UNIVERSITY *of the*
WESTERN CAPE

Tashia Moodley

An *in-vitro* study of a modified bioactive orthodontic cement

A thesis submitted in partial fulfilment of the requirements

for the degree of Master of Sciences (Dental)

in the Department of Restorative Dentistry

University of the Western Cape.

November 2017

KEYWORDS

Chitosan

Orthodontic cement

White spot lesions

Demineralization

Nanoparticles

Bioactive

Remineralization

Antibacterial

Shear Bond Strength

Biocompatibility

ABSTRACT

AN IN-VITRO STUDY OF A MODIFIED BIOACTIVE ORTHODONTIC CEMENT

Dr Tashia Moodley

MSc Thesis (Restorative Dentistry), Department of Restorative Dentistry, University of the Western Cape

Introduction: Demineralization of the enamel surface adjacent to bonded orthodontic brackets during treatment is an ongoing drawback, especially when manifested as white spot lesions. Enamel demineralization is initiated by organic acids produced mainly by *Streptococcus mutans*. A favourable substitute is the development of orthodontic bonding agents with antibacterial or microbial properties. Chitosan is an interesting candidate in this respect. Thus, the rationale of this study was to incorporate chitosan into an orthodontic cement to create a novel orthodontic cement and assess whether the incorporation of chitosan to an existing orthodontic cement would affect its biological and physical properties. **Aim:** To investigate the biological and physical properties of a chitosan modified bioactive orthodontic cement. **Methodology:** To achieve the chitosan modified orthodontic cement (test sample), chitosan nanoparticles were added to Transbond™ orthodontic cement (3M ESPE). To obtain a 10% concentration, Transbond was first extruded from the manufacturer's syringe onto a sterile glass slab and 1g chitosan nanoparticles was homogenously mixed to the 10g Transbond. For the control, unmodified Transbond was used. The microbiology testing was done using the dilution method after culturing *Streptococcus mutans*. Shear bond strength test was done by

bonding brackets to 20 premolars using the chitosan modified cement and 20 with unmodified cement. Bond strengths were evaluated using a universal testing machine. The Adhesive Remnant Index system was done to evaluate the amount of adhesive left on the tooth after de-bonding and examined under 20x magnification. Surface Hardness testing was determined using a Vickers Hardness Machine. 3T3 cells were cultured for cytotoxicity testing in 96 well plates and exposed to the test specimens. The MTT dye method was used to analyze cell survival. Surface morphological study of the chitosan nanoparticles was studied using field emission scanning electron microscopy. **Results:** The chitosan modified Transbond completely eradicated the microorganisms after 8 hours exposure compared to unmodified Transbond. Chitosan modified Transbond had a mean shear bond strength value of 14.5 MPa which was significantly higher than the Transbond cement mean shear bond strength of 10.2 MPa ($p<0.05$; Wilcoxon Rank Sum Test). The majority of the specimens from both the control (70%) and test groups (90%) showed an adhesive remnant index score of 1 (half the amount cement remaining on the bracket), which may indicate bond failure at the bracket-adhesive interface. Although the mean microhardness of chitosan modified cement was lower than the unmodified orthodontic cement, there was no statistically significant difference (Mann-Whitney Test, $p>0.05$). Thus, the addition of chitosan to the orthodontic cement did not significantly affect the surface hardness of the cement. When tested for bioactivity on human dental pulp cells the chitosan showed increase growth of these cells compared to the control. The surface morphology reflects spherical structures with evenly distributed hexagonal depressions on the surface. **Conclusion:** Chitosan presents as evenly shaped spherical particles that can

be incorporated into Transbond orthodontic cement without negatively impacting on its physical properties but at the same time having a positive effect on its biological properties in that it showed antibacterial effects and a positive influence of cells in culture.

10 November 2017

DECLARATION

I declare that: *"An in-vitro study of a modified bioactive orthodontic cement"*, is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Full name: Tashia Moodley Date: 10th November 2017

Signed 

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

CHAPTER I

Literature Review

1. Introduction

Orthodontic treatment aims to correct both function and aesthetics by establishing balanced occlusal and jaw relationship; with positive effects on the oral health and quality of life of patients (Aghoutan *et al.,* 2015). However, the placement of brackets on the tooth surface has complications. Enamel surface demineralization or white spots lesions (WSL) remain by far one of the leading undesirable ramifications of fixed orthodontic appliance treatment, despite techniques and material advances in preventive dentistry and orthodontics (Aghoutan *et al.*, 2015). Demineralization of the enamel surface adjacent to bonded orthodontic brackets during treatment is an ongoing drawback, especially when manifested as white spot lesions. The development of white spot lesions is due to prolonged plaque accumulation adjacent to the brackets (Gorelick *et al.,* 1982). Opportunities of white spot lesions are established when fixed orthodontic appliances induce continual growth and retention of dental plaque during orthodontic treatment (Norevall *et al.*, 1996). These lesions develop during orthodontic treatment; and may impede the successful result of the treatment which occasionally result in the early suspension of treatment (Aghoutan *et al., 2*015). These lesions can continue after orthodontic treatment.

The assessment of demineralized white spot lesions throughout orthodontic treatment is essential for both clinicians and researchers (Benson, 2008). Clinicians need to detect enamel lesions early as to advise patients concerning modifications in oral hygiene and diet, in addition to executing appropriate preventive treatment (Benson, 2008). Correct assessment of demineralization is also important to researchers to evaluate new products or interventions, as it could possibly assist in inhibiting the development of demineralized lesions during orthodontic treatment by developing new antibacterial materials to prevent demineralization (Benson, 2008). Inhibiting these lesions during treatment is a significant concern for orthodontists because these lesions are undesirable (Ogaard, 1989).

2. Enamel Demineralization

There are several drawbacks to the attachment of orthodontic brackets to teeth. The attachment of orthodontic appliances to the tooth surfaces makes oral hygiene very difficult, limits salivary self-cleaning and generates more active areas for plaque; promoting a decrease of plaque pH in the presence of carbohydrates and forming a physical barrier to prevent remineralization by calcium and phosphate ions from the saliva (Aghoutan *et al*., 2015). All these instabilities in the oral environment support colonization of aciduric bacteria, ensuing in a growth of *Streptococci mutans* and lactobacilli, mainly adjacent to the bonding adhesives (Jung *et al.,* 2014). This can interrupt the balance between the processes of demineralization and remineralization shifting towards demineralization, which would then progress to the more permanent formation of white spot lesions. Considering the duration of orthodontic treatment: “The longer the time of

oral appliances' wearing is; the more extended the caries risk is" (Aghoutan *et al.,* 2015). Early carious lesions in the enamel around the orthodontic brackets are clinically seen as a white opaque spot (Ogaard *et al.,* 1988). The area is slightly softer than the surrounding normal enamel (Ogaard *et al.,* 1988). The whitish appearance is caused by an optical phenomenon and increases when the tooth surface is air dried (Ogaard *et al.,* 1988).

Orthodontic patients are more susceptible to the developments of the WSL than untreated patients (Ogaard, 1989) because they carry brackets, bands, and different types of archwires for long period, which impairs oral hygiene and increases plaque retention sites (Gorelick *et al.,* 1982).

Clinical studies have shown a sharp increase in the number of white spot lesions during the first 6 months of treatment that continued to rise at a slower rate to 12 months; supporting the idea that the presence of fixed orthodontic appliances and greater treatment lengths serve as a risk factor for white spot lesion formation (Chapman *et al.,* 2010). The occurrence of new decalcifications amongst orthodontic patients with fixed appliances is reported to range from 13% to 75% (Gorelick *et al.,* 1982; Wenderoth *et a*l., 1999). The incidence of enamel demineralization after fixed orthodontic appliance therapy can be seen in about 50% of the patients (Gorelick *et al.,* 1982). Orthodontic brackets have an elevated risk of caries, and enamel lesions can occur within a month, irrespective of mechanical plaque control measures and whether a fluoridated dentifrice is used or not *(*Ogaard *et al.* 1988*;* Pascotto *et al.,* 2004*;* Wenderoth *et al.*, 1999). Reports of the white

spot lesions have been made by a number of investigators and have been recorded as early as one month after the start of treatment (Gorelick *et al.* 1982; O'Reilly and Featherstone, 1987). Hence, oral hygiene status of patients should be evaluated during the initial months of treatment and, if necessary, measures to prevent demineralization should be implemented.

Studies have shown a significant increase in the prevalence of WSL on the cervical and middle thirds of the crowns (Aghoutan *et al*., 2015). These lesions can generally extend over the tooth surface and may involve proximal extensions (Figure 1). Early caries lesions usually develop adjacent to the bracket, often around the gingival margin and buccal surfaces of teeth (Gorelick *et a*l., 1982). These white lesions are generally often identified when teeth are dry, and appear clinically as an opaque whitish or greyish halo under bands and around the bracket base margin and frequently at the interface between the orthodontic cement and the enamel (Aghoutan *et al.,* 2015). Premolars have been reported to have greater occurrence of white spot lesions (Chapman *et al.,* 2010), and the posterior area in the maxillary teeth having the lowest incidence. The maxillary anterior teeth exhibited larger mean surface area demineralization than anterior teeth in the mandibular arch (Willmot, 2008). Regardless of white spot lesion treatment advancements, these conditions are challenging to correct and restore to some extent based on the magnitude of their severity (Aghoutan *et al.,* 2015).

According to Mattousch *et al.*, (2007), the area of white spot lesions shows a notable decline during the first and second years following bracket removal. The most plausible reason for this clinical healing can be explained by eradication of the principle etiologic factor which is the cariogenic plaque adhered to fixed orthodontic elements, combined with enamel surface wear during tooth brushing and also by remineralization (Shungin *et al.*, 2010).

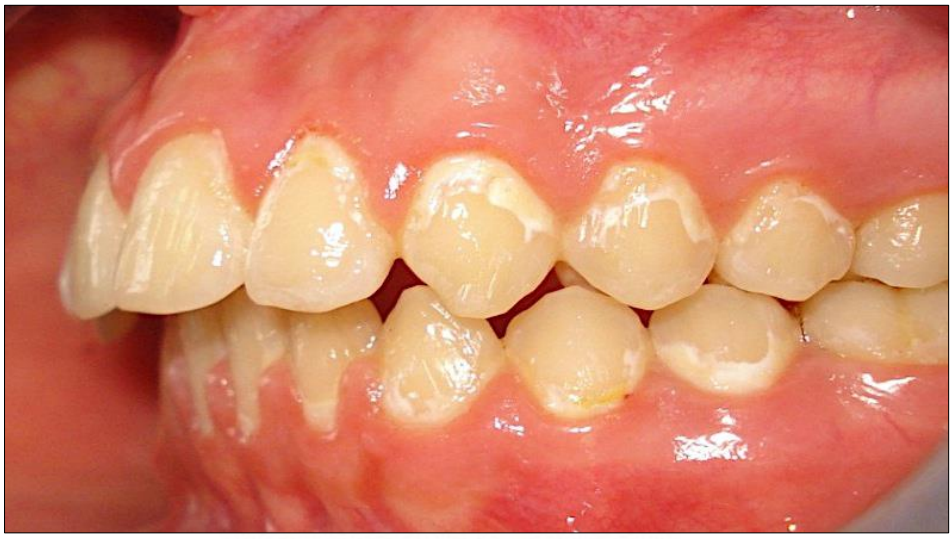

Figure 1. White spot lesions after orthodontic treatment (Aghoutan et al., 2015).

White spot lesions seem to be associated with the interaction of several factors including poor eradication of dental plaque, due to metallic brackets of fixed appliances. This prevents the self-cleansing mechanisms of the oral musculature and saliva, patient's altering factors and change in bacterial flora during fixed appliances (Hadler-Olsen *et al.*, 2012). Fixed orthodontic appliances encourage a rapid growth in the volume of dental

plaque. Furthermore, plaque has a lower pH than that present in non-orthodontic patients (Gwinnett and Ceen, 1979). Therefore, the plaque-retentive properties of the fixed appliance predispose the patient to an increased cariogenic risk. Additionally, there is a swift shift in the structure of the bacterial flora of the plaque following the placement of orthodontic appliances (Bishara and Ostby, 2008). The levels of acidogenic bacteria, such as *Streptococcus mutans*, become notably higher in orthodontic patients (Bishara and Ostby, 2008). If bacteria have a suitable source of fermentable carbohydrates, acid by-products will be produced, lowering the pH of the plaque (Bishara and Ostby, 2008). As the pH drops below the threshold for remineralization, carious decalcification takes place. The initial clinical indication of demineralization is recognized as a white spot lesion. Such lesions have been clinically induced within a span of 4 weeks, which is typically within the time period between one orthodontic appointment and the next (Ogaard *et al.*, 1988). In the highly cariogenic environment adjacent to orthodontic appliances or under loose bands, these lesions can rapidly progress (Bishara and Ostby, 2008). Widespread arrangements of plaque buildup reveals an association with bonded orthodontic brackets (Gwinnett and Ceen, 1979). Patients with orthodontic brackets have a higher risk of caries and enamel lesions which can occur within a month, regardless of mechanical plaque control and whether fluoridated dentifrice is applied (Ogaard *et al.*, 1988). Demineralized enamel, the foundation to caries development, can be credited to fixed orthodontic appliances (Gwinnett and Ceen, 1979) and continued contact to bacterial plaque thus appliances are connected to an increased threat of developing white spot lesions (Wenderoth *et al.*, 1999). The continual accumulation and retention of bacterial plaque, in

which fixed appliances induce, establishes a risk of white spot lesion development during treatment (Gorelick *et al.*, 1982; Mizrahi, 1982; O'Reilly and Featherstonem, 1987; Ogaard *et al.*, 1988).

Enamel demineralization is initiated by organic acids produced primarily by *Streptococcus mutans* (Featherstone, 2000). *S. mutans* is known to be the main influencing organism of dental caries (Wisth and Nord, 1977). Variations in the oral environment are triggered by metallic brackets, such as a decrease in pH and an increase in plaque accumulation. The area is slightly softer than the surrounding sound enamel (Ogaard *et al.*, 1988). Therefore, not only is this pathological but also unaesthetic. Roughness of the cement surface predisposes to rapid attachments and further growth of oral micro-organisms (Gwinnett and Ceen, 1979). Thus, the cement used to bond brackets to the tooth surface may also contribute to plaque accumulation.

Overall management of white spot lesions involve methods of both preventing demineralization and encouraging remineralization of existing lesions (Sudjalim *et al.*, 2007). Even with recent improvements to enhance the practice of orthodontics, white spot lesions are still a major risk associated with fixed appliance treatment (Robertson *et al.*, 2011). Inhibiting these lesions during treatment is a significant concern for orthodontists because the lesions are undesirable (Ogaard, 1989). The presence of fixed orthodontic

appliances often impedes both active and passive oral hygiene causing plaque accumulation and in many cases a high cariogenic challenge (Ogaard *et al.,* 1988).

Once lesions are left untreated, white spot lesions may develop carious cavitations which will need a restoration (Bishara and Ostby, 2008). Since these lesions progress rapidly; inhibition, early diagnosis and treatment continues to be a major challenge confronting orthodontists and involves detailed understanding of the caries process and the risk factors particular to each patient (Aghoutan *et al.,* 2015). Early diagnosis during orthodontic treatment would allow orthodontists to perform preventive methods to manage the demineralization development before lesions advance (Aghoutan *et al.,* 2015). Therefore, the prevention, diagnosis, and treatment of white spot lesions is necessary to avert tooth decay as well as reduce tooth discolouration that could compromise the aesthetics of the smile (Bishara and Ostby, 2008). For a better aesthetic result, it is important to implement remineralizing agents as early as possible, as white spot lesions can take up stains and become discolored after many years (Aghoutan *et al.,* 2015).

3. Prevention of Demineralization

At risk patients are those wearing orthodontic appliances, for whom a preventive approach should be implemented before, during and after orthodontic treatment (Aghoutan *et al.,* 2015). General management of white spot lesions consist of methods of preventing demineralization and promoting remineralization of existing lesions. Though, the efficacy of these approaches has been restricted by challenges, such as the effectiveness of fluoride

releasing products, localizing the fluoride to areas where it is needed and, most importantly, the lack of predictability related to patient compliance (Robertson *et al.*, 2011). Several methods have been used to prevent or reduce enamel demineralization during orthodontic treatment (irrespective of patient's oral hygiene limitations), including fluoride application in various forms, enamel sealants, rigorous oral-hygiene regimens and glass-ionomer cement for bonding bracket and modified appliance designs (Chung *et al.*, 2011). Prevention of demineralization around brackets can be roughly divided into those methods that are patient dependent and methods implemented by the clinician.

3.1 Patient Dependent Methods

Patient education would be the first line of defense against the progression of incipient caries lesions, stressing the significance of optimal oral hygiene. Possibly, the most significant prophylactic measure to prevent the incidence of white spot lesions in orthodontic patients is executing a good oral hygiene routine involving proper tooth brushing with a fluoridated dentifrice (Bishara and Ostby, 2008). However, it is still difficult for young patients to maintain adequate oral hygiene during orthodontic treatment with fixed appliances (Ngan *et al.*, 2009). Even if the patient is compliant, orthodontic attachments make the mechanical removal of plaque difficult, resulting in increased cariogenic challenge adjacent to orthodontic brackets and beneath the bands. Therefore, novel methods of preventing white spot lesions around orthodontic appliances and orthodontic bands are needed (Moreira *et al.*, 2015).

The combined application of fluoride regimens, oral hygiene instructions and dietary control can contribute significantly to inhibition of demineralization during fixed appliance treatment. Preventive approaches to avoid the increased caries risk for patients with fixed orthodontic treatment include adequate oral hygiene. However, patient compliance is a limiting factor and is not reliable in achieving an effective outcome (Melo *et al.*, 2014). While the proper use of these fluoride treatments provides the patient with increased caries protection, patient compliance is required and such cooperation can be difficult to obtain in some patients (Bishara and Ostby, 2008).

However, while fluoride treatments seem important when used in patients with orthodontic brackets bonded with composite resins, the added advantage is not apparent if the brackets are bonded with a resin-modified glass ionomer (RMGI). This is because the RMGI materials are able to release fluoride ions that have a beneficial effect of their own (Bishara and Ostby, 2008). Despite the fact that this group of bonding materials is able to release fluoride ions that have a favourable effect of their own (Bishara and Ostby, 2008). Approaches to inhibit white spot lesions are essential and should not be reliant on the patient's limitations.

3.2 Non Patient Dependent Methods

Alternative strategies that do not depend on patient compliance have also been more effective for preventing early demineralization. In general, the duration of orthodontic treatment places the patient at an increased caries risk for a prolonged period of time (Bishara and Ostby, 2008). As a result, continuous fluoride release from the bonding

system around the bracket base would be extremely beneficial. Thus, using fluoride containing sealants and adhesives to bond brackets has been attempted (Bishara and Ostby, 2008). For the less compliant orthodontic patients, the use of a fluoridated dentifrice alone is ineffective in preventing the development of carious lesions (O'Reilly and Featherstone, 1987). The in-office application of a high concentration of fluoride in the form of a varnish may be beneficial and should be considered by the clinician (Bishara and Ostby, 2008). These products offer the combined benefit of delivering a high concentration of fluoride during the regular orthodontic visit while eliminating the need for patient cooperation that is required with fluoride rinses (Bishara and Ostby, 2008). However, since the application of the varnish usually occurs in the clinician's office only, there is a limitation on the frequency of exposures that the patient will receive (Bishara and Ostby, 2008). In addition, the repeated varnish applications may increase costs to the patient and/or chair time to the clinician. A disadvantage of varnish application is the temporary discoloration of the teeth and gingival tissue (Bishara and Ostby, 2008).

Fluoride releasing materials, such as glass ionomer cements (GIC) and fluoride-releasing composite materials can also be used as orthodontic adhesive cements (Rogers *et al.*, 2010). Wilson and Kent were the first to describe glass ionomer cements (Millett and McCabe, 1996). Their physical properties are a combination of silicate and polycarboxylate cements (Millett and McCabe, 1996). Glass ionomer cements slowly releases fluoride over several months thereby, helping to prevent enamel demineralization (Swartz *et al.*, 1984). However, the low bond strength to tooth structure is a major

disadvantage of glass ionomer materials, which consequently results in high rates of de-bonding and subsequent bracket failures (Chin *et al*., 2009). Mixing GICs, however, is technique sensitive, and the hydrogels desiccate and crack in dry environments (Ewoldsen and Demke, 2001).

The breakdown of inorganic glasses by acid solution is generally undesirable. However, with ionomer glasses the glass composition is arranged to be degradable by moderately weak acids in order to produce a cement (Lohbauer, 2010). Usually an aqueous polyacid, such as polyacrylic acid or polyalkenoic acid is reacted with the finely powdered fluoroaluminosilicate glass (Wilson *et al.*, 1972). The acid – base setting is schematically shown in Figure 2.

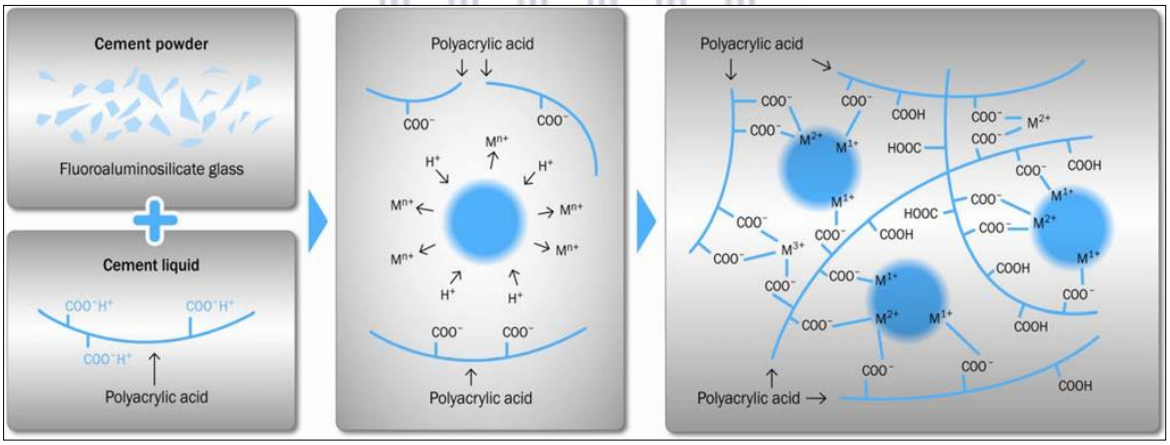

Figure 2. Setting reaction of a conventional glass ionomer cement (Lohbauer, 2010).

The acid invades and attacks the glass network which results in the release of cations, predominantly Aluminium (Al^{3+}) and Calcium (Ca^{2+}) or Strontium ions (Sr^{2+}). The cations

consequently assist in the creation of salt bridges amongst the polyacid chains and result in the development of silica hydrogel, the calcium polyacrylate formation demonstrating faster reaction kinetics than that of aluminum polyacrylate (Lohbauer, 2010).

The carboxylic acid attacks the surface layer of glass powder, although the glass interior core remains intact. The glass core acts as filler in the cement matrix. The reactivity of the glass surface governs the caliber of the set cement. A silica gel layer with gradient characteristics is developed at the junction amongst the glass particle and the cement matrix. The polycarboxylic acid is usually used in aqueous solution at a concentration of 45% wt. In order to control the GIC setting kinetics, a certain amount of dried polycarboxylic acid is added to the glass powder (Lohbauer, 2010).

Water plays an important role in the setting process (Lohbauer, 2010). Throughout the initial steps of the setting process, the water from the cement liquid is entirely integrated into the cement structure (Lohbauer, 2010). During cement setting, the cement paste has to be protected from increased water in order to avoid from dissolution of metal cations. Once the cement has set into a solid state, water can seize several locations, for example coordination sites around metal cations or hydration regions around the polyanion chain (Lohbauer, 2010). Deficiency of water can lead to cracking and crazing of the cement surface, developing a chalky surface appearance (Nicholson and Wilson, 2000). As the cement matures the proportion of loosely bound water declines relatively to the proportion

of tightly bound water. The setting process is observed to continue with time (Crisp *et al.*, 1979).

GIC are clinically desirable dental materials and have particular properties that make them beneficial as both for restorative and adhesive materials (Lohbauer, 2010). This involves adhesion to moist tooth structure and base metals, anticariogenic properties due to the release of fluoride, thermal compatibility with tooth enamel, biocompatibility and low toxicity. There are many advantages associated with GIC as an orthodontic cement. Firstly, they adhere to both enamel and metal surfaces (Bishara and Ostby, 2008). Secondly, they release fluoride and hereby prevent enamel demineralization (Bishara and Ostby, 2008). Finally, they can be removed more easily than composite at time of debond (Norevall *et al.*, 1996). GC Fuji Ortho is a typical GIC orthodontic cement widely used in orthodontics.

