Antibacterial Agent Incorporation in Orthodontic Bonding Cements: Impact on Biofilm and White Spot Lesion Formation

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DECLARATION OF INTEREST

None of the authors have any interest, financial or otherwise, in any of the orthodontic cements utilized in this study.

Premolar brackets were donated by American Orthodontics.

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Abstract

White spot lesions are an unfortunate but common side effect of orthodontic treatment. Since treatment of white spot lesions after their formation is not guaranteed to be successful and is often invasive, being able to prevent their formation would be preferable. This study aimed to evaluate the effectiveness of orthodontic cements with an antimicrobial monomer (C14-MAM) and an ion releasing bioactive glass (BAG) on biofilm and white spot lesion (WSL) formation. Experimental cements were made by adding antimicrobial entities (C14-MAM, BAG, C14-MAM+BAG) to an experimental resin cement formulation, and compared to a commercial material (Transbond XT). Cements were evaluated for biofilm formation by *S. mutans* quantification via luciferase assay, image analysis of WSL area on enamel via light microscopy, shear bond strength (SBS) and anti-bacterial monomer leached during storage in solution via nuclear magnetic resonance (NMR) spectroscopy.

Growth of *S. mutans* biofilm was significantly less for C14-MAM and C14-MAM+BAG cements than control and BAG cements, but there were no significant differences among cements for enamel protection against demineralization by *S. mutans* biofilms. SBS of experimental cements were not significantly different than the control cement, except for C14-MAM + BAG group, which was significantly lower. C14-MAM showed no leaching of the antimicrobial monomer. Future studies should focus on clinical effectiveness of the experimental cements and possible synergistic effects of the antimicrobial adjuncts on more complex oral biofilms.

Introduction

White spot lesion (WSL) can be an unfortunate side effect of orthodontic treatment. Observed in 40-60% of patients with a history of orthodontics, [1] these lesions can result in negative perceptions of the treatment and of the treating orthodontist [2] aside from the unesthetic damage to the tooth surface. White spot lesions are created by the same demineralizing mechanism as caries [1]. Tooth demineralization is caused by acid generated by bacterial biofilms in the presence of fermentable carbohydrates [3]. The demineralization process creates pores between the enamel rods and causes light to scatter, leading to the characteristic chalky, opaque appearances [4-6]. The orthodontic bracket acts as a surface upon which biofilms can thrive, ultimately causing white spots to form in an outline of where the orthodontic bracket was bonded. In addition to the brackets themselves, the resin composites used as the cement inherently enhance bacterial growth [7, 8]. Hence there is a potential impact of these resin cements on the ecology of microorganisms in dental plaque biofilm due to an increased biofilm buildup on the resin adhesives.

There are currently many options for the treatment of white spot lesions (WSL) but these can be costly and cannot guarantee remineralization of tooth structure [2]. Use of one or a combination of fluoride, casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), microabrasion, bleaching, restoration and resin infiltration may improve the appearance of the WSL blemish. As resolution of WSL is not guaranteed, various methods of prevention of WSL have been attempted by practicing orthodontists [9]. Fluoride, chlorhexidine and sealants are tools patients can use to prevent the demineralization process, but all require compliance with home care and good oral hygiene. To reduce reliance on patient compliance, it is of interest to explore the development of orthodontic cements that may aid in preventing WSLs.

There currently exist dental materials that have shown antibacterial properties [10]. Calcium Phosphate (CaP) works by remineralizing and neutralizing acids. Quaternary ammonium (QA) monomers physically disrupt the cell membrane of bacteria. 2 methacryloloxyethylphosphorylcholine (MPC) prevents protein binding which in turn prevents bacterial colonizers from adhering and metabolizing. 12-methacryloyloxydodecypyridiniumbromide (MDPB) is synthesized by combining the antibacterial agent quaternary ammonium and a methyacryloyl group and works as a contact bactericide [11]. Bioactive glasses release cations, such as calcium, that have an antibacterial as well as pH neutralizing effect and have been shown to reduce white spot lesions around brackets [12]. Zinc is a known bactericidal metal that works by damaging bacterial cell membranes, generating intercellular reactive oxygen species (ROS) that interact with bacterial cells and induce ROS within the bacterial cytoplasm [13]. Although these materials have been tested in experimental settings, there is a lack of commercially available products that have incorporated these antimicrobial monomers or compounds. Using an antibacterial dental material would be a way for orthodontists to provide patients with another tool for decreasing the likelihood to develop WSL. To ensure long lasting effects, the ideal cement would not leach the antibacterial agent, but have it permanently bound within such that it produces its antibacterial effect through direct contact with oral microorganisms.

The purpose of this study was to evaluate the effect of two different antibacterial strategies, antimicrobial monomer and ion releasing bioactive glass (BAG), incorporated into orthodontic cements on the formation of white spot lesions and tooth demineralization around orthodontic brackets. The primary aim was to evaluate the materials' impact on biofilm formation and their ability to prevent white spot lesion development *in vitro*. Secondary aims were to test bond strength

of these cements to enamel to ensure their adequacy, and to quantify leachates that could contribute to the antibacterial effect or an adverse biological reaction. The hypotheses to be tested were that the experimental cement mixtures would allow less cariogenic biofilm production and produce less white spot lesions on enamel while not releasing the antimicrobial monomer and not falling below clinically acceptable bonding strength values.

Materials & Methods

Experimental group

Experimental resin composite cements were created to ensure the same resin formulation for each of the four orthodontic cement groups studied. This formulation contained Bisphenol A Glycidyl Methacrylate (BisGMA) and triethylene glycol dimethacrylate (TEGDMA) (50:50 weight%) (both obtained from Esstech Inc., Essington, PA, USA). Filler was added at 70 wt% (methacrylate silanized barium silicate glass particles, 0.7 μm, Esstech Inc.) and mixed by hand with a spatula, and then mixed further with the use of a speed mixer at 2300 revolutions per minute for one minute (DAC 150, FlackTek, Landrum, SC). To enable light-curing activation, camphoroquinone and ethyl-4-dimethylaminobenzoate 0.2 and 0.8 wt%, respectively (purchased from Sigma Aldrich, Milwaukee, WI, USA) were then added. Butylated hydroxytoluene (BHT) at 0.05 wt% was added as the stabilizer. This formulation served as the control resin composite cement. The experimental cements were prepared by mixing in the antimicrobial entity with the control cement. The first of two different antibacterial entities, the antibacterial monomer to be co-polymerized within the resin cement, was a quaternary ammonium monomer containing a 14-carbon chain length functionalized with a methacrylamide (C14-MAM, Figure 1).

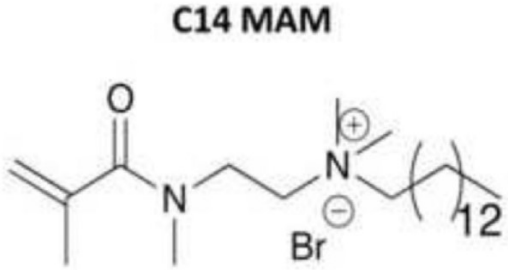


Figure 1: 14 carbon chain length quaternary ammonium monomer

This monomer was mixed into the resin formulation at 10 wt%, prior to filler addition. The second antibacterial entity was ion-releasing bioactive glass containing zinc (Zn-BAG), which had the following formulation: 65% Si; 29% Ca; 4% P, 2% Zn, and was synthesized in our lab according to the procedure outlined in a previous publication [14]. A commercially available cement (Transbond XT, denoted "TB"; 3M, St. Paul, MN) was used to compare with the bond strength of the four experimental cements. The following nomenclature was used for the orthodontic cements:

1. C: no addition to formulated resin cement (control)
2. C + M1: control cement + 10 wt% C14-MAM
3. C + M2: control cement + 15 wt% Zn-BAG
4. C + M1 + M2: control cement + 10 wt% C14-MAM +15 wt% Zn-BAG
5. TB: Transbond XT

Experimental design

Experimental and control cements were evaluated for their impact on biofilm and ability to impede formation of white spot lesions in two ways:

1. Quantification of *Streptococcus mutans* biofilm growth on experimental cements to determine antibacterial properties and range of effectiveness
2. Image analysis of area of white spot lesions on bonded enamel surface through light microscopy

In addition, other clinically relevant properties of the cements were evaluated:

3. Shear bond strength of bracket bonded to enamel using experimental, control and commercially available cements
4. Anti-bacterial monomer leached during storage in solution

Biofilm Quantification

Bioluminescent JM10 *Strepococcus mutans (S. mutans)* bacteria (donated by Dr. Justin Merritt's Lab), were grown on agar plates (BD Bacto Todd (BT) Hewitt Broth (Fisher scientific)) inside a 5% CO_2 incubator at 37ºC for 2 days. The JM10 modification is a derivative of type UA159 specifically designed to enable luminescence of adenosine triphosphate to quantify viable bacterial cells [15-17]. This assay is described as Luciferase assay because it utilizes the firefly luciferase reporter gene. A single colony was taken from this plate to inoculate 1 mL of sterile BT media and then incubated for another 24 hours under the same conditions. The bacteria were then added to 10 mL of media at a 500x dilution along with a 40% sucrose solution (1% sucrose final).

Cement discs were fabricated by placing uncured cement in a 1 mm thick, 10 mm diameter rubber mold secured between two glass slides. The discs were then cured with a light emitting diode (LED) curing light with an irradiance of 500 mW/cm^2 (7.5 mm diameter tip, Demi 500x, Kerr, Detroit, MI). The irradiance was measured using a radiometer (Demetron/Kerr, Brea, CA). The light tip was placed directly against the top of the glass slide, approximately at the center of the disc and cured for 20 seconds. Five cement discs were created for each experimental cement, as well as the control and TB cements.

Five cement discs for each of the four orthodontic cement mixtures and TB (total 25 discs) were distributed, one disc per well, one group per row of two sterile 24-well plates (Figure 2). Five milliliters of the inoculated BT + sucrose solution was added to the wells to cover each

sample disc for four of the five discs from each group. One of the discs from each group was used as the negative control and was covered in media and sucrose only without bacteria. The plate was incubated at 5% CO_2 and 37ºC for 24 hours on an orbital shaker. The media was removed and replenished with plain BT media for one hour prior to analyzing to provide nutrients to the bacterial cells and ensure that they were in an active state (i.e. not starving) (Figure 2). Each cement disc and its associated biofilm was moved into an individual well on a different plate with blacked out bottoms containing fresh media for the Luciferase Assay analysis. Ten microliters of coelenterazine-h solution (0.75 mg/mL diluted in ethanol; NanoLight Technologies, Prolume Ltd, Pinetop, AZ) was added by pipette to each well and vigorously agitated with the pipette to introduce oxygen and fuel the Luciferase reaction. The 24-well plate was then placed in a plate reader (SpectraMax iD3, Molecular Devices, LLC, San Jose, CA) and measured in luminescence mode between 300 and 850 nm. The data were reported in relative light units (RLU).

White Spot Lesion Quantification

Fifty extracted bovine incisors with intact labial surfaces, without noticeable discoloration or caries were selected. The teeth were divided into groups of ten for each cement mixture group including commercially available TB. Labial enamel surface debris was cleaned using a rubber prophy cup and plain pumice operating in a slow-speed handpiece. Surfaces were rinsed with water for 30 seconds and dried for 10 seconds with an air syringe. Two coats of nail polish (Kleancolor Metallic Nail Polish, Santa Fe Springs, CA) were applied to the facial enamel, with 3 minutes of air-drying time in between, leaving a 9 mm x 9 mm window of uncoated enamel (Figure 3). The teeth were photographed using a digital camera system (Edge Eyepiece Camera, Dino-Lite,

Torrance, CA) on a stereomicroscope (SMZ-10, Nikon, Tokyo, Japan) with lighting from opposite sides set at 60 degree angles to have as reference for future comparisons.

A template of a 3 mm x 3 mm window was made within a 1 mm thick rubber mold to create a uniform application of a given cement mixture on each tooth (Figure 4). The template was placed over each tooth and the facial surface was etched with 37% phosphoric acid for 30 seconds before being rinsed for 10 seconds and air-dried with an air-water syringe (A-dec, Newberg, OR) for an additional 10 seconds. Primer (Assure, Reliance Orthodontic Products, Itasca, IL) was placed into the 3 mm x 3 mm window, air thinned and cured using a dental curing light with a 7.5 mm diameter light guide, that emitted 700 mW/cm^2 (Satelec Mini LED ScanWave, A-dec, Newberg, OR). A cement was applied to the 3 mm x 3 mm window created by the template, pressed down with a glass slide to create a uniform thickness and surface area equivalent to that under a bracket, and light-cured for 40 seconds with the light placed directly over the cement, flush with the glass slide. The template was then removed. These bovine incisors with "cement slab" samples were stored in deionized water until subjected to the testing environment.

The teeth were incubated for 19 days under the same conditions described in the biofilm quantification process, with the media solution being replenished every 48 hours. After removal of the biofilm using dry cotton gauze, each tooth was thoroughly air dried using an air-water syringe for 10 seconds to reveal any surface changes. The teeth were then photographed exactly as before and the images were imported into imaging software (Image J; NIH-LOCI, Madison, WI). The image greyscale intensity levels were adjusted with the imaging software to further improve white spot visualization (Figure 5A-D). The non-demineralized area was then traced and measured using the initial photo of the same tooth as a comparison during simultaneous viewing.

