Oral Science*. 2015 Nov 1;23(6):591–8.
285. Krause TA, Liewehr FR, Hahn C Lo. The Antimicrobial Effect of MTAD, Sodium Hypochlorite, Doxycycline, and Citric Acid on *Enterococcus faecalis*. *J Endod*. 2007 Jan;33(1):28–30.
286. Arias-Moliz MT, Camilleri J. The effect of the final irrigant on the antimicrobial activity of root canal sealers. *J Dent*. 2016 Sep 1;52:30–6.
287. Pérez-Heredia M, Ferrer-Luque CM, González-Rodríguez MP, MartínPeinado FJ, González-López S. Decalcifying effect of 15% EDTA, 15% citric acid, 5% phosphoric acid and 2.5% NaOCl on root canal dentine. *Int Endod J*. 2008 May;41(5):418–23.
288. Marciano MA, Duarte Marco Antonio Hungaro, Camilleri Josette. Dental discoloration caused by bismuth oxide in MTA in the presence of sodium hypochlorite. *Clin Oral Investig*. 2015 Dec 1;19(9):2201–9.
289. Yildirim T, Oruçoğlu H, Çobankara FK. Long-term Evaluation of the Influence of Smear Layer on the Apical Sealing Ability of MTA. *J Endod*. 2008 Dec;34(12):1537–40.
290. Meschi N, Li X, Van Gorp G, Camilleri J, Van Meerbeek B, Lambrechts P. Bioactivity potential of Portland cement in regenerative endodontic procedures: From clinic to lab. *Dental Materials*. 2019 Sep 1;35(9):1342–50.

291. Moinzadeh AT, Aznar Portoles C, Schembri Wismayer P, Camilleri J. Bioactivity potential of endo sequence BC RRM putty. *J Endod*. 2016 Apr 1;42(4):615–21.
292. Cardinali F, Camilleri J. A critical review of the material properties guiding the clinician's choice of root canal sealers. *Clinical Oral Investigations*. Springer Science and Business Media Deutschland GmbH; 2023;27:4147–55.
293. An B, Zhang D. Bioinspired toughening mechanism: lesson from Dentin. *Bioinspir Biomim*. 2015 Jul 9;10(4):046010.
294. Lin, M., Genin, G. M., Xu, F., and Lu, T. Thermal Pain in Teeth: Electrophysiology Governed by Thermomechanics. *ASME*. May 2014; 66(3): 030801.