Glass ionomer cements were initially introduced as orthodontic bonding adhesives to take advantage of some of their desirable characteristics, namely, their ability to chemically bond to tooth structure (Bishara and Ostby, 2008), in addition to their sustained fluoride release following bonding (Artun and Bergland, 1984). Unfortunately, the bond strength for GIC, (Fricker, 1992), having a higher bond failure rate compared with composite and it's use for bonding orthodontic brackets became practically limited (Bishara and Ostby, 2008). Yet, shortcomings in their applications may affect the strength resulting in low mechanical strength and toughness (Cattani-Lorente *et al.*, 1994). As for glass ionomer

cement's low bond strengths, it has been suggested that it is not applicable for routine use in bonding orthodontic brackets (Millett and McCabe, 1996). The low adhesive strength of this conventional glass ionomer cements has been a limitation of its clinical use (Fajen *et al.,* 1990).

According to Miguel *et al.,* (1995), the clinical ability of a glass ionomer cement for direct bonding of orthodontic brackets was compared with a composite resin. Low fracture resistance limits their orthodontic use primarily for orthodontic band cementation (Ewoldsen and Demke, 2001). After 12 months, composite bonded brackets showed a statistically significantly lower failure rate (7.96%) than the glass ionomer cement (50.89%), irrespective of the dental arch tested (Miguel *et al.,* 1995). Glass ionomer cement showed statistically significant greater frequency of failures in all situations tested. Most of the failures in the composite group seem to have occurred in the bracket/composite interface, however a large amount of the failures of the GIC seem to have occurred within the cement. The association of the properties of the glass ionomer cement-fluoride release, resistance to acid erosion, and biocompatibility (Norris *et al.*, 1986), with bonding orthodontic brackets, theoretically appears ideal. Unfortunately, the high failure rate (50.9%) of the study showed a low clinical performance of the cement, when compared with the composite (8%) (Miguel *et al.*, 1995). GICs used for orthodontic bracket bonding has poor bracket retention compared with resin controls (Ewoldsen and Demke, 2001). GICs lack the physical properties necessary to retain brackets throughout treatments (Ewoldsen and Demke, 2001).

In an attempt to increase the bond strengths of GICs, resin particles were added to their formulation to create resin modified glass ionomer (RMGI) bonding systems. RMGICs are adhesive cements with improved physical properties and more stable hydrogels compared with GICs (Ewoldsen and Demke, 2001). These adhesives release fluoride like conventional GICs, but can furthermore be used to bond orthodontic brackets effectively because of their fairly higher bond strengths (Diaz-Arnold *et al.,* 1995). The recently introduced polyacid-modified composite and resin-modified glass ionomer cements (RMGICs) have a stronger bond strength compared with GICs while maintaining some ability to release fluoride and can be recharged (Cohen *et al.,* 2003). However, their fluoride-releasing ability varies between products which may be combined with a high bracket failure rate. Also, similar rates of demineralization have been reported with a RMGIC compared with a composite resin (Gaworski *et al.,* 1999). Although early studies showed that RMGICs have lower shear bond strength compared with composite resins (Coups-Smith *et al.,* 2003), specifically within the first half hour after bonding (Fricker, 1994), more recently these products were found to have an increased shear bond strength and are able to bond orthodontic brackets successfully (Coups-Smith *et al.,* 2003). Additionally, in vivo studies have shown no substantial changes in bracket failure rates between the RMGICs and composite adhesives (Summers *et al.,* 2004).

Supplementary to the chemical properties of RMGICs, resin monomers infiltrate the surface roughness to achieve a micromechanical interlock (bond) after polymerization

(Ewoldsen and Demke, 2001). Besides the benefit of operator-controlled setting, light-activated polymerization proceeds notably faster than acid-base (cement forming) reactions, resulting in improved early physical properties, specifically fracture resistance (Ewoldsen and Demke, 2001). Maturation hardening, continuous fluoride release, and caries inhibition are comparable in comparison with conventional GICs and RMGICs. Additionally, the performance and capabilities of both GICs and RMGICs are comparable to chemically bond in the presence of a wet environment (Diaz-Arnold *et al.,* 1999). RMGIs have notably lesser shear bond strength in comparison to composite resins designed for orthodontic bonding (Yassaei *et al.,* 2014).

Hybrid glass ionomer cements have been developed that combine the desirable properties of composite resin bond strength and glass ionomer fluoride release (Vorhies *et al.,* 1998). Usually the fluoride is maximally released during the first few days, and in some cases, it can still be measured in minor quantities after 2 or 3 months (Basdra *et al.,* 1996). Properties of hybrid glass ionomer cements appear to improve on some of the disadvantages of composite resins and traditional glass ionomer cements (Miller, 1996). That is, the bond strength of hybrid resin ionomers is greater than glass ionomer cements, whereas the property of fluoride release lacking in composite resins is maintained (Ashcraft *et al.,* 1997).

Adequate bond strength is essential for orthodontic success; without the ability to hold brackets on teeth, any other advantageous properties of a orthodontic bonding material

may be lost (Vorhies *et al.*, 1998). An in vitro study, by Basdra *et al.* (1996), indicate that fluoride-releasing adhesives may inhibit enamel decalcification adjacent to orthodontic brackets during the examined period of 4 weeks. An *in vivo* study, (Pascotto *et al.*, 2004), showed that a resin-modified glass ionomer cement could decrease enamel demineralization around a bonded bracket. However, considering that the findings of the study were observed after 30 days and that orthodontic treatment takes a longer period of time, during which fluoride release by glass ionomer decreases, as it is higher in the first few days. According to Ewoldsen and Demke (2001), resin adhesives release fluoride and the amount is quite low and most likely has no effect on caries. Therefore, this is questionable as to whether the amount of fluoride released by orthodontic adhesives is sufficient to prevent decalcification (Cacciafesta *et al.*, 2007).

The ideal orthodontic bracket cementing/bonding agent should provide continuous, prolonged fluoride release (throughout orthodontic treatment), and have adequate bond strength to satisfactorily retain orthodontic brackets (Vorhies *et al.*, 1998).

Since several studies showed a significant increase in oral bacteria during orthodontic treatment (Bloom *et al.*, 1964), hence a favourable approach is the development of orthodontic bonding agents with antibacterial or antimicrobial properties (Eliades *et al.*, 2004) in order to prevent demineralization around brackets. In addition, any efforts in producing an antibacterial orthodontic cement should not cause adverse effects on the

mechanical or biological properties of the cement such as the enamel bond strength or be toxic to the body.

Chitosan and modified chitosan are interesting possibilities in this respect. Orthodontic bonding agents are in direct contact with the vulnerable enamel surface and the properties of chitosan with regard to bacterial adhesion may play a key role in prevention of white spot lesions (Melo *et al.*, 2014).

4. Chitosan

4.1 Chemistry

Chitosan is derived from chitin (Figure 3), which is a naturally occurring linear polysaccharide (Dutta, 2016). Chitosan, $(C_6H_{11}O_4N)_n$ consists of a linear co-polymer polysaccharide consisting of β (1–4)-linked 2-amino-2-deoxy-D-glucose (D-glucosamine) and 2-acetamido-2-deoxy-D-glucose (N-acetyl-D-glucosamine) units (George and Abraham, 2006). The term chitosan is used to describe a series of polymers of different degrees of deacetylation, defined in terms of the percentage of primary amino groups in the polymer backbone, and average molecular weights (George and Abraham, 2006).

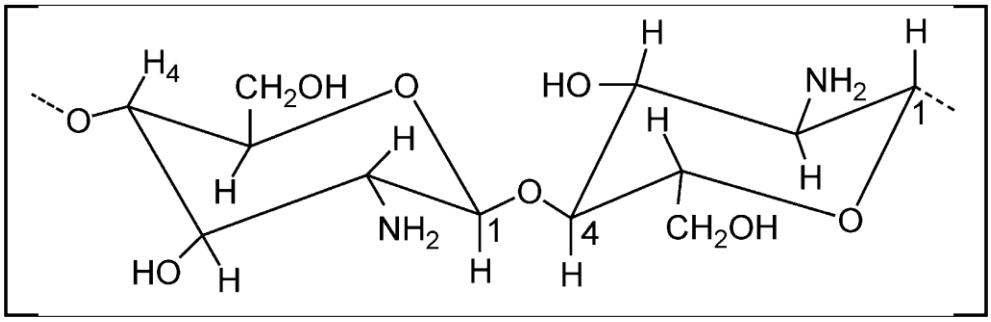

Figure 3. The chemical structure of chitosan (Van Der Merwe et al. 2004).

The chitin structure can be modified by removing the acetyl groups, which are bonded to amine radicals in the C2 position on the glucan ring, by means of a chemical hydrolysis in concentrated alkaline solution at elevated temperature to produce a deacetylated form (Figure 4) (Goy *et al.,* 2009).

When the fraction of acetylated amine groups is reduced to 40-35%, the resultant copolymer, (1 → 4)-2-amine-2-deoxy-β-D-glucan and (1 → 4)-2-acetamide-2-deoxyβ-D-glucan, is then referred to as chitosan. Chitosan is primarily distinguished by its molecular weight (MW) and the degree of acetylation (DA) (Goy *et al.,* 2009).

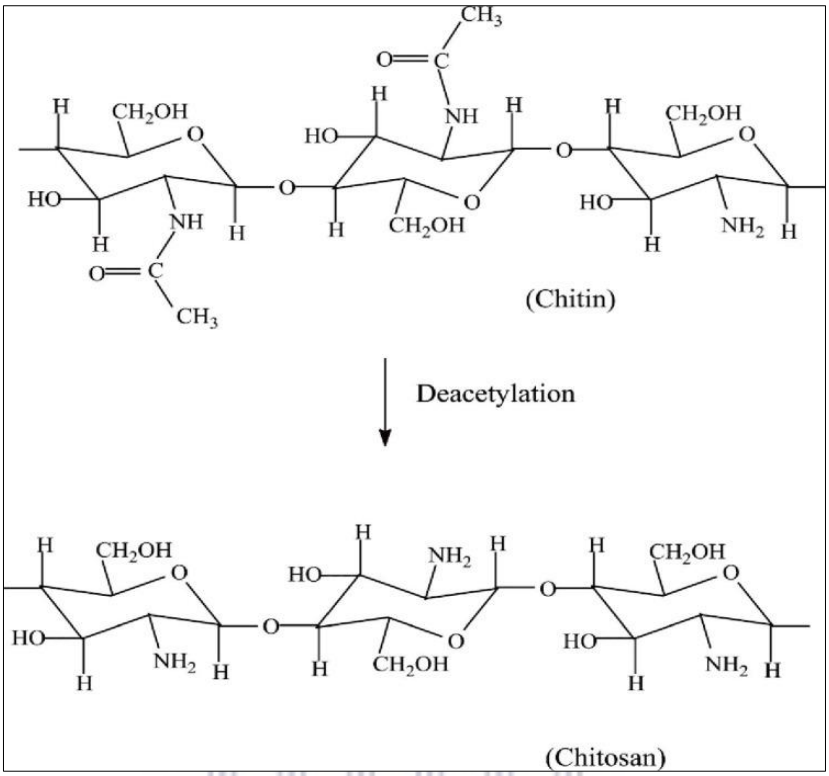

Figure 4. Chitosan derived from deacetylation of Chitin (Gomathysankar et al. 2014).

Chitosan is obtained by N-deacetylation of chitin, which is a naturally abundant muco-polysaccharide that forms the exoskeleton of crustaceans (Rani *et al.*, 2010). Chitosan is a polysaccharide that is individually characterised by the ratio of acetylated to deacetylated units as well as their high molecular weight, both parameters being equally responsible for the properties of the polymer (Aspden *et al.*, 1995). It is regarded as such a valuable natural biocompatible polymer because it is nontoxic, biodegradable (George and Abraham, 2006), mucoadhesive (He *et al.,* 1998) and also possesses gel-forming ability at low pH (Tseng *et al.,* 1995). Interaction between positively charged chitosan molecules and negatively charged microbial cell membranes leads to leakage of

proteinaceous and other intercellular constituents (Young *et al.,* 1982) that causes death of the microorganism.

The major properties of this polymer chitosan include biodegradability, biocompatibility, non-toxicity, high bioactivity, selective permeability, antimicrobial activity, ability to form gel and film, chelation ability and absorptive capacity (Mirani *et al.,* 2015). Particular properties of chitosan are especially important from a dental perspective. A pH of 6.3, which is suitable to buffer the oral pH value high enough to prevent the harmful action of organic acids on the tooth surface (Sano *et al.*, 2003). This material is also biocompatible and biodegradable. Positively charged chitosan, combines with the bacterial cell wall and membrane, resulting in bacteriostatic and bactericidal effects (Arnaud *et al.*, 2010). Muzzarelli *et al.* (1990), demonstrated that chitosan exhibits bactericidal action against several pathogens, including *Streptococcus mutans.* This is especially important since *S. mutans* is known to be the principal microbe responsible for dental caries (Bae *et al.,* 2006).

The viscosity of chitosan solution depends on a number of factors, such as: degree of deacetylation (DD) value, average molecular weight, concentration, ionic strength, pH and temperature. Increase in the DD value with decrease in molecular weight strongly reduces the viscosity of a chitosan solution. Increase of temperature, ionization or ionic strength usually decreases viscosity of chitosan (Struszczzyk, 2002). In low ionic strength media, chitosan adopts an extended conformation because of electrostatic repulsion

between chain segments (Struszczzyk, 2002). Electrostatic repulsion forces disappear; and chitosan conformation becomes a compact sphere regardless of molecular weight differences. The degree of deacetylation is one of the more essential chemical consideration differentiating chitosan and chitin, being a statistic analysis of the product obtained after the deacetylation process and characterizing the macromolecular composition of biopolymer chains. The quantity of DD governs the properties of this natural polymer, such as its solubility in aqueous acid solutions, extent of swelling in water, susceptibility to biodegradation, bioactivity or biocompatibility (Struszczzyk, 2002).

Chitin occurs as two allomorphs, specifically the α and β structures, which can be distinguished by infrared and solid-state Nuclear Magnetic Resonance (NMR) spectroscopies together with X-ray diffraction (Younes and Rinaudo, 2015). In the solid state, chitin chains are connected by the H-bonds arrangement which regulates the solubility, swelling and reactivity (Younes and Rinaudo, 2015). α-Chitin isomorph is considerably profuse; it occurs in fungal and yeast cell walls, in krill, lobster and crab tendons and in shrimp shells, as well as in insect cuticle. In addition to the native chitin, α-chitin can be methodically produced by: recrystallization from chitin solution (Helbert and Sugiyama, 1998), by *in vitro* biosynthesis (Ruiz-Herrera *et al.,* 1975) or enzymatic polymerization (Sakamoto *et al.,* 2000) due to high thermodynamic stability of this isomorph.

The uncommon β-chitin is noticed in association with proteins in squid pens and in the tubes synthesized by pogonophoran and vestimetiferan worms (Blackwell *et al.,* 1965). The crystallographic parameters of the two isomorphs allow us to accept that there are two antiparallel molecules per unit cell in α-chitin but only one in β-chitin in a parallel organization. In these two arrangements, the chains are structured in sheets and held by intra-sheet hydrogen bonds. In addition, in α-chitin, inter-sheet hydrogen bonds avert distribution of small molecules into the crystalline phase. No inter-sheet hydrogen bonds are found in the crystal structure of β-chitin.

The chains correlate to one another by very strong hydrogen bonding between the amide groups and carbonyl groups of the adjunct chain. Hydrogen linkages account for the great insolubility of chains in water and for the formation of fibrils (Struszczzyk, 2002). Water-soluble, reduced chitosan was used as a mouth-rinse agent, and displayed an antibacterial and plaque-reducing action (Bae *et al.,* 2006). Furthermore, recent studies have shown that chewing chitosan-containing gum effectively inhibited the growth of cariogenic bacteria in saliva (Mirani *et al.,* 2015).

4.2 Properties of Chitosan

Molecular weight is another significant factor that influences the physicochemical and biological properties of chitosan (Yuan *et al.,* 2011). It differs with the chitin source from which chitosan is obtained and decreases with increase in degree of deacetylation (Yuan *et al.,* 2011). For a lower chitosan MW (LMW), the detected effect is superior on the

reducing microorganism growth and multiplication (Goy *et al.*, 2009). The size and arrangement appears to be crucial to recognize the ability of LMW chitosan. The mobility, attraction and ionic synergy of small chains are easier than of big ones aiding the adoption of an extended arrangement and an effective binding to the membrane surface (Kumar *et al.*, 2005).

Chitin and Chitosan (CS) are fascinating polysaccharides as for the presence of the amino functionality, which could be suitably modified to impart desired properties and distinctive biological functions including solubility (Rinaudo, 2006). Even though chitin is insoluble in most organic solvents, chitosan is steadily soluble in dilute acidic solutions below pH 6.0 (Pillai *et al.*, 2009). This is due to the fact that chitosan can be deemed a strong base as it acquires primary amino groups with a pKa value of 6.3 (Pillai *et al.*, 2009). The occurrence of the amino groups suggests that pH significantly modifies the charged area and properties of chitosan (Yi *et al.*, 2005). At low pH, these amines get protonated and become positively charged and that makes chitosan a water-soluble cationic poly-electrolyte (Pillai *et al.*, 2009). On the other hand, as the pH increases above 6, chitosan's amines become deprotonated and the polymer loses its charge and becomes insoluble (Pillai *et al.*, 2009). The soluble–insoluble transition occurs at its pKa value around pH between 6 and 6.5. As the pKa value is highly dependent on the degree of N-acetylation, the solubility of CS is dependent on the degree of deacetylation and the method of deacetylation used (Cho *et al.*, 2000). The degree of ionization depends on the

pH and the pK of the acid with respect to studies based on the role of the protonation of CS in the presence of acetic acid and hydrochloric acid (Rinaudo *et al.,* 1999).

Chitosan is a functional material with evident antimicrobial action (Goy *et a*l., 2009). There are three antibacterial processes that have been determined: i) the ionic surface interface and as a consequent, cell wall leakage; ii) the inhibition of the mRNA and protein synthesis via the infiltration of chitosan into the nuclei of the microorganisms; and iii) the development of an external barrier, chelating metals and provoking the concealment of fundamental nutrients to microbial growth (Goy *et al.,* 2009). It is likely that all events occur concurrently but at unlike intensities. The molecular weight (MW) and the degree of acetylation (DA) are significant aspects in governing its antimicrobial action. Overall, the lower the MW and the DA, the higher will be the efficacy and success on diminishing microorganism development and proliferation (Goy *et al.,* 2009).

4.3 Antibacterial properties of Chitosan

Chitosan has been researched as an antimicrobial material against a broad variety of target organisms like algae, bacteria, yeasts and fungi in tests including in vivo and in vitro interfaces with chitosan in diverse forms (solutions, films and composites) (Goy *et al.,* 2009). Initial studies depicting the antimicrobial capability of chitin, chitosan, and their derivatives goes back to 1980-1990s (Shahidi *et al.,* 1999). In general, in these investigations and conclusions the chitosan is considered to be a bactericidal (kills live bacteria) or bacteriostatic (impedes the development of bacteria but does not suggest

whether or not bacteria are destroyed), frequently with no inconsistency amongst both these events (Goy *et al.,* 2009). Regardless of the discrepancy amongst gram-negative and gram-positive bacterial cell walls, antibacterial approaches equally initiate with contact at the cell surface and disturb the cell wall (Tan *et al.,* 2013). For gram-positive bacteria, lipoteichoic acids may supply a molecular connection for chitosan derivatives at the cell surface, permitting it to compromise membrane actions (Raafat *et al.,* 2008). Lipopolysaccharide and proteins in the gram-negative bacteria outer membrane are kept collected by electrostatic interactions with divalent cations that are necessitated to balance and secure the outer membrane. Polycations may oppose with divalent metals, such as Mg^{++} and Ca^{++} ions present in the cell wall, which will disturb the integrity of the cell wall or effect the action of degradation of enzymes (Kong *et al.,* 2010).

Interaction amongst chitosan derivatives and the cell membrane, which is fundamentally a negatively charged phospholipid bilayer, may vaguely alter the membrane permeability (Tan *et al.,* 2013). Additional contact may denature membrane proteins and introduce diffusion into the phospholipid bilayer (Tan *et al.,* 2013). The increased membrane permeability precedes to disruption of the cell membrane, leakage of intracellular substances and, eventually, the killing of cells (Kong *et al.,* 2010). Consequently, as a result of its biocompatible and functional benefits chitosan demonstrations widespread prospective for function as a biomaterial (Arnaud *et al.,* 2010). Once combined with composite cements physically crosslinked hydrogels are designed by physical interactions such as electrostatic, hydrophobic, or hydrogen bonding between the polymer chains

(Dutta, 2016). Hydrogel development can be stimulated by mixing the components within appropriate settings and conditions to begin the gelation (Dutta, 2016). Physical crosslinking is generally activated by stimuli such as pH and temperature (Dutta, 2016). Monitoring the concentration of chitosan being considerate to that of other constituents and thus controlling the polymer interactions has been demonstrated to considerably control the gel properties (Bhattarai *et al.*, 2010; Croisier and Jérôme, 2013)

This may describe its growth in the incidence of polar guest molecules (ranging from water to alcohol and amines) which infiltrate the crystal lattice without disrupting the sheet organization and the crystallinity of the sample. The elimination of the guest molecule permits us to return to the original state of anhydrous β-chitin. The reactivity of β-chitin isomorph is superior than the α-isomorph, which is vital for enzymatic and chemical changes of chitin (Kurita *et al.*, 1993).

Biodegradability of chitosan is dependent on different factors such as the degree of deacetylation, distribution of amine groups, the presence of acetyl groups, and molecular weight of the polymer (Croisier and Jérôme, 2013). Chitosan can be degraded using enzymes such as lysozyme, which is a glycosidic hydrolase present in the human body (Dutta, 2016). Lysozyme is reported to hydrolyze the β linkages between N-acetylglucosamine and glucosamine (Han *et al.*, 2012).Therefore, the degree of acetylation plays an important role in enzyme-mediated degradation of chitosan (Dutta,

2016). Chitosan with higher degree of deacetylation undergoes limited degradation, whereas increasing acetylation results in higher degradation (Verheul *et al.,* 2009). Antimicrobial property of chitosan stems from its interaction with the negatively charged cell surfaces by affecting cellular permeability or by chitosan's interaction with DNA, thereby inhibiting microbial RNA synthesis (Chung and Chen, 2008). Mucoadhesive property of chitosan also arises from its positively charged amine groups that can interact with the negatively charged groups in the mucin molecule (He *et al.,* 1998). Hemostatic property is due to the presence of positively charged groups in chitosan that interact with the negatively charged surfaces of blood cells (Croisier and Jérôme, 2013). Chitosan-based hemostatic bandage called Hemcon® has been FDA approved (Dutta, 2016). Both physical and chemical crosslinking methods can be used to develop chitosan hydrogels (Dutta, 2016).

4.4 Chitosan Nanoparticles

Both chitin and similarly occurring cellulose are significant naturally occurring polymeric materials as for their abundance in addition to the unusual molecular arrangements rather dissimilar from those of synthetic polymers (Kurita, 1998). Chitosan comprises of sufficient amino and hydroxyl groups, which allow nanoparticle preparation through both physical and chemical cross-linking (López-León *et al.,* 2005). Covalent cross-linking is generally produced by treatment of glutaraldehyde, which responds with the amino groups to form Schiff bases (Liu and Gao, 2009). Ionic cross-linking of chitosan is a typical non-covalent interaction, which can be recognized by association with negatively charged

multivalent ions such as tripolyphosphate (TPP) (Kawashima *et al.*, 1985). For pharmaceutical uses, physical cross-linking is more favourable since the cross-linking is reversible and may essentially prevent possible toxicity of the substances (Liu and Gao, 2009).

Two unlike methods are generally utilized to achieve chitosan micro- or nanoparticles: Chitosan chains can be chemically cross-linked steering to rather constant matrixes, where the strength of the covalent bonds is distinct (López-León *et al.,* 2005). Glutaraldehyde is largely used as a cross-linking molecule in covalent formulations (López-León *et al.*, 2005). Conversely, chitosan hydro-gels can be furthermore achieved by ionic gelation, where micro-or nanoparticles are created by means of electrostatic interactions among the positively charged chitosan chains and polyanions employed as cross-linkers (López-León *et al.,* 2005). The most significantly used polyanion is the tripolyphosphate (TPP) (López-León *et al.,* 2005).