Bond Strength

Fifty extracted human premolars were divided into groups of 10 for each cement, including a group to test the commercial cement (Transbond XT). Metal mandibular premolar brackets (Mini Masters Series™, American Orthodontics Corporation, Sheboygan, WI) bonded to the middle of the buccal surface using enough force to express as much cement out the side of the brackets as possible (Figure 6). When there was no more cement extruding from the sides of the bracket, it was considered fully seated. The cements were cured by holding the dental curing light source against the gingival aspect of the bracket at a 45 degree angle. Each tooth was inspected for resin flash visually and tactilely with a dental explorer; any flash on or around the bracket pad was then removed with a scaler.

The bonded premolars were immersed in deionized water for 14 days to ensure that the cements had likely equilibrated with water. The premolars were then prepared for shear bond strength (SBS) testing by embedding the root and lingual half of the crown in dental acrylic (Bosworth Company, Myerstown, PA, USA). Each tooth was placed root-down into a rectangular mold made of aluminum foil, 15 mm x 7 mm by 7 mm. Acrylic was mixed following manufacturer's guidelines (29g of powder per 6mL of liquid) and poured into the mold for each sample. Each tooth was placed with the root and lingual half of the crown in the acrylic and at an angle so that the buccal half of the crown and cementoenamel junction were exposed (Figure 6). The acrylic was allowed to set for 1 hour before removal from the mold. The mounted teeth were stored in deionized water at 21°C until they were tested for SBS.

The debonding force was measured with a universal testing machine (MTS Centurion, Eden Prairie, MN) equipped with a customized "loop" attachment to engage the bracket under the

wings (Figure 7). The load was applied in tension from the gingival to occlusal at a crosshead speed of 0.01 mm/second until the bracket debonded from the enamel surface (Figure 7). Shear bond strength was calculated as the debonding force divided by the bracket base surface area (A) provided by the manufacturer's product specifications and verified by digital caliper measurements ($A=6.84\ mm^2$)

Monomer Leaching

Cement discs were fabricated by placing uncured cement in a 1 mm thick, 10 mm diameter rubber mold secured between two glass slides (Figure 8A). Twelve cement discs were created per experimental and control groups. Near-infrared spectroscopy (NIR) was used to measure the area of the peak corresponding to the methacrylate vinyl absorbance at 6165 cm^{-1} (Nicolet 6700, ThermoFisher Scientific, Madison, WI, USA). The discs were then cured with a LED light (7.5 mm diameter tip, Demi 500x, Kerr, Detroit, MI). The light tip was placed directly against the top of the glass slide, approximately at the center of the disc and cured for 20 seconds (Figure 8B). The vinyl peak area was again measured using NIR and the percent degree of conversion (DC) was calculated according to Equation 1.

$$DC = \left(1 - \frac{polymer\ vinyl\ peak\ area}{monomer\ vinyl\ peak\ area}\right) \times 100 \qquad \text{Equation (1)}$$

The polymerized discs were checked for uniform hardness and then roughened on 600-grit silicon carbide paper on both the top and bottom surfaces to create a uniform surface for evaluating leaching properties (Figure 8C). The mass of each disc was recorded prior to testing using an analytical balance (Mettler Toledo AL204, Columbus, OH). Mass was measured to 0.0001 gm.

Three millimeters of purified water, one cement disc and 5 borosilicate glass beads were placed in a polypropylene vial (Figure 9). The glass beads were used to elevate the disc from the bottom surface of the vial so the entire surface area of the specimen was exposed to the solution. For each experimental and control cement group, the four discs were immersed for different time periods: 24 hours, 72 hours, 7 days and 14 days in a 37^{o}C oven. Three separate vials for each time period was used. At the set time point each disc was removed and patted dry with a lab tissue before mass was recorded. Next, the water was evaporated from the vials by placing these in a desiccator for 14 days. The leachate in each vial was recovered and identified by nuclear magnetic resonance (NMR). The 24-hr aqueous leachates were dissolved in 0.5 mL deuterium oxide containing a 10 mM dimethyl sulfoxide internal standard. Routine ^{1}H NMR spectra were acquired using a spectrometer (Bruker (Billerica, MA) Avance NEO 600 MHz, located at Lewis & Clark College, Portland, OR) equipped with a room temperature inverse triple resonance (TXI) probe and with a sample temperature of 25°C. For each sample (n=3), integration values peaks in the vinyl region (6.25-5.25 part per million) were compared to the integration values of the dimethyl sulfoxide (DMSO) standard to calculate the concentration of monomer found in the leachates. Results for each group were reported as total mass of leachates and leachate composition (%TEGDMA). The discs were then placed in a desiccator for 7 days for final weight measurement.

Reaction Kinetics

Kinetics of photopolymerization were measured using real-time NIR on specimens of the same geometry as those used for monomer leaching. Samples (n = 3) were photocured for 20 s with a LED curing light (7.5 mm diameter tip, Demi 500x, Kerr, Detroit, MI), at a distance of 1

cm from the surface of the sample delivering an irradiance of 500 mW/cm^2. The degree of conversion was monitored for 300 s and calculated according to Equation 1.

Statistical Analysis

The quantities of *S. mutans* measured via Luciferase assay, and the shear bond strengths for the experimental and control cement groups were compared using a one-way Analysis of Variance (ANOVA) on Ranks and Student Newman Keuls test. The area of WSL and the leaching quantities were analyzed by one-way ANOVA, followed by Student Newman Keuls test. All analyses were performed at the significance level of $\alpha = 0.05$.

Results

Biofilm Quantification

The numbers of viable bacteria grown on the cement discs as assessed by the luciferase assay were on average 2200 RLU and 8100 RLU for C+M1+M2 and C+M1 groups, respectively and not significantly different (Figure 10 and Table 1); these values were three orders of magnitude and significantly lower than the averages for C+M2, TB, and Control groups, which were 2500 x 10^3, 3000 x 10^3, and 5100 x 10^3, respectively, and not significantly different from one another..

White Spot Lesion Quantification

The mean non-demineralized areas of the bovine teeth were not found to be significantly different among the cement groups and are shown in Figure 11 and Table 2. The percent of the surface that remained intact was low, ranging from 4.7-8.7%. There was no significant difference in protected area for any of the experimental groups whether outliers (outside of two standard deviations) were included ($p=0.545$) or excluded ($p=0.258$) in the dataset.

Bond Strength

The shear bond strengths of the cements when used to bond premolar brackets to human enamel are shown in Figure 12 and Table 3. TB group had the highest SBS value, 27.42 MPa, which was not significantly higher than the average for the control group, 23.23 MPa. The cement groups with antimicrobial adjuncts exhibited significantly decreased SBS than the commercial cement. C+M1, C+M2, C+M1+M2 exhibited SBS values of 17.18, 19.14 and 15.09 MPa, respectively. There was no significant difference between the control cement and C+ M1 and

between control and C+ M2 group; however, there was a significant difference between the control and the C+M1+M2 group.

Degree of Conversion

The rate of conversion showed the C + M1 + M2 group exhibited significantly slower rate at average of 0.031%/sec compared to the 0.048, 0.052, and 0.051 for the C+M2, C+M1 and C groups, respectively (Figure 13-14). The final degree of conversion for the four cement groups showed that the C + M1 + M2 group exhibited significantly lower degree of conversion, 56.17%, compared to that for C + M2 (66.27%), C (66.99%) and C + M1 (67.30%) (Figure 15).

Monomer Leaching

NMR spectra showing the components leached over the 14 day period are shown in figures 16-17. The reference spectra for TEGDMA is shown in figure 18. Identification of characteristic peaks showed the leachates consisted of mostly TEGDMA (~6.15 ppm for vinyl groups) with some methacrylic acid (~5.73 and ~5.64 for vinyl groups). There was no evidence of methacrylamide peaks in the leachates from the groups containing the C14-MAM monomer in their formulation. The weight change of the composite discs in comparison to the initial are noted in Table 4. Only one disk from each group showed a measurable weight loss. Total water uptake was calculated by subtracting the dry, desiccated weight of the discs from the wet weight and the total loss of eluted components found as leachates was calculated by subtracting dry, desiccated weight from the initial weight the. These weights are also found in Table 4.

Discussion

New cements for bonding orthodontic brackets that have antimicrobial entities incorporated have been formulated with the purpose of targeting bacteria known to cause white spot lesions in treatment. Addition of quaternary ammonium monomer and bioactive glass have been previously utilized in dental materials to hinder carious activity [8, 18]. The purpose of this study was to evaluate how the presence of these materials in orthodontic bonding cements influenced white spot lesion formation. A genetically modified *Streptococcus mutans* biofilm was grown on discs of each cement for one day to assess any antibacterial effect of the experimental cements.

Quaternary ammonium has a positively charged quaternary amine (N+) that interacts with the negatively charged membrane of bacteria to lead to cytoplasmic leakage. It has been postulated that the long chain alkylic substituent can pierce and disrupt the cell envelope, adding to its antimicrobial properties [19], though this remains as conjecture. The length of the side alkyl chain has been shown to be critical in defining the antimicrobial potential. Longer carbon side chain lengths, up to 16-18 carbons, have shown greater antimicrobial properties. This is likely due to increased hydrophobicity and charge in the area which enhances the likelihood of interaction with the hydrophobic bacterial membrane [20, 21]. In our study, we used a monomer with a chain length of 14 carbons because it was found to show statistically greater antimicrobial properties without passing the critical threshold at which point the chain can curl and hinder the positively charged quaternary ammonium group from electrostatic interactions [20-22]. We expected a decrease in biofilm when using our experimental resin polymerized with C14-MAM. Our results show that resin polymerized with C14-MAM did indeed significantly decrease bacterial activity bound to the resin (Figure 10). The biofilm was viable as can be seen in the photos in preparation for the

luciferase assay (Figure 2), but the biofilm was less complete and robust, as evidenced by the lower RLU values recorded. This supports the finding by Fugolin et al (2019) who found no biofilm adhesion to quaternary ammonium monomers with 14 carbon chain length [22]. Our study supports the finding that when quaternary ammonium monomers are polymerized and covalently bonded in resin, it retains its antimicrobial properties and provides contact inhibition of a common species of oral bacterial [18].

Previous research has shown that bioactive glasses possess an antibacterial effect against common oral bacteria, partly due to their high pH production upon dissolution and partly due to the release of potentially toxic ions [12, 23]. In our study we incorporated BAG formulated with zinc in order to produce a material that not only released calcium and caused local pH to rise, but also released antimicrobial zinc ions. Zinc has been proven in medicine and dentistry to have antibacterial activity through promotion of production of reactive oxygen species. Direct contact of ZnO with the bacterial cell wall leads to membrane destabilization and an increase in permeability [13]. We expected to see a decrease in activity when bacteria were exposed to the experimental cements containing Zn-BAG. Our luciferase assay results showed a trend towards this, that is, less bacterial adhesion or activity was shown for the Zn-BAG cement compared to the commercial cement and the control cement (2500×10^3, 3000×10^3, and 5100×10^3, respectively), but these differences were not statistically significant. Previous studies showed that gram positive bacteria such as *Streptococcus mutans* are susceptible to ZnO [24, 25]; however, the concentration of ZnO might not have met a critical threshold in our study.

The control cement was used during the biofilm quantification portion of the study to be able to compare our experimental cements. The only difference between the control and the C14-MAM and Zn-BAG groups were the addition of the antimicrobial entities. Transbond XT was used

as the commercially available control group. Lastly, we created a cement that incorporated both C14-MAM and Zn-BAG to see if there would be a difference in antimicrobial property when both components were present. Our results show that the group with both C14-MAM and Zn-BAG had significantly less viable bacteria compared to the control. The luciferase value for this group was not significantly different from the group with C14-MAM alone (C + M1), but was significantly different from the group with Zn-BAG alone (C + M2). The statistical analysis verified that there was a significant effect attributed to adding C14-MAM monomer to the experimental cement, but not for the addition of the Zn-BAG. In addition, adding Zn-BAG did not significantly enhance the antimicrobial activity of C14-MAM ($p<0.05$). Again, based on the fact that Zn ions have known anti-microbial properties, the lack of an affect was most likely due to the fact that the concentration in the glass itself was low, and this was further diminished by its incorporation into a polymer resin cement, where leaching is a time dependent phenomenon. The rate and amount of release may have been too low to produce the desired effect, especially when the medium was changed every two days and the ion concentration could not build up.

Bovine teeth with slabs of experimental and control cements were exposed to *Streptococcus mutans,* a bacteria known to contribute to white spot lesions and caries in the oral environment. The extent of demineralization, or the lack of intact enamel, was not significantly different for any of the cements ($p=0.258$). The presence of intact enamel was evident, but there were outliers (values outside of two standard deviation units) in area of intact enamel in most groups (Table 2; figure 11). This adjunctive protection against demineralization may have been from the priming agent that might have spread during the bonding process and remained covering the enamel during the storage period, thus protecting it from the acid producing biofilm. The experiment to quantify WSL was conducted for 19 days, which does not represent the full extent

of orthodontic treatment, and therefore may underestimate the potential damage that a thriving biofilm may cause in the clinical situation. However, WSLs have been found to manifest as quickly as the time between a single orthodontic appointment [26] and it was obvious in this study that the bovine teeth had been demineralized to a significant extent after the 19-day study period. The experimental formulations were unable to significantly resist demineralization in the study period. The expectation was that C14-MAM cement would provide protection for the enamel via contact killing of *S. mutans.* The C14-MAM monomer was expected to be bound within the polymer network of the resin and not be leached out [18, 27], thereby not having any long-range effect. Due to this limitation, we did not expect intact areas to be present at any clinically important distance from the cement slabs for cements containing C14-MAM. Although Zn-BAG was expected to have long distance range effect due to the leaching of the ions into the local environment, thus providing greater protection against demineralization [9, 28, 29], our results did not reflect this. It may be that a threshold for ion release was not reached and therefore the demineralization of the majority of the tooth surface was unimpeded. In a 2019 study by Sergi et al., it was found that the bacterial reduction effect from bioactive glass was dose and time dependent [25]. The inhibitory effect reached statistical significance only when 7-day eluates of BAG were tested, which means the inhibitory effect could have decreased past the 7 day mark in our 19 day study. It is possible that had we terminated the storage period earlier, the extent of demineralization may have been less complete, allowing us to determine any potential beneficial effects of the ion release.

