Interfacial Characteristics and Cytocompatibility of Hydraulic Sealer Cements

Kebudi Benezra, Mira; Schembri Wismayer, Pierre; Camilleri, Josette

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Corresponding Author: Professor josette camilleri,

Corresponding Author's Institution: university of malta

First Author: Mira Kebudi Benezra

Order of Authors: Mira Kebudi Benezra; Pierre Schembri Wismayer; josette camilleri

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Abstract: Objective: The stability and long term success of a root canal obturation depends on the choice of sealer as the sealer bonds to the dentine and stabilises the solid cone. Furthermore the sealer needs to be non-toxic as sealer toxicity will certainly led to treatment failure. The aim of this study was to assess the sealer to dentine interface of three hydraulic root canal sealers and assess their cytocompatibility compared to AH Plus.
Methodology: Four dental root canal sealers were assessed. AH Plus, MTA Fillapex, BioRoot RCS and Endoseal were characterized by scanning electron microscopy and energy dispersive spectroscopy The sealer to tooth interface was assessed by confocal microscopy and scanning electron microscopy and the biocompatibility was measured by assessing the cell metabolic function by direct contact assays and alkaline phosphatase activity.
Results: The tricalcium silicate-based sealers presented a diverse microstructure and elemental composition regardless their similar chemistry and classification. The BioRoot RCS was free of aluminium and all sealers presented different radiopacifying elements. The sealer penetration in the dentinal tubules and interfacial characteristics was different. Migration of silicon was evident from sealer to tooth for all sealers containing tricalcium silicate. The MTA Fillapex and BioRoot RCS exhibited the best cytocompatibility in both the direct contact test and the alkaline phosphatase activity.
Conclusions: The use of hydraulic calcium silicate based sealers has introduced a different material type to endodontics. These materials are different to the classic sealers mostly due to their hydraulic nature and their interaction with the environment. Further investigation is necessary on how these sealers interact with dentine and their mechanism of bonding. Furthermore proper material characterisation is necessary and also the role each component plays on the mechanism of bonding.

Interfacial characteristics and cytocompatibility of hydraulic sealer cements

[1]Mira Kebudi Benezra, [2]Pierre, Schembri Wismayer, [3, 4]Josette Camilleri

[1]Department of Endodontics, Faculty of Dentistry, Bezmialem Vakif University, Istanbul, Turkey, [2]Department of Anatomy, Faculty of Medicine and Surgery, University of Malta, Msida, Malta, [3]Department of Restorative Dentistry, Faculty of Dental Surgery, University of Malta, Msida, Malta, [4]School of Dentistry, College of Medical and Dental Sciences, University of Birmingham, Edgbaston, Birmingham, U.K.

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Correspondence

Prof J. Camilleri

School of Dentistry

College of Medical and Dental Sciences

University of Birmingham

5, Mill Pool Way

Edgbaston B5 7EG

Birmingham

United Kingdom

J.Camilleri@bham.ac.uk

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Interfacial characteristics ~~Assessment of sealer to dentine interface~~ and cytocompatibility of hydraulic sealer cements

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Conclusions: The use of hydraulic calcium silicate-~~-~~based sealers has introduced a different material type to endodontics. These materials are different to ~~the classic~~other sealers mostly due to their hydraulic nature and their interaction with the environment. Although the sealers tested had similar chemistry their cytocompatibility and bonding mechanisms were diverse.

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1. Introduction

The success of root canal obturation depends on the sealer characteristics. The sealer stabilises~~holds~~ the solid cone ~~in place~~ and bonds to the dentine~~ resulting in obturation stability~~. At the root apex the root canal sealer is in contact with the apical tissues thus its biocompatibility is also an important property.

The ~~classic~~traditional root canal sealers were classified depending on their chemical composition. They are inert and the interaction of the sealer to the dentine occurs by sealer tags penetrating in the dentinal tubules. Thus the bond is effected by the efficacy of the smear layer removal. During the last decade the use of mineral trioxide aggregate (MTA) has been extended to fill the root canal either entirely or in conjunction with gutta-percha (1, 2). The MTA was not indicated for use as a root canal sealer. Filling the root canal with MTA resulted in higher leakage apically than gutta-percha-sealer obturations (2). Eventually sealers based on MTA were developed and a number are available clinically.

The bonding mechanism of MTA was not very well investigated and the bonding mechanism was never reported. However the bond strength was dependant on the environment humidity (3) with higher values reported in contact with simulated tissue fluid (4). The biomineraliztion ability of MTA is responsible for the enhanced bond strength (5). Subcutaneous implantation of ~~The use of~~ MTA ~~as a sealer~~ resulted in the formation of mineralised tissues (6). The bonding mechanism was first described for Biodentine and bonding occurred by alkaline etching and the development of mineral infiltration zone in the material in contact with the tissues (7). In this research it was shown using confocal laser

microscopy and fluorescent markers that mineral ions from the material cross over to the dentine and a layer is deposited at the interface. The mineral infiltration zone was also shown for BioRoot RCS also a tricalcium silicate-based material developed to be used as a sealer (8). The bonding characteristics of BioRoot RCS were shown to be different to those of AH Plus as the latter only bonded by sealer tags while the hydraulic calcium silicate-based materials also demonstrate the mineral ion rich layer at the interface. –No phosphate-based phases were shown in BioRoot RCS in contact with tooth structure however beta calcium phosphate was deposited on the sealer surface when the material was immersed in simulated tissue fluids (9). This shows the the mineral infiltration in dentine is unlikely to be hydroxyapatite.

A number of hydraulic calcium silicate sealers are premixed. Thus their setting depends on the humidity of the root canal. One type the Endosequece BC sealer has been investigated and it showed complete setting (9). Since this sealer contained a phosphate phase its interaction with dentine and the development of mineral infiltration zone could not be assessed (9). There is lack of knowledge on how hydraulic sealer cements interact with the dentine and whether changes in the presentation and sealer chemistry effects the interfacial characteristics of the materials. The aim of this study was to characterise three hydraulic calcium silicate sealers which had diverse chemistry and presentation and assess the interfacial zone of these sealers. Furthermore the biocompatibility of the sealers was also investigated as sealer toxicity also effects the clinical success of endodontics. AH Plus an epoxy resin-based sealer was used as a control.

2. Materials and Methods

The following root canal sealers were used in this study:

1. AH Plus (Dentsply, DeTrey GmbH, Konstanz, Germany)

2. MTA Fillapex (Angelus, Londrina, Brazil)

3. BioRoot RCS (Septodont, Saint-Maur-des-Fossés, France)

4. Endoseal (Maruchi, Wonju-si, Gangwon-do, South Korea)

All sealers were mixed and manipulated in accordance with the manufacturers' instructions, except for Endoseal, a premixed root canal sealer that was syringed.

2.1 Material characterization

The sealers were mixed following manufacturer's instructions and were allowed to set at 100% humidity for 48 hours at 37ºC. The endoseal was covered with a moist gauze. Three discs 10 mm in diameter were prepared for each sealer type. The set sealers were characterized by scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS). Disc-shaped specimens (10 mm in diameter and 2 mm high) were prepared from each sealer type. They were vacuum impregnated in resin (Epoxyfix, Struers GmbH, Ballerup, Denmark). The resin blocks were then ground with progressively finer diamond discs and pastes using an automatic polishing machine (Tegramin 20, Struers GmbH, Ballerup, Denmark). Specimens were mounted on aluminum stubs, carbon coated (Agar Scientific, Stansted, UK) and viewed under the scanning electron microscope (SEM; Zeiss MERLIN Field Emission SEM, Carl Zeiss NTS GmbH, Oberkochen, Germany). Scanning electron micrographs at high magnification of the different material microstructural components in back-scatter electron mode were captured and energy dispersive spectroscopy was carried out.

2.2. Assessment of interfacial characteristics

The root dentine–sealer interface was assessed using confocal microscopy with 0.1% fluorescein dye (Sigma Aldrich, Dorset, UK) and scanning electron microscopy and energy dispersive mapping.

2.2.1 Tooth Preparation

No ethical approval or patient consent was sought as the country legislation did not restrict the collection of extracted teeth. Sixteen single-rooted human teeth with fully formed apices (including bicuspids, canines, and incisors) were collected anonymously from dental offices (general dentists, oral surgeons, and periodontists) and stored in distilled water until use. All teeth were decoronated standardizing the root length to 15-mm length. Roots were prepared by using ProTaper Universal instruments (Dentsply Maillefer, Ballaigues, Switzerland) in a modified crown-down manner up to F4 as the master apical file, 1 mm shorter than the root length (14 mm).

The canals were irrigated with 2 mL of 5% NaOCl between the changes of the rotary files using a 30-gauge Miraject Endotec Luer (Hager & Werken GmbH & Co. KG, Duisburg, Germany) tip attached to the plastic syringe and introduced 3 mm shorter than working length. The final rinse was performed with 5 mL of 5% NaOCl for 5 min, followed by 5 mL of distilled water and 5 mL 17% EDTA followed by 5 ml saline. The root canals were dried with paper points. Then randomly divided for SEM and confocal examination.