4.5 Chitosan applications in Medicine

In health care environment and hygienic applications, biocidal polymers may be combined into fibres, membrane, or hydrogel, and used for contact disinfectants in many biomedical applications, including wound dressing, orthopaedic tissue engineering, drug delivery carrier and haemodialysis (Kong *et al.,* 2010). Chitosan has been widely investigated for a variety of applications in the biomedical industry (Dutta, 2016). The beneficial properties of chitosan are its biocompatibility, biodegradability (Rossi *et al.,* 2012),

antimicrobial activity (Chien and Shah, 2012), mucoadhesivity, wound healing and haemostatic properties and low toxicity (Rossi *et al.*, 2012).

A broad range of medical applications for chitin and chitin derivatives have been described over the last three decades (Wang *et al.*, 1996). Due to these distinctive biological properties, considerable research has been done to improve chitosan-based hydrogels for biomedical applications (Dutta, 2016).

Chitin and chitosan have many distinctive biomedical properties and have been applied in many different industrial areas already, such as food, cosmetic and pharmaceutical industries (Le *et al.*, 1998). It has been suggested that chitosan may be used to impede fibroplasia in wound healing and to stimulate tissue growth and differentiation in tissue culture (Le *et al.*, 1998). Fibres made of chitin and chitosan are useful as absorbable sutures and wound-dressing materials (Le *et al.*, 1998). These chitin sutures resist attack in bile, urine and pancreatic juice, which are difficult with other absorbable sutures (Dutta *et al.*, 2004). It has been claimed that wound dressings made of chitin and chitosan fibres (Le *et al.*, 1998) accelerate the healing of wounds by about 75 percent (Dutta *et al.*, 2004).

Due to its physical and chemical properties, chitosan is being used in a vast array of widely different products and applications, ranging from pharmaceutical and cosmetic products to water treatment and plant protection (Dutta *et al.*, 2004). Chitosan is a promising candidate for burn treatment. This is true since chitosan can form tough, water-absorbent, biocompatible films (Dutta *et al.*, 2004). These films can be formed directly on the burn

by application of an aqueous solution of chitosan acetate. Another advantage of this type of chitosan treatment is that it allows excellent oxygen permeability. This is important to prevent oxygen-deprivation of injured tissues (Dutta *et al.*, 2004).

Chitosan has been found to have an accelerative consequence on wound healing and wound bandage procedures (Dutta *et al.*, 2004). Regenerated chitin fibres, non-woven mats, sponges and films reveals a growth in wound healing by over 30 percent (Dutta *et al.*, 2004). Chitin can also be used as a coating on normal biomedical materials. Standard silk and catgut sutures coated with regenerated chitin or chitosan demonstrate wound healing activities only slightly lower than the all-chitin fibres (Dutta *et al.*, 2004). Surgical gauze coated with regenerated chitin shows a significantly greater quantity of activity than an uncoated control group (Dutta *et al.*, 2004).

4.6 Chitosan application in Dentistry

Chitosan has fascinating attributes that have been described in the literature such as: drug carrier of controlled release (Hamman, 2010), antibacterial (Feng and Xia, 2011), prevention of decalcification of dental enamel (Arnaud *et al.*, 2010) by inhibiting plaque formation and these properties expose its prospective in numerous commercial products (Ali, 2015). There is data that proves that adding chitosan in dental materials, enhances the biocompatibility of materials and hinders the adsorption of oral bacteria to the tooth surface (Ríos and Recio, 2005). The antimicrobial activity of the chitosan products is also built on the physical conditions and molecular weight (Tan *et al.*, 2013). Generally,

chitosan derivatives with an elevated molecular-weight and solid particles cannot go through cell membranes and merely associate with the cell surface to adjust cell permeability or form a covering throughout the cell that keeps cells against nutrient passage through the microbial cell membrane (Eaton *et al.,* 2008). However, low-molecular-weight, water-soluble chitosan or nanoparticles could infiltrate the cell walls of bacteria, combine with DNA and prevent the synthesis of mRNA and DNA transcription (Tan *et al.,* 2013). Thus, due to chitosan's unique bioactive properties, chitosan can be used in both medicine and dentistry.

Therefore, it is postulated that incorporating chitosan into an orthodontic cement may prove promising because of its antibacterial properties and can thus be used to prevent demineralization of enamel around orthodontic brackets. The continual antibacterial effect of chitosan incorporated into an orthodontic cement therefore, may be beneficial in preventing unsightly white spot lesions.

Thus, the rationale of this study was to incorporate chitosan into an orthodontic cement to create a novel orthodontic cement and assess whether the incorporation of chitosan to an existing orthodontic cement would affect its biological and physical properties.

The ultimate goal of this study was to create a novel chitosan modified orthodontic cement that can be used clinically to prevent demineralization around orthodontic brackets.

CHAPTER II

Aims and Objectives

1. Aim

The aim of this study was to investigate the biological and physical properties of a chitosan modified orthodontic cement.

2. Objectives

1. To determine the antibacterial effects of chitosan modified orthodontic cement and compare it to the antibacterial effects of an unmodified orthodontic cement.
2. To determine the shear bond strength of a chitosan modified orthodontic cement and compare it to the shear bond strength of an unmodified orthodontic cement.
3. To determine and compare the Adhesive Remnant Index (ARI) on the teeth bonded with chitosan modified cement to that of unmodified orthodontic cement.
4. To evaluate the surface hardness of chitosan modified orthodontic cement and compare this to the surface hardness of an unmodified orthodontic cement.
5. To determine the cytotoxicity of a chitosan modified orthodontic cement and compare the cytotoxicity to an unmodified orthodontic cement.
6. To examine the surface morphology of chitosan nanoparticles using a Scanning Electron Microscope.

CHAPTER III

Methodology

1. Materials

Chitosan (Sigma Aldrich, USA) and glacial acetic acid (Merck, Germany) was used according to the manufacturer's instructions. Chitosan with the following properties was used in this study:

- Degree of de-acetylation 87%.
- Low Molecular Weight of 50 -190 KDa.
- Iso-electric point of 4.0 – 5.0 d.
- High purity.

2. Preparation of Chitosan Nanoparticles

The preparation of chitosan nanoparticles as described by Shrestha *et al.,* (2010) was used in this study. The method involves an ionic interaction between the positively charged amino groups of chitosan and the polyanion tripolyphosphate (TPP), which acts as chitosan cross-linker (Figure 5).

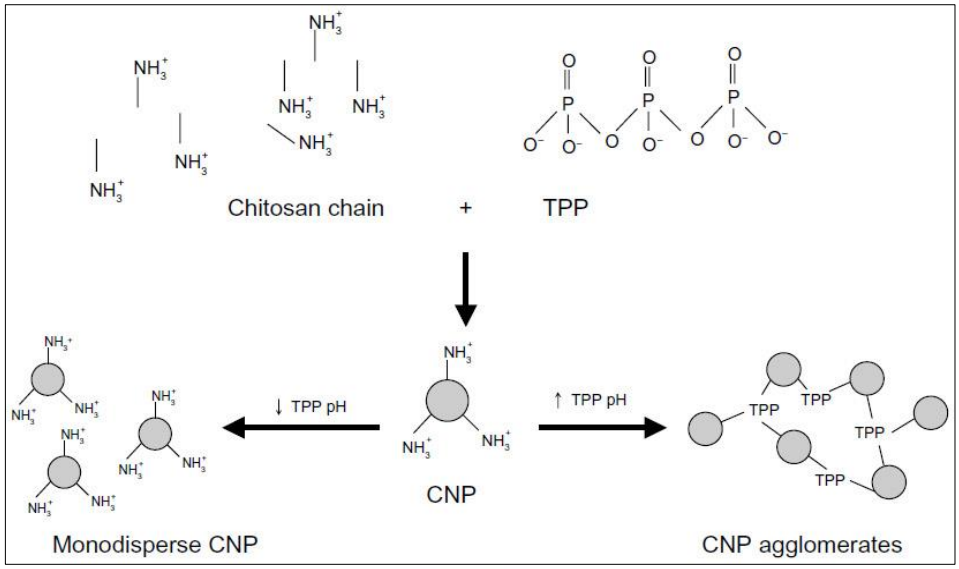

Figure 5. Diagrammatic explanation of the formation of chitosan nanoparticles (CNP) through the addition of tripolyphosphates (TPP) to chitosan (Dutta, 2016).

Nanoparticle formation takes place immediately after the addition of a TPP solution to a solution of chitosan, under mild stirring, at room temperature (Figure 5). Chitosan (0.2g) was dissolved in 200ml of 1 v/v% acetic acid to obtain a chitosan hydrogel concentration of 0.1w/v% stirring at speed of 1000rpm for 2 hours. The pH of the chitosan hydrogel, thus formed was raised to 5 with 1 mol/L NaOH (Shrestha *et al.*, 2010).

To obtain a concentration of 0.1% sodium tripolyphosphate (TPP), 0.6g of TPP was mixed in 600ml distilled water, stirring at a speed of 1000rpm for 2 hours.

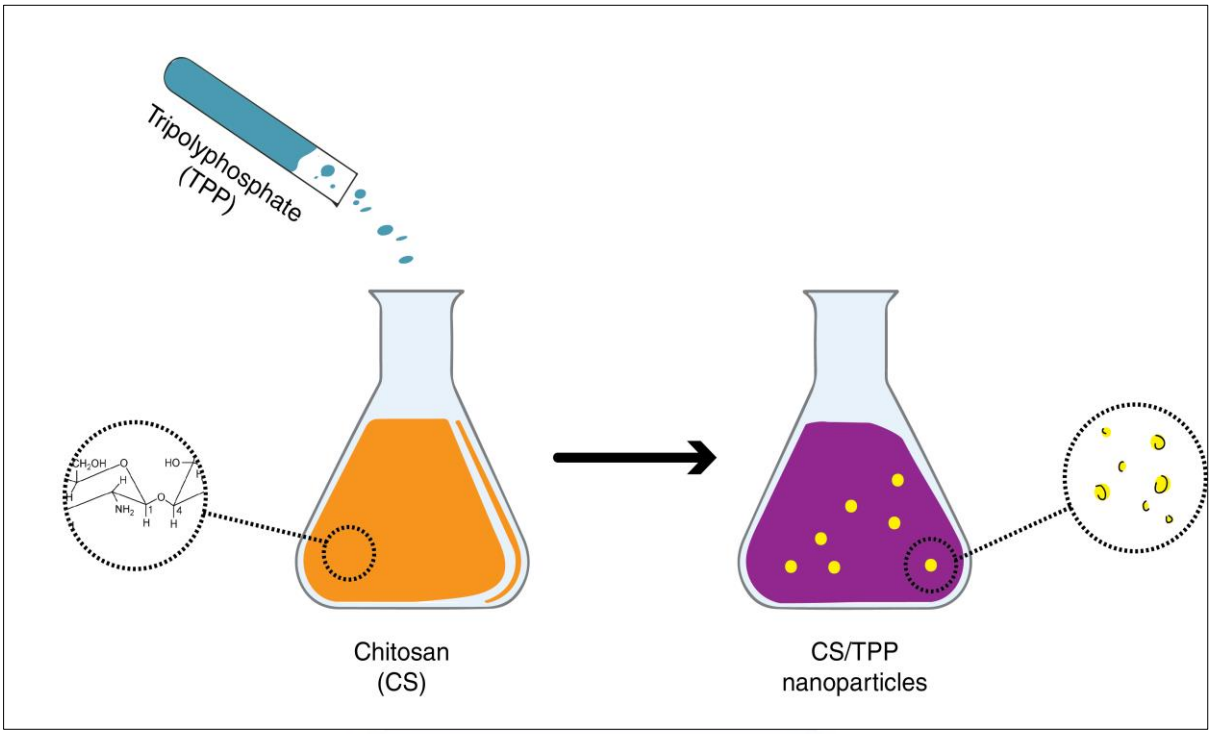

Figure 6. Schematic representation of the method of ionic gelation/polyelectrolyte complexation.

Chitosan nanoparticles was formed by adding 0.1% sodium tripolyphosphate (obtained as described above) in distilled water to chitosan hydrogel solution in a ratio of 3:1 while stirring at a speed of 1000 rpm (Figure 6). Chitosan nanoparticles were then separated by centrifugation at 15,000 rpm for 30 minutes. The supernatant was discarded, and chitosan nanoparticles was extensively rinsed with deionized water to remove any residual NaOH and then freeze-dried prior to further use.

3. Preparation of chitosan modified orthodontic cement

To achieve the chitosan modified orthodontic cement (test sample), chitosan nanoparticles (manufactured as described above) was added to Transbond™ orthodontic cement (3M ESPE Oral Care, 2510 Conway Avenue, St Paul, MN 55144-100 USA) to obtain a final concentration of 10% chitosan concentration in the new cement. Transbond was extruded from the manufacturer's syringe onto a sterile glass slab and 1g chitosan nanoparticles was mixed evenly to the 10g Transbond. This was done in a dark area to avoid any polymerization that may occur if the cement is exposed to light.

4. Sample preparation

Sample discs (n=10) were prepared by extruding the Transbond orthodontic cement into polytetrafluoroethylene Teflon mould (PTFE) with 7mm diameter and 2mm height in between 2 cover slides and light cured using an Elipar™ DeepCure-S (3M ESPE) and cured for 10 seconds (according to manufacturer's guide). Samples were also prepared with chitosan modified Transbond cement. The light intensity of the curing light was tested using a CureRite Light Meter (Dentsply, USA) and found to be 1400 mW/cm^2. The curing light was tested after every 5 samples. The cured cement was removed from the Teflon mould and the specimen was stored in an incubator for 24 hours at 37^{0}C and 100% humidity before sterilization (Ibrahim *et al.*, 2015). Sample discs were weighed in order to normalize each material in both the test and the control group.

The following properties of this newly formed chitosan modified cement was compared to unmodified orthodontic cement as positive control namely; antibacterial properties, shear bond strength, surface hardness and biocompatibility testing.

The null hypothesis in this study stated that there were no differences between the test and the control groups.

5. Microbiological Investigation

Streptococcus mutans (ATCC UA159 strain) was obtained from the American Type Culture Collection (Manassas, USA) and used as the test bacterium for analyzing the antibacterial activity of the test and control samples.

Specimen preparation and biofilm formation

The sample discs were sterilized in an ethylene oxide gas sterilizer (SSS; Sterile Services, Singapore). The antibacterial activity of the newly formed test cement and control was used to test against a *Streptococcus mutans* bacteria (ATCC UA159 strain). A single colony was cultured in 3mL of Brain Heart Infusion (BHI) broth (Sigma-Aldrich, St. Louis, MO, USA) and was grown overnight at 37^0C in 5% CO_2 incubator. To form the inoculation medium, bacterial suspensions containing 10^8 cells/mL was adjusted to optical density at 0.68 at a 600nm wavelength using a spectrophotometer (UV-1700, Shimadzu, Tokyo, Japan). The inoculum was prepared by suspending the bacterial cells from a single

colony from an overnight culture into a sterile saline solution to match a 0.5 McFarland standard (containing about $10^7 – 10^8$ colony forming units/ml).

The antibacterial tests were performed using the Agar Dilution Method (Balouiri et al. 2016). Test samples and control samples were placed in a BHI broth containing *S. mutans* in 12 well subculture plates and placed in an Orbital Shaker Incubator (Biocom Biotech, USA) at 37^0C in 5% CO_2, so as to expose the bacterial culture to the test specimen and the control specimen. Samples were then taken at time intervals of 0 minutes, 30 minutes, 1 hour, 2 hours, 4 hours and 8 hours. 100µl of each well broth (test and control samples) was pipetted in a 96 well plate (Figure 7) and diluted using 50µl phosphate buffer saline (Sigma-Aldrich, St. Louis, MO, USA). This was all done in the lamina flow under sterile working conditions. 2µl of the broth of each test were streaked onto agar plates, labeled according to the time interval at which the sample were taken and placed in an incubator 37^0C in 5% CO_2 incubator. Following 24 hours, the colony forming units at the various time interval agar plates were then counted using a cell counter.

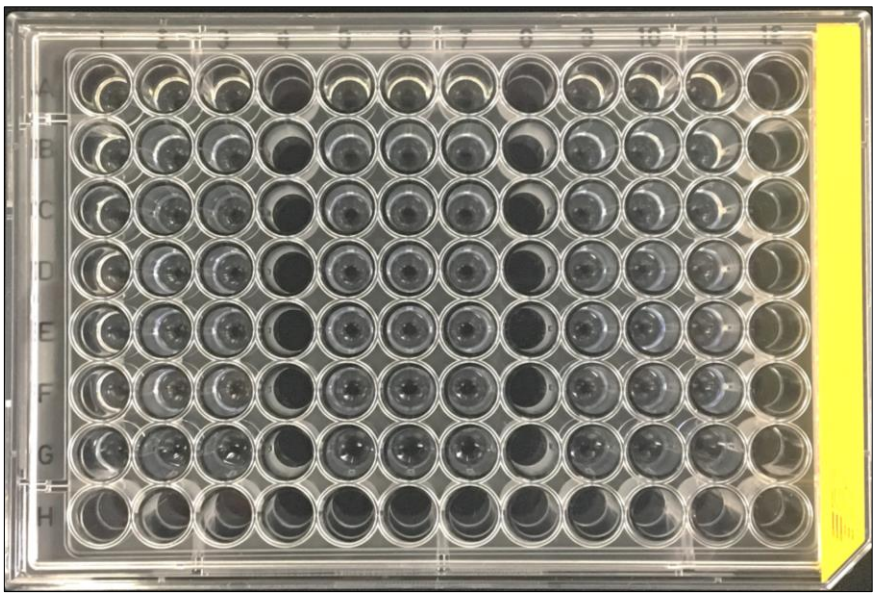

Figure 7. Showing 96 well plate containing dilutions of the broth exposed to the test specimen.

Figure 8. Lamina Flow hood with 96 well plates and agar plates during dilution test method.

All work was done in the Lamina Flow hood (Bio Flow, USA) to maintain sterility (Figure 8). All microbiological experiments were repeated 4 times. Cell counts were plotted at the various intervals to determine and compare the growth patterns of the bacteria for the test sample and the unmodified Transbond (control).

6. Shear Bond Strength

A total of 40 extracted human upper and lower premolars (with no restorations, cracks or caries) were used for this part of the study. Extracted teeth were obtained from the Department of Oral Surgery, Dental Faculty, University of the Western Cape following ethical clearance (Ethics clearance no. BM/16/5/20) and after signed patient consent was obtained (Patient Information and Consent Form, Appendix 1 & 2). Teeth extracted for orthodontic purposes from 14- to 17-year-old patients undergoing orthodontic treatment, were used in this study. After extractions, the teeth were carefully inspected and only intact teeth were used for this study. Teeth that had caries, filling, previous root canal treatment, cracks or fractures were excluded from this study. Enamel structure of the teeth was examined at 20x magnification, and teeth with hypoplastic areas or surface irregularities were excluded from the study. The teeth were cleaned and stored in saline in an incubator at a standardized temperature of 37^{o}C until use. The teeth were randomly divided into two groups: Group 1 (Test Group) included orthodontic brackets bonded to the enamel surfaces of 20 extracted teeth using the chitosan modified Transbond™ (3M ESPE) orthodontic cement (as prepared and described previously). Group 2 included orthodontic brackets bonded to the enamel surfaces of 20 teeth using the unmodified Transbond™ (3M ESPE) orthodontic cement (Control Group). The buccal surfaces of all teeth were cleaned and polished with an oil free pumice paste using a slow handpiece and rinsed with water. The enamel surface was etched with 37% phosphoric acid solution (Wright Health Group Ltd, RSA) for 15 seconds and rinsed off for 10 seconds with water according to manufacturer's instructions. The teeth were then dried using an air syringe.

Transbond™ XT Primer (3M ESPE, USA) was applied to the tooth surface and left to air dry. Roth stainless steel brackets for upper and lower premolars were used in this study (slot size 0.022). Transbond™ XT orthodontic cement was applied onto the bracket base in the control group and the bracket bonded to the buccal surface of the premolar teeth. In the test group the brackets were bonded to the teeth using the newly formed chitosan modified orthodontic cement. To ensure standardized positioning of the brackets, each bracket was positioned on the tooth using Bracket Holding Tweezers, over the midpoint of the clinical crown on buccal surfaces of the prepared premolar teeth and pressed firmly onto the surface. Bracket height of 3.5mm was determined using an Orthodontic Gauge, above the incisal tip of crown to standardize the positioning of the bracket onto the buccal surface of the teeth. All excess adhesive was removed from around the bracket. The cement under the bracket was cured using Elipar™ DeepCure-S (3M ESPE) curing light for 10 seconds. The Elipar curing light was tested for light output using a CureRite light intensity meter (Dentsply, USA) and found to be 1400 mW/cm^2. The light intensity was checked after every 5 samples and was found to be stable at 1400 mW/cm^2.

For the shear bond strength test preparation, the teeth containing the bonded brackets were embedded in specially designed moulds of PVC with chemically cured acrylic resin. Teeth were positioned in the PVC containers with their long axis of the crown being parallel to the direction of the shear force to be applied in testing machine (Markovic *et al.,* 2008*).* Samples were placed in an incubator at a standardized temperature of 37^{o}C for 48 hours prior to shear bond strength testing.

After 48 hours, the samples were subjected to a shear bond strength test in an Olsen Universal Testing Machine (Tinius Olsen, H10KT, Horsham, USA) at crosshead speed of 0.5 mm/min. The circular PVC test cylinders were mounted in the universal testing machine, with the brackets positioned perpendicular to the application of force. The angulation was defined and the direction of the de-bonding force was parallel to the enamel surface in an occluso-gingival direction. A stainless-steel flat end application rod was used for de-bonding the brackets with its chisel end placed onto the enamel/cement interface. The machine was activated with a crosshead speed of 0.5 mm/minute until a fracture was noticed. The force required to break the bond was recorded and the bond strength was calculated in megapascals (MPa). The mean shear bond strength values of the test samples were compared to the mean shear bond strength values of the unmodified cement.

Finally, after breakage, the bracket base, as well as the enamel surface of each tooth, was visually inspected under a microscope at 20x magnification, in an attempt to identify the weakest point in orthodontic bonding using the Adhesive Remnant Index (Surmont et al. 1992).

7. Adhesive Remnant Index

The Adhesive Remnant Index (ARI) system was used to evaluate the amount of adhesive left on the tooth after de-bonding (Montasser and Drummond 2009). The ARI score according to Artun and Bergland (1984), was used in this study to evaluate the adhesive

remaining on the tooth structure. This original index system was developed on the basis of cement remaining on extracted teeth, and the criteria are as follows (Artun and Bergland, 1984):

Score 0 = No adhesive left on the tooth.

Score 1 = Less than half of the adhesive left on the tooth.

Score 2 = More than half of the adhesive left on the tooth.

Score 3 = All adhesive left on the tooth, with distinct impression of the bracket mesh.

Excess resin outside the bracket base area was not considered. Two operators were used to record the scores. The ARI scoring index was first discussed amongst the operators to determine accuracy of scores. When consensus could not be reached between the two operators a third examiner was used to verify the scores to avoid intra operator variability. The tooth base area on which the bracket was de-bonded from was examined under 20x magnification. The residual adhesive was measured, and the percentage of the adhesive remaining on the tooth surface was calculated.

8. Surface Hardness

In this part of the study, the surface hardness of the newly formed chitosan modified cement was compared to the surface hardness of unmodified orthodontic cement (Transbond, 3M ESPE, USA) as positive control. A total of 14 samples discs were prepared *i.e.* 7 sample discs for the chitosan modified cement (test sample) and 7 samples for the unmodified cement (control) was prepared. Test discs with 7mm diameter and 2mm height were prepared in a PTFE mould between 2 cover slides and light cured using

an Elipar Deep Cure light curing unit (3M ESPE, USA). The slide was removed and the top surface of the specimen was identified with a permanent marker. As previously described the light intensity was checked using a CureRite Light Meter (Dentsply, USA). The Elipar curing light was tested for light output and found to be 1400 mW/cm^2. The light intensity was checked after every 5 samples and was found to be stable at 1400 mW/cm^2.

The cured cement test material was pushed out of the mould and the specimens were stored for 24 hours at 37°C and 100% humidity before use. The top and the bottom surfaces were marked with three longitudinal pencil lines to indicate the position of the indentation. Surface hardness was measured using a Vickers Hardness Machine (Zwick, Germany) (Figure 9).

Figure 9. Vickers Hardness Machine used to measure surface hardness of the specimens.

The Vickers hardness machine was adjusted to a load of 300g for 15 seconds according to guidelines set out in ISO 4049 (ISO 2009a) . Each sample was placed on the stage of the machine and a magnification of 40x was used to adjust and bring into focus the center of the test material in the disc to identify a smooth surface, devoid of irregularities or other voids (Figure 10).