The shear bond strength to enamel of the experimental cements were also compared to the control and commercial control. There was no significant difference between the control and commercial cement. This result verified that our base formulation was acceptable in that it produced a bond strength that was comparable to a successful commercially available material,

Transbond. All of the experimental formulations exhibited significantly lower SBS relative to the commercially available cement. The formulation with both C14-MAM and Zn-BAG exhibited significantly lower SBS relative to the experimental control. Since the cements with just one of the antibacterial entities did not exhibit a significant difference in SBS when compared with the experimental control, the results suggest that the addition of both impacted the resin formulation enough to lower bond strength. Based on the lower degree of conversion data for the C+M1+M2 formulation, this reduced bond strength may be attributed to reduced properties of this cement owing to the lower degree of conversion. This reduced conversion may have resulted in a cement with slightly reduced properties, and this may have been reflected in the lower bond strength.

It has been suggested that a clinically adequate bond strength for a metal orthodontic bracket to enamel should be 6-8MPa [30]. The mean SBS values of all formulation were within this range or exceeded these limits and therefore could be considered sufficient for clinical applications. One limitation to this study is that the bonded premolars used in the SBS testing were not exposed to an aging procedure simulating the oral condition prior to testing. Storage in media simulating the oral environment with bacteria may have led to decreased SBS of the cements, including the controls. Follow up studies should examine the adequacy of the bond strength clinically, either through storage in water or other media for an extended period of time, or in a split-mouth design clinical study.

Each experimental cement along with the control were formed into discs and submerged in water for various periods of time. A limitation to the fabrication of the discs was that the light curing unit guide diameter was smaller than the disc diameter. This meant that curing of the edges of the disk depended upon light scattering within the composite disc to cure the cement. After the initial light curing, the discs were subjectively checked to be sure they were hardened throughout

the edges and did not feel tacky from unreacted monomer, and they did not crumble during sanding. Thus, it was concluded that the discs had undergone at least adequate, if not optimal, conversion.

After preparation, the discs were weighed and then agin after each specific time point during water exposure, verifying an increase in weight due to water absorption. The weight gain reached its peak at 72 hours and remained stable at 7 and 14 days. The discs were weighed after desiccation and showed no significant differences in weights of the discs pre- and post-leaching test. The NMR analysis (Figures 16-17) showed that there was no leachate attributable to C14-MAM. This was an important finding and confirmed that the antimicrobial monomer had co-reacted with the other monomers and was tightly bound within the cement, and thus capable of exerting a perpetual contact antimicrobial effect The NMR analysis of the leachates from the cements in comparison to the TEGDMA reference NMR spectrum (Figure 18) showed that the majority of the leachates consisted of TEGDMA. Although TEGDMA was lost, the loss was not sufficient to be detectable by the mass loss measurements, likely due to the limited sensitivity of the balance used (lower limit equal 0.0001 gm). The scale used measured to the ten thousandths of a gram, but the loss was likely in the hundred thousandths of a gram. Using a balance with greater sensitivity may have shown that weight loss occurred and correlated with the appearance of the TEGDMA monomer in the leachates verified by NMR. However, it is important to note that the NMR is more highly sensitive for identifying any leachates than the weighing experiment.

IR spectra results before and after polymerization were used to calculate rate of polymerization and degree of conversion. The cement with C14-MAM and Zn-BAG exhibited significantly lower degree of conversion and a slower cure rate, which as noted earlier may have contributed to the lower bond strength (Figure 12). The reason for the

reduced cure and cure rate for the C+M1+M2 cement is not clear at this point, especially since the addition of the monomer alone or the Zn-BAG alone had no effect.

Conclusion & Future Research

Certain biomaterials have shown protective properties in cariogenic environments. The object of this study was to evaluate the effect of two different antibacterial strategies, antimicrobial monomer (C14-MAM) and ion releasing bioactive glass (BAG), when incorporated into orthodontic cements on the formation of biofilms and the formation of white spot lesions from tooth demineralization around orthodontic brackets. Addition of C14-MAM showed significantly less bacterial growth while addition of BAG did not. This, however, did not result in less demineralization, as all cement groups showed areas of intact enamel that were not significantly different from each other. Addition of both C14-MAM and BAG together significantly decreased shear bond strength to human enamel in comparison to the commercial cement, but addition of either one of those alone did not significantly impact the SBS of the cement. The addition of both C14-MAM and BAG also showed significantly lower degree of conversion and slower rate of conversion compared to all other groups. NMR spectra identified leachates consisted mostly of TEGDMA, but there was no evidence of methacrylamide peaks in the leachates from the groups containing the C14-MAM monomer in their formulation, verifying that the methacrylamide monomer was tightly bound, likely via copolymerization with the resin matrix. Further studies should aim to test the long term antimicrobial effect of the cements on biofilm, maintenance of shear bond strength, depth of protection of enamel and ultimately their ability to reduce or prevent white spot lesion development during orthodontic treatment.

Comprehensive Literature Review

White Spot Lesions

White Spot Lesions (WSLs) are the first clinical observation of progression of dental caries [31]. WSLs can be defined as a demineralization of the enamel surface and subsurface without cavitation [32]. Clinically these lesions are characterized by a white, chalky, opaque appearance and can be located in pits, fissures and smooth surfaces of teeth [31]. The white appearance is due to an optical phenomenon which is caused by mineral loss in the surface or subsurface enamel [33-35]. The demineralization process creates pores between the enamel rods and alters the refractive index in the affected area. The voids cause light to scatter after penetrating a shorter distance into the enamel, leading to decreased refractive index and results in greater enamel opacity [26, 33-35]. Because the voids of the crystalline structure are filled with water, when the area is dried, water is replaced with air and cause further reduction of the refractive index [26]. Drying a suspect tooth can help visualize WSL with ease.

The process of caries involves demineralization and remineralization of enamel and dentin. And depends on a combination of four major etiologic factors:

1. Microorganisms
2. Metabolic substrates
3. Teeth and their environment
4. Time

White Spot Lesions in Orthodontics

White spot lesions remain the most common complication of fixed orthodontic appliance therapy[2] and can manifest as quickly as between one orthodontic appointment [36]. Orthodontic patients develop significantly more WSLs than patients without fixed appliances [37] and the percentage of patients developing at least one WSL can be as large as 72.9 % [38]. Fixed appliance creates a greater surface area for plaque to be retained. Without adequate hygiene, the induced rapid increase in the amount of dental plaque around the orthodontic brackets will lower the pH and result in carious decalcification that is seen as a WSL [39-41]. Aside from serving as a physical plaque trap, Beyth et al. found that composites tend to accumulate more biofilm than other restorative materials [7]. Khalichi et al. found resin composites inherently enhance bacterial growth [8]. These studies support Bourbia et al.'s conclusion that there is a potential impact of composite resins on the ecology of microorganisms in dental plaque biofilm due to an increased biofilm buildup on composites [42]

Streptococcus Mutans in Orthodontics

Tooth demineralization is caused by acid generated by bacterial biofilms in the presence of fermentable carbohydrates [3]. *Streptococcus mutans* is a gram positive coccus found in the oral cavity that colonize dental surface and cause damage to hard tooth structure [43]. *S.mutans* have many characteristics that make it cariogenic[44]:

1. They have excellent sugar transport at low pH
2. They are homolactic acid fermenters
3. They are aciduric and acidophilic

4. They utilize glucose to make insoluble glucans which protects them from saliva and allows them to increase the acid in their local environment
5. They utilize fructose to make levans to metabolize between meals

The critical pH of demineralization of enamel is 5.5. Being homolactic acid fermenters, *S. mutans* almost exclusively produce lactic acid as a by-product of sucrose fermentation which has a pH of 3.8. *S. mutans* are able to thrive in an acid environment and their ability to make insoluble glucans to protect themselves protect them from the buffering effect of saliva and allow them to maintain a localized acidic environment. All of these factors make *S. mutans* a keystone cariogenic bacteria.

Prevention of White Spot Lesions

With such a high prevalence rate, practitioners have been looking for ways to prevent WSLs forming. Good oral hygiene including tooth brushing regularly with a toothpaste containing fluoride concentration of 0.1% or greater can help prevent WSLs [45]. An experiment with a modified Stephans Curve illustrated that by simply disrupting the biofilm on teeth, amount of acid is greatly reduced [46]. Widely used methods for prevention of white spot lesions are discussed below:

Fluoride

For patients with less than ideal compliance, simple act brushing with fluoridated tooth brush is not enough [47]. Studies have found that fluoride rinses significantly reduced the number of WSLs in patients that complied with prescribed use [47, 48]. Mouth rinses with 0.05% sodium

fluoride should be used on daily basis for it to have an impact [45]. However, poor patient compliance is well documented in dental and medical literature. That is why more sustained fluoride supplementation that is independent of patient's cooperation may be more useful [45]. Fluoride varnish has shown to decrease the amount of enamel demineralization in orthodontic patients [49, 50].

Aside from supplementation, using dental materials that have sustained released of fluoride have shown to decrease the extent of demineralization in patients with fixed orthodontic appliances [51-53]. Resin-modified glass ionomer (RMGI) has fluoride releasing capabilities and Hallgren et al. has found the plaque adjacent to brackets bonded with RMGI had higher concentration of fluoride than plaque adjacent to brackets bonded with resin composite [54].

Chlorhexidine

Chlorhexidine is the most commonly used antiseptic in dentistry and has proven to be effective in control and management of biofilms in gingivitis. It is available as mouthwash, gel, or varnish. It is shown to decrease count of mutans streptococci. There currently are conflicting reports of the efficacy of chlorhexidine in decreasing the prevalence of caries during orthodontic treatment [55]

Sealants

Sealants can be applied to patients without relying on patient compliance for effectiveness. Although use of sealants in orthodontics for the prevention of decalcification is not new, there have been conflicting findings off the effectiveness of sealing smooth enamel surfaces.[56-58] O'Reilly et al in their 2013 study assessed the efficacy of a new sealant Biscover LV. This new

formulation of sealant was developed to eliminate the formation of oxygen inhibited layer by chemical means and displayed low wear to abrasion and no effect on bond strength. The study found that the sealant did not prevent all white spot lesions for full duration of treatment but demonstrated a clinically small but statistically significant ability to prevent white spot lesions compared with untreated control teeth [59].

Motivational Teaching

In a meta-analysis of studies by Rubak et al., they found that strategies to increase patient's intrinsic motivation out-performed traditional advice-giving strategies in 80% of the studies [60]. Motivating patients to maintain good oral hygiene habits may be the most effective preventive measure.

Treatment of White Spot Lesions

WSLs are difficult to treat and often are permanent regardless of the treatment approach [2]. That is why it is important to stress to orthodontic patient the importance of preventing the development of white spot lesions. As a clinician, all options must be given to the patient to reduce the look of WSL and to achieve better esthetics.

Fluoride

Many clinicians consider the application of topical fluoride to WSLs to be the first step in treatment [61]. However, many studies do not advocate application of high concentration of fluoride due to the possibility of it creating an undesirable esthetic effect [36, 62, 63]. The high concentration of fluoride react mainly with outer surface of the lesion, arresting the development

[36, 64] but not repairing them. The lesions can then stain to worsen the esthetics [45]. Bishara et al suggested applying lower concentration of fluoride or letting saliva remineralize the lesion slowly over time [63].

CPP-ACP

In the 1980s casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) was found to be capable of absorbing through the enamel surface and could influence the carious process [65]. CPP- ACP is a delivering system that allows freely available calcium and phosphate ions to attach to enamel and reform into calcium phosphate crystals. In their systematic review, Lapenaite et al [65] found that there have been conflicting reports of the effectiveness of CPP-ACP. While some clinical studies reported decrease in WSL with the use of CPP-ACP agents post orthodontic treatment, some studies saw no significant change over time.

Microabrasion

Microabrasion consists of a chemical and mechanical processing of the enamel surface by applying an abrasive slurry or hydrochloric acid with a brush. This can be a useful method for treatment of postorthodontic WSLs but the depth of the lesion should be under 0.2mm and maybe used with a bleaching technique. As microabrasion is relatively invasive, delayed application after saliva based remineralization and spontaneous surface abrasion is recommended [55]

Bleaching

The esthetic results of bleaching procedures are limited and they may give rise to tooth sensitivity and decreased enamel microhardness. A recent study showed that bleaching incipient

enamel caries with 10% carbamide peroxide could camouflage WSLs with no effect on chemical and mechanical properties of the enamel. The study also considered application of CPP-ACP as an adjunct treatment for promotion of mineral gain in the subsurface lesion [66].