2.2.2 Confocal Microscopy Examination

The sealers were mixed according to the manufacturer's instructions. Fluorescein (Sigma-Aldrich, Dorset, UK) was added to the sealers in a 0.1% proportion. The sealers were placed inside the root canals using a lentulo spiral. The coronal and apical access was restored with glass ionomer cement (Fuji IX; GC Europe, Leuven, Belgium). Two teeth were prepared for each material and were immersed in HBSS for 28 days at 37°C. At the end of the immersion period the teeth were removed from solution, dried and were embedded in resin, sectioned longitudinally using a hard tissue microtome (Accutom 50, Struers GmbH, Ballerup, Denmark) and polished using an automatic polishing machine as indicated above. The root dentine–cement interface was assessed using a confocal microscope (Nikon Eclipse, Tokyo, Japan) with

an oil immersion × 60 magnification objective lens. The fluorescein was visible at an excitation/emission wavelength of 494/521 nm.

2.2.3 Scanning Electron Microscopy Examination

The teeth used for scanning electron microscopy (SEM) investigation were filled with sealers as mentioned above but without fluorescein, and immersed in HBSS for 28 days at 37°C. They were processed in a similar way to the previous experiment. The sections were then mounted on aluminium stubs and carbon coated. The root dentine–cement interface at different levels along the root canal was then viewed with an SEM (Zeiss MERLIN Field Emission SEM, Carl Zeiss NTS GmbH, Oberkochen, Germany) in back-scatter electron mode at x 2000 magnification. EDX analysis was performed over the materials and tooth structure in order to determine the elemental constitution. Furthermore elemental maps across the interface were performed with each element being mapped in a different color.

2.3 Investigation of sealer biological properties

The biocompatibility was assessed by evaluating the cell activity and proliferation of gingival fibroblasts in contact with the different sealers. Human gingival fibroblasts were obtained from gingival tissue from healthy patients who underwent oral surgery. The were isolated and grown in cell culture medium (DMEM) supplemented with 10% fetal bovine serum, 100 mg/mL penicillin G and 50 mg/mL streptomycin at 37°C in air with 5% CO_2 in a humidified incubator under ambient atmospheric pressure. At 70 to 80% confluence, cells were detached using 0.25% trypsin and 0.05% EDTA for 5 minutes at 37°C and replated or counted.

The cytocompatibility of the test materials was evaluated *in vitro* according to ISO 10993-5;2009 (10) using a direct testing method. The 3-(4,5 dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess cell metabolic function (11).

The four sealers were mixed in strict compliance with manufacturers' instructions and shaped with 1-mm thick non-reactive plastic molds with a diameter of 10 mm under aseptic conditions.

For direct testing, 1.5x 10^5 gingival fibroblast cells were seeded in 1 ml in a 24 well plate, after including the discs in each well. This was done in triplicate. Following 1 day of incubation, the disc was removed from each well, put into another 24 well plate and incubated with 1ml medium and 200µl MTT. After this the medium was aspirated and 500µl of DMSO to dissolve off any formazan crystals formed. 100 µl of this DMSO was added to a 96 well plate and absorption was read at 405 nm (Spectrostar nano BMG labtech).

The cell metabolic function was assessed by alkaline phosphatase activity, in case of any stimulation to osteoblastic differenciation. Briefly, 15,000 cells were plated into each well of a 24 well plate, and exposed to the conditioned medium from the different sealers over 3 days at a dilution of 1:32. After removal of the medium each well was washed twice in PBS, and 50 ul alkaline phosphatase substrate, p-Nitrophenyl Phosphate (p-NPP) was added and incubated at 37 ° C for 45 minutes. The activity was then immediately read at 405 nM on a spectrophotometer.

2.4 Statistical Analysis

The data were evaluated using Statistical Package for the Social Sciences software (PASW Statistics 18; SPSS Inc, Chicago, IL). One-way analysis of variance and Tukey post-hoc tests at a significance level of P = 0.05 were used to perform multiple comparison tests.

3. Results

3.1 Material characterisation

The scanning electron micrographs and EDS plots of all sealers tested are shown in Figure 1. The AH Plus was composed of rounded and slightly elongated particles which exhibited different levels of brightness. The EDS analysis of the material showed peaks for calcium, zirconium and tungsten (Figure 1). The MTA Fillapex, BioRoot RCS and Endoseal were all composed of a cement and radiopacifier phase. The material matrix was dense and the phases were uniformly distributed. The cement particles exhibited peaks for calcium and silicon while the radiopacifiers appeared whiter on the scanning electron micrograph (Figure 1). Furthermore the cement particles in Endoseal exhibited high levels of aluminium indicating an aluminate phase present. Aluminium and sulphur was also shown in MTA Fillapex. Both sealers are derived from Portland cement thus show traces of magnesium, aluminium and sulphur which are typical of Portland cement as opposed to BioRoot RCS which did not include these trace elements as it is composed of pure tricalcium silicate (Figure 1).

3.2 Assessment of interfacial characteristics

The confocal micrographs assessing the material to tooth interface are shown in Figure 2a. The sealers ~~were well compacted and~~ exhibited low porosity and dentinal tubule penetration was evident for all sealers particularly for AH Plus. The BioRoot RCS also exhibited an interfacial zone, which was distinct from the rest of the sealer. This interfacial zone was composed of an area, which was devoid of larger particles but included smaller particles interspersed in the interfacial region. This was only evident in BioRoot RCS. The dentinal tubule penetration was less in BioRoot RCS compared to the other sealers.

The scanning electron micrographs and elemental maps for all test sealers are shown in Figure 2b and the EDS analyses in Figure 2c. There was some gaps shown in AH Plus and BioRoot RCS and this was also evident in the elemental maps. The BioRoot RCS had an area of structureless morphology at the interface. The microstructure of the MTA Fillapex at the

interface in contact with the tooth also exhibited a different microstructure to the material bulk and this was more evident than for BioRoot RCS. The interface lacked the general features of the material and had brightly coloured dots, which would be the tantalum as indicated in the EDS analyses (Figure 2c). The interface appeared black with no elements in the elemental map. However tantalum peaks coincide with silicon as shown in the EDS analyses (Figure 2c). Similar peak overlaps can be seen also in AH Plus and BioRoot RCS for zirconium and phosphorus.

3.3 Investigation of biological activity

In the direct contact test, the MTA Fillapex exhibited the best cell growth followed by BioRoot RCS. Both AH Plus and Endoseal did not encourage cell growth on the material surface (Figure 3a). The alkaline phosphatase activity (Figure 3b) showed highest activity after 1-day exposure for MTA Fillapex, which reduced in the 28-day exposure. In comparison Endoseal maintained the same activity for both 1 and 28 day exposures. The AH Plus and BioRoot RCS both exhibited reduced activity when compared to the Endoseal and MTA Fillapex.

4. Discussion

The current study investigates three hydraulic sealers ~~to~~ assessing the interfacial characteristics with dentine. Although the sealers were all based on tricalcium silicate cement they exhibited diverse compositions and presentations. The MTA Fillapex was Portland cement-based as shown by the EDS analysis, the BioRoot RCS was tricalcium silicate-based while the Endoseal exhibited high levels of aluminium showing high proportions of an aluminate phase. The radiopacifiers were also different as shown in the EDS analysis. The MTA F~~f~~ill~~-~~apex investigated in this study was the new version just launched by Angelus and it was bismuth oxide free but contained calcium tungstate. Zirconium oxide was found in both

BioRoot RCS and Endoseal. However Endoseal contained also bismuth oxide. The high levels of aluminium present in the Endoseal, which was even higher than the MTA Fillapex could be of concern. Recent studies investigating a number of dental Portland cement-based materials reported ~~high levels~~presence of aluminium in the plasma and liver of test animals (12). Furthermore oxidative stress was reported in the animal brain (13). The Endoseal had not been characterised so far and the MTA Fillapex was a new recently launched version so also not tested previously.

The sealers also had different presentations. The MTA Fillapex is presented in a 2-paste system and uses a salicylate resin matrix. The matrix is meant to allow fluid movement thus enabling hydration of the cement particles. The reaction of the salicylate occurs in the presence of calcium ions thus enabling the formation of calcium salicylate and material hardening. The BioRoot RCS had a powder/liquid formulation thus its setting was independent on the environmental factors. The Endoseal was premixed and its setting and hydration depend on the wetness of the root canal.

Although both the test sealers are tricalcium silicate-based only BioRoot RCS exhibited evidence of an interfacial zone using confocal microscopy. This interfacial zone has been reported using similar methodologies for Biodentine (7) and BioRoot RCS (8). The interfacial zone was different for all the material tested. The AH Plus and Endoseal exhibited no changes to the material in contact with the dentine. The sealer exhibited the same microstructure at the interface as it did in the sealer bulk. The BioRoot RCS exhibited some changes in microstructure at the interface and poor adaptation of the sealer with a gap present in contact with the dentine. A previous study using X-ray diffraction analysis to determine the composition of the sealer in contact with the dentine showed no formation of calcium phosphate phase in the BioRoot RCS in contact with dentine (9). Thus the mineral infiltration zone is unlikely to be formed regardless the changes seen in confocal microscopy. This is in

accordance with a previous study using scanning electron microscopy and elemental analyses along a line at the interface where no movement of ions was observed for Biodentine (14). The MTA Fillapex showed a change in microstructure of the material at the interface. A phase change in the material was also served in the previous study (9).