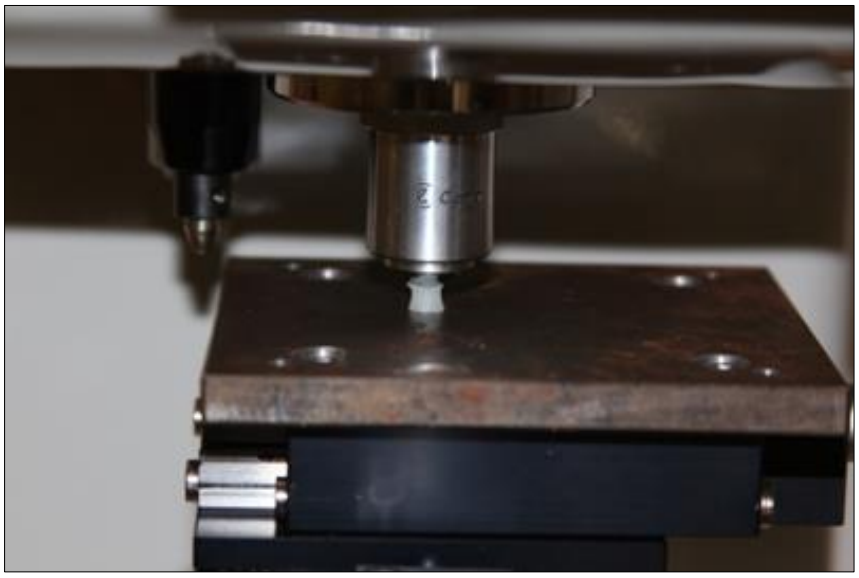

Figure 10. Specimen placed on platform and observed using 40x magnification.

The Vickers hardness test method indented the sample with a diamond indenter, in the form of a pyramid with a square base and an angle of 136 degrees between opposite faces subjected to a test force of 300g (Figure 11). The full load was applied for 15 seconds.

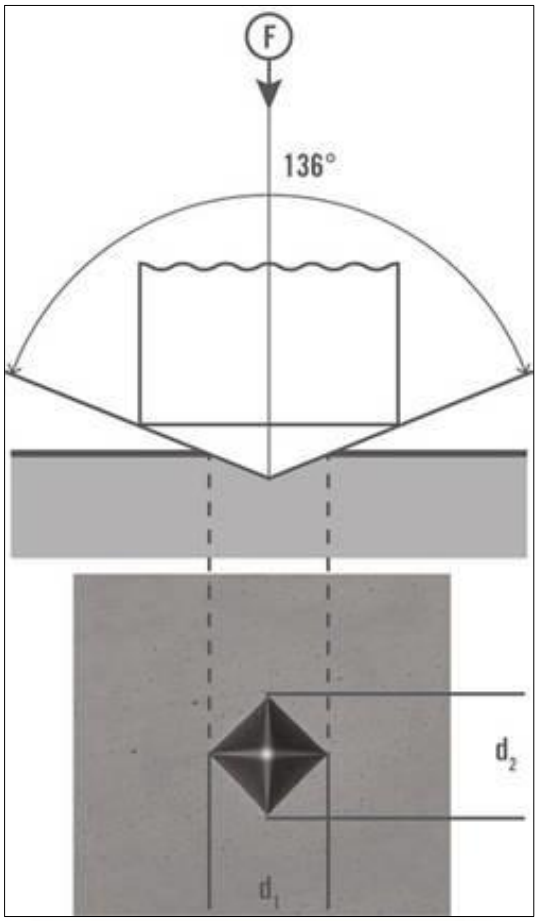

Figure 11. Diagrammatic representation of the indentation created on specimen.

The area of the sloping surfaces of the indentation is calculated by the machine. The Vickers hardness is the quotient obtained by dividing the Kgf load by the square mm area of indentation. The mean values of the chitosan modified cement was compared to the mean values of the unmodified cement for statistically significance differences. A p value of <0.05 was considered a significant difference.

9. Cytotoxicity Testing

Cytotoxicity of the orthodontic cement with and without chitosan was tested using Balb/c 3T3 mouse fibroblast cells. Balb/c 3T3 mouse fibroblast cell line was obtained from the National Repository for Biological Materials (Sandringam, RSA) and maintained and cultured under standard conditions (37^{o}C under 5% carbon dioxide and 95% humidity) in Dulbecco's Modified Eagles Medium (DMEM). The medium was supplemented with 10% fetal bovine serum, 1% penicillin, 1% streptomycin and 0.2% fungizone, changed every second day and cells sub-cultured using routine trypsin/EDTA procedures.

Ten control bottles containing DMEM were exposed to the unmodified cement while a further 10 bottles was exposed to chitosan modified cement for cytotoxicity testing. Three repetitions (30 bottles in total for each material) were done on each test group and the mean values were recorded. The plated cells were incubated for 24 hours and the widely used MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) colorimetric assay was used to evaluate cell viability (Grobler *et al.*, 2004; Grobler *et al.*, 2008; Perchyonok *et al.*, 2013).

Balb/c 3T3 Mouse Fibroblast Cell Survival Rate

To test the possible influence of the various cements on the survival rate of 3T3 cells, the cells were grown to near confluency as described by Grobler *et al.* (2008). The 3T3 cells were diluted to a final cell suspension containing approximately 3×10^5 cells/ml and plated out in a 96 well plate.

Two hundred µl of each group was added to each of 20 wells in the 96 well plate. Chitosan modified Transbond XT was the test sample. The negative control was Transbond XT cement. After 24 hours, the MTT colorimetric assay was used to evaluate cell growth. This assay involves the ability of viable cells to use mitochondrial dehydrogenase enzymes to convert MTT (a soluble tetrazolium salt) to a blue/violet formazan end-product (Mosmann, 1983). Twenty µl MTT (5mg/ml in phosphate- buffered solution) was added to each well and left for a further 3 hours to incubate at 37°C. The medium was discarded to eliminate the MTT and the precipitated formazan crystals was solubilized with 100 µl/well of di-methylsulfoxide (DMSO). Absorbance was measured at wavelength 540 nm on a spectrophotometer to determine the number of viable cells. Each test was repeated five times, for each material. The mean values of cell viability were expressed as a percentage of the control values to show increase or decrease in the number of viable cells as an indication of the cytotoxicity of the test material.

10. Surface morphology determination

The surface morphological study of the chitosan nanoparticles was performed by Field Emission Scanning Electron Microscope (FESEM), (Auriga Field Emission Scanning Electron Microscope, Zeiss, Germany) (Figure 12).

Figure 12. Auriga Field Emission Scanning Electron Microscope (Zeiss, Germany).

A pinch of the chitosan powder was gently sprinkled onto the carbon paper. The stud was gently tapped to loosen and to evenly spread the powder (Figure 13).

Figure 13. Specimen attached to the stud using carbon adhesive tape.

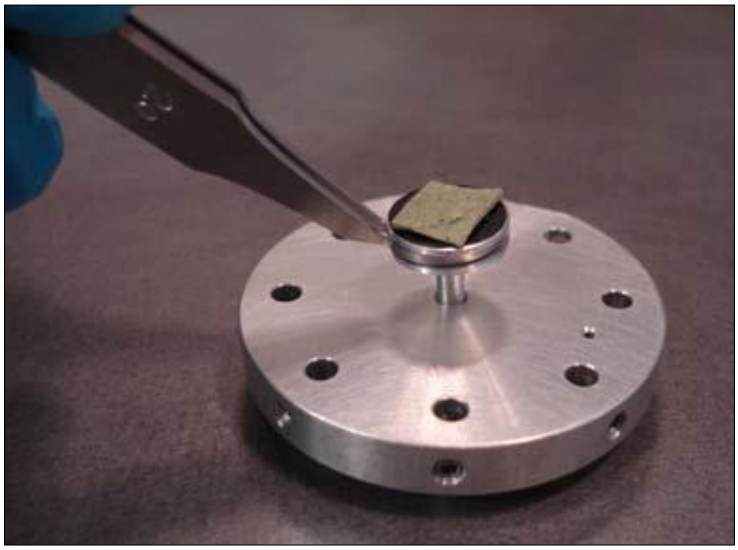

Figure 14. Tweezers were used to carefully attach the stud to the sample holder.

A blower was then used to gently remove excess powder. The specimens obtained were mounted on aluminum stubs, with a double-stick carbon tape (Figure 14) and then sputter-coated with gold/palladium and observed using a field emission scanning electron microscope,

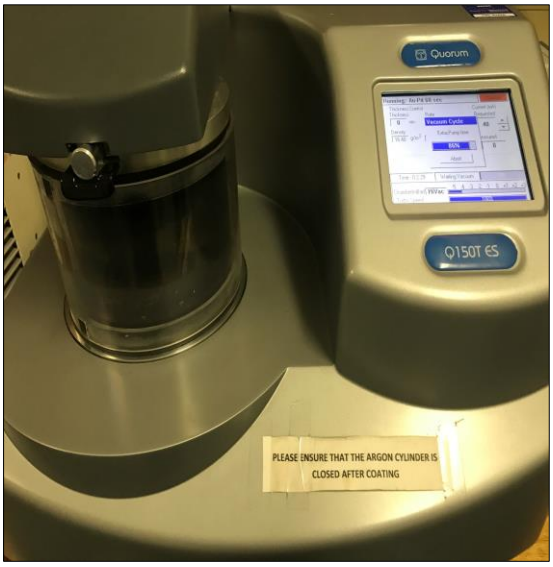

Figure 15. Quorum Sputter Coater (Q150T ES, Quorum Technologies Ltd, UK).

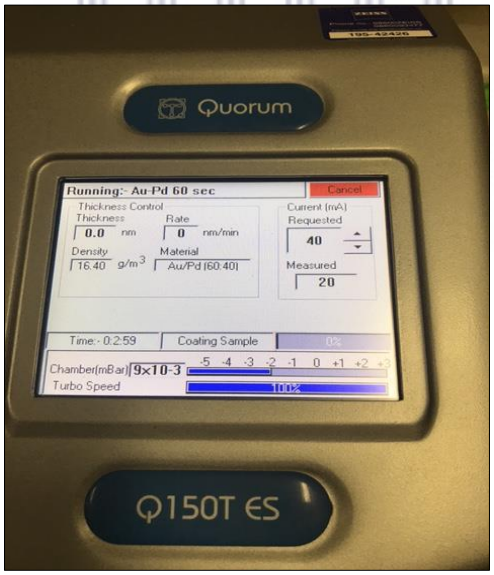

Figure 16. Settings on Quorum Sputter Coater (Q150T ES).

The specimens were sputter coated with gold/palladium for 60 seconds (Figure 15 & 16)) under a vacuum in a Quorum Sputter Coater (Q150T ES, Quorum Technologies Ltd, UK). The specimens were then placed on a platform ready for SEM analysis (Figure 17).

Figure 17. Specimens sputter coated with gold/palladium.

CHAPTER IV

Results

1. Microbiological study of chitosan modified cement

Growth of the bacterial colonies formed on the agar plates were observed, photographed and counted at 0 minutes, 30 minutes, 1 hour, 4 hour and 8 hour time intervals and shown in Figures 18 to 38. In all 4 experiments, in the test sample group containing cement and chitosan showed a steady decline in cell counts starting from baseline (0 minutes) to final count at 8 hours. There was complete eradication of all bacteria after 4 hours exposure of the bacteria to the test sample to final count at 8 hours. On the other hand, in the control group in all four test repetitions it was observed a steady increase in bacterial cell numbers shown on the agar plate when exposed to the control cement (unmodified cement) from 0 minutes (baseline) to final count at 8 hours after exposure.

Figure 18. Control sample at 0 minutes showing initial microorganism growth seeded on the agar plate (agar plate labelled 0 min Control).

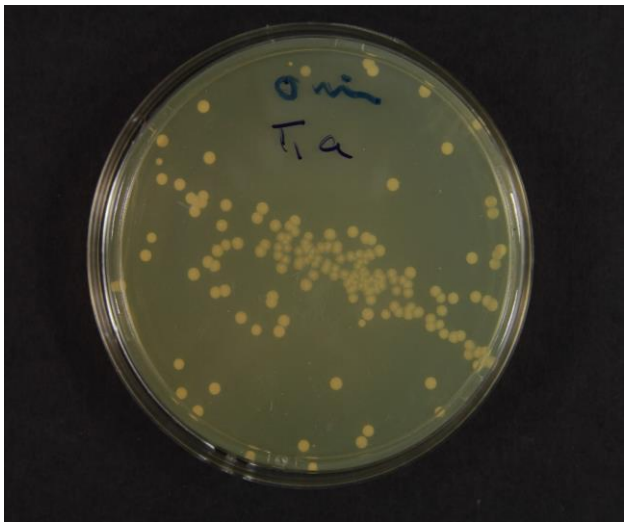

Figure 19. Test sample 1 at 0 minutes showing initial microorganism growth seeded on agar plate (agar plate labelled 0 min T_1a).

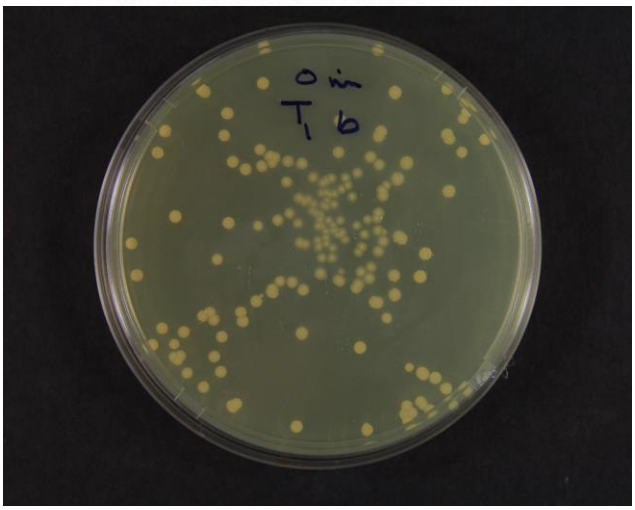

Figure 20. Test sample 2 at 0 minutes showing initial growth seeded on agar plate (agar plate labelled 0 min T_1b).

Figure 21. Control sample at 30 minutes showing microorganism growth seeded on the agar plate (agar plate labelled 30 min Control).

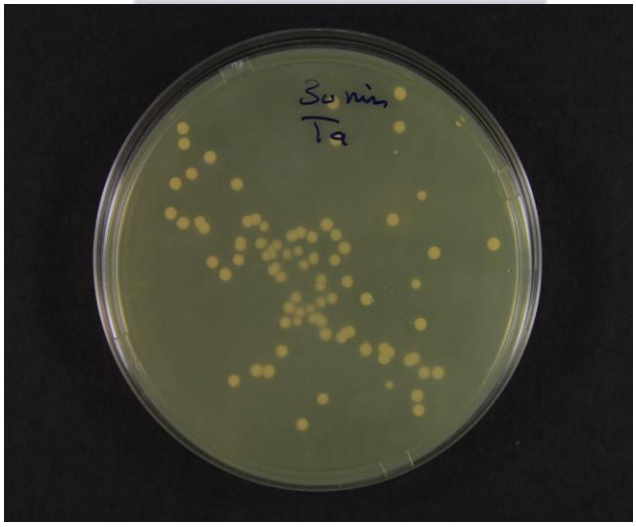

Figure 22. Test sample 1 at 30 min showing microorganism growth on the agar plate (agar plate labelled 30 min Ta).

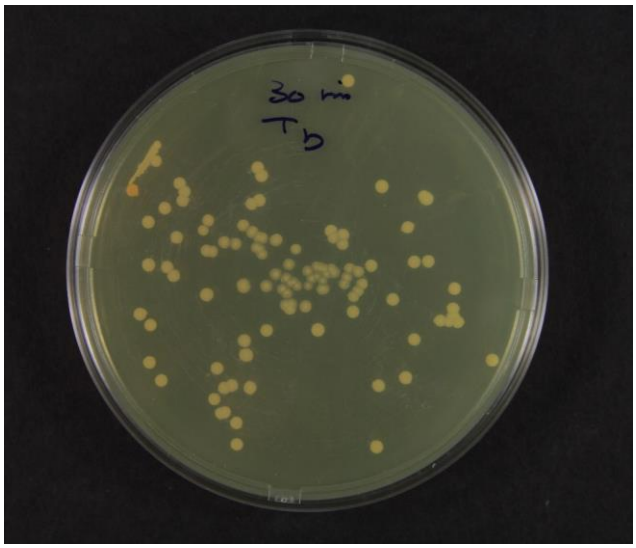

Figure 23. Test sample 2 at 30 minutes showing microorganism growth on the agar plate (agar plate labelled 30 min Tb).

Figure 24. Control sample at 1 hour showing microorganism growth on the agar plate (agar plate labelled 1 hour Control).

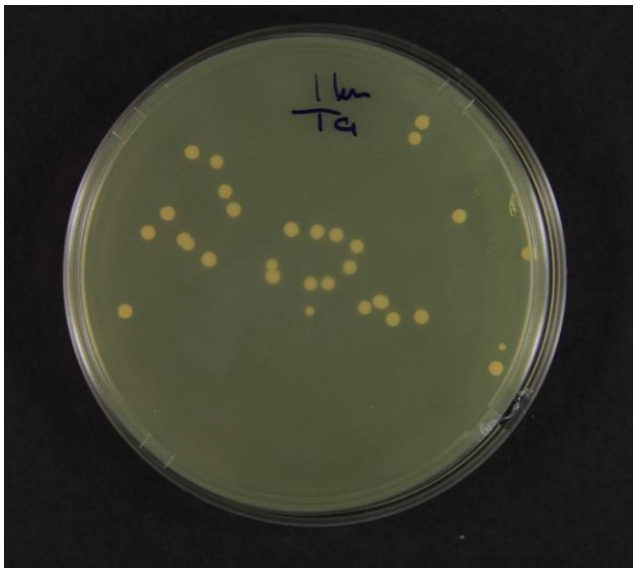

Figure 25. Test sample 1 at 1 hour showing microorganism growth on the agar plate (agar plate labelled 1 hour Ta).

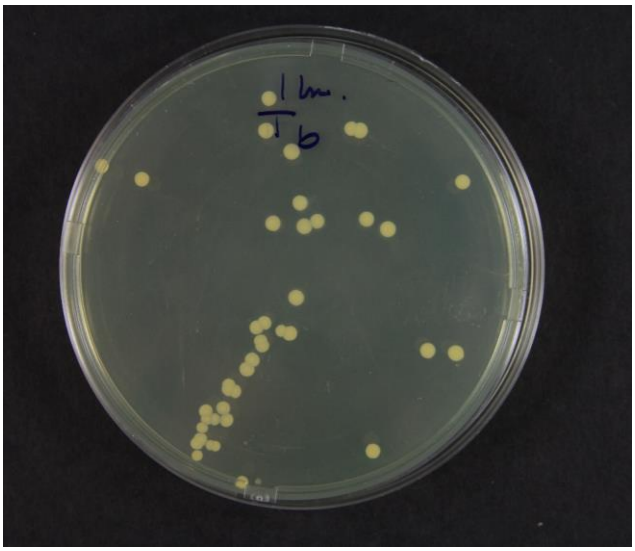

Figure 26. Test sample 2 at 1 hour showing microorganism growth on the agar plate (agar plate labelled 1 hour Tb).

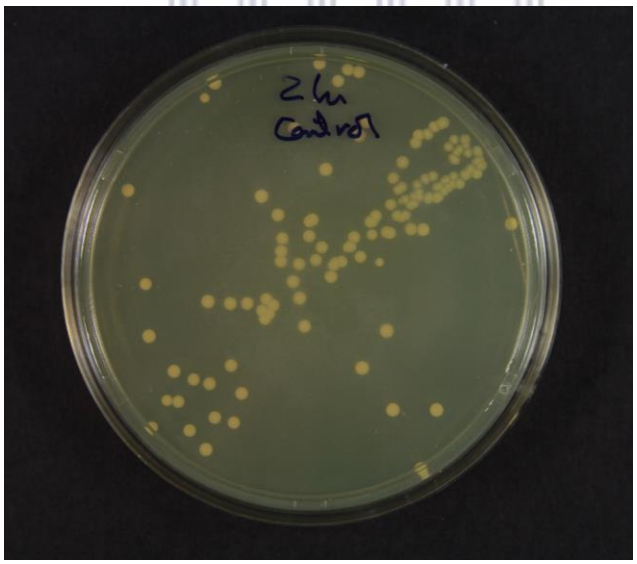

Figure 27. Control sample at 2 hour showing microorganism growth on the agar plate (agar plate labelled 2 hour Control).

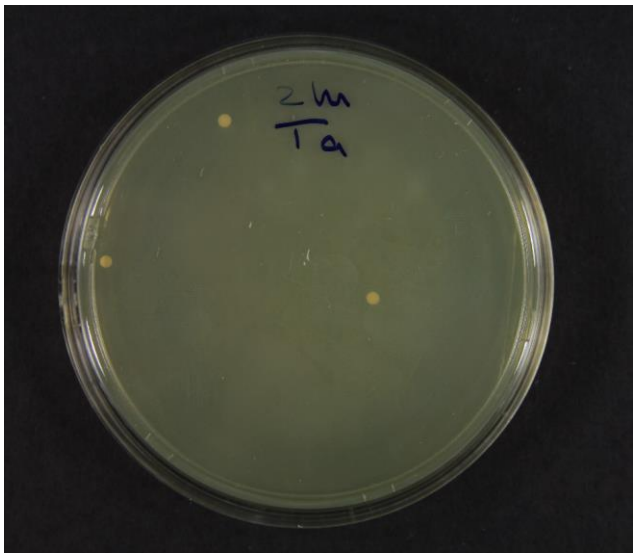

Figure 28. Test sample 1 at 2 hours showing microorganism growth on the agar plate (agar plate labelled 2 hour Ta).

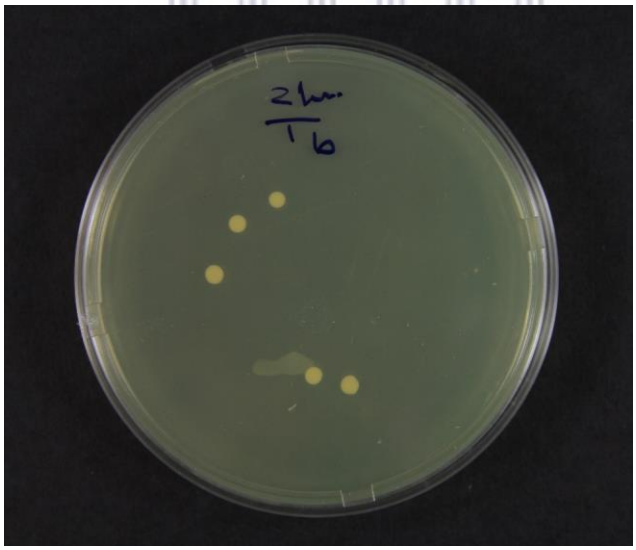

Figure 29. Test sample 2 at 2 hours showing microorganism growth on the agar plate (agar plate labelled 2 hour Tb).

Figure 30. Control sample at 4 hour showing microorganism growth on the agar plate (agar plate labelled 4 hour Control).

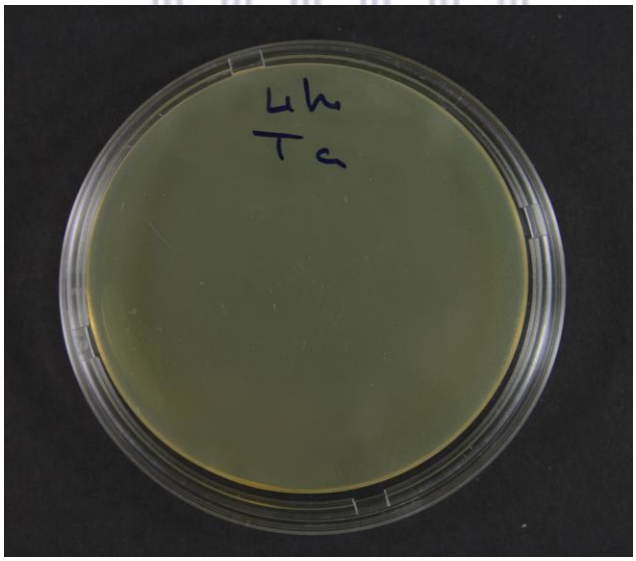

Figure 31. Test sample 1 at 4 hours showing microorganism growth on the agar plate showing complete elimination of bacteria (agar plate labelled 4 hour Ta).