Resin infiltration

A minimally invasive treatment technique in which the WSL is infiltrated with the use of low viscosity resin has been introduced in recent years. The procedure involves the penetration of the resin through etching to make the outer surface more permeable. The resin stops the progression of caries and camouflage the WSL by reinforcing the compromised enamel prism structure and providing a refractive index close to that of sound enamel. The camouflage effect depends on the depth of the lesion. Although there are limited clinical experience available in relation to orthodontic WSLs, current studies show that this method can create an enduring esthetic improvement of post-orthodontic WSLs [55].

Antimicrobial Compounds in Dental Materials

As dental caries remains a prevalent disease worldwide, efforts have been devoted to developing bioactive dental materials containing additives that have remineralizing and antimicrobial capabilities

Calcium Phosphate (CaP)

Calcium phosphate (CaP) neutralizes acid in demineralizing conditions and enhances remineralization by providing Ca and P ions [10]. CaP filler particles ranging from about 1 μm to 55μm in size were incorporated into dental resin. This composite released supersaturating levels

of calcium and phosphate which were shown to remineralize tooth lesions in vitro [67, 68]. One draw back to CaP composites was that they were mechanically too weak to be used in restorations [67, 68]. Nanoparticles of CaP (NACP), approximately 100nm, were synthesized and loaded into dental resins[69, 70] and were found to process greater mechanical properties. In an in vitro study by Weir et al, cyclic demineralization at pH4 and remineralization at pH7 showed NACP nanocomposite achieved enamel remineralization four times greater than that of commercial fluoride release composite control [71]. A significant limitation of CaP containing resins is that the ion release is short term, lasting only a couple of months[67-70, 72]. Rechargeable CaP composite was developed that showed ion re-release did not decrease with increasing the number of recharge cycles [73]. Although possible, this composite would require intermittent recharging for it to remain effective.

Quaternary ammonium (QA) monomers

Quaternary ammonium methacrylate (QAM) can be polymerized and covalently bonded in resin matrix to provide long term contact inhibition with oral bacteria [18]. QAM resins possess positively charged quaternary amine N+, which can interact with the negatively charged membrane of bacteria, leading to membrane disruption and cytoplasmic leakage [19]. Being able to hold the charge at the surface via formation of a polymer network decreases the likelihood that the QA compound will leach out, potentially increasing the longevity of the antibacterial effect and decreasing cytotoxicity concerns[27].

The long chain alkylic substituent is responsible for piercing and disrupting the cell envelope [19]. The length of the side alkyl chain has a difference in antimicrobial effect. Longer carbon side chain lengths (up to 16-18 carbons) have shown greater antimicrobial properties [20,

21]. This is likely due to increased hydrophobicity and charge in the area which enhances the likelihood of interaction and penetration of the side chain into hydrophobic bacterial membrane. However, if the chain is longer than the critical threshold, antibacterial effect is decreased as the chain can curl and hinder the positively charged quaternary ammonium group from electrostatic interactions [21].

QA monomers polymerized with ester- containing methacrylate are highly susceptible to enzymatic and hydrolytic degradation [74]. To combat degradation, the ester group can be substituted with more resistant methacrylamides [21, 27, 74] to form quaternary ammonium methacrylamides (QAMAM). This would make its bactericidal capabilities more applicable in dental settings.

Paper by Fugolin et al in 2019 [22] synthesized experimental composite incorporating QA methacrylate, acrylamide and methacrylamide compounds and studied their kinetics of polymerization, mechanical properties, antimicrobial and antifouling properties. A composition without any QA monomer was used as the control. The study found all quaternary ammonium monomers with 14 carbons on the side alkyl chain showed statistically greater antimicrobial properties than monomers with 9 carbons on the side chain. This supports the findings by Tiller and Li [20, 21]. Fugolin et al found no biofilm adhesion to any of the monomers with 14 carbons (C14) on the side chain. This was important since disabling early colonizers to adhere to the surface means later stage colonizers, which tend to be more desctructive, cannot bind. Acrylamide and methacrylamide showed statistically lower maximum rate of polymerization than methacrylates. ^{1}H-NMR analysis of uncured monomers showed that amide C14 versions were most stable with virtually no degradation even after 30 days in extremely acidic conditions. All three types of compositions were found to have no significant difference in flexural strength and elastic modulus

after 24h dry storage. The study supported previous postulations that biofilm inhibition is driven by mechanism related to inhibition of bacterial adhesion in combination with cell disrupting contact kill [22].

2 methacryloloxyethyl phosphorylcholine (MPC)

2-methacryloloxyethyl phosphorylcholine (MPC) is a methacrylate with phospholipid polar group in the side chain that makes it a hydrophilic protein repellent [10, 75]. Oral bacteria, including *s.mutans*, need a pellicle to adhere to tooth or resin surface [76]. Pellicle is a layer of adsorbed salivary proteins [76] and by preventing this initial step in biofilm formation, MPC resists bacterial adhesion. In an experimental dental composite, 3% MPC and 1.5% dimethylaminohexadecyl methacrylate (DMAHDM) were incorporated. The composite showed significantly decreased amount of protein adsorption, lactic acid production by biofilm, and biofilm CFU in comparison to commercial control [77]. In another study, MPC was incorporated into resin-modified glass ionomer (RMGIs) to be used as orthodontic cement. The incorporation of MPC reduced protein adsorption [78]

12-methacryloyloxy dodecypyridinium bromide (MDPB)

MDPB was created to be incorporated into dental resin-based materials to give antibacterial properties [79, 80]. MDPB was synthesized by combining the antibacterial agent quaternary ammonium and a methacryloyl group. The mechanism of antibacterial effect of quaternary ammonium compounds, as discussed above, is believed to be due to the cationic binding to cell wall components which disturbs the membrane function and subsequently disturbs the bacterial membrane[19]. It has antibacterial activity against oral bacteria before being polymerized and can

be chemically bound to polymer matrix to have a bactericide effect [11]. Studies by Imazato et al have shown inhibition of bacterial growth on the surface of cured resin with MDPB by means of non-released immobilized bactericide [79, 81, 82].

In the 2003 paper by Imazato et al, the team investigated the in vitro antibacterial activity of an adhesive resin incorporating MDPB after curing and compared bonding properties of adhesive systems with and without incorporation of MDPB [82]. Experimental adhesives were prepared by addition of monomer MDPB at various wt% to commercially available resin LB bond. Results showed that when incubated on control adhesive, S.mutans showed normal growth while growth of bacteria on the experimental adhesive was inhibited by approximately 97%. The tensile bond strength did not show significant difference between the adhesive systems with and without MDPB. The study concluded that a comprehensive antibacterial adhesive system employing MDPB containing primer and adhesive would contribute to prevent harmful effects caused by oral bacteria in vivo. Although further study is needed to test this monomer in vivo, it is believed that reduction in cariogenic bacteria can lower risk of demineralization and cavitation.

Bioactive Glass – Zinc

The discovery of mesoporous glasses led to a new class of biomaterials acting as bioactive platforms for the local release of organic or inorganic agents eliciting an antimicrobial effect. Introduction of the first gel derived bioactive glasses (BAGs) took place in early 1990s by Larry Hench and his students Li and Pereira [83]. The typical melt-derived, surface reactive BAG usually contains SiO_2, Na_2O, CaO, and P_2O_5. Sol-gel BAG is a three-dimensional cross-linked matrix of hydrolyzed alkoxides of SiO_2, CaO, and P_2O_5 that releases ions such as calcium, phosphate and

fluoride [9]. Studies have shown that bioactive glasses possess an antibacterial effect on common bacteria due to high pH produced upon dissolution and via the released cations [12].

Although it has been hypothesized that antimicrobial effect of BAGs are related to its ability to increase the pH in bacterial suspensions [23], study by Salehi et al found that BAG solutions are capable of exerting a significant decrease of bacteria colonies even at neutral pH [12]. This proved that the antibacterial activity was at least in part due to release of ions from the glasses.

Mesoporous bioactive glass (MBAGs) were developed in 2004 by Zhao [84]. Main advantages of MBAGs over melt-derived glasses are ultrahigh specific surface areas (>100m^2/g) and high pore volumes [85].The mesoporous structure offers opportunities for loading and delivering different dugs and bioactive molecules [86]. MBAGs are formed by a combination of sol-gel and supramolecular arrangement routes. The result is a mesoporous material with composition similar to conventional sol-gel glasses but with an ordered mesoporous configuration of micelles [85]. Manfred et al tested novel bioactive glass containing orthodontic cements against commercially available cement (Transbond XT) and compared their ability to reduce enamel softening surrounding orthodontic brackets [9]. The study found that all experimental adhesives containing BAG outperformed Transbond XT at maintaining superficial enamel hardness surrounding orthodontic brackets with the adhesive with the highest BAG surface area having the most preventive effect against WSL. The study concluded that key factors for combating demineralization include release of fluoride and increased BAG surface area.

MBAGs and NMBAGs (non-mesoporous BAGs) can inhibit the growth or viability of bacterial cells through release of antibacterial metallic ions incorporated in their glass network [28]. Antibacterial activity of zinc is proposed by following mechanisms [13]:

1. Promote the production of reactive oxygen species (ROS) and thereby cause DNA, RNA and protein damages
2. Direct contact of ZnO particles to bacterial cell wall leading to destabilization of their membranes and enhanced permeability
3. Partial dissolution of ZnO particles and release of bactericidal Zn^{2+} ions in the water

Antimicrobial effect of zinc has been previously studied. Lim et al in 2018 [29] studied the penetrating and bactericidal activity of zinc compound against *Streptococcus mutans* biofilms. Mature biofilms are surrounded by a protective three dimensional matrix of extracellular polymeric substance (EPS) that inhibit penetration of external molecules [29]. In utilizing antimicrobial materials in the mouth, one must consider potential toxicity. Although large amounts of zinc ions are potentially toxic [87], small amounts are essential for metabolic process [88] and have a positive impact on bone formation[89]. Lim et al hypothesized that biofilm disrupting efficacies of zinc and erythritol could be synergistically enhanced by formation of complexes. Zinc chloride was dissolved in various sugar alcohol and the bactericidal activity against *S.mutans* was assessed via Alamar blue assay. The team concluded that zinc chloride and erythritol found cationic complexes that weakened intra- and inter- molecular interactions of EPS in biofilms. The complex penetrated into biofilm and were effective at killing bacteria while not increasing the cytotoxicity of zinc chloride.

In combining releasing ability of BAGs and antibacterial effect of zinc, the 2019 study by Sergi et al [25] investigated biological performance of bioactive glasses with different amount of zinc oxide (ZnO). A previously developed bioactive glass was modified by adding 2, 3.8 and 5mol% of ZnO with the aim of obtaining a novel glass with bioactivity, antibacterial effects and high crystallization temperature at the same time. Bactericidal assays were carried out using

Staphylococcus epidermidis (gram positive), *Escherichia coli* and *Pseudomonas aeruginosa* (both gram negative). When tested against *S. epidermidis,* the bioactive glass caused a dose and time dependent reduction of bacterial growth. The inhibitory effect reached statistical significance only when 7 day eluates of modified BAG with 3.8mol% were tested. Eluates of BGs tested against the gram negative species showed no evident inhibition of bacterial growth. The result was supported by previous studies reporting gram positive bacteria are more susceptible to ZnO than gram negative bacterial [24]. Additional investigation is needed to test for potential use of BAGs modified with biologically active elements such a zinc for antibacterial effects.

Figures and Tables

Figure 1. C14 MAM

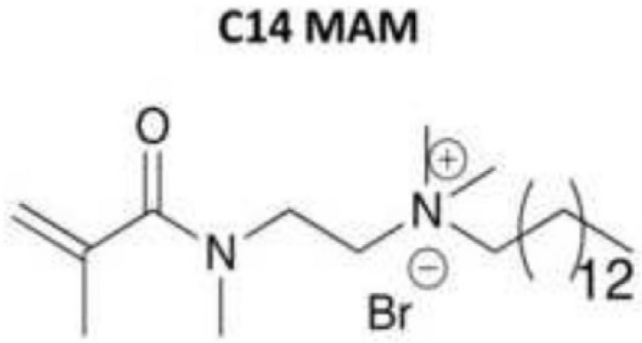


Figure 2. Cement disc samples in wells of 24-well plate, prepared for Luciferase Assay