The movement of calcium from the sealer to the tooth could not be monitored by the elemental mapping since both sealers and tooth structure contain calcium. In contrast sealer tags rich in apatite were shown for Endoseal in a previous study (15). Silicon was shown to migrate from the sealers to the tooth. Silicon migration has also been reported *in vitro* in an animal model (16). The interference in mapping certain elements due to peak overlap in elemental analyses has already been reported (17).

The MTA Fillapex was shown to enhance cell attachment and proliferation. The MTA Fillapex used in this study was different to the one used in previous studies as the material used in the current study has been launched recently and was shown to have a different material composition namely the absence of bismuth oxide and its replacement with calcium tungstate. Thus no comparisons can be made to previous research. The BioRoot RCS in comparison was shown to be cytotoxic. This is in contrast to previous research showing BioRoot RCS to be biocompatible tested using periodontal ligament stem cells (18). Furthermore the BioRoot RCS was shown to enhance the stem cells better than the Endoseal also in contrast to the findings in the current study. Previous research on biocompatibility of Endoseal implanted in subcutaneous tissues of rats showed Endoseal to have a similar reaction to MTA and better than AH Plus (19). This is also inferred in the current study at the cellular level. Furthermore Endoseal was shown to enhance cell activity better than MTA Fillapex (20). However the data cannot be compared to the current study since the MTA Fillapex used in the previous research may have been the bismuth-containing MTA Fillapex.

Material characterization is necessary in every research work to make sure that the materials are well characterized to enable comparison to further research.

Conclusions

The use of hydraulic calcium silicate based sealers has introduced a different material type to endodontics. These materials are different to the ~~classic~~traditional -sealers mostly due to their hydraulic nature and their interaction with the environment. Regardless the similar chemistry the sealers exhibited a different bonding mechanism and biological properties. Further investigation is necessary on how these sealers interact with dentine and their mechanism of bonding. Furthermore proper material characterisation is necessary and also the role each component plays on the mechanism of bonding.

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Declaration

I affirm that I/We have no financial affiliation (e.g., employment, direct payment, stock holdings, retainers, consultantships, patent licensing arrangements or honoraria), or involvement with any commercial organization with direct financial interest in the subject or materials discussed in this manuscript, nor have any such arrangements existed in the past three years. Any other potential conflict of interest is disclosed

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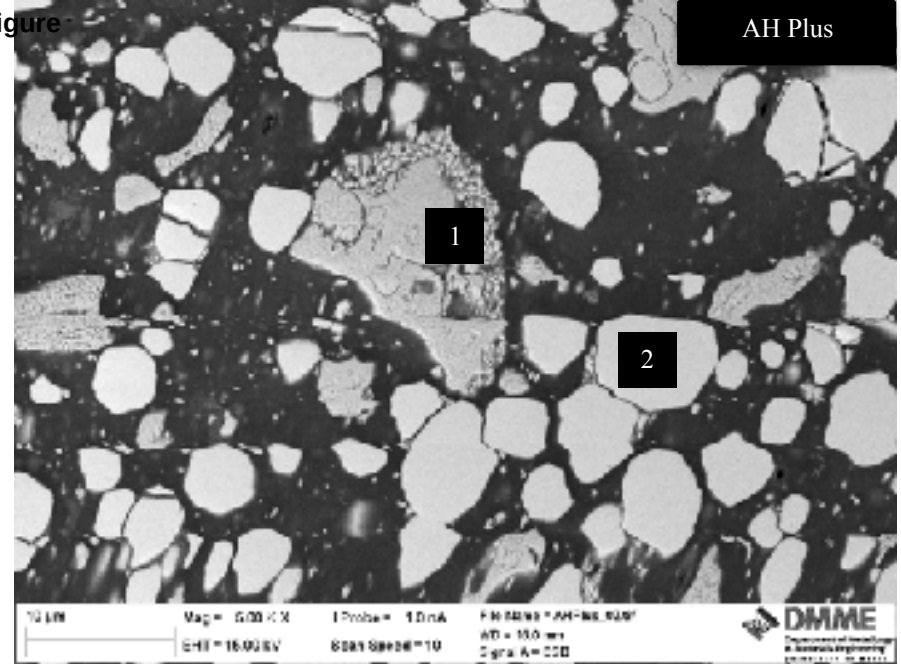
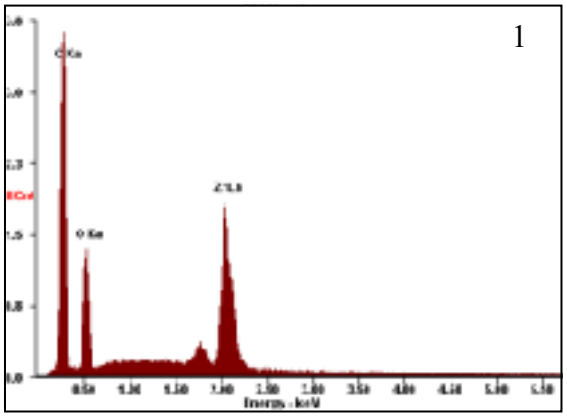
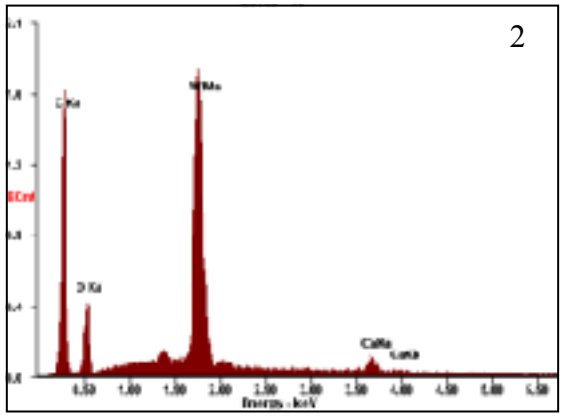
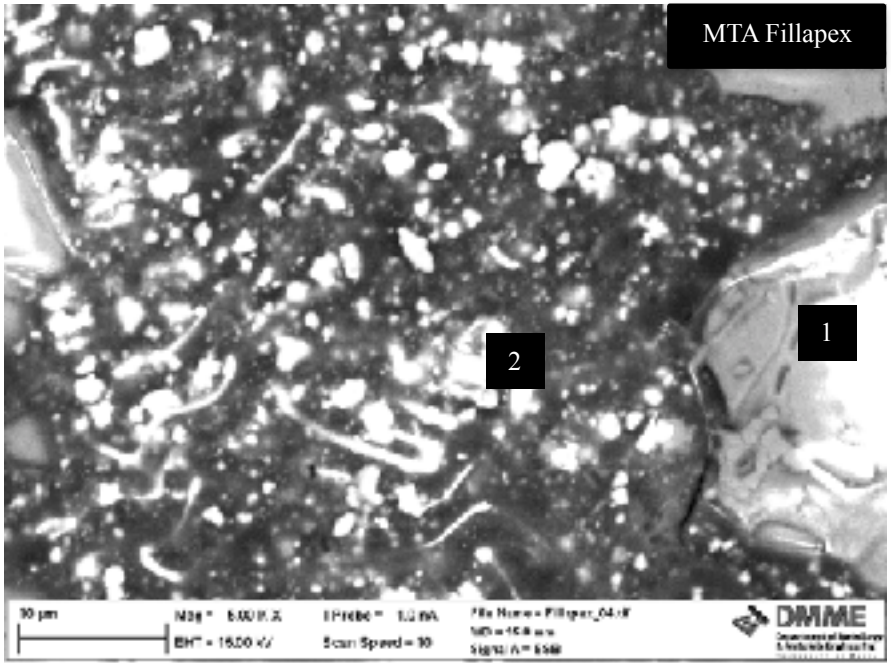
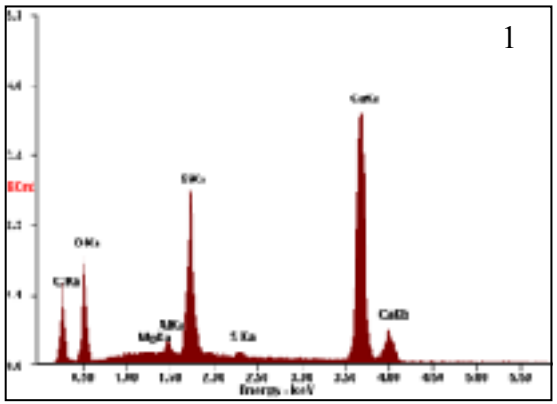
Figure