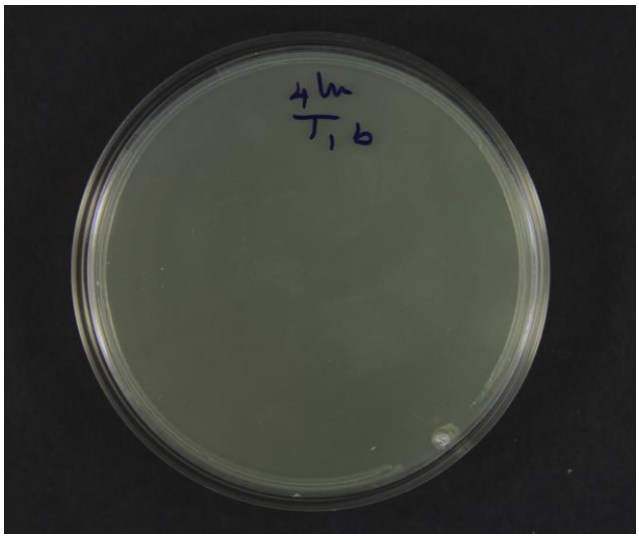

Figure 32. Test sample 2 at 4 hours showing microorganism growth on the agar plate showing complete elimination of bacteria (agar plate labelled 4 hour T1b).

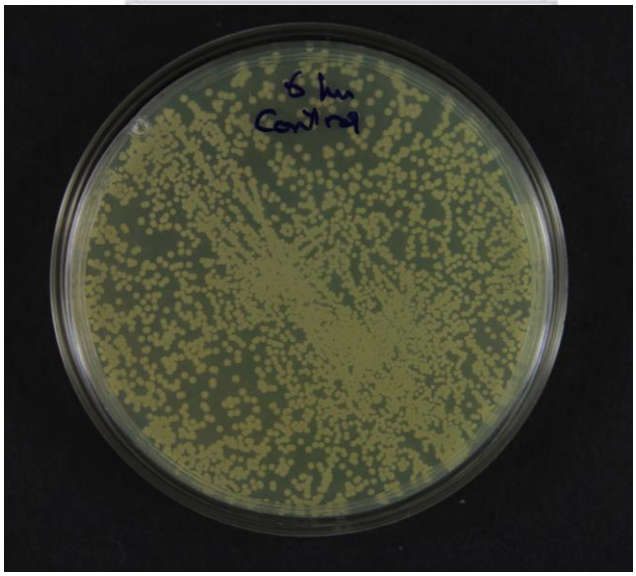

Figure 33. Control at 6 hours showing bacterial growth which is too numerous to count (agar plate labelled 6 hour Control).

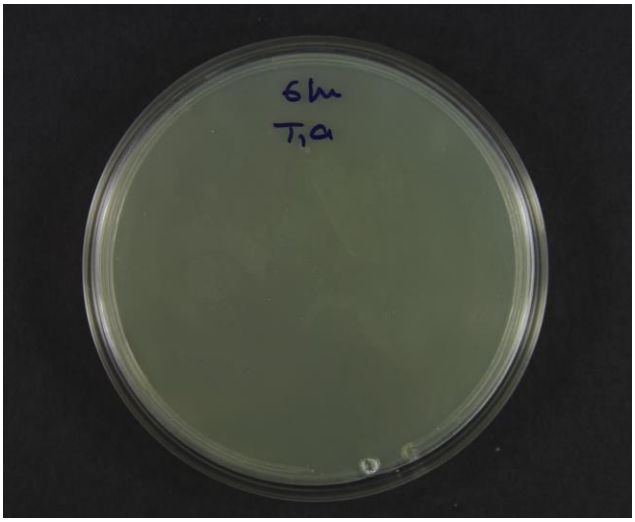

Figure 34. Test sample 1 at 6 hours showing microorganism growth on the agar plate showing complete elimination of bacteria (agar plate labelled 6 hour T1a).

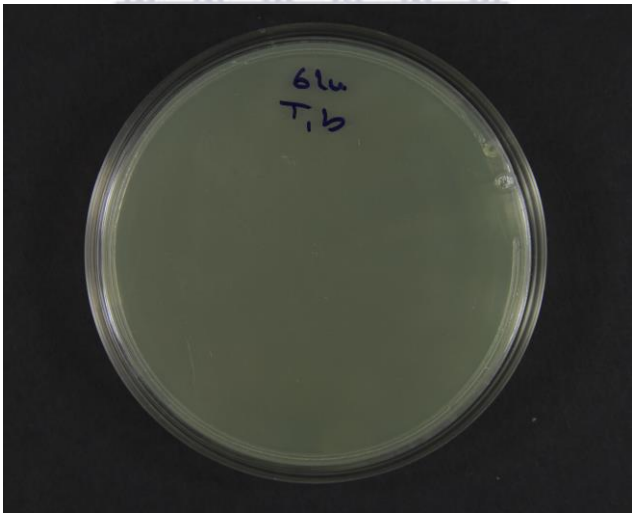

Figure 35. Test sample 2 at 6 hours showing microorganism growth on the agar plate showing complete elimination of bacteria (agar plate labelled 6 hour T1b).

Figure 36. The control at 8 hours had complete bacterial growth which is too numerous to count (>300).

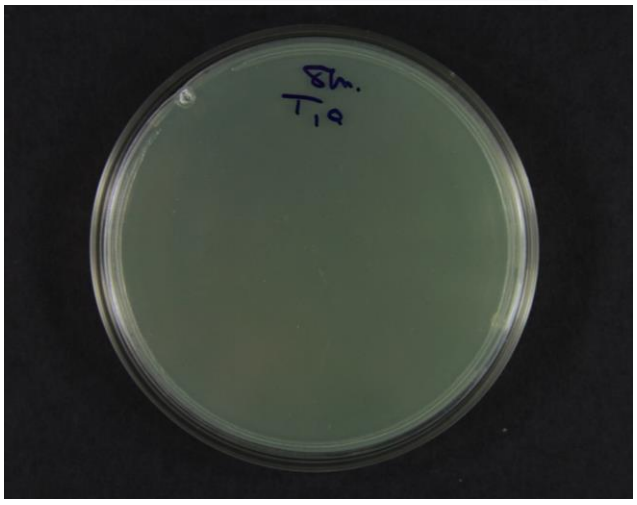

Figure 37. Test sample 1 at 8 hours showing microorganism growth on the agar plate showing complete elimination of bacteria (agar plate labelled 8 hour T1a).

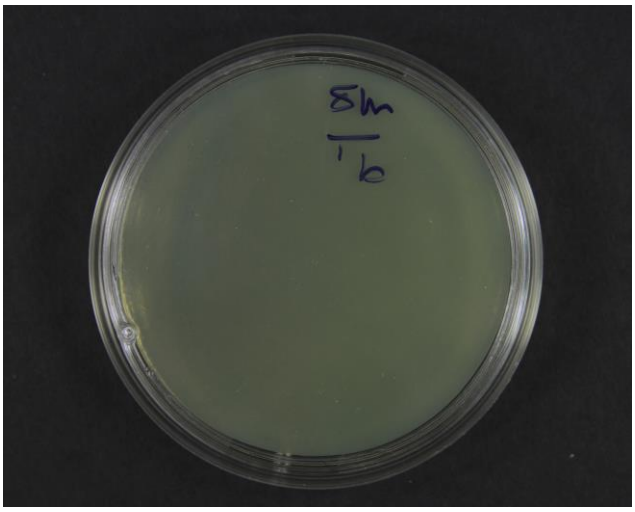

Figure 38. Test sample 2 at 8 hours showing microorganism growth on the agar plate showing complete elimination of bacteria (agar plate labelled 8 hour Tb).

The number of viable microorganisms present in the test sample i.e. chitosan modified cement was plotted and compared to the number of viable cells in the control group i.e. unmodified cement and plotted against the various time intervals (Figure 39). For the test sample i.e. chitosan modified cement, the graph (Figure 39) shows from baseline 0 minutes a steady decline in microorganism count where they become completely eradicated from 2 hours to 8 hours final count.

For the control sample i.e. cement without chitosan, the microorganisms showed an increase in number from baseline to 8 hours. This indicates that the chitosan modified cement killed bacteria in culture. In the control sample the drop in cell counts noted at 1 hour time interval could possibly be explained by the acclimatization of the

microorganisms to the environment and a drop in the cell numbers. Once acclimatization of the microorganisms to the environment was established the cell counts show a steady growth and this may be typical of cell growth curves of cells in culture.

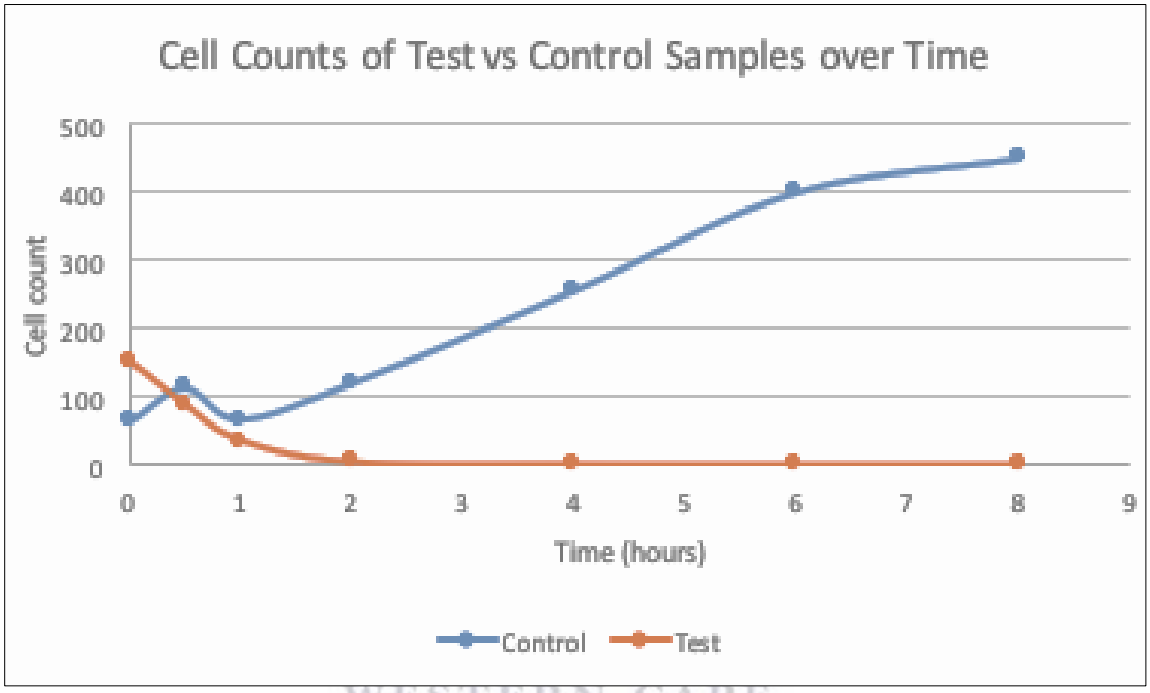

Figure 39. Graph showing mean cell counts of agar plates exposed to control versus test of cell counts of agar plates exposed taken at various time intervals.

2. Shear Bond Strength

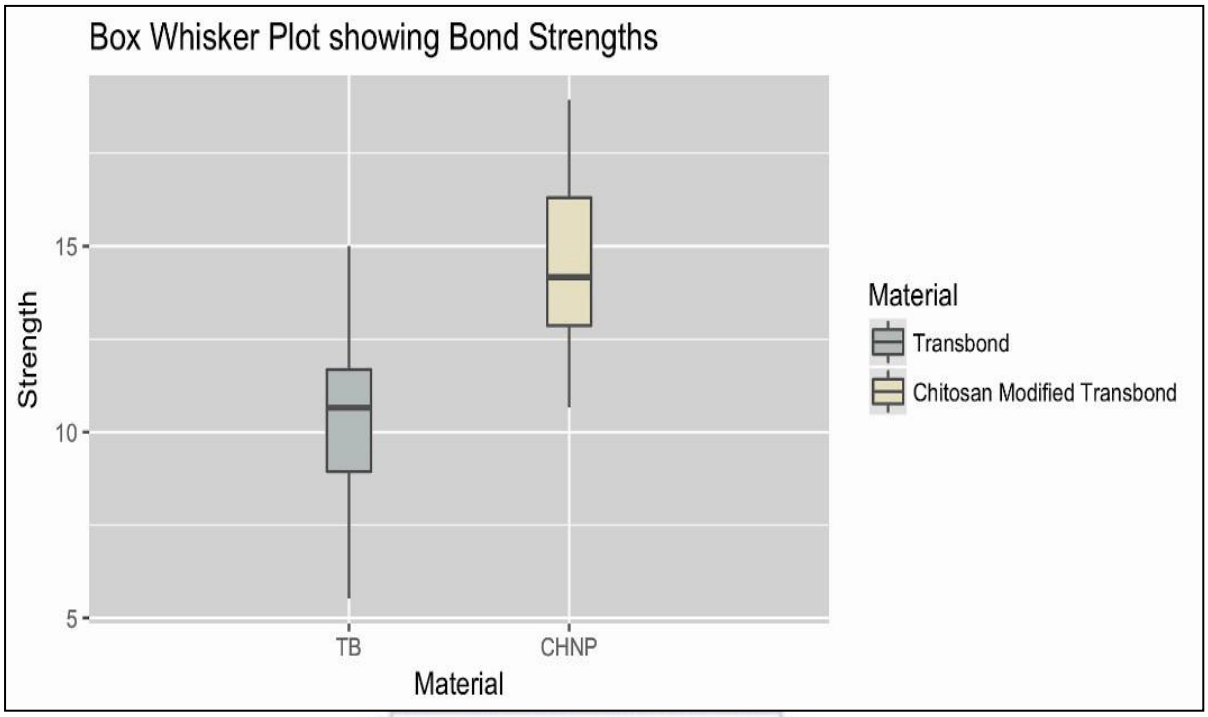

Figure 40. Box Whisper Plot showing the distribution of shear bond strength data in MPa including the median, maximum, minimum, upper and lower quartiles of the shear bond strength values for unmodified Transbond (TB) versus chitosan modified Transbond (CHNP).

Figure 40 shows the distribution of shear bond strength values obtained in the two groups. Chitosan modified Transbond (CHNP) had mean shear bond strength value of 14.5 MPa which was significantly higher than the unmodified Transbond cement with a mean shear bond strength of 10.2 MPa ($p < 0.05$, Wilcoxon Rank Sum Test).

The mean shear bond strength of Transbond cement showed a wider distribution of the data compared to chitosan modified cement indicating that the addition of chitosan not only produced a higher shear bond strength but also showed a narrower distribution of the data set.

When the upper half of the data sets *i.e.* the set of all values above the median values for both the test and the control samples were compared, the test samples showed higher distribution of values above the median.

3. Adhesive Remnant Index (ARI)

Artun and Bergland (1984), used an Adhesive Remnant Index (ARI) system to evaluate the amount of adhesive left on the tooth after de-bonding (Montasser and Drummond, 2009).

Table 1. Table showing ARI Scores 0, no adhesive on the tooth; 1, less than 50% adhesive on the tooth; 2, more than 50% adhesive on the tooth, and 3, all adhesive remained on the tooth.

	Adhesive Remnant Index Scores			
	0	1	2	3
Group 1 (Control)	4	14	2	-
Group 2 (Test)	-	18	2	-

.

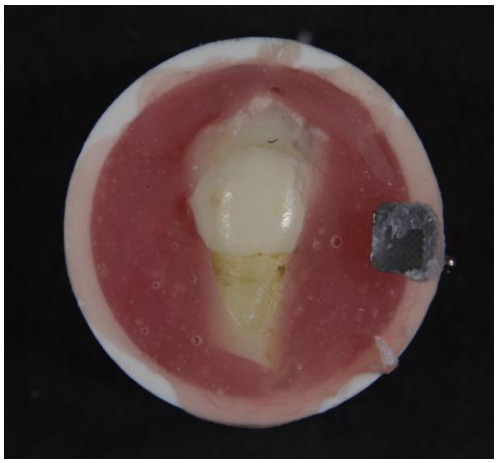

Figure 41. Picture showing the adhesive remaining on the tooth surface after bracket was broken off using the Control cement. The bracket is shown adjacent to the tooth.

Figure 42. Picture showing a bracket that was broken off, with the Control cement remaining on the bracket with an ARI score of 1.

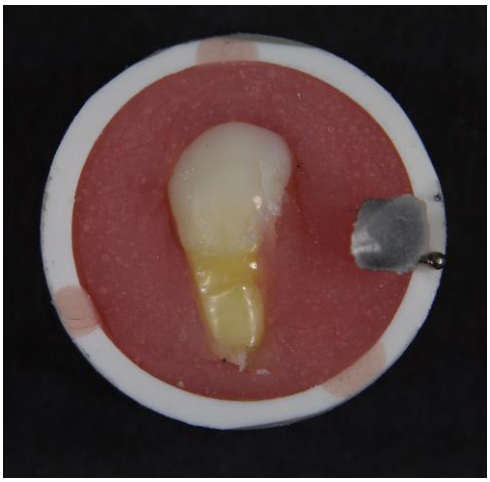

Figure 43. Picture showing the adhesive remaining on the tooth surface after bracket was broken using the Test cement. The bracket is shown adjacent to the tooth.

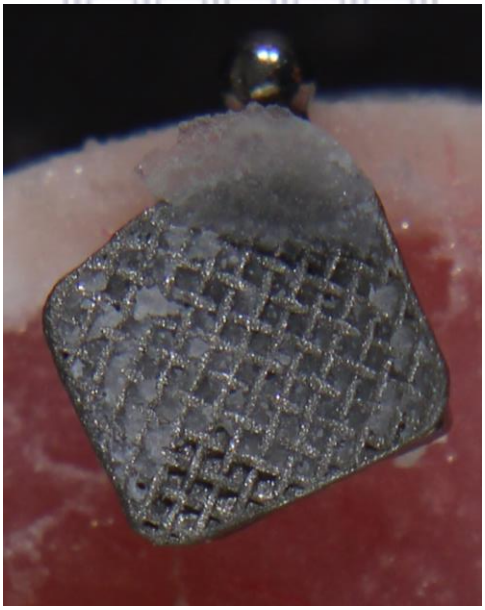

Figure 44. Picture showing a bracket broken off from the tooth using the Test cement with an ARI of 1.

The ARI scores showed only 4 specimens from the control group showed presence of cement on the bracket and nothing on the tooth surface *i.e.* a score of 0. The majority of the specimens for both the control and test group showed 50% cement remaining on the tooth surface *i.e.* a score of 1. Two specimens each from both groups showed more than 50% cement on the tooth surface *i.e.* a score of 2. There was no specimens showing 100% cement remaining on the tooth surface *i.e.* score of 3 (Table 1).

4. Surface Hardness

Although the mean micro-hardness of chitosan modified cement was lower than the unmodified orthodontic cement, there was no statistically significance difference (Mann-Whitney Test, $p>0.05$). When the medians were compared, the median surface hardness of the top surface of chitosan-modified cement (Figure 45) was 47.61 (95% confidence interval for mean-upper bound 58.26; lower bound 36.95). The median surface hardness of the top surface of unmodified orthodontic cement was 43.22 (95% confidence interval for mean-upper bound 50.88; lower bound 35.56).

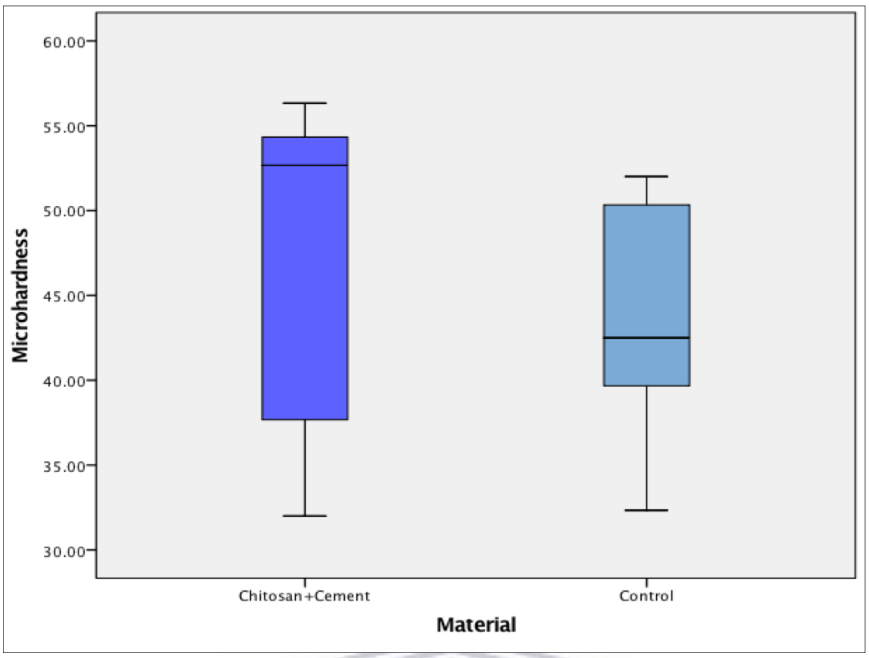

Figure 45. Box plot showing microhardness of chitosan modified Transbond cement compared to microhardness of unmodified Transbond (control).

5. Cytotoxicity

When the mouse fibroblast cells were exposed to the test sample *i.e.* the chitosan modified Transbond (Figure 46), there was in increase in the cells numbers (114.8%) compared to the controls.

Bar Chart showing Cell Viability

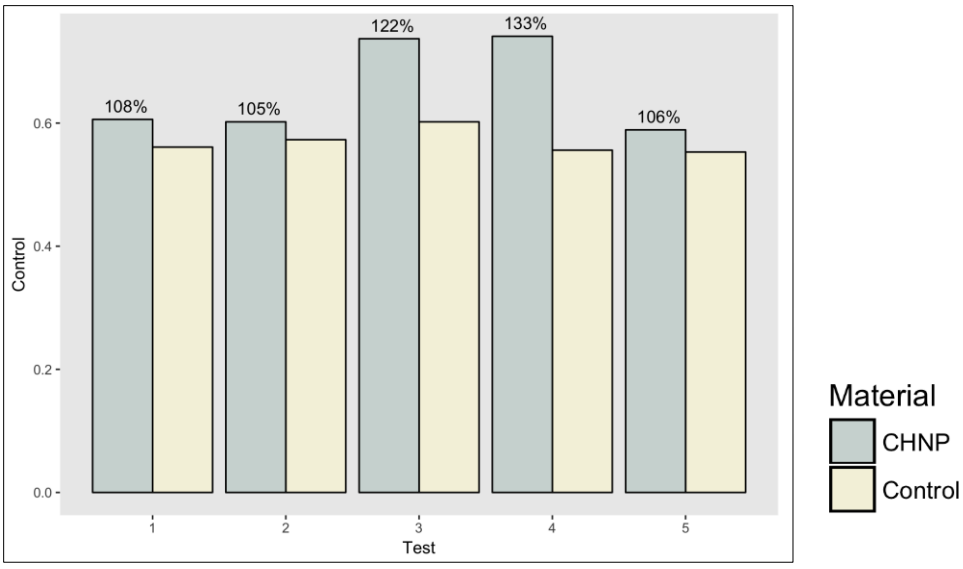

Figure 46. Graph showing increase growth of cells when cultured were exposed to Test (CHNP) compared to Control (unmodified Transbond cement).

6. Scanning electron microscopy analysis

Chitosan nanoparticles were observed under scanning electron microscopy at 1000, 5000 and 10 000 times magnification (Figures 47, 48, 49). Particles appear to be spherical in shape with almost evenly distributed depressions on the surface of the particles.

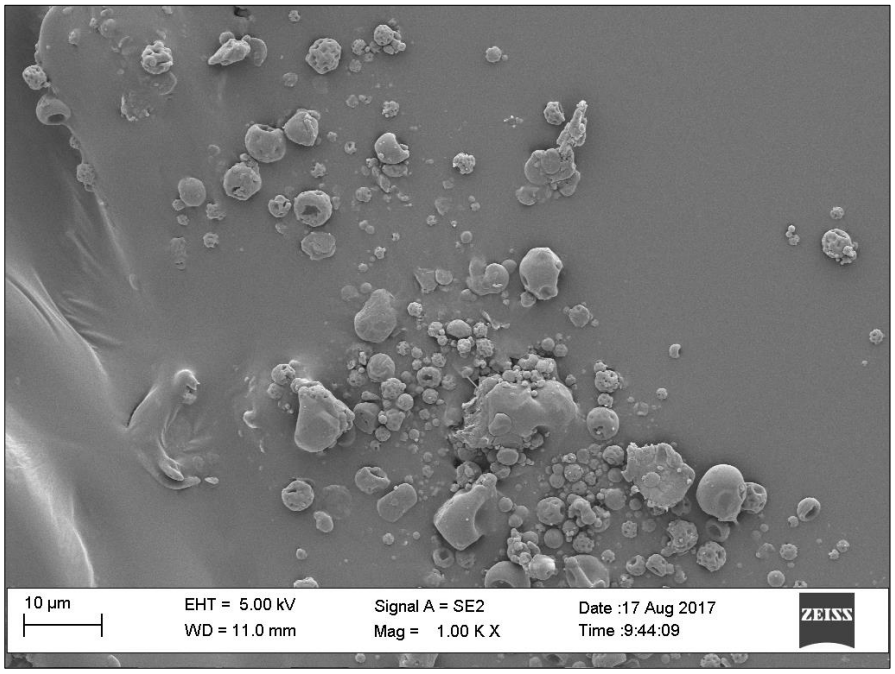

Figure 47. Scanning electron image of chitosan at 1000 magnification.