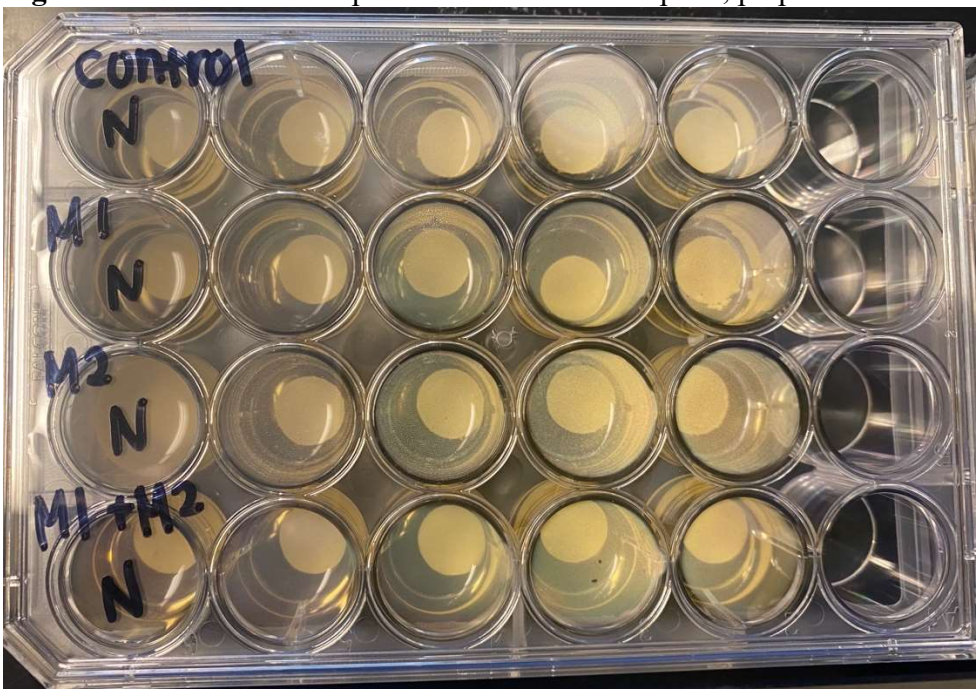


Figure 3. 9 mm x 9 mm window of enamel in otherwise painted bovine incisor tooth

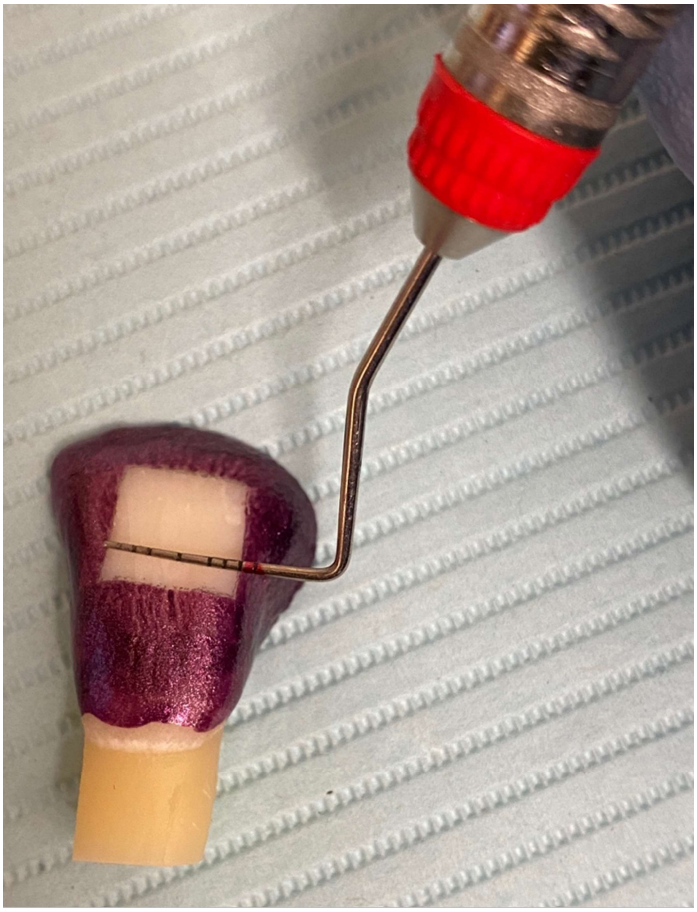


Figure 4. Black rubber template with window used to produce 3 mm x 3 mm button of cement on the bovine incisor tooth

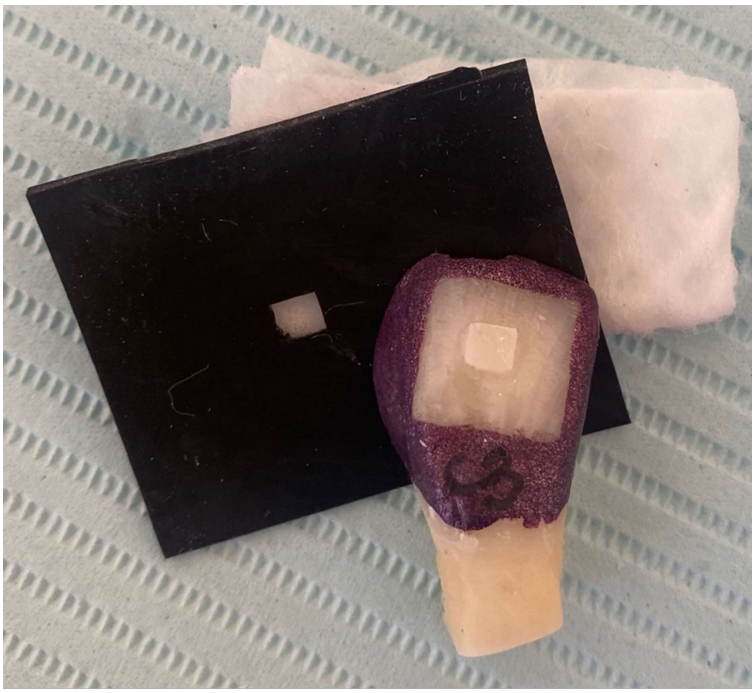
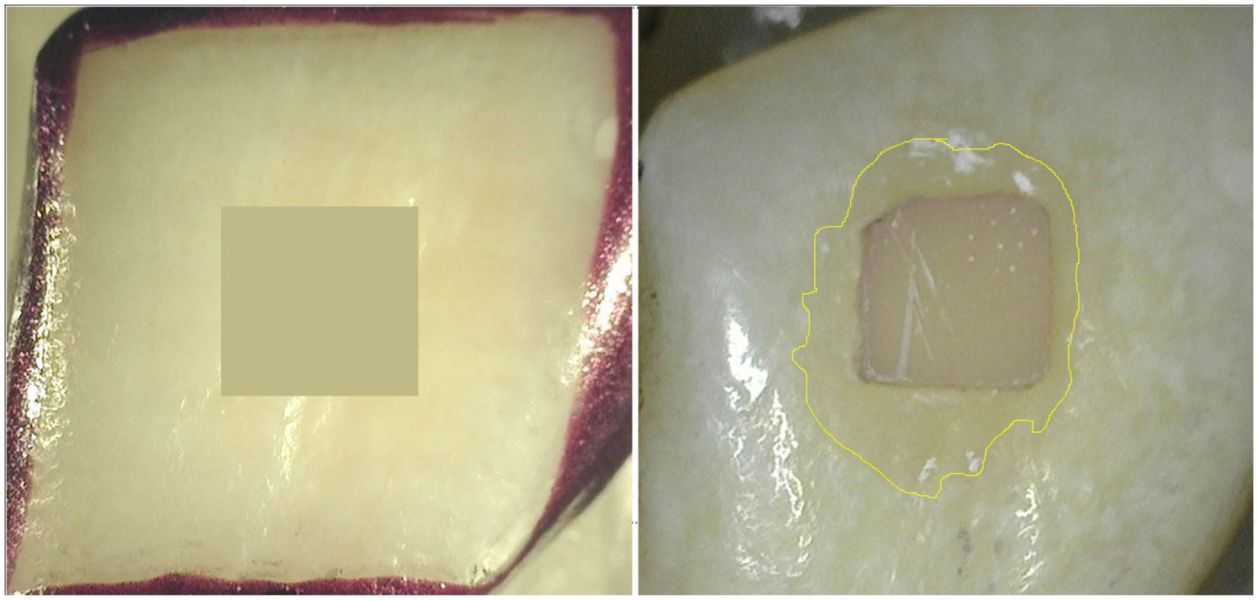


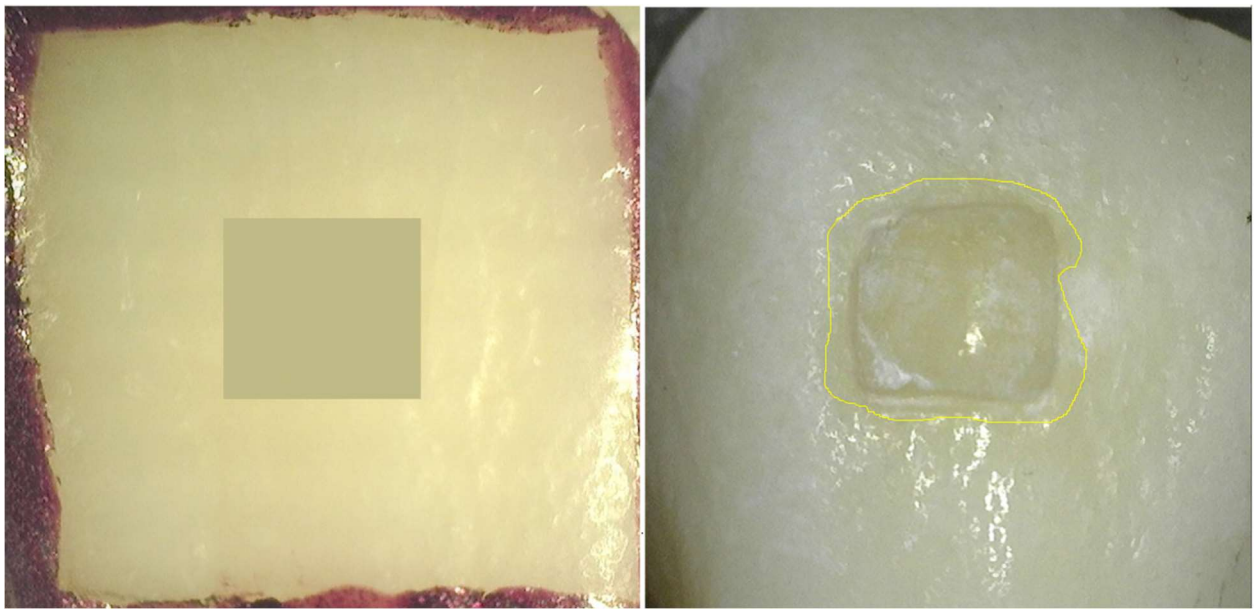
Figure 5A-D. Before and after demineralization of bovine teeth with outline of intact enamel

A.Control



B. C+M1

C. C +M2



D. C+ M1+M2

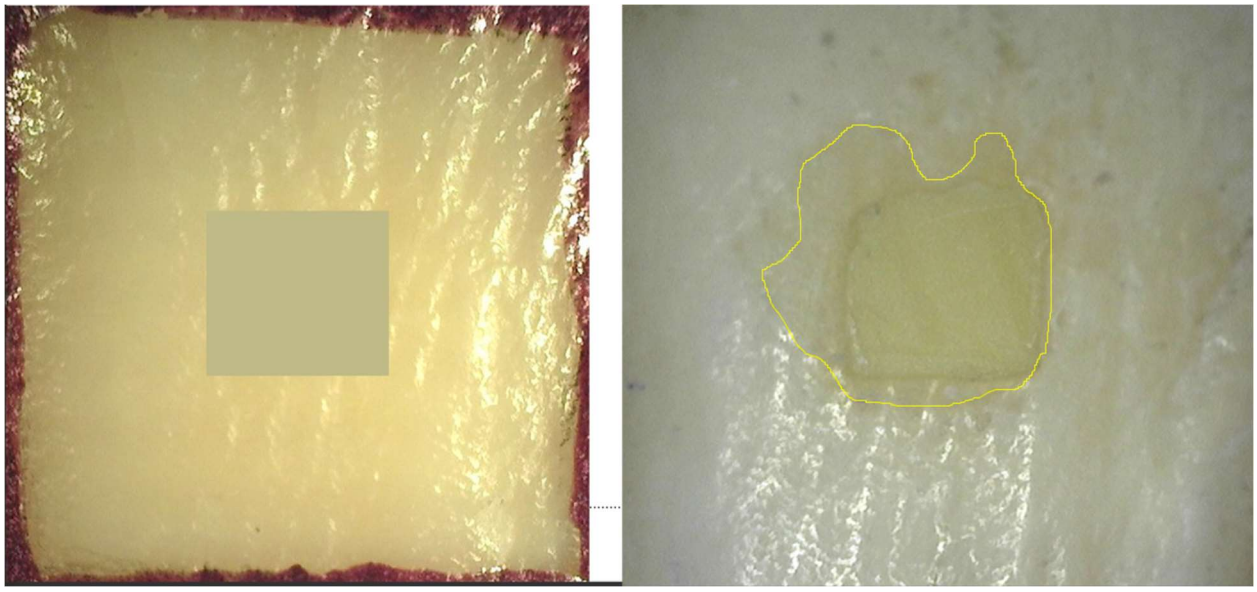


Figure 6. Bonded Human Premolar embedded in acrylic

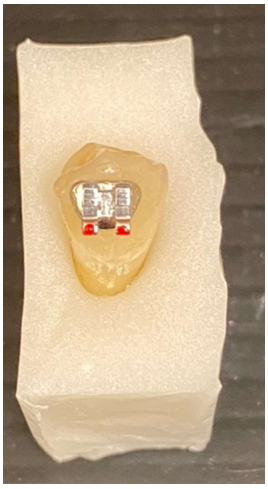


Figure 7. Mounted human premolar with bonded bracket for shear bond testing in universal testing machine.