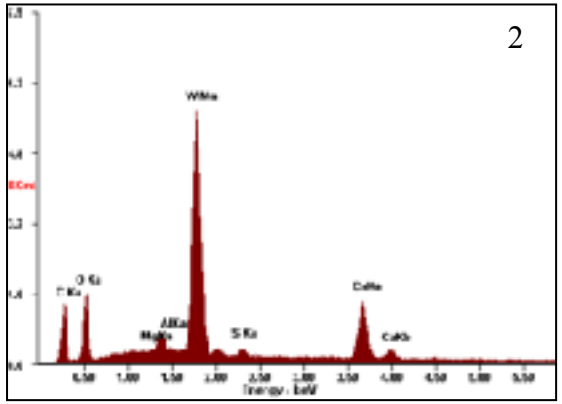












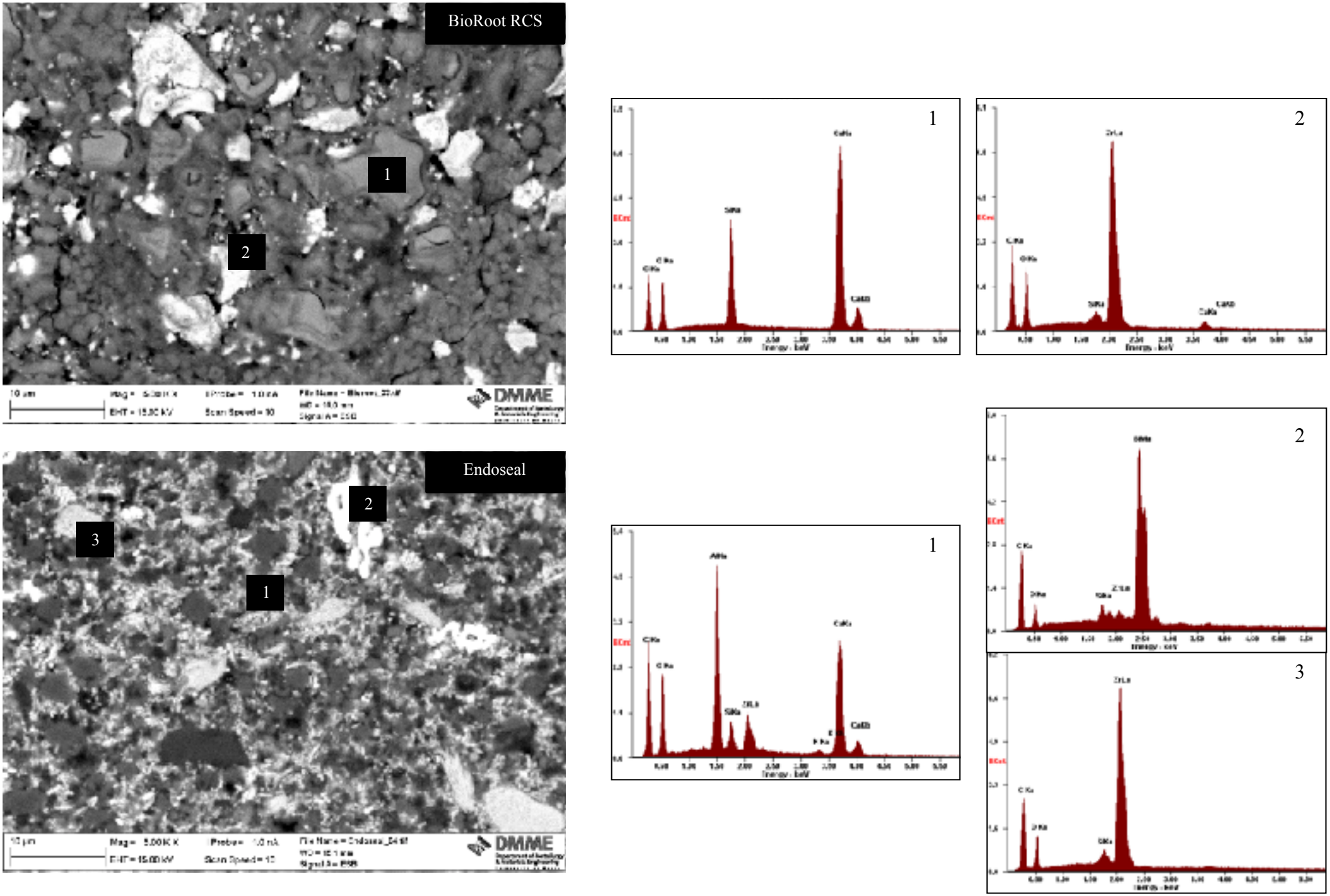


Figure 1:Back-scatter scanning electron micrographs of polished sections of test sealers showing microstructural components and EDS analyses of the material and the specific phases identified and labeled

Figure

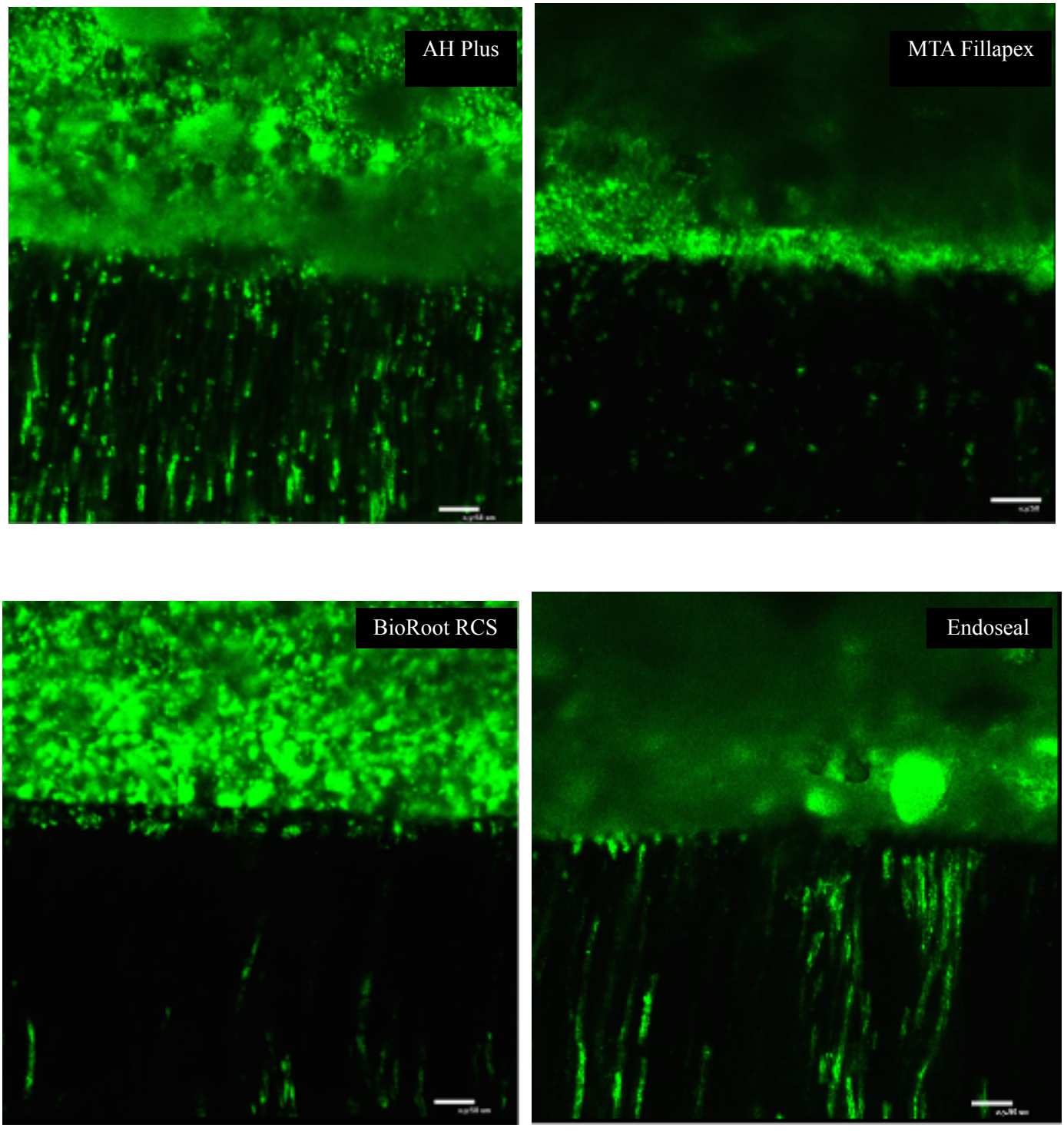
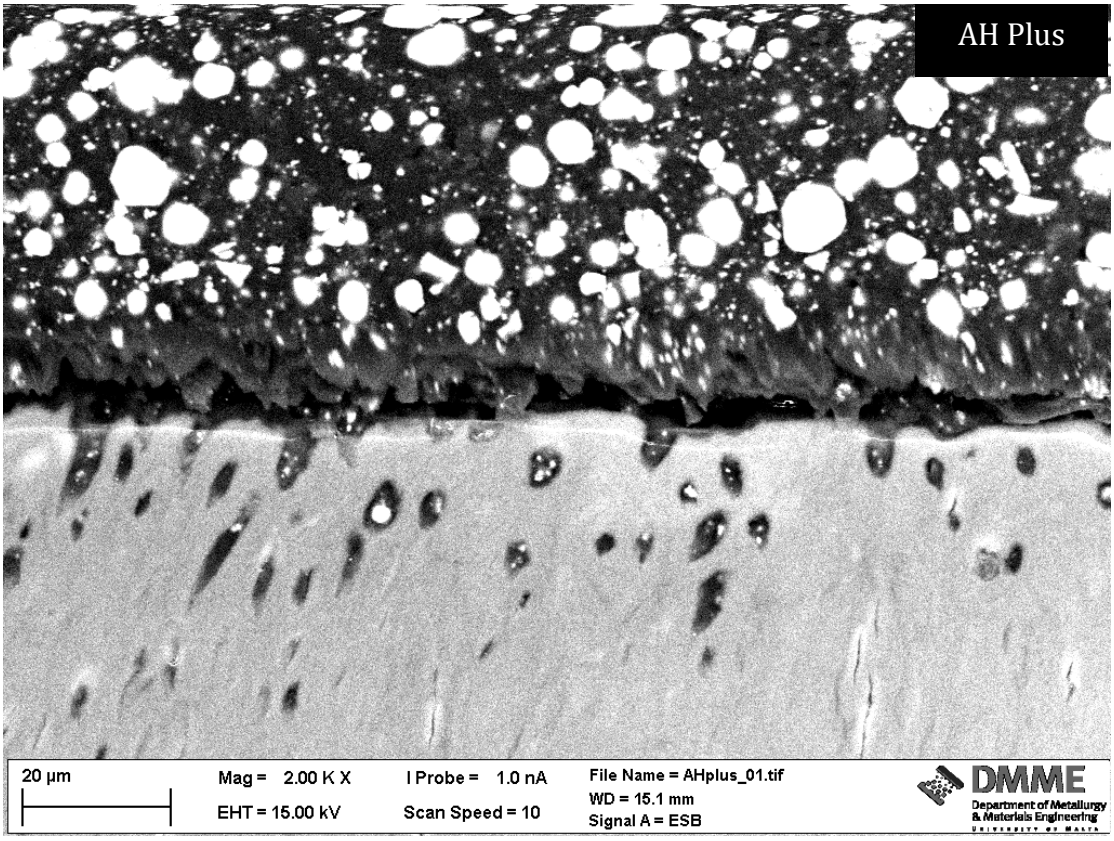