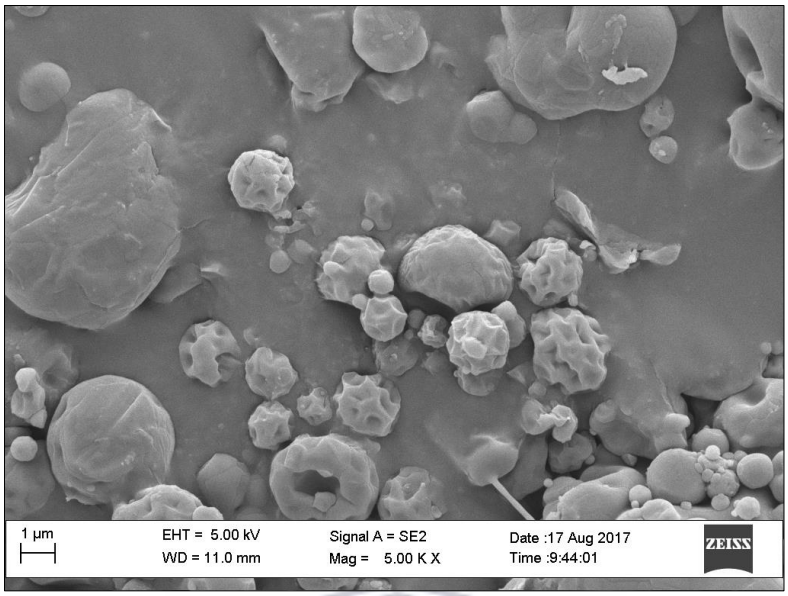

Figure 48. Scanning electron image of chitosan at 5000 magnification

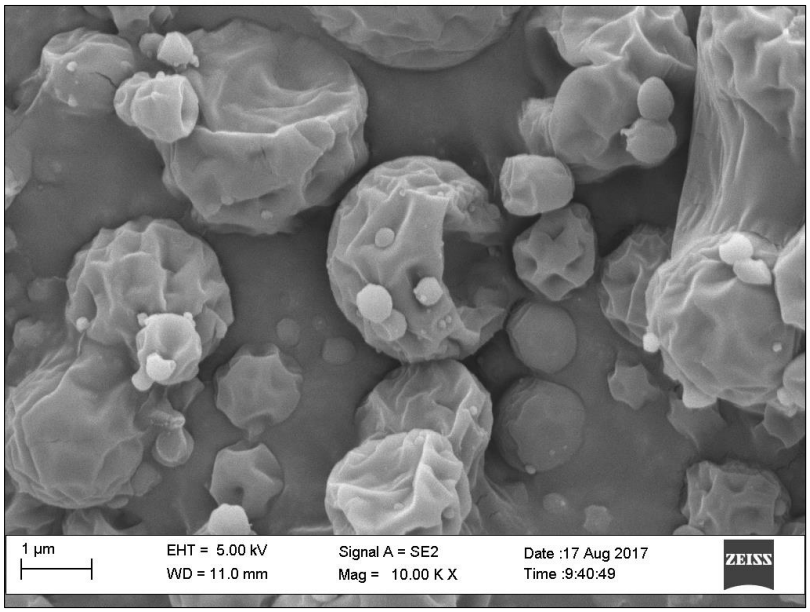

Figure 49. Scanning electron image of chitosan at 10000 magnification

CHAPTER V

Discussion

The evolution of Dentistry, is to a large extent, closely linked to advancements in dental materials. The introduction of dentine bonding agents saw dentistry catapult from GV Black's radical approach to cavity design to a more conservative, minimally invasive approach. Most of these so called synthetic materials are relatively inert materials. However, recently, there is an increased interest in studying the interactions between dental materials and tooth tissues that can promote bioactivity. Recently, with the introduction of bioactive materials there has been another shift in dental material science. Dental material science is in the midst of a major transition in terms of refocusing and embracing new and exciting biological technologies and might be viewed as the death of conventional dental materials (Bayne, 2005). This new shift in emphases from traditional synthetic materials toward options that involve truly biologically active materials is a field of much research in dental material science.

In 1969, Hench introduced the concept of bioactivity as "a bioactive material is one that elicits a specific biological response at the interface of the material which results in the formation of a bond between the tissues and the material" (Asthana and Bhargava, 2014). Biomaterial is therefore, any matter, surface or construct that interacts with biological systems onto which it is placed. The ideal properties of bioactive materials should be

bactericidal and bacteriostatic, sterile, stimulate dentine formation and maintain pulp vitality (Asthana and Bhargava, 2014).

This trend of studying bioactivity and biomaterials has found its way into dentistry as well. In dentistry, thus far, much of the focus on bioactive materials have been on regenerative procedures especially in the field of endodontics with materials like MTA (Dentsply, USA) and Biodentine (Septodont, USA) aimed at regeneration of dentine, for example repair of pulpal exposures. In restorative dentistry, a modification in bioactive chemically bonded cements has been introduced in the form of a calcium aluminate glass ionomer luting cement (Ceramir, Doxa Dental, USA).

Historically, orthodontic cements are inert and mainly meant to attach brackets to either bands or tooth structure. In the early days of fixed-appliance orthodontic treatment, orthodontic brackets were welded to gold or stainless-steel bands. Many developments have occurred in the decades that followed, including many newer adhesives, newer bracket materials, faster or more efficient curing methods, self-etching primers, fluoride-releasing agents, and sealants (Gange, 2015). These newer cements and bracket showed improved bonding to tooth structure. However, these developments failed to address the one major drawback of orthodontic brackets *in vivo,* and that is, the accumulation of plaque around these brackets which can lead to demineralization of the enamel upon which the bracket is cemented onto. Therefore, the purpose of this study was to address this concern of the untoward effects of orthodontic cementation of brackets with its

resultant demineralization and white spot lesions by modifying an existing orthodontic cement to a bioactive cement.

In this study, the addition of a naturally occurring cationic biopolymer, chitosan to an existing orthodontic cement (Transbond) was aimed at converting an inert cement into a novel bioactive orthodontic cement.

Biopolymers are preferred over synthetically derived polymers in regenerative medicine and dentistry because of their structural similarities with the extracellular matrix, chemical versatility, and better biocompatibility (Dutta, 2016). The International Union of Pure and Applied Chemistry (IUPAC) defines biopolymers as "macromolecules formed by living organisms". The four important classes of biopolymers are: (a) polysaccharides; (b) proteins (c) lipids and (d) specialty polymers (McNaught and Wilkinson, 2014). Chitosan belongs to the polysaccharide group of biopolymers. Chemically, chitosan is a polymeric biopolymer comprising of N-acetylglucosamine and glucosamine copolymer units.

These naturally formed biopolymers have major advantages: biodegradability, biocompatibility, non-toxicity, cost-effectiveness, abundance, renewability, greenness and immunogenicity (Thandapani *et al.,* 2017). Among the biopolymers, chitosan a polysaccharide, offers remarkable biological properties, which have paved the way for its applications in the pharmaceutical and biomedical fields, particularly in new drug delivery systems (Dutta *et al.,* 2012; Rani *et al.,* 2010; Thandapani *et al.,* 2017). Chitosan is safe,

non-toxic and biocompatible polymer therefore can be used in medical and pharmaceutical applications. It is also approved by Food and Drug Administration (USA) for wound dressing (Thandapani et al. 2017).

Adding chitosan on its own to the Transbond cement will make the cement rough, as the particle size of the chitosan is fairly large. Although, the lowest molecular weight chitosan commercially available *i.e*. 50 -190 KDa was used in this study, this still presented with a challenge of large molecular size particle. Therefore, the chitosan was first converted to chitosan nanoparticles and then incorporated into the Transbond orthodontic cement. This was meant to ensure that nanoparticle size chitosan added to Transbond would not affect the surface roughness as a larger particle size may make the cement more susceptible to staining and plaque accumulation.

This utilization of nanoparticle size chitosan into the dental cement merges the fields of material science and biology. Nanoparticles hold large surface area to volume ratio which means larger surface area for higher bonding capacity. The application of larger or macro size molecules in therapy is frequently hindered by stability and/or permeation issues (Grenha, 2012). Nanoparticles have potential to easily conjugate with biomolecules and have wide application in drug delivery systems and in medicine (Rather *et al.,* 2013). Nanotechnology, nowadays is a commonly used buzzword in numerous fields of science. In the health field, the introduction of nanotechnology gave rise to a novel concept 'nanomedicine'.

Nanotechnology is the branch of technology that deals with dimensions of nanometer size particles, especially the manipulation of individual atoms and molecules.

A variety of nanofillers have already been used in dentistry, especially in composite resin cements. 3M ESPE uses a sol-gel technology to produce tiny nanospheres which are agglomerated into nanoclusters, and either the spheres or clusters can become filler particles for composite resins (3M Technical Data, 2014). These filler particles are inert and act more to strengthen the dental composite. These particles do not chemically bond to the resin composite and hence, has to be coated with organosilanes. The inclusion of chitosan nanoparticles into Transbond cement acts as "nanofillers" in the composite resin cement but not in the traditional sense of filler particles in composites which are inert. Chitosan on the other hand is a bioactive biopolymer that forms polymer chains with the resin cement.

Chitosan nanoparticles has the advantage in that it can form a chemical network within the composite resin. Possible explanation for this is through the cationic polymerization, a type of chain growth polymerization in which a cationic initiator transfers charge to a monomer which then becomes reactive. This reactive monomer goes on to react similarly with other monomers to form a polymer. Interactions of this kind has been applied to build up polymer/polymer ionic complexes involving chitosan and negatively charged polymers.

Traditional composite resins, on the other hand, polymerize through free-radical polymerization by which polymers form by the successive addition of free-radical building blocks. Free radicals in composite resins requires the addition of separate initiator molecules like champhorquinone (Anusavice *et al.,* 2013). Owing to the cationic polymerization of the chitosan, the chitosan nanoparticles within the newly formed orthodontic cement may behave as one unit rather it simply being "inert" filler particles within the cement. Behaving a "single unit" may account for its improved physical properties in this study.

In this study, chitosan nanoparticles were produced through tripolyphosphate (TPP) ionic gelation method. Several methods have been developed to convert chitosan to chitosan nanoparticles which include emulsion-droplet coalescence, emulsion solvent diffusion, reverse micellar method, ionic gelation, polyelectrolyte complexation, spray-drying, template polymerization, precipitation and ionotropic gelation method (Grenha 2012; Thandapani *et al.,* 2017). In this study, the ionic gelation was used to produce chitosan nanoparticles and this method involves an ionic interaction between the positively charged amino groups of chitosan and the polyanion tripolyphosphate, which acts as chitosan cross-linker. Sodium tripolyphosphate (TPP) crosslinker is generally used for preparing chitosan nanoparticles. Sodium triphosphate or sodium tripolyphosphate is an inorganic compound with formula $Na_5P_3O_{10}$ (McNaught and Wilkinson 2014). The polyphosphates are hydrolyzed into simpler phosphates, which is similar to adenosine triphosphates or

ATP, that is present within living cells (Thandapani *et al.,* 2017). Thus, the toxicity of polyphosphates is low and it has neither mutagenic or carcinogenic effects nor adverse reproductive effects (Thandapani *et al.,* 2017).

Nanoparticle formation takes place immediately after the addition of a TPP solution to a solution of chitosan (Figure 50). The production of nanoparticles by ionic gelation results in smaller particles for higher amounts of cross-linker (Grenha *et al.*, 2005; Teijeiro-Osorio *et al.,* 2009).

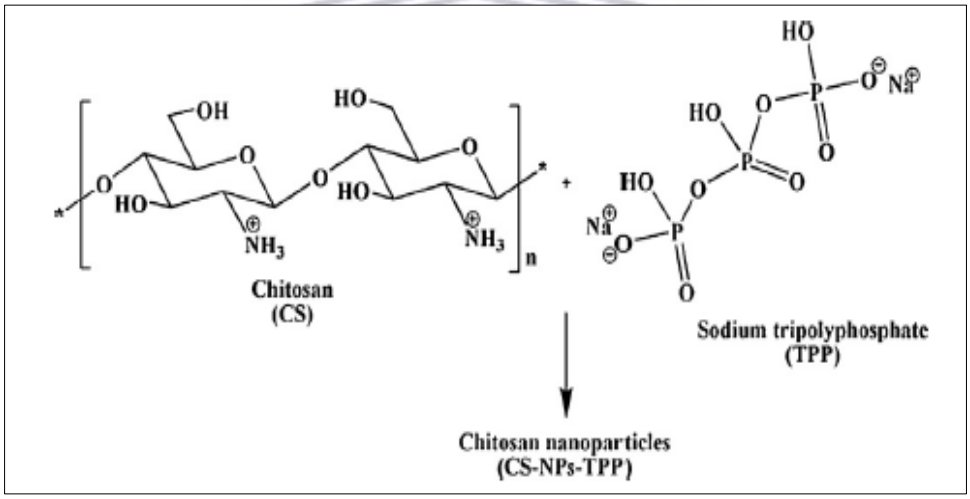

Figure 50. Preparation of chitosan nanoparticles (CS-NPs) using sodium tripolyphosphate (TPP) from chitosan (CS) (Thandapani *et al.*, 2017).

Once the chitosan nanoparticles were formed and incorporated into the cement, the question then arises, does the chitosan still maintain its biological properties within this newly formed resin cement?

1. *Antimicrobial effects of chitosan modified orthodontic cement*

Several bioassays for antimicrobial screening methods are available such as disc diffusion, well diffusion, broth dilution or agar dilution. Methods such as flow cytofluorometric and bioluminescent methods are not widely used because they require specified equipment and their reproducibility and standardization still needs to be evaluated (Balouiri *et al.*, 2016). The agar disc diffusion method to determine the Minimum Inhibitory Concentration (MIC), is not appropriate as it is not possible to quantify the amount of the antimicrobial agent diffused into the agar medium (Balouiri *et al.,* 2016). In this study, because the actual antimicrobial effect of the chitosan needed to be quantified by counting the remaining colony forming units, the agar dilution method was used. The protocols as provided by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for this method was followed (Kahlmeter *et al.,* 2006). These guidelines provide a uniform procedure for testing that is practical to perform in most clinical microbiology laboratories. The agar dilution method is the most appropriate for the determination of minimum inhibitory concentration values, since they offer the possibility to estimate the concentration of the tested antimicrobial agent in the agar (Balouiri *et al.,* 2016). This assay does allow the bioassay to be performed in a standardized approach in order to evaluate the clinical relevance of results (Pfaller *et al.,* 2004).

In this study, all the test samples exposed to chitosan modified Transbond showed no bacterial growth after 8 hours while the control showed an increase in bacterial growth

from baseline to 8 hours. Compared with the unmodified Transbond, the chitosan modified Transbond had higher antibacterial activity showing that the addition of chitosan to Transbond renders the cement antibacterial. Complete eradication of the bacteria was noted following 4 hours of exposure to the chitosan (Figure 31 & 32).

When the bacterial growth curves (Figure 39) were compared, the control group showed a steady increase in cell growth from baseline to final count at 8 hours. However, in the control group there was an initial drop in cell numbers after 30 minutes. This may be explained by the fact that the microorganisms needed to establish and acclimatize themselves to the new environment. The normal growth pattern was then followed after this initial adjustment period and growth occurred from 1 hour to final count at 8 hours. This steady growth in cell numbers in the control group may indicate that the Transbond cement does not possess any antibacterial properties. When the test group growth pattern was studied it showed a steady decline in cell numbers from baseline to final count at 8 hours. Complete eradication of the bacteria was noted after 4 hours of exposure to the chitosan modified cement. This decline in the cell numbers in the test group indicates that the newly formed chitosan modified Transbond orthodontic cement possesses sufficient antibacterial properties to eradicate *Streptococcus mutans* in-vitro.

Several mechanisms explaining antimicrobial activity of chitosan has been postulated. The most acceptable mechanism is the interaction between the positively charged chitosan molecules and negatively charged microbial cell membranes (Perchyonok *et al.,* 2015).

This interaction is mediated by the electrostatic forces between the protonated NH_3+ groups of chitosan and the electronegative charges of the microbial cell surfaces (Perchyonok *et al.,* 2015). Chitosan can also penetrate the bacterial cell membrane and then bind to the DNA, inhibiting its transcription and mRNA synthesis. Another alternative hypothesis for the antibacterial mechanism of chitosan is thought to be as a result of its ability to bind to the negatively charged bacterial cell membrane, increasing its permeability and ultimately resulting in leaking of the cytoplasmic contents and bacterial cell death (Qi and Xu, 2004). Chitosan binds to the negatively charged components of the bacterial cell wall forming an impermeable layer and blocking transportation into the cell (Rabea *et al.,* 2003). Others postulated that as chitosan has the ability to chelate metals, microbial growth will be inhibited by reducing enzyme activity through metal chelation (Cuero *et al.,* 1991).

2. Shear bond strength of chitosan modified orthodontic cement

Higher adhesiveness of orthodontic cements is desirable to maintain an intimate contact between orthodontic brackets and enamel thereby avoiding debonding. The purpose of the shear bond strength testing in this study was to determine whether the addition of chitosan to the bonding cement would affect the bond strength of the bracket to the enamel tooth structure.

Transbond XT composite cement was specifically developed for bonding orthodontic brackets to the enamel. According to the manufacturer (3M ESPE), the main advantages offered by this material are: reduced working time, no need for mixing, and good

adhesion to enamel thus being largely used in clinical orthodontics and experimental studies as controls and hence used in this study. When Transbond XT was used alone it showed good bond strength to enamel (10.5 MPa), which is similar to other studies done on Transbond XT (Bishara *et al.,* 2004). When Transbond XT was modified with chitosan in this study the shear bond strength increased further. Thus, the addition of chitosan to the Transbond cement improved its adhesive properties to tooth structure in this study. This improvement in adhesive properties may be explained possibly as a result of the intrinsic biodhesive property of chitosan (Perchyonok *et al.,* 2015). The term "bioadhesion" refers to any bond formed between two biological surfaces or a bond between a biological and a synthetic surface (Perchyonok *et al.,* 2015). The water absorption capacity together with the cationic nature of chitosan which promotes binding to subsurface may be responsible for the higher bond strengths obtained when chitosan was used with Transbond cement in this study. According to Cafaggi *et al.,* (2007), hydration of the polymer causes mobilization of the polymer chains which influences polymeric adhesion (Alaçam *et al.,* 2000). Appropriate swelling is important for increase in adhesivity. However, over hydration can negatively impact on the adhesive property (Battino *et al.,* 2002). Hence, in this study the bonded teeth were stored in distilled water for 24 hours prior to testing to ensure that sufficient hydration took place prior to testing and that there was no negative effect of this hydration as this may more closely mimic the oral environment. A further advantage of addition of chitosan lies in the molecular arrangement of the polymeric chains that can interact further with the substrate (Perchyonok *et al.,* 2015) increasing adhesion to the substrate. Due to the presence of

amine groups in its structure, chitosan is converted to a polyelectrolyte in acidic media. Since many minerals and cells carry negative charges, the positive charge of chitosan interacts strongly with these negatives surfaces (Kaş, 1997).

Chitosan can bind to many materials such as cholesterol, fats, proteins and tumor cells. It has also shown an affinity for proteins. Owing to its cationic nature, electrostatic complexes are used for encapsulation of drugs, immobilization of enzymes and as a gene carrier (Kaş, 1997). For instance, fibroblasts which exhibit a more negative charge surface when compared to keratinocytes, exhibit a higher adhesion to chitosan.

When testing shear bond strength of orthodontic brackets, the location of the contact point of the de-bonding force has a significant influence on shear bond strength measurements and bond failure pattern (Klocke and Kahl-Nieke, 2005). Hence it is important to take this parameter into consideration at the start of the study design and it is especially important when comparing results with other *in-vitro* bracket bond strength results (Klocke *et al.*, 2004).

The position of the rod of the Universal Shear Bond Strength machine relative to the bracket can be positioned in 3 different contact areas as shown in Figure 51. The contact area where the force can be applied to the bracket base can be in any of these 3 locations:

1. close to the enamel/adhesive interface
2. force applied to the ligature wire groove between bracket base and wings
3. force applied to the occlusal bracket wings

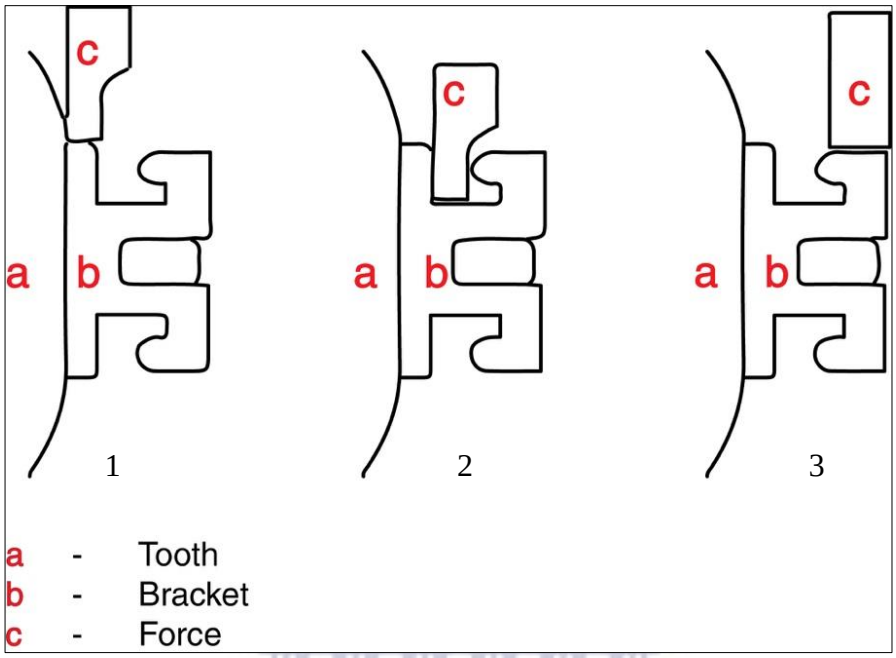

Figure 51. Diagram showing the direction of force application during de-bonding at various positions: 1. close to the enamel/adhesive interface, 2. force applied to the ligature wire groove between bracket base and wings and 3. force applied to the occlusal bracket wings. a represents the tooth surface on which the bracket b is bonded onto while c represents the position of the tip on the bracket.

In this study, the force was applied to the area close to the enamel/adhesive interface as in the clinical situation this will be where actual breakage may occur. The further the force applied from the interface, lower is the bond strength (Klocke and Kahl-Nieke, 2006). Using a similar force application point as this study, Klocke and Kahl-Nieke (2006) showed similar results to this study in which a mean shear bond strength of 11.52 MPa

was obtained compared to 10.2 MPa obtained in this study. This study further showed that the inclusion of chitosan to the orthodontic cement improved its adhesive properties.

3. Adhesive remnant index (ARI)

The term de-bonding typically suggests the removal of orthodontic brackets as well as the remaining adhesive/cement from the enamel of the tooth surface. Numerous studies in the literature give several procedures for removal of remaining adhesive and their consequences on the enamel surface (Oliver, 1988). Throughout the years, Adhesive Remnant Index or ARI scores have been one of the most commonly assessed components in research on orthodontic adhesives and quantifies the amount of remaining adhesive either on the bracket or on the tooth surface. Since the adhesive remnant score system is qualitative and subjective, various efforts have been made to adjust the original system, or to improve different quantitative approaches that can be used to more precisely evaluate the adhesive remnant (Montasser and Drummond, 2009). To more accurately assess the adhesive remnant qualitatively, several studies expanded the ARI system that was developed by Artun and Bergland (1984) including 5 or 6 scales (Uysal and Sisman, 2008). According to O'Brien *et al.* (1988), research designed to present a more accurate system for characterizing the resin remnant; this study used a quantitative method whereby a magnified image of the enamel is digitized and the amount of remaining resin is expressed as a percentage of bracket base area (Montasser and Drummond, 2009).