Figure 8A. Cement Disc Fabrication

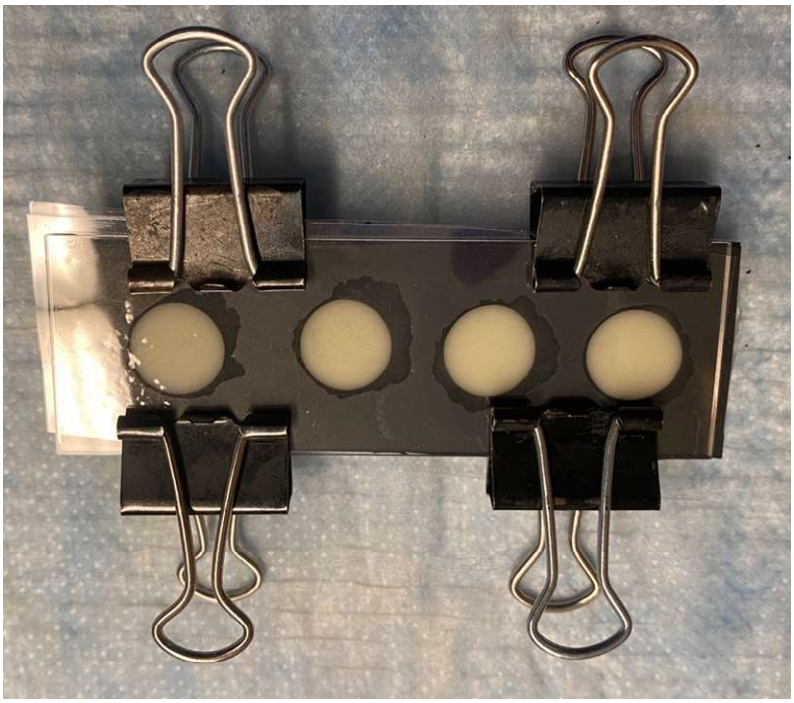


Figure 8B. Cement Disc Polymerization

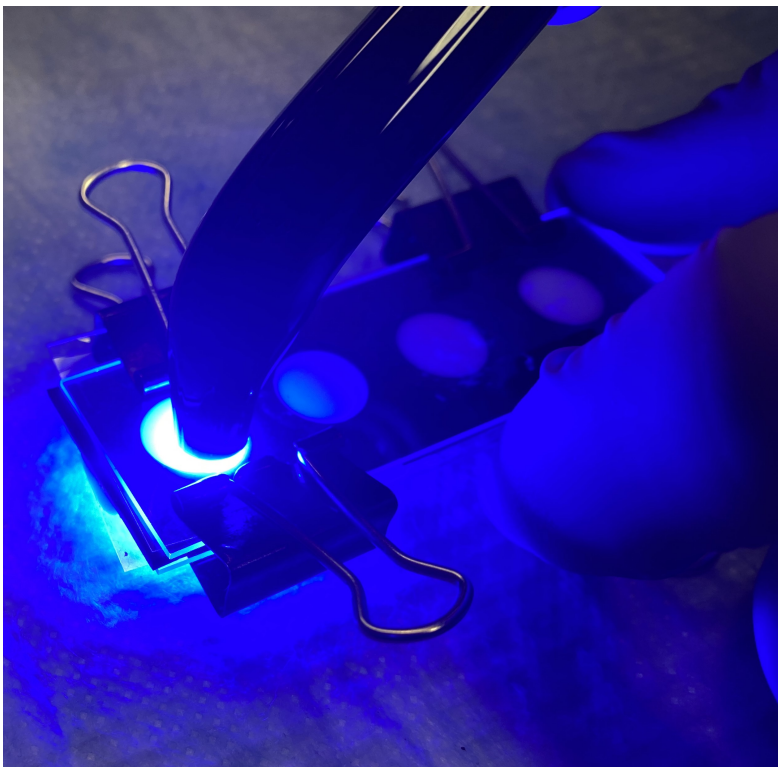


Figure 8C. Cement Disc Roughened Surface

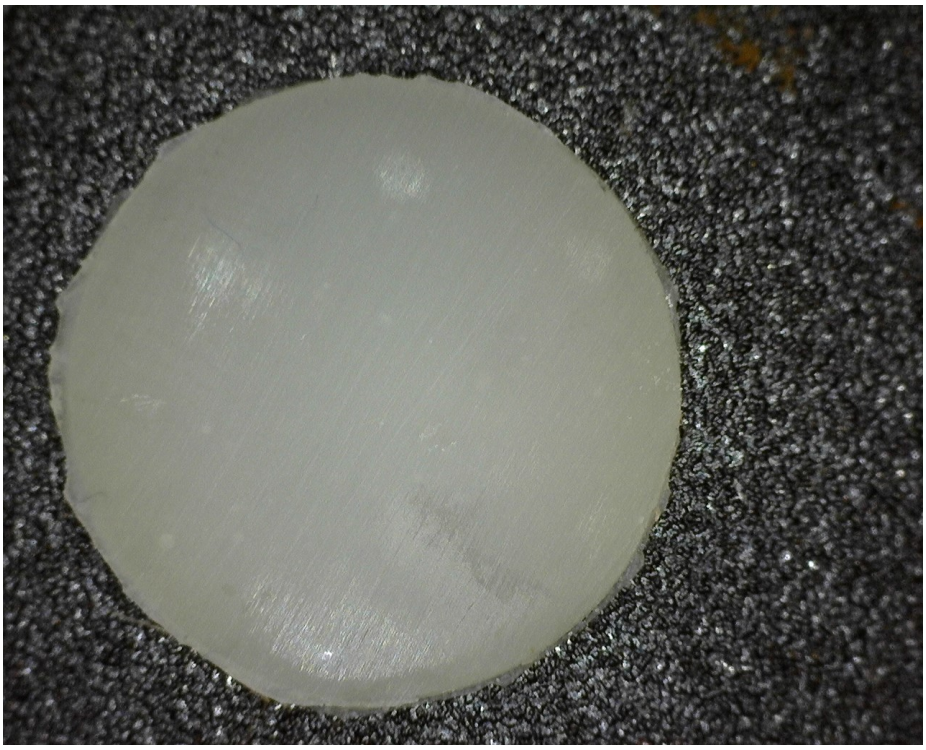


Figure 9. Cement discs in vials for leaching analysis

Figure 10. Biofilm quantification

Relative light units (RLU) quantified by the luciferase assay after one day of biofilm growth on a composite disc. Each value is mean ± SD; n =4.

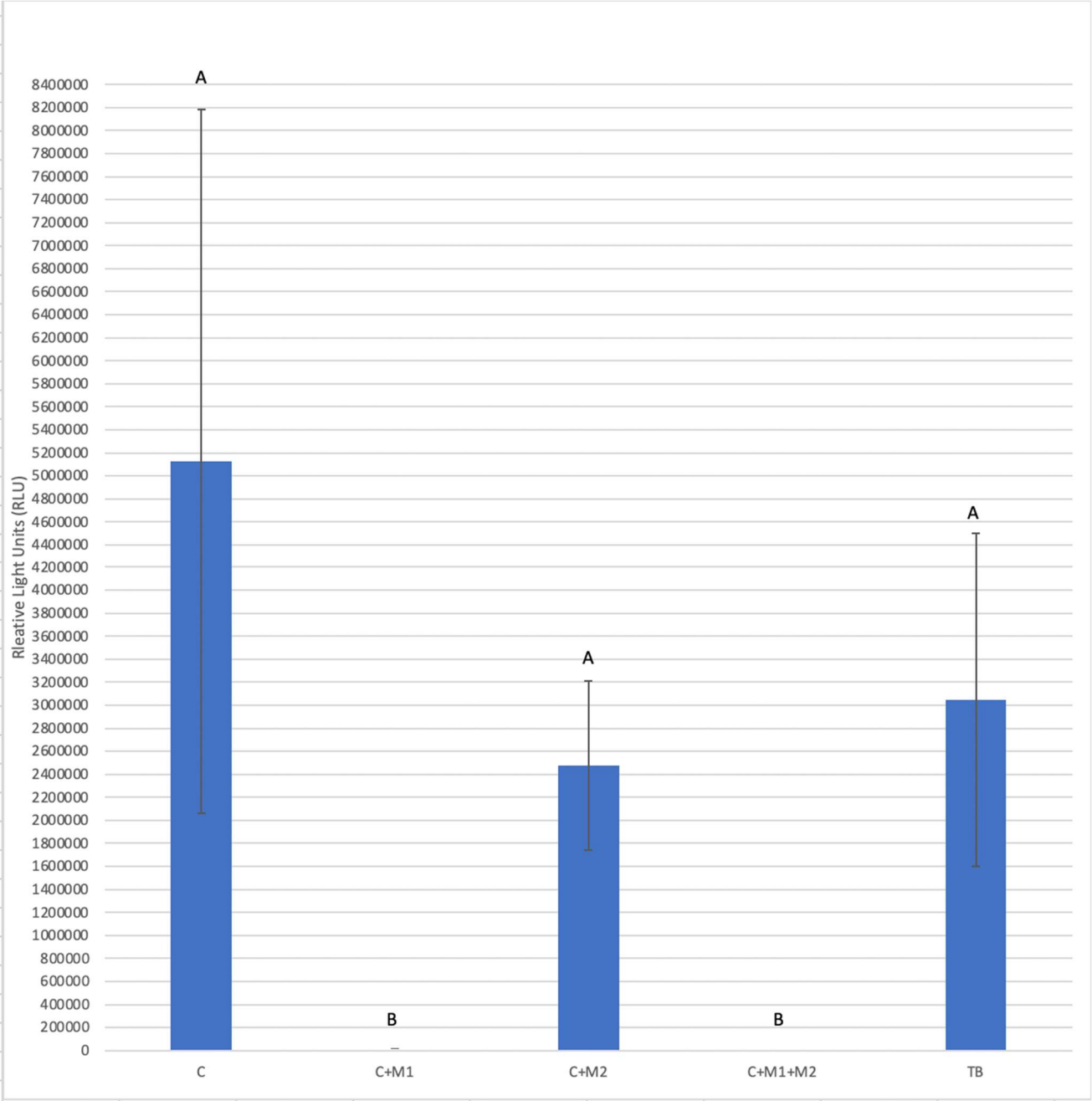
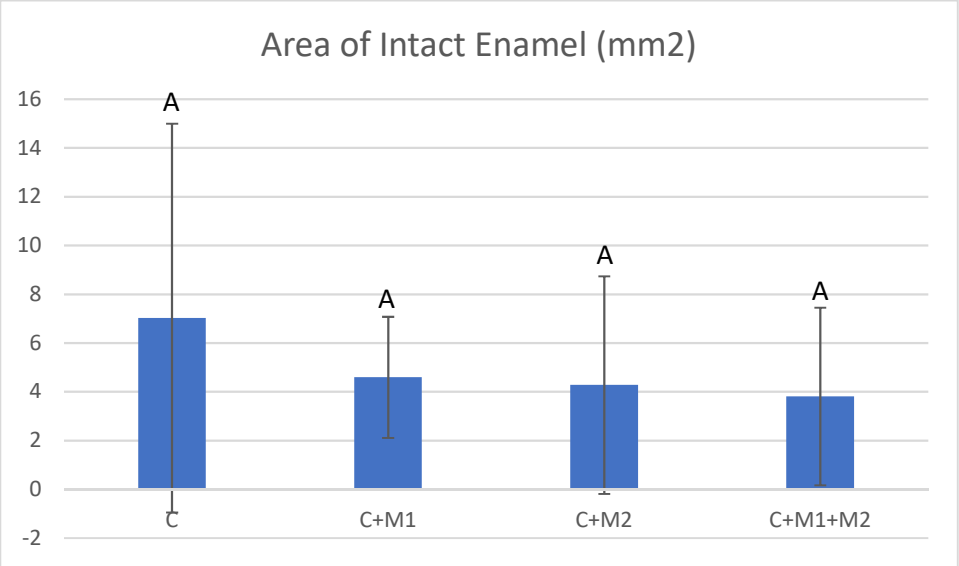


Figure 11. Area of intact enamel

Intact area of enamel (mm^2) in the exposed enamel of the bovine teeth bonded with experimental cement. Each value is mean ± SD (n=9-10). There was no statistically significant differences in the area of intact enamel (P = 0.545)



The graph below shows the area of intact enamel without the outliers. The area measurement was considered an outlier when it was outside two standard deviations. (P=0.258)