Figure 2a: Confocal micrographs showing interface of root canal sealers in contact with root dentine showing interaction of sealers with dentine and sealer penetration in the dentinal tubules.

Figure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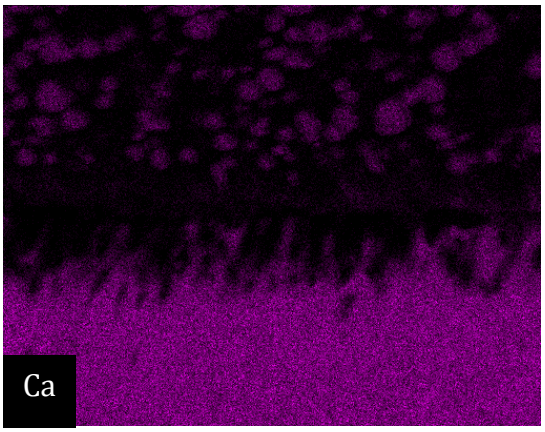
DMME
Department of Metallurgy & Materials Engineering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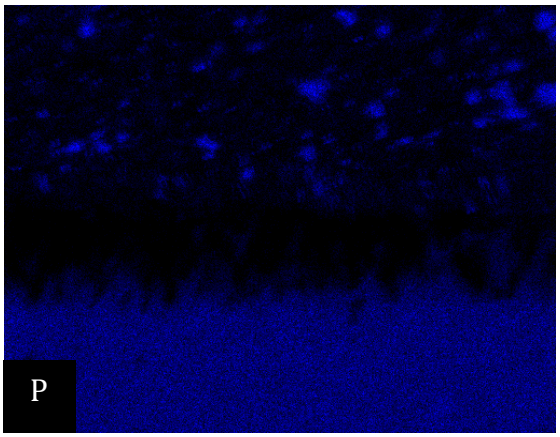
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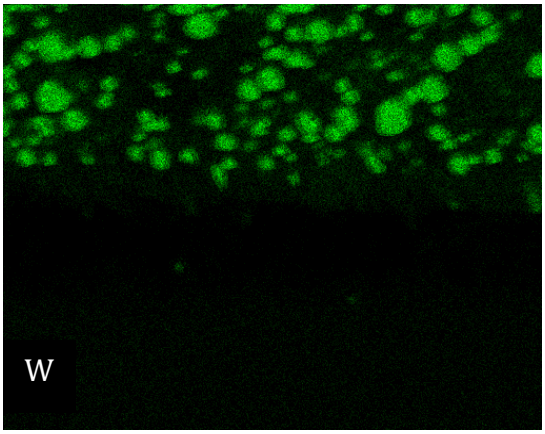
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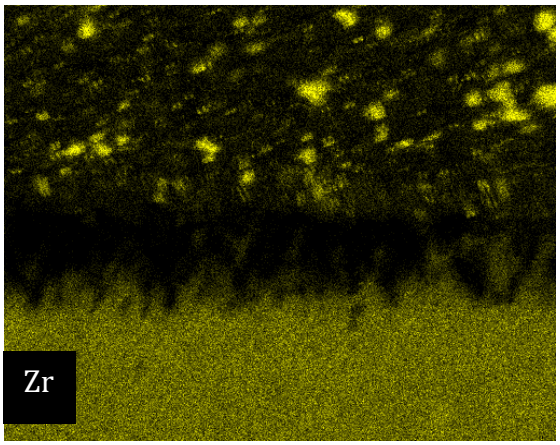
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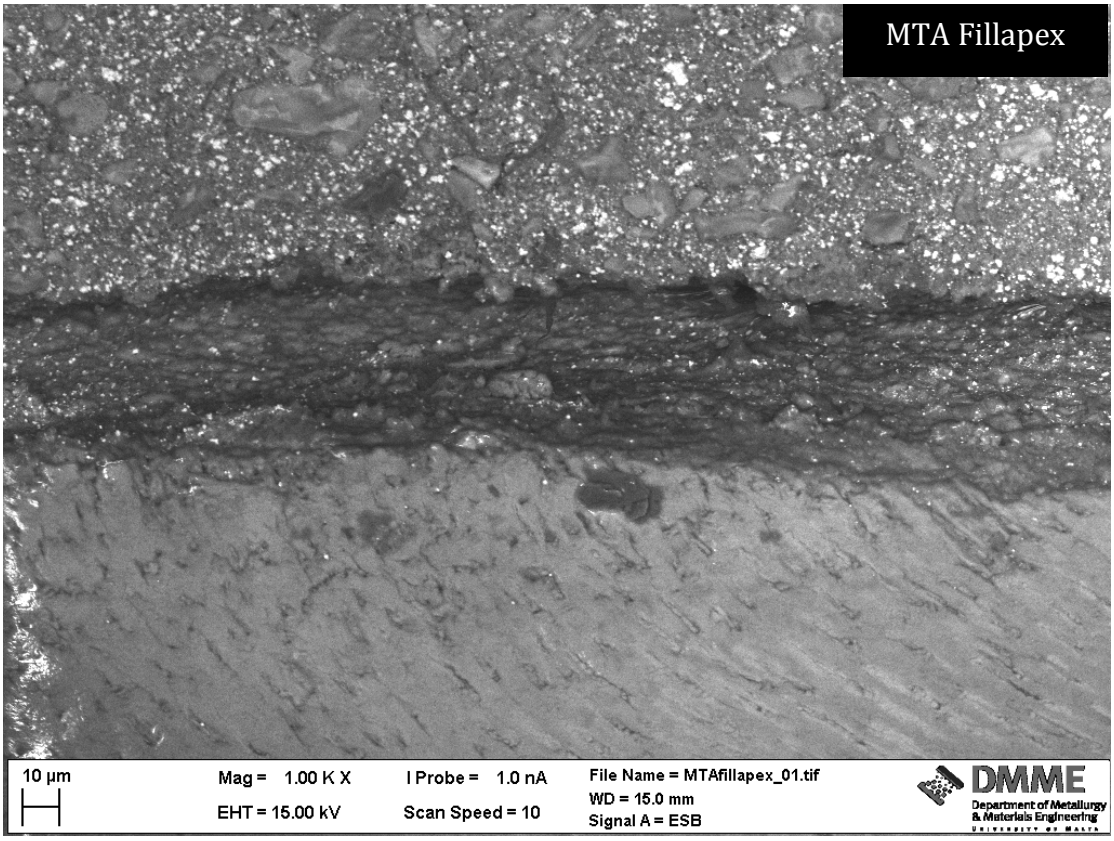
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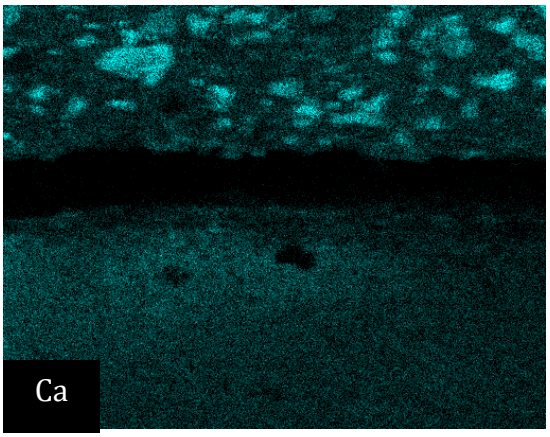
Department of Metallurgy & Materials Engineering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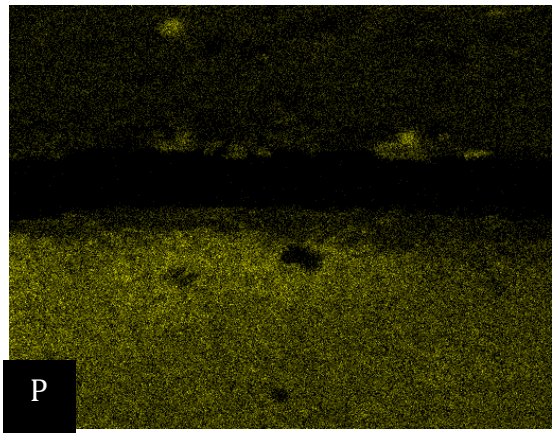