To precisely score the ARI is essential as it is a significant aspect to be investigated in

the variety of orthodontic adhesive (Montasser and Drummond, 2009). Precise assessment of the adhesive remnant, which is critical in the conclusive procedure of enamel clean up after de-bonding, is required for acceptable removal and restoration of the enamel surface to as close to pre-treatment condition as possible (Montasser and Drummond, 2009). Majority of laboratory analyses on the bond strength of orthodontic brackets have examined teeth and brackets under 10x magnification to evaluate and score the adhesive remnant (Uysal and Sisman, 2008), although laboratory studies aimed for assessment of the enamel surface after de-bonding and cleaning of the surface have used more sophisticated methods such as scanning electron microscope, finite element analysis, and 3-dimensional profilometry (Chen *et al.*, 2008). Clinically, evaluation of the adhesive remnants on the enamel surface after bracket de-bonding and enamel cleaning usually is done by visual assessment under a dental operating light (Montasser and Drummond, 2009).

There are many variations of the ARI scoring. According to Shamsi *et al.* (2006), the ARI scoring system is as follows:

Score 0 = more than 75% of adhesive was left on tooth;

Score 1 = 75% of adhesive left on tooth;

Score 2 = 50% of adhesive left on tooth;

Score 3 = 25% of adhesive left on tooth;

Score 4 = less than 25% of adhesive left on tooth; and

Score 5= no adhesive left on the tooth image.

The modified ARI was extended from the original ARI that considered adhesive remaining on the tooth surface (Artun and Bergland, 1984). The original index system (described earlier) was introduced on the basis of studies on de-bonding of extracted teeth. The ARI score based on Artun and Bergland (1984) classification was used in this study to evaluate the adhesive remaining on the tooth structure.

Even though the variations in adhesive remnant scores suggest the bonding strength, adhesive systems that show less residual resin may be preferable because they are easier to remove and safer to clean up from the enamel surface after de-bonding procedures (Oz *et al.,* 2014). As adhesive resin tags infiltrate the enamel surfaces, reaching depths up to 50 μm, this may apply irreparable damage to the enamel surfaces once orthodontic brackets have been removed (Eminkahyagil *et al.,* 2006). Thus, an assessment system to evaluate the adhesive remnant could be helpful for researchers as well as clinicians. If adhesive remnants are not adequately detected, ARI scores could be inaccurate (Oz *et al.*, 2014).

During bracket removal, bond failure can occur at the adhesive-enamel interface or at the adhesive-bracket interface (adhesive failure), or within the adhesive (cohesive failure) (Bonetti *et al.,* 2011). Usually, bracket failure is a combination of adhesive and cohesive failures, the latter resulting in retention of material on the enamel and bracket surfaces (mixed failure). When adhesive failure amongst the adhesive resin and the enamel surface appears, a certain quantity of enamel damage is almost certain because of the

micromechanical bond between the composite resin bonding agent and the acid-etched enamel (Bonetti *et al.,* 2011). An adhesive remnant index score of 0 suggests that bond failure happened at the adhesive-enamel interface, resulting in a greater risk for tooth enamel damage (Bonetti *et al.,* 2011). In this study, 4 specimens from control group received an adhesive remnant index score of 0 where all the cement was present on the bracket. This may not be desirable as cement de-bonding may be a risk for enamel fractures. The majority of the specimens from both the control (70%) and test groups (90%) showed an adhesive remnant index score of 1 (half the amount cement remaining on the bracket), which may indicate bond failure at the bracket-adhesive interface (Bonetti *et al.,* 2011). This may be desirable as there may be minimum risk for enamel damage during de-bonding.

The ARI score system has proved to be of value in studies of orthodontic adhesive systems (Montasser and Drummond, 2009). It is a quick and simple method that needs no special equipment. However, its reliability requires investigation, with special attention on the effects of magnification on evaluation of the adhesive remnant (Montasser and Drummond, 2009). However, Delport and Grobler (1988) have questioned whether the differences in ARI scores indicate a variance in bond strength amongst the enamel and the adhesive for the different adhesive systems, but adhesive systems that display a smaller amount of adhesive remnant on the tooth has been recommended for simpler and safer removal of remaining resin after de-bonding (Guan *et al.,* 2000).

To accurately score the ARI is important because it is an important factor to be considered in the selection of orthodontic adhesive (Montasser and Drummond, 2009). Studies, (Delport and Grobler, 1988), have debated whether the differences in ARI scores reflect a difference in bond strength between the enamel and the adhesive for the different adhesive systems, but adhesive systems that show less adhesive remnant on the tooth has been advocated for easier and safer removal of residual resin after de-bonding (Guan *et al.,* 2000). Accurate evaluation of the adhesive remnant, which is crucial in the final process of enamel cleaning after de-bonding, is needed for satisfactory removal and restoration of the enamel surface to as close to pretreatment condition as possible (Montasser and Drummond, 2009). Most laboratory studies on the bond strength of orthodontic brackets have examined teeth and brackets under 10x magnification to assess and score the adhesive remnant (Uysal and Sisman, 2008), although laboratory studies designed for evaluation of the enamel surface after de-bonding and cleaning of the surface have used more sophisticated methods such as scanning electron microscope, finite element analysis, and 3-dimensional profilometry (Chen *et al.,* 2008). Clinically, evaluation of the adhesive remnant and the enamel surface after bracket de-bonding and enamel cleanup generally is done by visual inspection under a dental operating light (Montasser and Drummond, 2009).

4. *Surface hardness*

Since, surface hardness is a measure of how resistant a material is to change in shape when a compressive force is applied, it was important to determine whether the addition

of chitosan to the cement would affect its physical property. Hence, the surface hardness of the test material was determined and compared to the control using Vickers Hardness. Although the mean microhardness of chitosan modified cement was lower than the unmodified orthodontic cement, there was no statistically significant difference (Mann-Whitney Test, $p>0.05$). Thus, the addition of chitosan to Trasnbond did not significantly affect the surface hardness of the cement.

5. *Biocompatibility of chitosan modified cement*

Biocompatibility refers the compatibility of manufactured materials and devices with body tissues and fluids. Biocompatibility may be defined as the ability of a material to function in a specific application in the absence of any adverse host response (Schmalz, 1994). Dental materials, as with other fields of biotechnology, need to consider compatibility of materials with tissues as one of the most important properties. A variety of *in vitro* cytotoxicity assays (also called screening tests) are available to determine the biocompatibility of materials (Freshney, 2006) . In this study, guidelines as set out by The International Organization for Standardization (ISO) published the ISO 10993-5 document in 1999 to assess biological reaction to materials was used (ISO, 2009).

Cytotoxicity is the ability of a substance or material to cause damage to tissue cells. Thus, the reaction of cultured cells in cytotoxicity testing of dental materials will depend mainly on:

1) the material or specimen tested and/or its components and to a lesser extent to

2) the type of cells on which the material is tested on.

A critical variable in cytotoxic testing of dental materials is the type of cell line that is used. Currently there are differences in opinion with regards to which cell line should be used. The International Standards Organization (ISO, 2009) stated that where specific sensitivity is required, primary cell cultures, cell lines and organo-typic cultures obtained directly from living tissue can only be used if reproducibility and accuracy of the response can be demonstrated. The ISO specification does allow other cell lines to be used if the same results can be shown (ISO, 2009). Grobler *et al.* (2008) has shown similar responses to human dental pulp cells and 3T3 cell lines on various dentine bonding agents. Hence, in this study the mouse 3T3 cell lines were used.

Both the test sample *i.e.* the chitosan modified orthodontic cement and the unmodified Transbond cement were exposed to 3T3 in culture. The median cell survival rates were found to be 114.8% when the cells were exposed to chitosan modified Transbond which seem to increase the cells numbers compared to the control *i.e.* unmodified Transbond cement. The results showed that chitosan in its own capacity stimulated the cell survival rate to a value of 114.8% which is 14.8% higher than that of the control sample. Thus, it can be deduced that the presence of chitosan in the cement seems to stimulate cell growth. This may suggest that the bioactive property of chitosan is still maintained even when added to Transbond as the chitosan seems to stimulate growth of the 3T3 in culture.

6. Surface microstructure

The surface microstructure was studied under scanning electron microscopy to evaluate the surface morphology of the chitosan nanoparticles. The particles were observed under 1 000, 5 000 and 10 000 times magnification and particles of varying sizes were observed. The surface morphology reflects spherical shapes structures with evenly distributed hexagonal depressions on the surface.

Thus, it seems that the chitosan presents as evenly shaped spherical particles that can be incorporated into Transbond orthodontic cement without negatively impacting on its physical properties but at the same time having a positive effect on its biological properties in that it showed antibacterial effects and a positive influence of cells in culture.

CHAPTER VI

Conclusion

In this study, it has been demonstrated that the newly prepared chitosan modified Transbond XT orthodontic cement possesses suitable antimicrobial activity yet at the same time also improving the adhesion to tooth structure. Thus, the addition of bioactive chitosan to Transbond presents a promising novel technology whereby the newly formed cement possesses sufficient antibacterial properties but at the same time does not compromise its bond strength.

Chitosan modified orthodontic cement demonstrated good biocompatibility by eliciting a positive growth response of 3T3 cells in culture thus demonstrating that the addition of chitosan to the orthodontic cement did not negatively affect its bioactivity.

The surface hardness between the chitosan modified cement was not negatively affected as both the unmodified cement and modified cement showed similar surface hardness.

In this study, a bioactive orthodontic cement was developed by modifying an existing orthodontic cement and evaluated for its biological and physical properties.

The science of bioactivity and nanotechnology was introduced to a dental cement to improve its antimicrobial and at the same time have superior adhesive and mechanical properties.

Further *in-vivo* studies or clinical trials will now be required to demonstrate its antimicrobial effectiveness in preventing demineralization around orthodontic brackets clinically thereby improving one of the major disadvantages of bonding brackets to tooth structure.

CHAPTER VII

Limitations

This is an *in-vitro* study and performed under ideal laboratory conditions where variables like conditions for microbiological growth and cell culture growth were all controlled under ideal conditions in an incubator. In the clinical situation, these conditions may vary. This study therefore, should be followed up with a randomized clinical trial.

This study was done in time intervals up to 8 hours, although complete eradication of microorganism was shown, orthodontic brackets are usually placed for prolonged periods of time. In an *in-vitro* study it is challenging to mimic the oral environment. Specimens were not aged.

This study was performed by modifying one orthodontic cement only. Further studies should be done testing chitosan on other orthodontic cements. This may then give a broader indication on how chitosan behaves with other cement formulations as well.

In this study, only *Streptococcus mutans* was used as the test bacterium. Although this may be the most commonly implicated bacterium for plaque formation, other bacteria may also be present in plaque formation. Hence, further *in-vitro* studies should investigate the effects of chitosan on other bacteria as well.

REFERENCES

3M Technical Data. (2014). *Filtek Supreme XTE 3M*. http://multimedia.3m.com/mws/media/668804O/filtek-supreme-xte-flowable-restorativetechnical-data-sheet-we.pdf. Accessed 8 November 2017

Aghoutan, H., Alami, S., Quars, F. El, Diouny, S., & Bourzgui, F. (2015). White spot lesions in orthodontic treatment and fluoride - clinical evidence. *Emerging Trends in Oral Health Sciences and Dentistry*, 317–339.

Alaçam, A., Tulunoglu, O., Oygür, T., & Bilici, S. (2000). Effects of topical Catalase application on dental pulp tissue: a histopathological evaluation. *Journal of dentistry*, *28*(5), 333–339.

Anusavice, K. J., Shen, C., & Rawls, H. R. (2013). *Phillips Science of Dental Materials 12th Edition*.

Arnaud, T. M. S., De Barros Neto, B., & Diniz, F. B. (2010). Chitosan effect on dental enamel de-remineralization: An in vitro evaluation. *Journal of Dentistry*, *38*(11), 848–852.

Artun, J., & Bergland, S. (1984). Clinical trials with crystal growth conditioning as an alternative to acid-etch enamel pretreatment. *American Journal of Orthodontics*, *85*(4), 333–340.

Ashcraft, D. B., Staley, R. N., & Jakobsen, J. R. (1997). Fluoride release and shear bond strengths of three light-cured glass ionomer cements fluoride release for Individual Bonding Agents. *American Journal of Orthodontics and Dentofacial Orthopedics*,

111(3), 260–265.

Aspden, T. J., Adler, J., Davis, S. S., Skaugrud, & Illum, L. (1995). Chitosan as a nasal delivery system: Evaluation of the effect of chitosan on mucociliary clearance rate in the frog palate model. *International Journal of Pharmaceutics*, *122*(1–2), 69–78.

Asthana, G., & Bhargava, S. (2014). Bioactive Materials : A Comprehensive Review. *Scholars Journal of Applied Medical Sciences (SJAMS)*, *2*(6E), 3231–3237.

Bae, K., Jun, E. J., Lee, S. M., Paik, D. I., & Kim, J. B. (2006). Effect of water-soluble reduced chitosan on Streptococcus mutans, plaque regrowth and biofilm vitality. *Clinical Oral Investigations*, *10*(2), 102–107.

Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). Methods for in vitro evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis*, *6*(2), 71–79.

Basdra, E. K., Huber, H., & Komposch, G. (1996). Fluoride released from orthodontic bonding agents alters the enamel surface and inhibits enamel demineralization in vitro. *American journal of Orthodontics and Dentofacial Orthopedics*, *109*(5), 466–72.

Battino, M., Ferreiro, M. S., Fattorini, D., & Bullon, P. (2002). In vitro antioxidant activities of mouthrinses and their components. *Journal of Clinical Periodontology*, *29*(5), 462–7.

Bayne, S. C. (2005). Dental Biomaterials: where are we and where are we going? *Journal of Dental Education*, *69*(5), 571–585.

Benson, P. (2008). Evaluation of White Spot Lesions on Teeth with Orthodontic Brackets. *Seminars in Orthodontics*, *14*(3), 200–208.

Bhattarai, N., Gunn, J., & Zhang, M. (2010). Chitosan-based hydrogels for controlled, localized drug delivery. *Advanced Drug Delivery Reviews*, *62*(1), 83–99.

Bishara, S. E., & Ostby, A. W. (2008). White Spot Lesions: Formation, Prevention, and Treatment. *Seminars in Orthodontics*, *14*(3), 174–182.

Bishara, S., Soliman, M., & Oonsombat, C. (2004). The effect of variation in mesh-base design on the shear bond strenght of orthodontic brackets. *Angle of Orthodontic*, *74*(3), 400–4.

Blackwell, J., Parker, K. D., & Rudall, K. M. (1965). Chitin in pogonophore tubes. *Journal of the Marine Biological Association of the United Kingdom*, *45*(3), 659–661.

Bloom, R. H., Brown, L. R., & City, K. (1964). A study of the effects of orthodontoc appliances on the oral microbial flora, *17*(5), 658–667.

Bonetti, G. A., Zanarini, M., Incerti Parenti, S., Lattuca, M., Marchionni, S., & Rosaria Gatto Bologna, M. (2011). Evaluation of enamel surfaces after bracket debonding: An in-vivo study with scanning electron microscopy. *American Journal of Orthodontics and Dentofacial Orthopedics*, *140*(5), 696–702.

Cacciafesta, V., Sfondrini, M. F., Tagliani, P., & Klersy, C. (2007). In-vitro fluoride release rates from 9 orthodontic bonding adhesives. *American Journal of Orthodontics and Dentofacial Orthopedics*, *132*(5), 656–662.

Cafaggi, S., Russo, E., Stefani, R., Leardi, R., Caviglioli, G., Parodi, B., et al. (2007). Preparation and evaluation of nanoparticles made of chitosan or N-trimethyl chitosan and a cisplatin-alginate complex. *Journal of Controlled Release*, *121*(1–2), 110–123.

Cattani-Lorente, M. ., Godin, C., & Meyer, J. (1994). Mechanical behavior of glass

ionomer cements affected by long-term storage in water. *Dental Materials*, *10*(Jan), 37–44.

Chapman, J. A., Roberts, W. E., Eckert, G. J., Kula, K. S., & Gonzlez-Cabezas, C. (2010). Risk factors for incidence and severity of white spot lesions during treatment with fixed orthodontic appliances. *American Journal of Orthodontics and Dentofacial Orthopedics*, *138*(2), 188–194.

Chen, C.-S., Hsu, M.-L., Chang, K.-D., Kuang, S.-H., Chen, P.-T., & Gung, Y.-W. (2008). Failure Analysis: Enamel Fracture after Debonding Orthodontic Brackets. *Angle Orthodontics*, *78*(6), 1071–1077.

Chien, K. B., & Shah, R. N. (2012). Novel soy protein scaffolds for tissue regeneration: Material characterization and interaction with human mesenchymal stem cells. *Acta Biomaterialia*, *8*(2), 694–703.

Chin, M. Y. H., Sandham, A., Rumachik, E. N., Ruben, J. L., & Huysmans, M. C. D. N. J. M. (2009). Fluoride release and cariostatic potential of orthodontic adhesives with and without daily fluoride rinsing. *American Journal of Orthodontics and Dentofacial Orthopedics*, *136*(4), 547–553.

Cho, Y. W., Jang, J., Park, C. R., & Ko, S. W. (2000). Preparation and solubility in acid and water of partially deacetylated chitins. *Biomacromolecules*, *1*(4), 609–614.

Chung, Y. C., Yeh, J. Y., & Tsai, C. F. (2011). Antibacterial characteristics and activity of water-soluble chitosan derivatives prepared by the Maillard reaction. *Molecules*, *16*(10), 8504–8514.

Chung, Y., & Chen, C. (2008). Antibacterial characteristics and activity of acid-soluble

chitosan. *Bioresource Technology*, *99*, 2806–2814.

Cohen, W. J., Wiltshire, W. A., Hons, B., Dawes, C., & Lavelle, C. L. B. (2003). Containing Fluoride. *American Journal of Orthodontics and Dentofacial Orthopedics*, *124*(5), 571–576.

Coups-Smith, K. S., Rossouw, P. E., & Titley, K. C. (2003). Glass ionomer cements as luting agents for orthodontic brackets. *Angle Orthodontist*, *73*(4), 436–444.

Crisp, S., Lewis, B. G., & Wilson, A. D. (1979). Characterization cements liquid component of glass-ionomer 5 . The effect of the tartaric acid concentration. *Journal of Dentistry*, (4), 304–312.

Croisier, F., & Jérôme, C. (2013). Chitosan-based biomaterials for tissue engineering. *European Polymer Journal*, *49*, 780–792.

Cuero, R. G., Duffus, E., Osuji, G., & Pettit, R. (1991). Aflatoxin control in preharvest maize: effects of chitosan and two microbial agents. *The Journal of Agricultural Science*, *117*(2), 165.

Delport, A., & Grobler, S. R. (1988). A laboratory evaluation of the tensile bond strength of some orthodontic bonding resins to enamel. *American Journal of Orthodontics and Dentofacial Orthopedics*, *93*(2), 133–137.

Diaz-Arnold, A. M., Holmes, D. C., Wistrom, D. W., & Swift, E. J. (1995). Short-term fluoride release/uptake of glass ionomer restoratives. *Dental Materials*, *11*(2), 96–101.

Diaz-Arnold, A., Vargas, M., & DR, H. (1999). Currebt status of luting agents for fixed prosthodontics. *Journal of Prosthetic Dentistry*, *81*(2), 135–141.

Dutta, P. K. (2016). *Chitin and Chitosan for Regenerative Medicine*. *Springer*.

Dutta, P. K., Duta, J., & Tripathi, V. S. (2004). Chitin and Chitosan: Chemistry, properties and applications. *Journal of Scientific and Industrial Research*, *63*(1), 20–31.

Dutta, Tripathi, S., & Dutta, P. K. (2012). Progress in antimicrobial activities of chitin, chitosan and its oligosaccharides: a systematic study needs for food applications. *Food and Science Technology International*, *18*(1), 3–34.

Eaton, P., Fernandes, J. C., Pereira, E., Pintado, M. E., & Xavier Malcata, F. (2008). Atomic force microscopy study of the antibacterial effects of chitosans on Escherichia coli and Staphylococcus aureus. *Ultramicroscopy*, *108*(10), 1128–1134.

Eliades, T., Eliades, G., Silikas, N., & Watts, D. C. (2004). Tensile properties of orthodontic elastomeric chains. *European Journal of Orthodontics*, *26*(2), 157–162.

Eminkahyagil, N., Aman, A., Ce4tinsahin, A., & Karabulut, E. (2006). Effect of Resin-removal Methods on Enamel and Shear Bond Strength of Rebonded Brackets. *Angle Orthodontist*, *76*(2), 314–321.

Ewoldsen, N., & Demke, R. S. (2001). A review of orthodontic cements and adhesives. *American Journal of Orthodontics and Dentofacial Orthopedics*, *120*(1), 45–48.

Fajen, V. B., Duncanson, M. G. J., Nanda, R. S., Currier, G. F., & Angolkar, P. V. (1990). An in vitro evaluation of bond strength of three glass ionomer cements. *American Journal of Orthodontics and Dentofacial Orthopedics*, *97*(4), 316–322.

Featherstone, J. D. B. (2000). the Science and Practice of Caries Prevention the Science and Practice of Caries Prevention. *Journal of American Dental Association*, *131*, 887–899.

Feng, Y., & Xia, W. (2011). Preparation, characterization and antibacterial activity of water-soluble O-fumaryl-chitosan. *Carbohydrate Polymers*, *83*(3), 1169–1173.

Freshney, R. (2006). Culture of animal cells: A manual of basic technique, fifth edition. *In Vitro Cellular & Developmental Biology - Animal*, *42*(5), 169.

Fricker, J. P. (1992). A 12-month clinical evaluation of a glass polyalkenoate cement for the direct bonding of orthodontic brackets. *American Journal of Orthodontics and Dentofacial Orthopedics*, *101*(4), 381–384.

Fricker, J. P. (1994). A 12-month clinical evaluation of a light-activated glass polyalkenoate (ionomer) cement for the direct bonding of orthodontic brackets. *American Journal of Orthodontics and Dentofacial Orthopedics*, *105*(5), 502–505.

Gange, P. (2015). The evolution of bonding in orthodontics. *American Journal of Orthodontics and Dentofacial Orthopedics*, *147*(4), S56–S63.

Gaworski, M., Weinstein, M., Borislow, A. J., & Braitman, L. E. (1999). Decalcification and bond failure: A comparison of a glass ionomer and a composite resin bonding system in vivo. *American Journal of Orthodontics and Dentofacial Orthopedics*, *116*(5), 518–521.

George, M., & Abraham, T. E. (2006). Polyionic hydrocolloids for the intestinal delivery of protein drugs: Alginate and chitosan - a review. *Journal of Controlled Release*, *114*(1), 1–14.

Gomathysankar, S., Halim, A. S., & Yaacob, N. S. (2014). Proliferation of keratinocytes induced by adipose-derived stem cells on a chitosan scaffold and its role in wound healing, a review. *Archives of Plastic Surgery*, 41(5), 452-457.

Gorelick, L., Geiger, A. M., & Gwinnett, A. J. (1982). Incidence of white spot formation after bonding and banding. *American Journal of Orthodontics*, *81*(2), 93–98.

Goy, R. C., Britto, D. De, & Assis, O. B. G. (2009). A review of the antibacterial actiity of chitosan, *Polímeros: Ciência e Tecnologia, 19(3)*, 241–247.

Grenha, A. (2012). Chitosan nanoparticles: a survey of preparation methods. *Journal of Drug Targeting*, *20*(4), 291–300.

Grenha, A., Seijo, B., & Remuñán-López, C. (2005). Microencapsulated chitosan nanoparticles for lung protein delivery. *European Journal of Pharmaceutical Sciences*, *25*(4–5), 427–437.

Grobler, S. R., Oliver, A., Moodley, D., & Van Wyk Kotze, T. J. (2008). Cytotoxicity of recent dentin bonding agents on mouse fibroblast cells. *Quintessence International*, *39*(6), 511–6.

Grobler, S. R., Olivier, A., Moodley, D., & van W Kotze, T. W. (2004). Cytotoxicity of two concentrations of a dentine bonding agent on mouse 3T3 and human pulp fibroblast cell-lines. *SADJ : Journal of the South African Dental Association*, *59*(9), 368–370,372.

Guan, G., Takano-Yamamoto, T., Miyamoto, M., Hattori, T., Ishikawa, K., & Suzuki, K. (2000). Shear bond strengths of orthodontic plastic brackets. *American Journal of Orthodontics and Dentofacial orthopedics*, *117*(4), 438–443.

Gwinnett, A. J., & Ceen, R. F. (1979). Plaque distribution on bonded brackets: A scanning microscope study. *American Journal of Orthodontics*, *75*(6), 667–677.

Hadler-Olsen, S., Sandvik, K., El-Agroudi, M. A., & Ogaard, B. (2012). The incidence of

caries and white spot lesions in orthodontically treated adolescents with a comprehensive caries prophylactic regimen - A prospective study. *European Journal of Orthodontics*, *34*(5), 633–639.