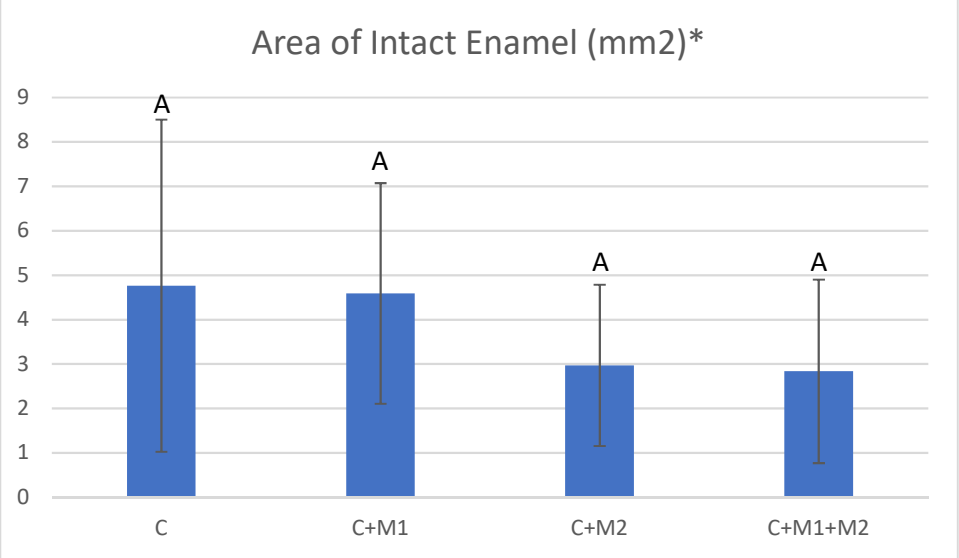


Figure 12. Shear Bond Strength

Shear bond strength of each cement. Each value is mean ± SD (n= 8-10). $p<0.050$

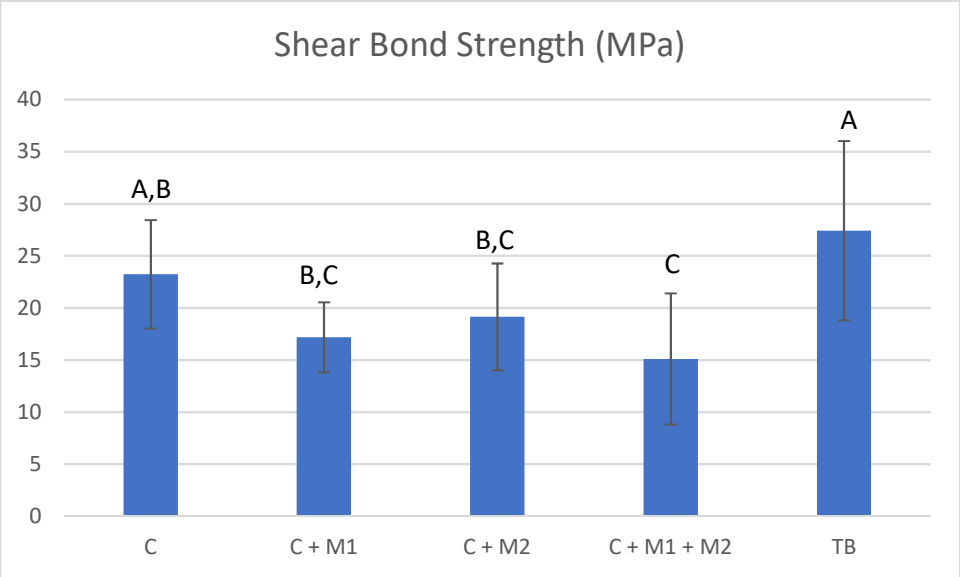
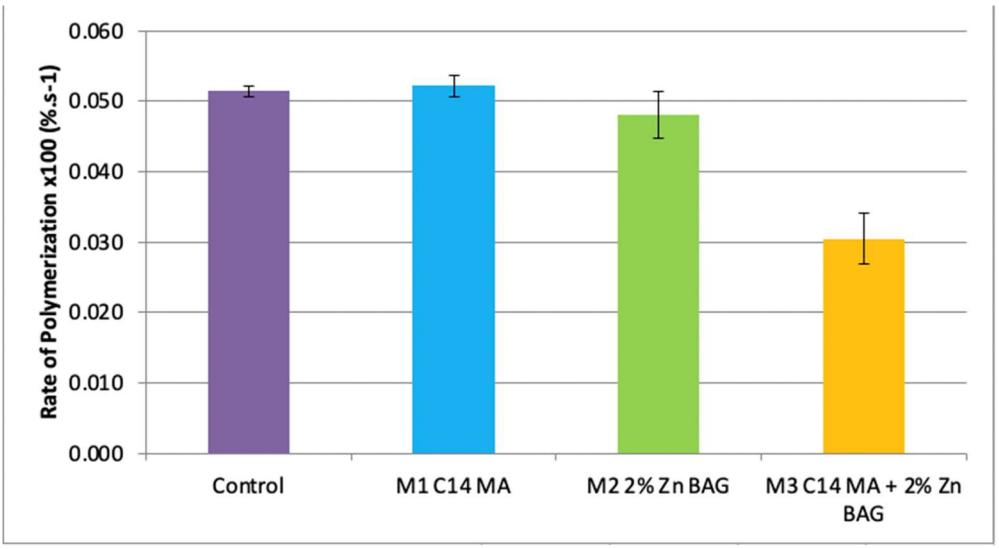


Figure 13. Maximum Rate of Conversion (Rpmax)

	Rpmax				
	sp1	sp2	sp3	average	std
Control	0.051429	0.052191	0.050662	**0.051**	**0.001**
M1 C14 MA	0.053892	0.051882	0.050804	**0.052**	**0.002**
M2 2% Zn BAG	0.046813	0.051882	0.045633	**0.048**	**0.003**
M3 C14 MA + 2% Zn BAG	0.034057	0.03052	0.026925	**0.031**	**0.004**



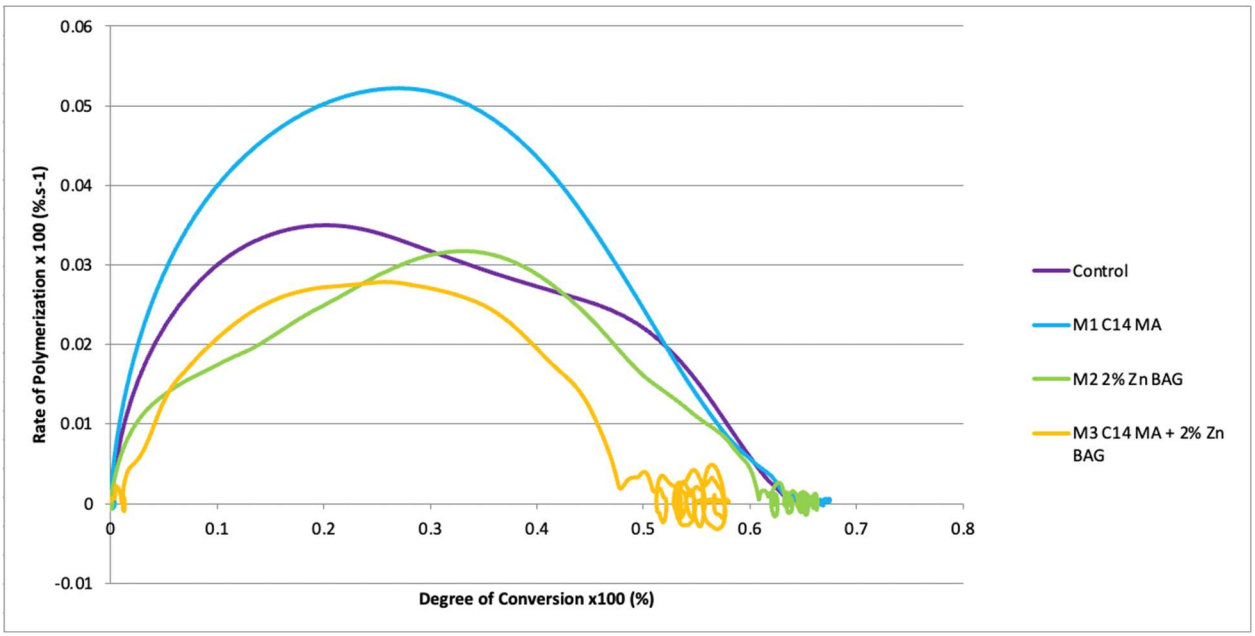
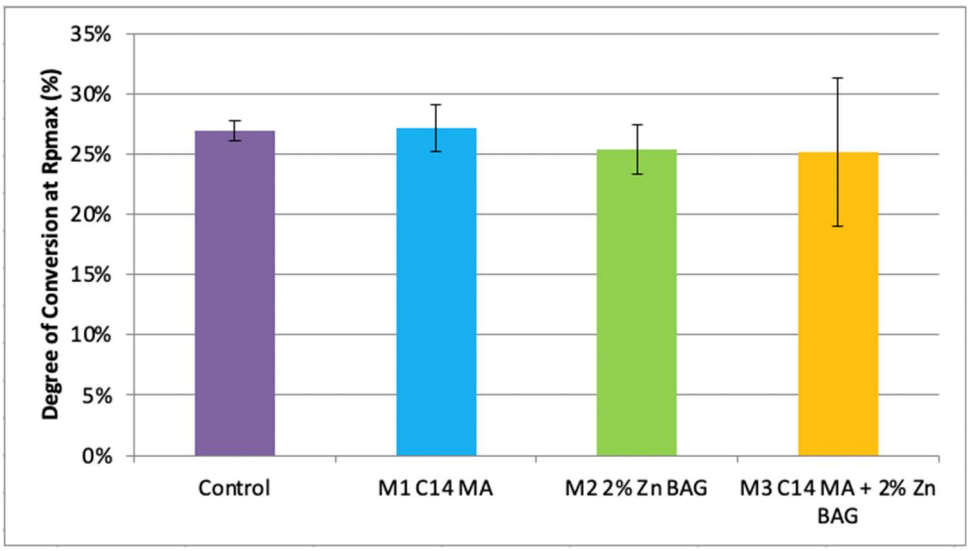


Figure 14. Degree of conversion at Rpmax

	DC at Rpmax				
	sp1	sp2	sp3	average	std
Control	0.261127	0.278032	0.268833	**26.93%**	**0.85%**
M1 C14 MA	0.289829	0.251833	0.273112	**27.2%**	**1.90%**
M2 2% Zn BAG	0.27668	0.248006	0.237604	**25.4%**	**2.02%**
M3 C14 MA + 2% Zn BAG	0.315171	0.248154	0.191821	**25.2%**	**6.18%**



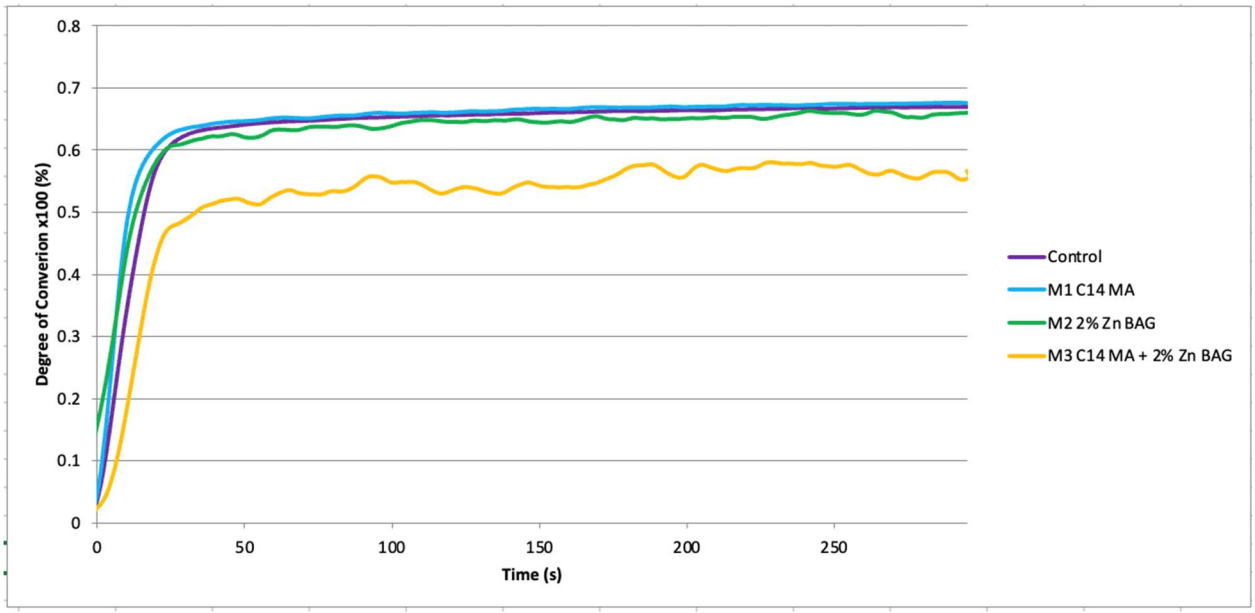
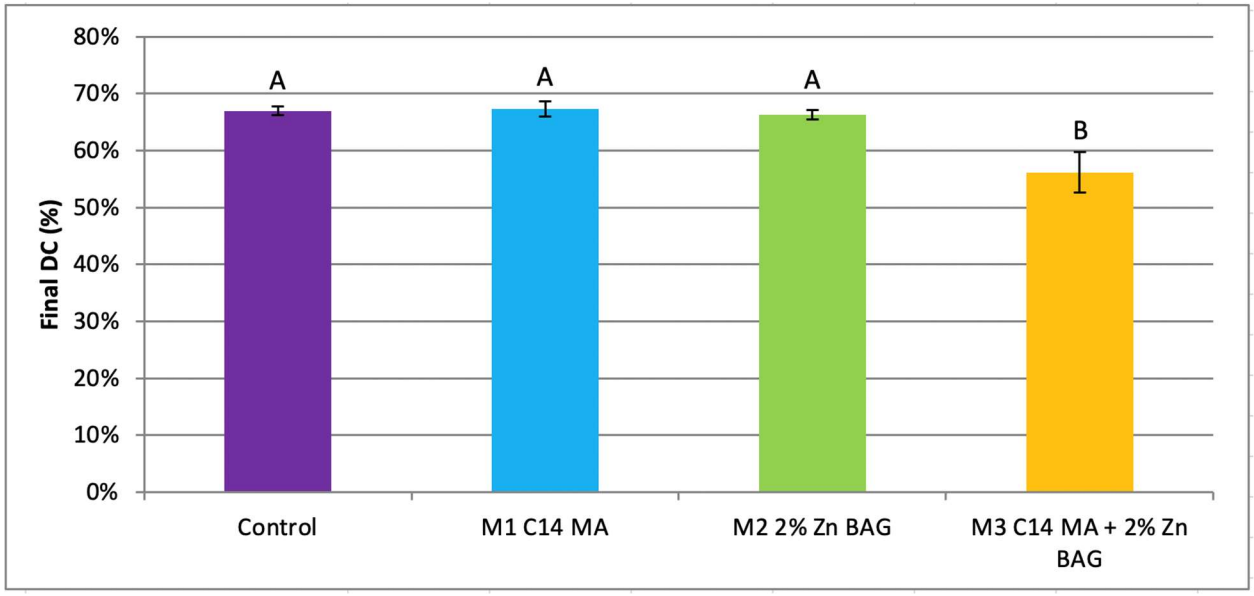