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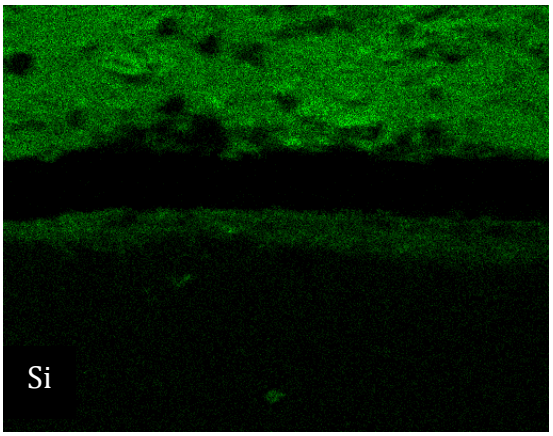
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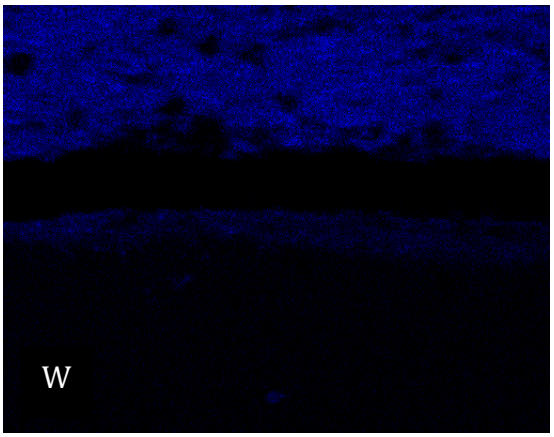
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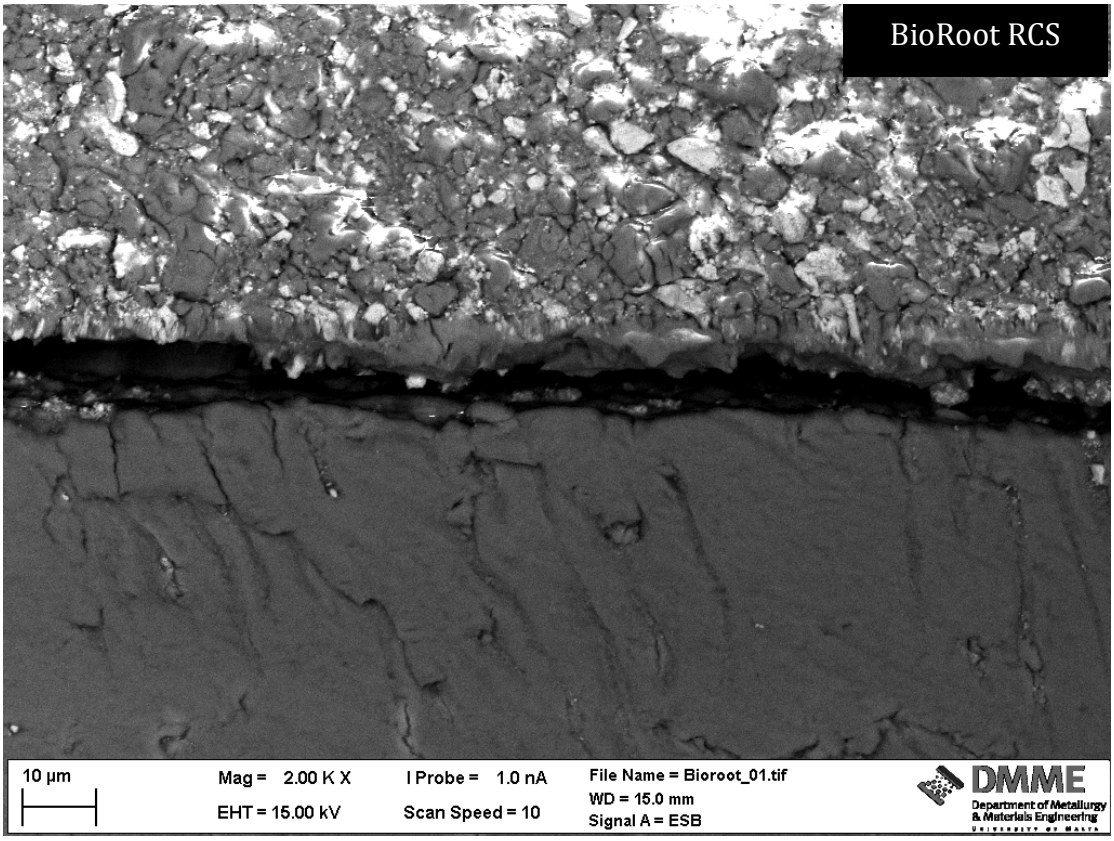
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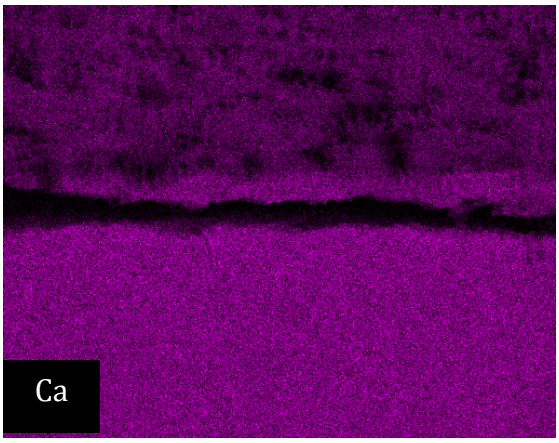
WD = 15.0 mm
Signal A = ESB
DMME
Department of Metallurgy & Materials Engineering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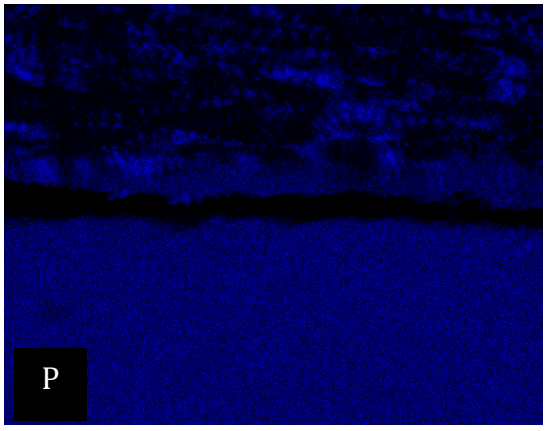
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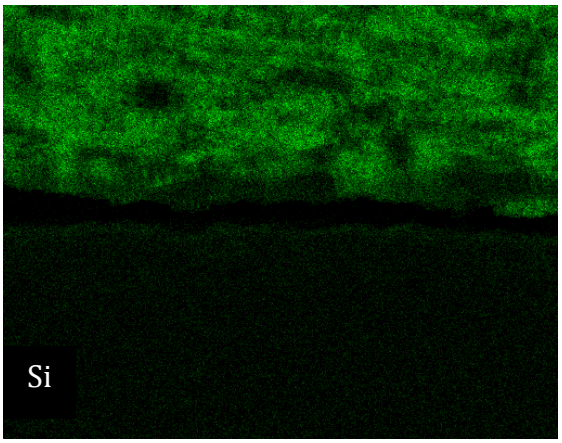
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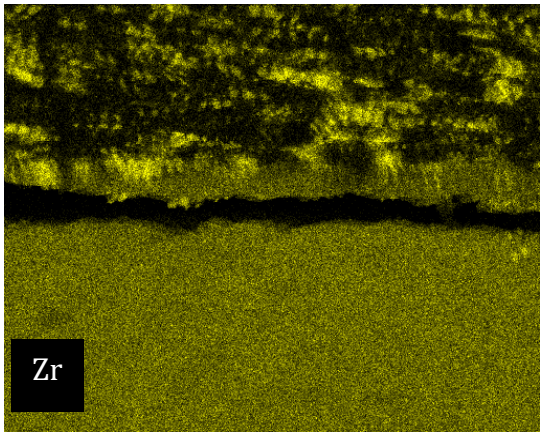
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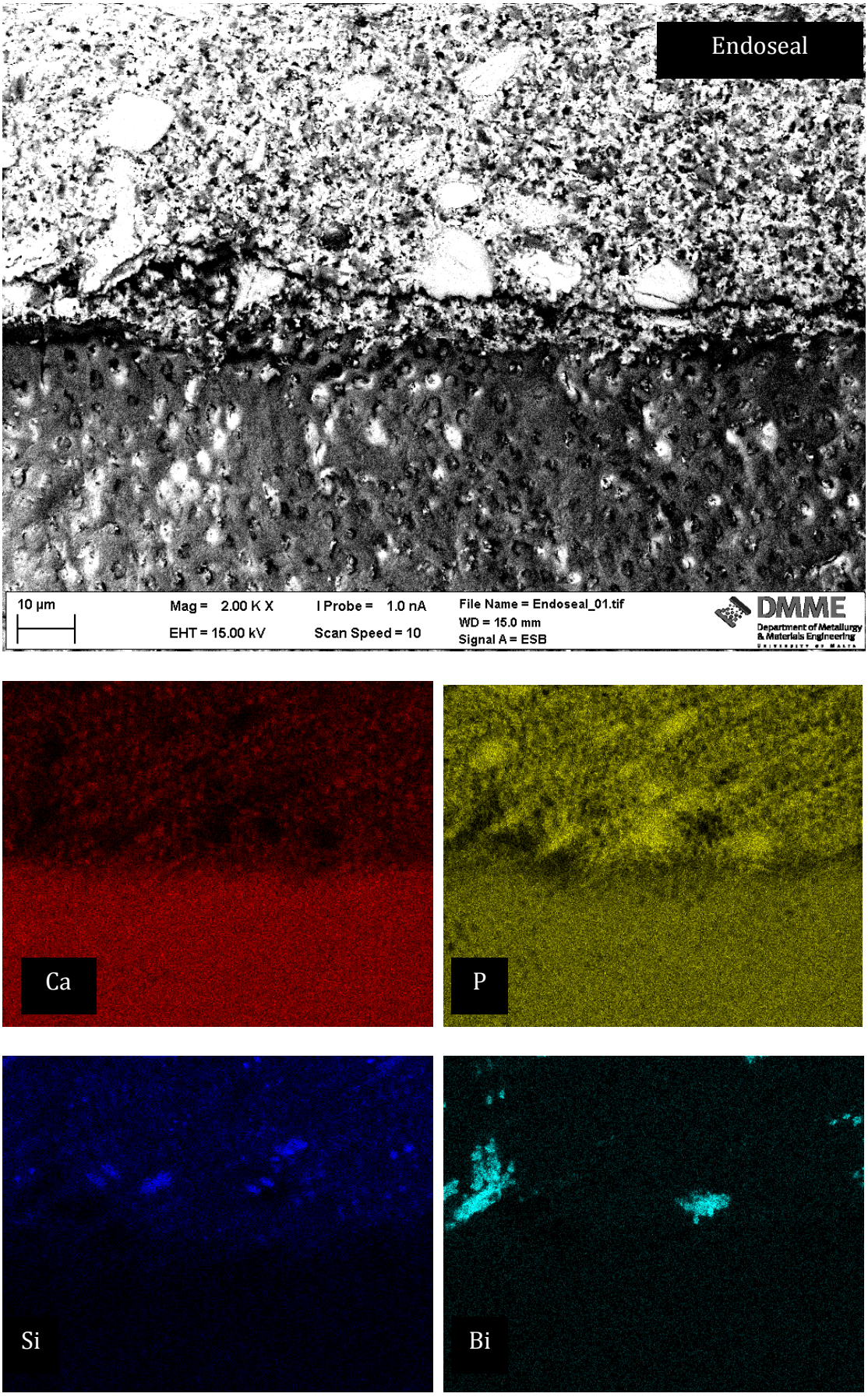