Hamman, J. H. (2010). Chitosan based polyelectrolyte complexes as potential carrier materials in drug delivery systems. *Marine Drugs*, *8*(4), 1305–1322.

Han, T., Nwe, N., Furuike, T., Tokura, S., & Tamura, H. (2012). Methods of N -acetylated chitosan scaffolds and its in vitro biodegradation by lysozyme. *J. Biomedical Science and Engineering*, *5*(January), 15–23.

He, P., Davis, S. S., & Illum, L. (1998). In vitro evaluation of the mucoadhesive properties of chitosan microspheres. *International Journal of Pharmaceutics*, *166*(1), 75–88.

Helbert, W., & Sugiyama, J. (1998). High-resolution electron microscopy on cellulose II and α-chitin single crystals. *Cellulose*, *5*, 113–122.

Ibrahim, M. a., Neo, J., Esguerra, R. J., & Fawzy, a. S. (2015). Characterization of antibacterial and adhesion properties of chitosan-modified glass ionomer cement. *Journal of Biomaterials Applications*, *0* (0), 1–11.

ISO. (2009a). ISO 4049:2009 - Dentistry -- Polymer-based restorative materials. https://www.iso.org/standard/42898.html.

ISO. (2009b). ISO 10993-5:2009 - Biological evaluation of medical devices -- Part 5: Tests for in vitro cytotoxicity. https://www.iso.org/standard/36406.html.

Jung, W.-S., Kim, H., Park, S.-Y., Cho, E.-J., & Ahn Seoul, S.-J. (2014). Quantitative analysis of changes in salivary mutans streptococci after orthodontic treatment. *American Journal of Orthodontics and Dentofacial Orthopedics*, *145*(5), 603–609.

Kahlmeter, G., Brown, D. F. J., Goldstein, F. W., MacGowan, A. P., Mouton, J. W., Odenholt, I., et al. (2006). European Committee on Antimicrobial Susceptibility Testing (EUCAST) Technical Notes on antimicrobial susceptibility testing. *Clinical Microbiology and Infection*, *12*(6), 501–503.

Kaş, H. S. (1997). Chitosan: properties, preparations and application to microparticulate systems. *Journal of Microencapsulation*, *14*(6), 689–711.

Kawashima, Y., Handa, T., Kasai, A., Takenaka, H., & Lin, S. Y. (1985). The effects of thickness and hardness of the coating film on the drug release rate of theophylline granules coated with chitosan-sodium tripolyphosphate complex. *Chemical & pharmaceutical bulletin*, *33*(6), 2469–2474.

Klocke, A., & Kahl-Nieke, B. (2005). Influence of force location in orthodontic shear bond strength testing. *Dental Materials*, *21*(5), 391–396.

Klocke, A., & Kahl-Nieke, B. (2006). Effect of debonding force direction on orthodontic shear bond strength. *American Journal of Orthodontics and Dentofacial Orthopedics*, *129*(2), 261–265.

Klocke, A., Shi, J., Vaziri, F., Kahl-Nieke, B., & Bismayer, U. (2004). Effect of time on bond strength in indirect bonding. *Angle Orthodontist*, *74*(2), 245–250.

Kong, M., Chen, X. G., Xing, K., & Park, H. J. (2010). Antimicrobial properties of chitosan and mode of action: A state of the art review. *International Journal of Food Microbiology*, 144(1), 51–63.

Kumar, A. B. V., Varadaraj, M. C., Gowda, L. R., & Tharanathan, R. N. (2005). Characterization of chito-oligosaccharides prepared by chitosanolysis with the aid of

papain and Pronase , and their bactericidal action against Bacillus cereus and Escherichia coli. *Biochemical Journal*, *175*, 167–175.

Kurita, K. (1998). Chemistry and application of chitin and chitosan. *Polymer Degradation and Stability*, *59*(1–3), 117–120.

Kurita, K., Tomita, K., Ishii, S., Nishimura, S. I., & Shimoda, K. (1993). B-chitin as a convenient starting material for acetolysis for efficient preparation of N-acetylchitooligosaccharides. *Journal of Polymer Science, Part A: Polymer Chemistry*, *31*(9), 2393–2395.

Le, Y., Anand, S. C., & Horrocks, A. R. (1998). Recent developments in fibres and materials for wound management, *Indian Journal of Fibre and Textile Research*, *22*(4), 337–347.

Liu, H., & Gao, C. (2009). Preparation and properties of ionically cross-linked chitosan nanoparticles. *Polymers for Advanced Technologies*, *20*(7), 613–619.

Lohbauer, U. (2010). Dental glass ionomer cements as permanent filling materials - Properties, limitations and future trends. *Materials*, *3*(1), 76–96.

López-León, T., Carvalho, E. L. S., Seijo, B., Ortega-Vinuesa, J. L., & Bastos-González, D. (2005). Physicochemical characterization of chitosan nanoparticles: Electrokinetic and stability behavior. *Journal of Colloid and Interface Science*, *283*(2), 344–351.

Markovic, E., Glišic, B., Ščepan, I., Markovi, D., & Vukoman, J. (2008). Bond strength of orthodontic adhesives. *Journal of Metallurgy*, *14*(2), 79–88.

Mattousch, T. J. H., Van Der Veen, M. H., & Zentner, A. (2007). Caries lesions after

orthodontic treatment followed by quantitative light-induced fluorescence: A 2-year follow-up. *European Journal of Orthodontics*, *29*(3), 294–298.

McNaught, A., & Wilkinson, A. (2014). *Compendium of Chemical Terminology 2nd ed*. (A. McNaught & A. Wilkinson, Eds.)*Blackwell Scientific Publications, Oxford* (2nd ed.). Oxford.

Melo, M. A. S., Wu, J., Weir, M. D., & Xu, H. H. K. (2014). Novel antibacterial orthodontic cement containing quaternary ammonium monomer dimethylaminododecyl methacrylate. *Journal of Dentistry*, *42*(9), 1193–1201.

Miguel, J. A. ., Almeida, M. ., & Chevitarese, O. (1995). Clinical comparison between a glass ionomer cement and a composite for direct bonding of orthodontic brackets. *American Journal of Orthodontics*, *107*(5), 484–487.

Miller, J. R. (1996). A three-year clinical trial using a glass ionomer cement for the bonding of orthodontic brackets. *Angle Orthodontist*. 66(4), 309-312.

Millett, D. T., & McCabe, J. F. (1996). Orthodontic bonding with glass ionomer cement - a review. *European Journal of Orthodontics*, *18*(4), 385–399.

Mirani, S. Ali, Sangi, L., Kumar, N., & Ali, D. (2015). Investigating the the antibacterial effect of chitosan in dental resin composites : a pilot study. *Pakistan Oral and Dental Journal*, *35*(2), 15–18.

Mitchell, C. A., O'Hagan, E., & Walker, J. M. (1995). Probability of failure of orthodontic brackets bonded with different cementing agents. *Dental Materials*, *11*(5–6), 317–322.

Mizrahi, E. (1982). Enamel demineralization following orthodontic treatment. *American*

Journal of Orthodontics, *82*(1), 62–67.

Montasser, M. A., & Drummond, J. L. (2009). Reliability of the Adhesive Remnant Index Score System with Different Magnifications. *Angle Orthodontist*, *79*(4), 773–776.

Moreira, D. M., Oei, J., Rawls, H. R., Wagner, J., Chu, L., Li, Y., et al. (2015). A novel antimicrobial orthodontic band cement with in situ-generated silver nanoparticles. *Angle Orthodontist*, *85*(2), 175–183.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, *65*(1–2), 55–63.

Muzzarelli, R., Tarsi, R., Filippini, O., Giovanetti, E., Biagini, G., & Varaldo, P. E. (1990). Antimicrobial Properties of N-Carboxybutyl Chitosan, *Antimicrobial Agents and Chemotherapy*, *34*(10), 2019–2023.

Ngan, P. W., Gladwin, M., Tanna, N., & Kao, E. (2009). Effects of sealant and self-etching primer on enamel decalcification. Part II: An in-vivo study. *American Journal of Orthodontics and Dentofacial Orthopedics*, *135*(2), 206–213.

Nicholson, J. W., & Wilson, A. D. (2000). The effect of storage in aqueous solutions on glass-ionomer and zinc polycarboxylate dental cements. *Journal of Materials Science*, *1*, 357–360.

Norevall, L. I., Marcusson, A., & Persson, M. (1996). A clinical evaluation of a glass ionomer cement as an orthodontic bonding adhesive compared with an acrylic resin. *European Journal of Orthodontics*, *18*(4), 373–84.

Norris, D., McInnes-Ledoux, P., Schwaninger, B., & Weinberg, R. (1986). Retention of

orthodontic bands with fluoride-releasing cements new. *American Journal of Orthodontics, 89*(3), 206–211.

O'Brien, K. D., Watts, D. C., & Read, M. J. F. (1988). Residual debris and bond strength—Is there a relationship, *American Journal of Orthodontics and Dentofacial Orthopedics*, *94*(3), 222–230.

O'Reilly, M. M., & Featherstone, J. D. B. (1987). Demineralization and remineralization around orthodontic appliances: An in vivo study. *American Journal of Orthodontics and Dentofacial Orthopedics, 92*(1), 33–40.

Ogaard, B. (1989). Prevalence of white spot lesions in 19-near-olds: A study on untreated and orthodontically treated persons 5 years after treatment. *American Journal of Orthodontics and Dentofacial Orthopedics*, *96*(5), 423–427.

Ogaard, B., Rolla, R., & Arends, J. (1988). Orthodontic appliances and enamel demineralization. *American Journal of Orthodontics and Dentofacial Orthopedics*, *94*(1), 68–73.

Oliver, R. G. (1988). The effect of different methods of bracket removal the amount of residual adhesive, *American Journal of Orthodontics and Dentofacial Orthopedics*, *93*, 196–200.

Oz, A., Yaziciog, S., Arici, N., Serdar Akdeniz, B., Murat, N., & Arıcı, S. (2014). Assessment of the Confidence of the Adhesive Remnant Index Score With Different Methods. *Turkish Journal of Orthodontics, 26*(4), 149–153.

Pascotto, R. C., Navarro, M. F. D. L., Filho, L. C., & Cury, J. A. (2004). In vivo effect of a resin-modified glass ionomer cement on enamel demineralization around

orthodontic brackets. *American Journal of Orthodontics and Dentofacial Orthopedics*, *125*(1), 36–41.

Perchyonok, V. T., Reher, V., Basson, N., & Grobler, S. (2015). Biomaterials for Functional Applications in the Oral Cavity via Contemporary Multidimensional Science. In V. K. Thakur & M. K. Thakur (Eds.), *Handbook of Polymers for Pharmaceutical Technologies*, (1st ed., pp. 365–412).

Pfaller, M. A., Sheehan, D. J., & Rex, J. H. (2004, April 1). Determination of Fungicidal Activities against Yeasts and Molds: Lessons Learned from Bactericidal Testing and the Need for Standardization. *Clinical Microbiology Reviews*, 17(2), 268-280.

Pillai, C. K. S., Paul, W., & Sharma, C. P. (2009). Chitin and chitosan polymers: Chemistry, solubility and fiber formation. *Progress in Polymer Science (Oxford)*, *34*(7), 641–678.

Qi, L., & Xu, Z. (2004). Lead sorption from aqueous solutions on chitosan nanoparticles. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *251*(1–3), 183–190.

Raafat, D., Von Bargen, K., Haas, A., & Sahl, H. G. (2008). Insights into the mode of action of chitosan as an antibacterial compound. *Applied and Environmental Microbiology*, *74*(12), 3764–3773.

Rabea, E. I., Badawy, M. E. T., Stevens, C. V., Smagghe, G., & Steurbaut, W. (2003). Chitosan as antimicrobial agent: Applications and mode of action. *Biomacromolecules*. American Chemical Society, 4(6), 1457-1465.

Rani, M., Agarwal, A., & Negi, Y. S. (2010). Review: Chitosan based Hydrogel polymeric

beads - as drug delivery system. *BioResources*, *5*(4), 2765–2807.

Rather, M. A., Sharma, R., Gupta, S., Ferosekhan, S., Ramya, V. L., & Jadhao, S. B. (2013). Chitosan-Nanoconjugated Hormone Nanoparticles for Sustained Surge of Gonadotropins and Enhanced Reproductive Output in Female Fish. *PLoS ONE*, *8*(2), e57094.

Rinaudo, M. (2006). Chitin and chitosan: Properties and applications. *Progress in Polymer Science*, *31*(7), 603–632.

Rinaudo, M., Pavlov, G., & Desbrières, J. (1999). Influence of acetic acid concentration on the solubilization of chitosan. *Polymer*, *40*(25), 7029–7032.

Ríos, J. L., & Recio, M. C. (2005). Medicinal plants and antimicrobial activity. *Journal of Ethnopharmacology*, *100*(1–2), 80–84.

Robertson, M. A., Kau, C. H., English, J. D., Lee, R. P., Powers, J., & Nguyen, J. T. (2011). MI Paste Plus to prevent demineralization in orthodontic patients: A prospective randomized controlled trial. *American Journal of Orthodontics and Dentofacial Orthopedics*, *140*(5), 660–668.

Rogers, S., Chadwick, B., & Treasure, E. (2010). Fluoride-containing orthodontic adhesives and decalcification in patients with fixed appliances: A systematic review. *American Journal of Orthodontics and Dentofacial Orthopedics*, *138*(4).

Rossi, F., Chatzistavrou, X., Perale, G., & Boccaccini, A. R. (2012). Synthesis and degradation of agar-carbomer based hydrogels for tissue engineering applications. *Journal of Applied Polymer Science*, *123*(1), 398–408.

Ruiz-Herrera, J., Sing, V. O., Van der Woude, W. J., & Bartnicki-Garcia, S. (1975).

Microfibril assembly by granules of chitin synthetase. *Proceedings of the National Academy of Sciences of the United States of America*, *72*(7), 2706–10.

Sakamoto, J., Sugiyama, J., Kimura, S., Imai, T., Itoh, T., Watanabe, T., & Kobayashi, S. (2000). Artificial chitin spherulites composed of single crystalline ribbons of α-chitin via enzymatic polymerization. *Macromolecules*, *33*(11), 4155–4160.

Sano, H., Shibasaki, K., Matsukubo, T., & Takaesu, Y. (2003). Effect of chitosan rinsing on reduction of dental plaque formation. *The Bulletin of Tokyo Dental College*, *44*(1), 9–16.

Schmalz, G. (1994). Use of cell cultures for toxicity testing of dental materials-advantages and limitations. *Journal of Dentistry*, *22*(SUPPL. 2), S6–S11.

Shahidi, F., Arachchi, J. K. V., & Jeon, Y. J. (1999). Food applications of chitin and chitosans. *Trends in Food Science and Technology*, *10*(2), 37–51.

Shamsi, A. Al, Cunningham, J. L., Lamey, P. J., & Lynch, E. (2006). Shear Bond Strength and Residual Adhesive after Orthodontic Bracket Debonding. *Angle Orthodontist*, *76*(4), 694-699.

Shrestha, A., Zhilong, S., Gee, N. K., & Kishen, A. (2010). Nanoparticulates for antibiofilm treatment and effect of aging on its antibacterial activity. *Journal of Endodontics*, *36*(6), 1030–1035.

Shungin, D., Olsson, A. I., & Persson, M. (2010). Orthodontic treatment-related white spot lesions: A 14-year prospective quantitative follow-up, including bonding material assessment. *American Journal of Orthodontics and Dentofacial Orthopedics*, *138*(2), 136–137.

Struszczzyk, M. H. (2002). Chitin and Chitosan. *Polimery*, *47*(5), 316–325.

Sudjalim, T. R., Woods, M. G., Manton, D. J., & Reynolds, E. C. (2007). Prevention of demineralization around orthodontic brackets in vitro. *American Journal of Orthodontics and Dentofacial Orthopedics*, *131*(6), 1–9.

Summers, A., Kao, E., Gilmore, J., Gunel, E., & Ngan, P. (2004). Comparison of bond strength between a conventional resin adhesive and a resin-modified glass ionomer adhesive: An in vitro and in vivo study. *American Journal of Orthodontics and Dentofacial Orthopedics*, *126*(2), 200–206.

Surmont, P., Dermaut, L., Martens, L., & Moors, M. (1992). Comparison in shear bond strength of orthodontic brackets between five bonding systems related to different etching times : An in vitro study, *American Journal of Orthodontics and Dentofacial Orthopedics*, 414–419.

Swartz, M. L., Phillips, R. W., & Clark, H. E. (1984). Long-term F release from glass ionomer cements. *Journal of Dental Research*, *63*, 158–160.

Tan, H., Ma, R., Lin, C., Liu, Z., & Tang, T. (2013). Quaternized chitosan as an antimicrobial agent: Antimicrobial activity, mechanism of action and biomedical applications in orthopedics. *International Journal of Molecular Sciences*, *14*(1), 1854–1869.

Teijeiro-Osorio, D., Remuñán-López, C., & Alonso, M. J. (2009). Chitosan/cyclodextrin nanoparticles can efficiently transfect the airway epithelium in vitro. *European Journal of Pharmaceutics and Biopharmaceutics*, *71*(2), 257–263.

Thandapani, G., Supriya Prasad, P., Sudha, P. N., & Sukumaran, A. (2017). Size

optimization and in vitro biocompatibility studies of chitosan nanoparticles. *International Journal of Biological Macromolecules, 104*(B), 1794–1806.

Tseng, H., Furuhata, K. ichi, & Sakamoto, M. (1995). Bromination of regenerated chitin with N-bromosuccinimide and triphenylphosphine under homogeneous conditions in lithium bromide-N,N-dimethylacetamide. *Carbohydrate Research*, *270*(2), 149–161.

Uysal, T., & Sisman, A. (2008). Can Previously Bleached Teeth Be Bonded Safely Using Self-etching Primer Systems, *Angle Orthodontist*, *78*(4), 711-715.

Van Der Merwe, S. M., Verhoef, J. C., Verheijden, J. H. M., Kotzé, A. F., & Junginger, H. E. (2004). Trimethylated chitosan as polymeric absorption enhancer for improved peroral delivery of peptide drugs. *European Journal of Pharmaceutics and Biopharmaceutics*, *58*(2), 225–235.

Verheul, R. J., Amidi, M., van Steenbergen, M. J., van Riet, E., Jiskoot, W., & Hennink, W. E. (2009). Influence of the degree of acetylation on the enzymatic degradation and in vitro biological properties of trimethylated chitosans. *Biomaterials*, *30*(18), 3129–3135.

Vorhies, A. B., Donly, K. J., Staley, R. N., & Wefel, J. S. (1998). Enamel demineralization adjacent to orthodontic brackets bonded with hybrid glass ionomer cements: an in vitro study. *American Journal of Orthodontics and Dentofacial Orthopedics*, *114*(6), 668–674.

Wang, H., Li, W., Lu, Y., Zhiliang, W., & Zhong, W. (1996). Studies on Chitosan and Poly (acry1ic acid) Interpolymer Complex . II . Solution Behaviors of the Mixture of Water-Soluble Chitosan and Poly (acry1ic acid). *Journal of Applied Polymer*

Science, *61*, 2221–2224.

Wenderoth, C. J., Weinstein, M., & Borislow, a J. (1999). Effectiveness of a fluoride-releasing sealant in reducing decalcification during orthodontic treatment. *American Journal of Orthodontics and Dentofacial Orthopedic*, *116*, 629–634.

Willmot, D. (2008). White Spot Lesions After Orthodontic Treatment. *Seminars in Orthodontics*, *14*(3), 209–219.

Wilson, A. D., Kent, B. E., Clinton, D., & Miller, R. P. (1972). The Formation and Microstructure of Dental Silicate Cements. *Journal of Materials Science Science*, *7*, 220–238.

Wisth, P. J., & Nord, A. (1977). Caries experience in orthodontically treated individuals. *Angle Orthodontist*, 47(1), 59-64.

Yassaei, S., Davari, A., Goldani Moghadam, M., & Kamaei, A. (2014). Comparison of Shear Bond Strength of RMGI and Composite Resin for Orthodontic Bracket Bonding. *Journal of Dentistry (Tehran, Iran)*, *11*(3), 282–289.

Yi, H., Wu, L. Q., Bentley, W. E., Ghodssi, R., Rubloff, G. W., Culver, J. N., & Payne, G. F. (2005). Biofabrication with chitosan. *Biomacromolecules*, *6*(6), 2881–2894.

Younes, I., & Rinaudo, M. (2015). Chitin and chitosan preparation from marine sources. Structure, properties and applications. *Marine Drugs*, *13*(3), 1133–1174.

Young, D. H., Köhle, H., & Kauss, H. (1982). Effect of Chitosan on Membrane Permeability of Suspension-Cultured Glycine max and Phaseolus vulgaris Cells. *Plant physiology*, *70*(5), 1449–54.

Yuan, Y., Chesnutt, B. M., Haggard, W. O., & Bumgardner, J. D. (2011). Deacetylation

of chitosan: Material characterization and in vitro evaluation via albumin adsorption and pre-osteoblastic cell cultures. *Materials*, *4*(8), 1399–1416.

APPENDIX 1

UNIVERSITY *of the*
WESTERN CAPE

Oral & Dental Research Institute
Faculty of Dentistry and WHO Oral Health Collaborating Centre
University of the Western Cap, Cape Town

Patient Information Sheet to be given to the patient to take home

I, Dr Tashia Moodley am a qualified dentist involved in research and training at the University of the Western Cape, Faculty of Dentistry.

I am doing research on new dental materials and how it sticks/bonds to teeth.

After the extraction of your teeth, they are either discarded or given to the students to practice on. I wish to use your teeth to be able to determine whether a new cement I made will stick/bond to the teeth. Donating your teeth in the study is on a voluntary basis. Donating your teeth for this study or refusing to participate will not harm or prejudice you in any way. The teeth supplied to me will not have your name on it as well as I will not be able to identify you in any way. Upon completion of this study the teeth will be discarded. Participating in the study will definitely benefit future studies and will add to our existing pool of knowledge. All information will be kept strictly confidential.

Dr Tashia Moodley (Researcher)

I, (Patient's name)..... fully understand the information supplied to me by Dr Tashia Moodley in this information sheet

Signature: .....

Date: .....

APPENDIX 2

UNIVERSITY *of the*
WESTERN CAPE

Oral & Dental Research Institute
Faculty of Dentistry and WHO Oral Health Collaborating Centre
University of the Western Cape, Cape Town

Consent form

I, Mr/Mrs/Miss.....

Date of Birth:..... File no./Hosp. Sticker.....

am willing to donate my extracted teeth in the study as described to me in the patient information letter by Dr T. Moodley. I understand that donating my teeth is voluntary.

The study is approved by the Ethical and Research Committee of the University of the Western Cape. I have been adequately informed about the objectives of the study. My rights will be protected and all my details will be kept confidential. No personal information will be published.

I hereby consent to donate my teeth for the research/study.

Patient's/patient's parent or guardian's name:.....

Patient's/patient's parent or guardian's signature:.....

Witness's name:.....

Witness's signature:.....

Researcher's signature:.....

Dr Tashia Moodley

Oral & Dental Research Institute Oral Health Centre Tygerberg

Contact details: Tel: (021) 937 3090

Mobile: 082 657 3948`

Date:.....

APPENDIX 3

a. Chitosan (Sigma Aldrich, USA)

b. Glacial acetic acid (Merck, Germany)

c. Transbond ™ Orthodontic Cement (3M ESPE)

d. Elipar™ DeepCure-S (3M ESPE)

e. CureRite light intensity meter (Dentsply, USA)

f. *Streptococcus mutans* (ATCC UA 159 strain, American Type Culture Collection Manassas, USA)

g. Brain Heart Infusion (Sigma-Aldrich, St. Louis, USA)

h. Ethylene oxide gas sterilizer (SSS; Sterile Services, Singapore).

i. Spectrophotometer (Shimadzu, Tokyo, Japan)

j. Phosphate Buffer Saline (Sigma-Aldrich, St. Louis, USA)

k. Orbital Shaker Incubator (Biocom Biotech, Pretoria, RSA

l. Lamina Flow (Bio Flow, USA)

m. Phosphoric acid solution (Wright Health Group Ltd, RSA)

n. Roth stainless steel brackets (OrthoShop, RSA)

o. Universal Testing Machine (Tinius Olsen, Horsham, USA)

p. Vickers Hardness Machine (Zwick, Germany)

q. Balb/c 3T3 mouse fibroblast cells (The National Repository for Biological Materials, Sandringam, RSA)

r. Field Emision Scanning Electron Microscope (Auriga, Zeiss, Germany)

s. Quorum Sputter Coater (Quorum Technologies Ltd, UK)

APPENDIX 4