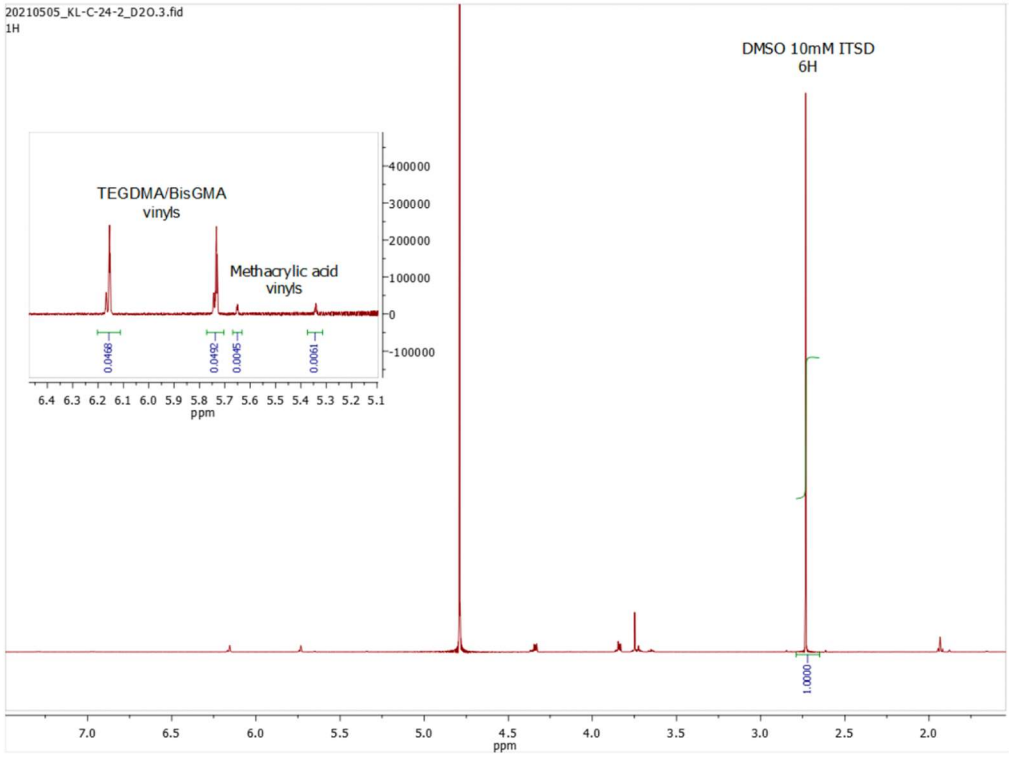
Figure 15. Final degree of conversion (DC)

	DC at 300s				
	sp1	sp2	sp3	average	std
Control	0.66469	0.679018	0.665961	**66.99%**	**0.79%**
M1 C14 MA	0.686597	0.660111	0.67227	**67.30%**	**1.33%**
M2 2% Zn BAG	0.67133	0.66157	0.655257	**66.27%**	**0.81%**
M3 C14 MA + 2% Zn BAG	0.601839	0.54895	0.534182	**56.17%**	**3.56%**

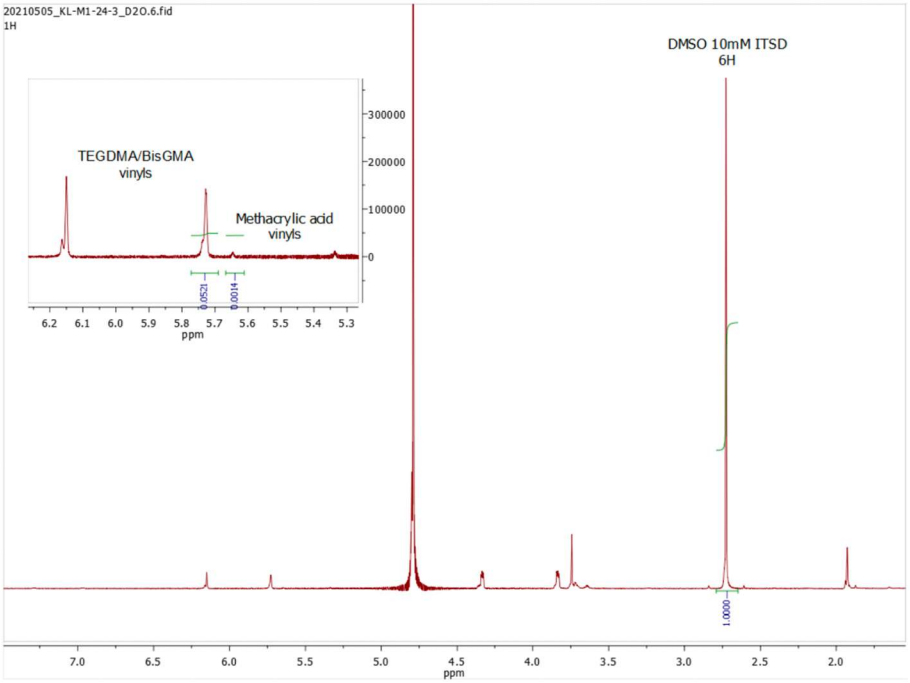


Figures 16-17. NMR spectra of leached monomer

Control Group:



M1 Group:



Figures 18. TEGDMA reference NMR spectrum

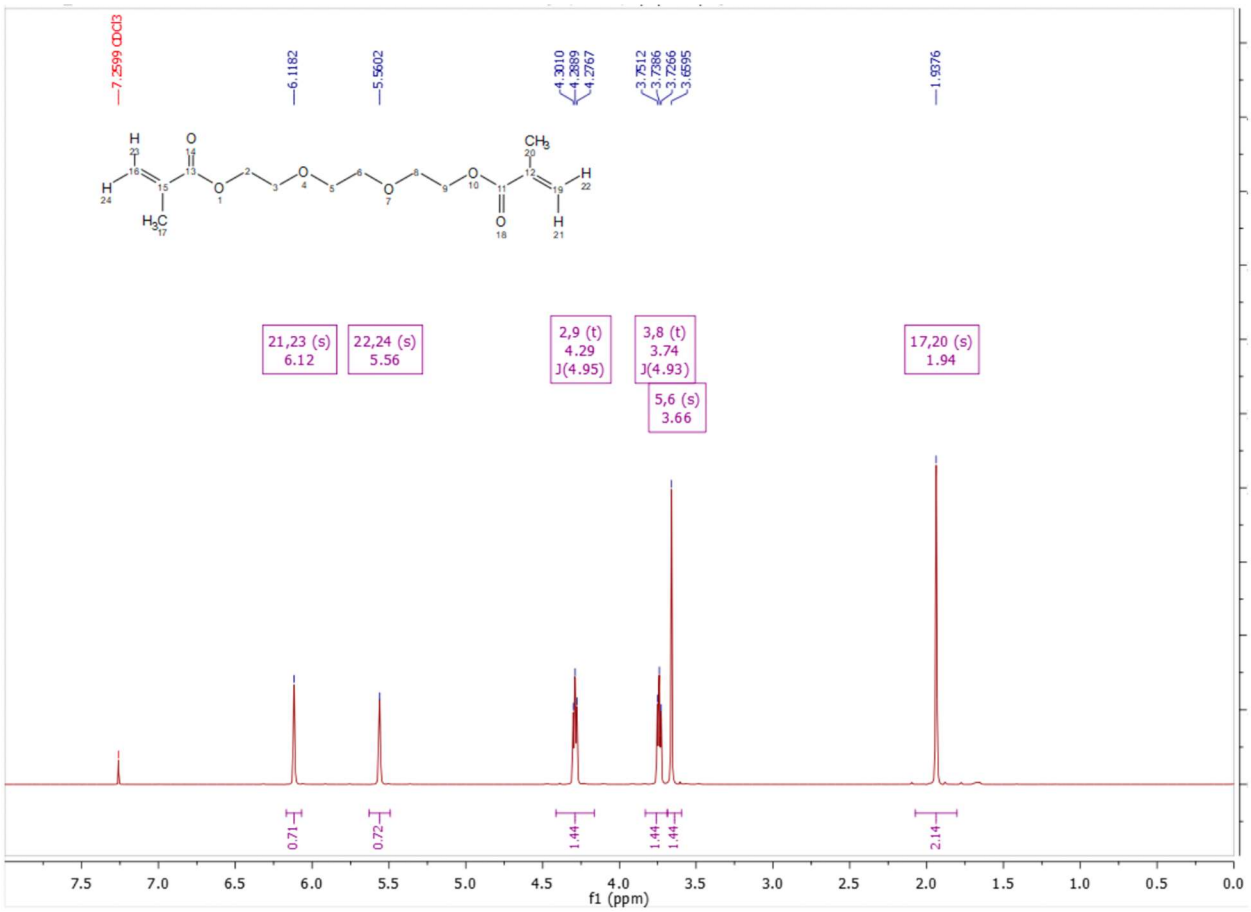


Table 1. Luciferase assay values for each composite (RLU)

Sample	**Luciferase Value**				
	Control	**C+M1**	**C+M2**	**C+M1+M2**	**TB**
Blank	1.9×10^4	3520	7814	1581	1.3×10^4
1	8.6×10^6	1.3×10^4	3.3×10^6	2403	4.1×10^6
2	2.5×10^6	9699	2.8×10^6	3198	4.5×10^6
3	6.8×10^6	6033	2.2×10^6	1700	1.9×10^6
4	2.6×10^6	3631	1.6×10^6	1476	1.7×10^6
Average	5.1×10^6	8090	2.5×10^6	2194	3.1×10^6
SD	3.1×10^6	4115	7.4×10^5	777	1.5×10^6

Table 2. Intact area of bovine teeth bonded with the cements

Sample	**Intact area (mm^2)**			
	Control	**C+M1**	**C+M2**	**C+M1+M2**
1	4.10	7.39	4.79	0
2	3.71	5.41	1.58	1.73
3	4.66	7.30	2.34	2.37
4	27.38*	1.47	3.10	2.58
5	2.91	4.69	6.38	2.60
6	1.78	1.00	16.00*	1.12
7	0	5.82	2.51	3.39
8	5.79	6.83	3.12	4.64
9	13.13	1.35	2.91	12.57*
10	6.76	4.57	0	7.08
Average	7.02	4.58	4.27	3.81
SD	7.97	2.48	4.46	3.64
Average w/o outliers *	4.76	4.58	2.97	2.83
SD w/o outliers*	3.73	2.48	1.81	2.06

*Statistics without outliers – area that was outside two standard deviations were considered outliers

Table 3. Shear bond strengths for each composite

Sample	**Shear Bond Strength (MPa)**				
	Control	**C+M1**	**C+M2**	**C+M1+M2**	**TB**
1	*broke	23.48	11.11	8.63	12.72
2	27.78	19.81	23.89	12.05	33.44
3	29.53	15.63	23.22	16.89	15.19
4	15.45	14.59	19.49	12.31	30.96
5	16.68	14.71	18.87	7.92	35.95
6	20.22	18.45	21.46	13.63	29.65
7	23.45	20.18	20.04	25.95	29.24
8	20.82	17.65	10.31	25.51	*broke
9	27.51	12.24	25.82	11.48	32.18
10	27.66	15.07	17.21	16.48	*broke
Average	23.23	17.18	19.14	15.09	27.42
SD	5.20	3.36	5.13	6.30	8.60

Table 4. Cement disc leaching - weight change (g)

Control Sample	Duration in water				After Desiccation	
	24hrs	72hrs	7 days	14 days	Total weight loss	Total Water Uptake
1	.0006	.0006	.0015	.0017	0	.0017
2	.0006	.0023	.0015	.0012	0	.0012
3	.0011	.0020	.0018	.0016	-0.0001	.0015
Average	.0008	.0016	.0016	.0015	0	.0015
SD	.0003	.0009	.0002	.0003	0	.0003
C + M1 Sample	Duration					
	24hrs	72hrs	7 days	14 days		
1	.0016	.0012	.0016	.0017	-0.0002	.0015
2	.0016	.0014	.0021	.0012	0	.0012
3	.0018	.0017	.0027	.0016	0	.0016
Average	.0017	.0014	.0021	.0015	0	.0014
SD	.0001	.0003	.0006	.0003	0	.0002
C + M2 Sample	Duration					
	24hrs	72hrs	7 days	14 days		
1	.0007	.0017	.0005	.0018	-0.0002	.0016
2	.0017	.0017	.0014	.0013	0	.0013
3	.0010	.0022	.0023	.0017	0	.0017
Average	.0011	.0019	.0014	.0016	0	.0015
SD	.0005	.0003	.0009	.0003	0	.0002
C + M1 +M2 Sample	Duration					
	24hrs	72hrs	7 days	14 days		
1	.0015	.0025	.0024	.0023	-0.0002	.0021
2	.0008	.0022	.0013	.0022	0	.0022
3	.0009	.0015	.0024	.0017	0	.0017
Average	.0011	.0021	.0020	.0021	0	.0020
SD	.0004	.0005	.0006	.0003	0	.0003

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