Figure 2b: Scanning electron micrographs and elemental maps of various elements to show elemental distribution in material matrix

Figure

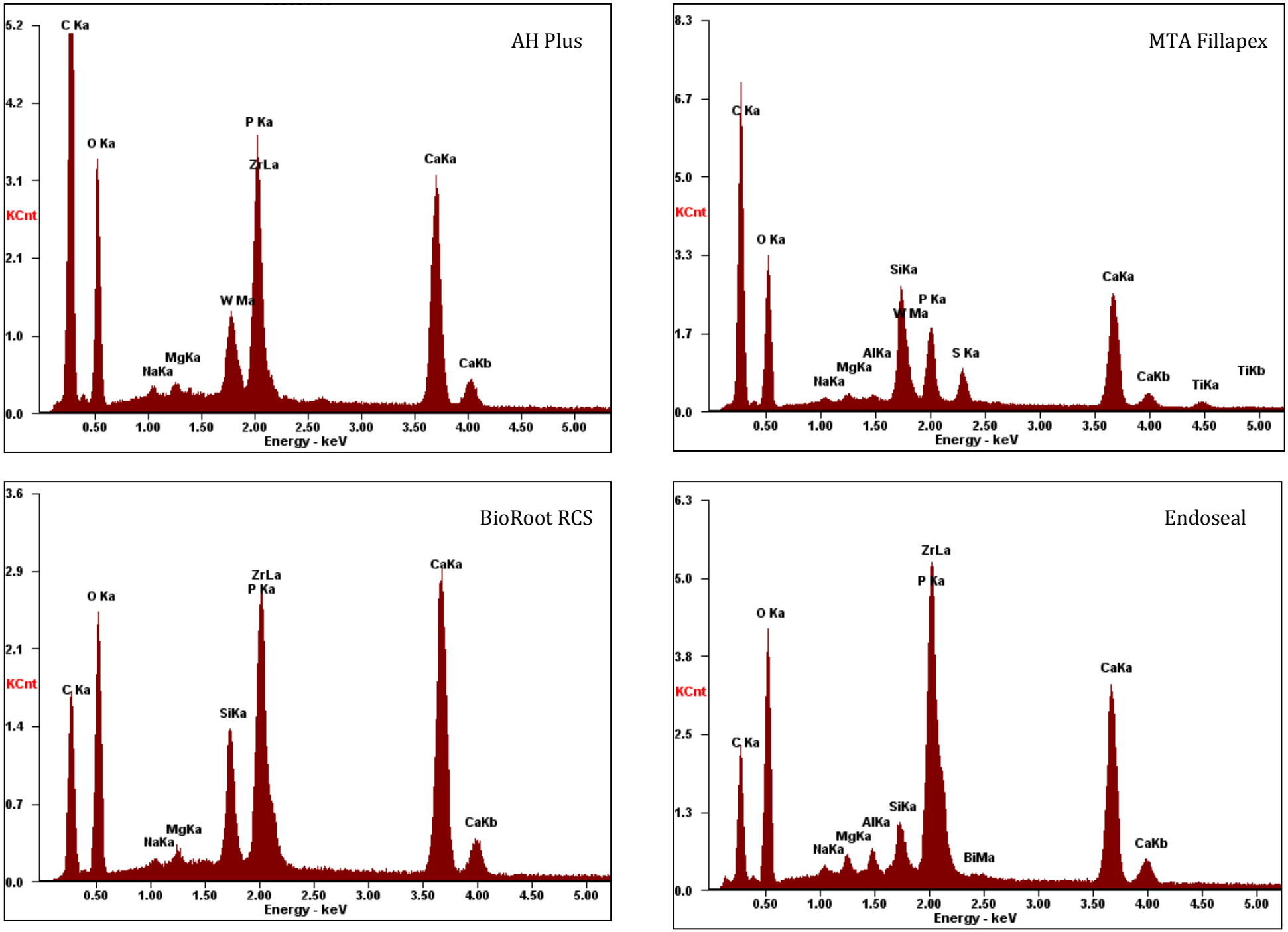

Figure 2c: Energy dispersive spectroscopy of the tooth to material interface to show elemental composition of the sealers and the tooth

Figure

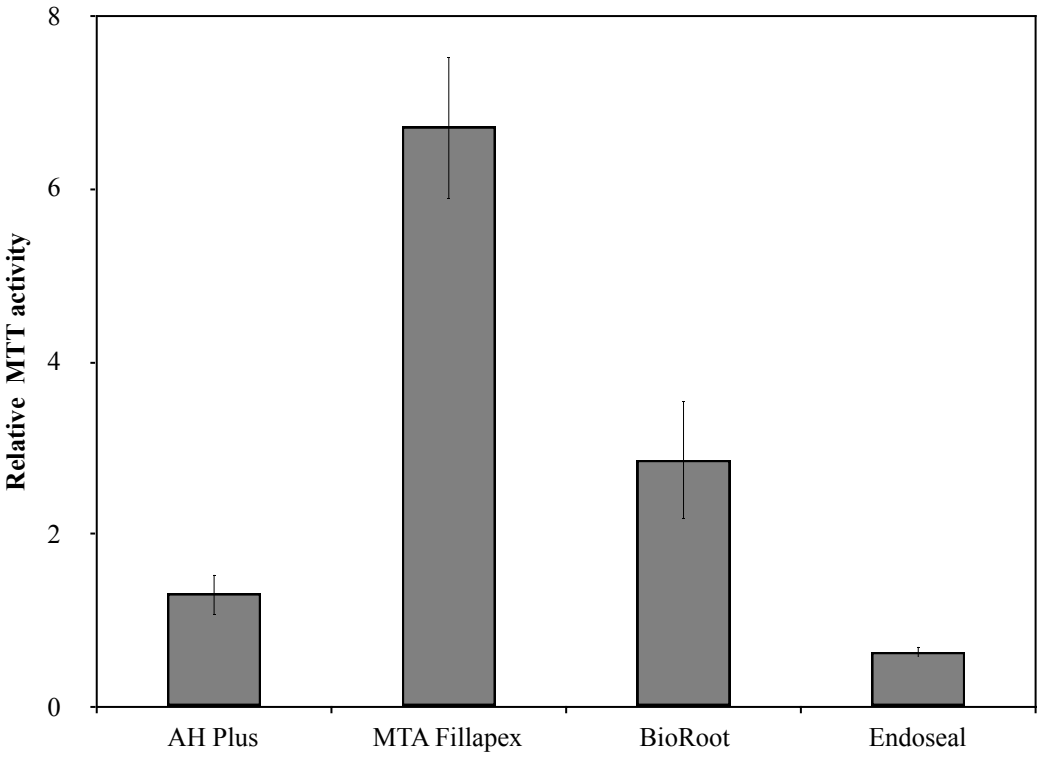

Figure 3a: Cell proliferation and expression of gingival fibroblasts in response to exposure to different sealers in direct contact test

Figure

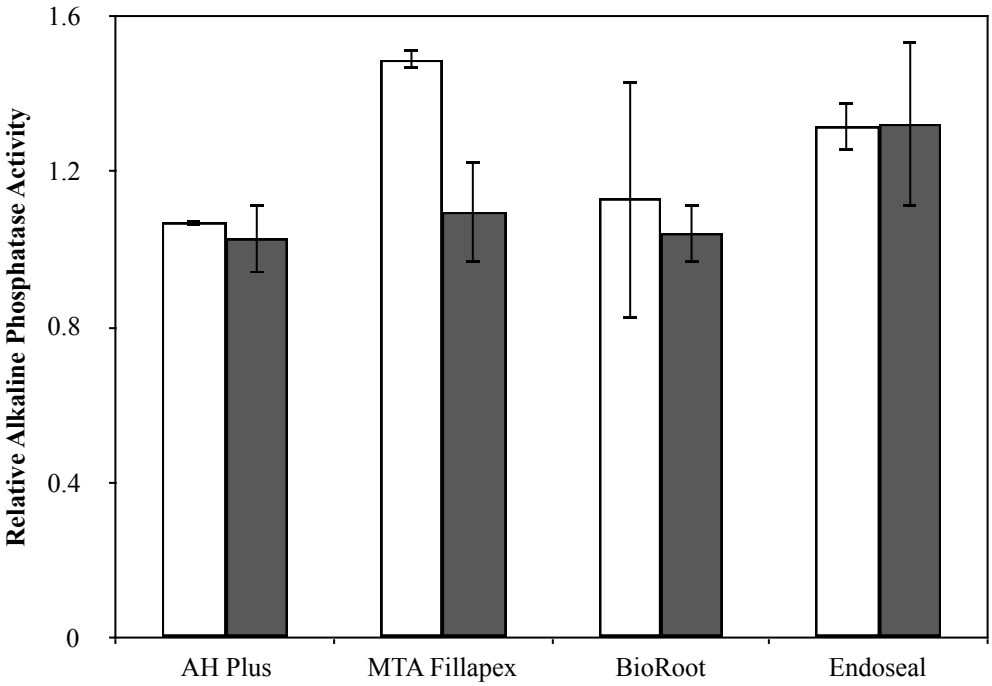

Figure 3b: Cell proliferation and expression measured by alkaline phosphatase activity of gingival fibroblasts in response to exposure to 1 and 28 day elution from different sealers

Highlights

- Although the sealers have a similar chemistry they present diverse properties and different interaction with the dentine and cytocompatibility.
- These hydraulic sealers are different to the classic sealers as they interact with the environment thus the sealers change depending on the specific use.
- In the current paper we investigate the effect of the specific formulation and also the presentation on the interfacial characteristics and biocompatibility.

***Statement of Clinical Relevance (max 40 words)**

Statement of clinical relevance

The hydraulic calcium silicate-based sealer cement properties depend on the environment they are placed in. The sealers are interactive rather than inert.

Replies to the reviewer comments

Reviewers' comments:
Reviewer #1: In this study, the authors tried to assess the sealer to dentine interface of three hydraulic root canal sealers and assess their cytocompatibility compared to AH Plus. The introduction builds up a 'story' to assess the sealer to dentine interface of three hydraulic root canal sealers and assess their cytocompatibility, was an issue worthy to be discussed in clinical aspect. The materials and methods were fine, but the protocol may need further modification.

Thank you very much for your comments. Can you kindly indicate what modifications are you expecting to the methodology.

The results did not show N and P values for each group.

Modified. If the data is qualitative we cannot include P values

The discussion should include the possible reasons of higher cytotoxicity of Bioroot in this study, which was in contrast to previous studies. The figures are too many and not in good quality. The authors should focus on the advantages as well as mechanisms of three hydraulic root canal sealers.

This was never claimed in the manuscript. In fact it was stated that both MTA Fillapex and Bioroot showed higher cytocompatibility than the rest of sealers tested.

We have submitted the original micrographs and if not clear we can resubmit.

A critical review of the literature reveals that the present study does not represent a novel approach. The results although interesting are incomplete and insufficient to support their conclusions. Correct use of English, such as grammar and proper wording is a problem. The manuscript should be proofread by a person who is fluent in English.

Can you kindly be more specific and indicate why you feel the results are incomplete and insufficient. The manuscript was written by a native English speaker.

Reviewer #2: Please refer to document returned to editor for in-depth suggestions.

Document was not supplied. Here are some general comments.

Shorten the title.
Modified as suggested

MTA Fillapex is not tricalcium-silicate based; it is resin based. Others have referred to Fillapex as MTA-based, but it is not. Only about 15% of the material is a

hydraulic powder; therefore, it cannot be called "tricalcium silicate-based" because TCS is the not the majority. The resin is the matrix.

There is no official classification for these materials. Some are resin based and others are water based. Some are tricalcium silicate based and others are Portland cement based. It is a matter of opinion. Thank you for sharing your opinion on this.

Make the conclusions more descriptive of your experiments, not future plans.
Does sealer need to "hold the cone in place"? This is not a Grossman criterion. Rephrase.
Conclusions modified. The hydraulic sealers are not in line with Grossman criteria. Furthermore I think it is a bit obvious that the cone has to be held in place. Why would anyone want a floating cone. Reworded as suggested.

Define classic sealers- do you include AH Plus as classic?
Reworded and called them traditional. This means the sealers that are non interactive. AH Plus is one of them. It is considered as the gold standard.

Reference 2 is inappropriately cited. In this dye leakage test, ProRoot MTA was used with gutta percha and that product is NOT indicated as a sealer. Furthermore, the samples were not exposed to physiological solution, so the bioactivity was not engaged for the coarse, but bioactive ProRoot MTA.

Kindly understand the context of the sentence. This was the first articles that have used a hydraulic calcium silicate inside the root canal. It does not mean that the material was a sealer. However I reworded for clarity. In fact in the following sentence I say that the sealers were developed later.

Reference 6 is inappropriate cited. Holland used MTA in subcutaneous implants, not as a sealer.
Reworded for clarity

Your use of "mineral infiltration zone" is not helpful to a reader. Please define it - are you saying that the MTA/TCS penetrates the tubules or are you saying the HA forms and penetrates the tubules? If you are meaning more tubule penetration- how does that differ from sealer tags? Please be considerate of readers who are not following every article you have written, and rewrite this section.
Just for the record I have not written this article but I am citing published work. And furthermore this is meant to be a blind review thus such comments are highly inappropriate.

Why was the most popular hydraulic sealer not tested- Endosequence?
How do you know it is the most popular? We chose a range of sealers. We are entitled to choose the sealers we are happy to work with. We opted for Endseal as one of the premixed materials.

2.1- How were the sealers “set"?
Added to the document

2.2.2 How were the teeth sectioned and how were they polished?
Give your carbon coater equipment.
What temperature was used for HBSS storage?
All added to manuscript

MTA Fillapex is resin-based, therefore its description as a cement and radiopacifier phases is incorrect.
As discussed above this is a matter of opinion until the materials receive an official classification

3.1 You are confusing the readers with your distinction of tricalcium silicate versus Portland cement. Portland cement, as you know, has many potential formulas, but always contains tricalcium silicate. Your distinction of the two is misleading. Also, a strictly tricalcium silicate material may contain dissolved magnesium oxide. Please stick with your observations succinctly without making unclear distinctions.

I think I am being very precise. There is a distinction between the materials made form Portland cement and these which are produced form tricalcium silicate. The manufacturing processes and indeed the material chemistry is different. The Portland cement ones can be modified through the aluminate reaction while the tricalcium silicate based ones cannot.

3.2 By compacted, do you mean the material was well compacted to make the samples, or the material had few pores?
Few pores. Reworded for clarity

What is structureless morphology? Featureless? Amorphous in appearance?
Amorphous means not crystalline so cannot be used in this context. Structureless is featureless.

As for the comments on aluminium, please note that the references you cited say "Al might have been released", and no neuronal damage was identified. The detriment of Aluminum from MTA is undetermined. It seems you are propagating a theory that is uncorroborated by the evidence of success of such products with no adverse effects reported to the responsible authorities to advantage one company. Perhaps you should cite work on Endosequence or DiaRoot materials.
When you mention high levels of Al, it is unclear to the reader- is this 1% of 20%. Please clarify.
There has been some initial research on this and I cite the research. The scope of writing scientific articles is to mention what other people are reporting as well not only to say your opinions. I am not propagating any theories but only reporting published work in a scientific way including citations. Endoseqence is not a material that is used in this aper thus I do not see why I should report what has been published on Endosequence. You are putting too much stress on Endosequence thus I fell you have a conflict of interest which is inappropriate when you are reviewing a paper. The high levels has been deleted.

Figure 1 was too low in resolution and could not be viewed. The magnifications couldn't be compared, the EDS peaks couldn't be seen for comments.

Please be more brief and direct in your writing. Don't be vague by using "different" frequently, without describing the differences.

Manuscript updated following reviewer comments and figures modified for clarity