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Evaluation of the Biocompatibility and Micromechanical Properties of Experimental Dental Adhesives

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Evaluation of the Biocompatibility and Micromechanical Properties of Experimental Dental Adhesives

by

Hoda Moussa

An Abstract of a Thesis
in
Forensic Science

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Master of Science

May 2018

State University of New York
College at Buffalo
Department of Chemistry

ABSTRACT

The purpose of this study was to investigate the biocompatibility and micromechanical properties of newly synthesized antibacterial monomer and cross-linker functional surfactants into a commercially available dental adhesive (Single Bond, 3M ESPE, Saint Paul, MN, USA) at three concentrations (0.05, 0.1, 0.25 mg/mg). All groups were analyzed by evaluation of micro-tensile bond strength, ultimate tensile strength, cell viability, antibacterial properties, and surface micro-hardness. Scanning electron microscopy (SEM) was used for interfacial characterization.

Human extracted molars were used as a substrate for bonding adhesives for the micro-tensile bond strength (MTBS) and scanning electron microscopy (SEM) studies. Twenty resin-dentin beams (0.9 ± 0.1 mm^2) per group were evaluated at 24 hrs and 6 mos for MTBS. Slabs of ~1 mm were analyzed in the SEM for surface characterization. For the ultimate tensile strength (UTS), ten hour-glass shaped specimens ($10 \times 2 \times 1$mm) per group were tested at 24 hrs, 1 wk, and 6 mos. To evaluate toxicity, four disc-shaped specimens (5×2 mm) per group were incubated with human gingival fibroblasts (HGF). Antibacterial properties were evaluated by incubating three disc-shaped specimens (8×1 mm) per group with a strain of caries-producing bacteria *S. mutans*.

Within the limitations of this *in vitro* study, it was concluded that incorporation of antibacterial monomer and cross-linker additives may be a viable option to help increase the longevity of tooth-colored adhesive restorations. Single Bond adhesive modified with 0.1 mg/mg monomer appears to provide the optimal balance for biocompatibility and micromechanical properties.

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CHAPTER 1
INTRODUCTION AND BACKGROUND

1.1. STATEMENT OF THE PROBLEM

Dental caries is a pandemic affecting millions of people. According to the Centers for Disease Control and Prevention, it was reported that in 2015, 91% of Americans over the age of twenty have had dental caries at some point and 27% of adults over twenty had untreated caries [1]. Patients who have suffered from caries and have had restoration, face the potential consequence of dental restoration failure overtime and/or the risk of developing secondary caries.

Dental restorations can fail due to several factors that continue to be explored in dental research. The longevity of tooth-colored restorations remains an issue as they are known to fail due to the potential formation of recurrent or secondary caries under the existing restoration [2]. Secondary caries can occur due to several reasons including poor oral hygiene, which leads to plaque formation, micro-leakage and others [3]. Micro-leakage around the restoration margins provides an open pathway for acid-producing bacteria, which then begins the gradual dissolution of tooth substrates once the pH of the micro-environment drops under a certain level leading to the formation of secondary caries [4].

In a study by Gordan, *et al.* [5], the authors concluded that if an original restoration had been repaired or replaced within the first year of treatment, an additional treatment was most likely needed. To overcome the issues of restoration failure, the dental adhesive would ideally be antibacterial as well as offer long-term strong bonding to tooth structure. A study by Hiraishi, *et al*. demonstrated that incorporation of 1% chlorhexidine (CHX) exhibited significant antibacterial activity without compromising the bond strength to dentin [6]. The incorporation of

the antibacterial monomer 12-methacryloyloxydodecylpyridinium bromide (MDPB) to a dental adhesive showed a stable bond strength to dentin after one year, and greater antibacterial activity as compared to other antibacterial agents such as CHX [7].

The present thesis intends to evaluate an experimental antibacterial monomer and cross-linker functional surfactant, incorporated into a resin adhesive, for their antibacterial properties. Their biocompatibility and effect on micromechanical properties of the adhesive including micro-tensile bond strength, ultimate tensile strength and surface micro-hardness will also be investigated. Nine study groups were evaluated by incorporation of an antibacterial monomer and cross-linker to a commercially available adhesive Singe Bond (3M ESPE, Saint Paul, MN, USA). Eight groups incorporated chemically modified monomers, cross-linkers, or a combination of both. Single Bond alone was used as a control. The following sections of this thesis will address background information on dental caries, basic mechanisms of adhesion, dental adhesive systems, and the laboratory techniques commonly used to evaluate adhesive blends for their antibacterial properties, biological response, and mechanical properties. Later, the different test methods used in this project as well as the results are presented; which are then followed by a discussion section intended to interpret the observed results, address the limitations of the present study, while also providing directions for future research. The following testing methods will be discussed: antibacterial assay, cytotoxicity, micro-tensile bond strength, ultimate tensile bond strength, and microstructure characterization by field-emission scanning electron microscopy.

1.2. DENTAL CARIES

Dental caries is an irreversible microbial disease, affecting millions of people, which results from the dissolution of the inorganic component of the tooth, enamel and dentin [8].

Fortunately, it is a treatable disease. However, once treated, restoration failure is a common issue, which then compromises adhesive longevity [9]. Inadequate salivary flow, poor oral hygiene, and insufficient fluoride exposure are some of the factors known to contribute to the development and progression of caries [10].

Caries can be classified as primary and secondary. Primary caries is the initial attack of the tooth substrates. This occurs when fermentable carbohydrates are processed by host bacteria normally present in the dental biofilm leading to the production of lactic acid, which in turn dissolves the inorganic component of the tooth [11]. Conversely, secondary caries form around and under existing dental restorations [12]. The formation of secondary caries suggests that the seal at the composite–dentin interface cannot withstand the physical, chemical, and mechanical stresses present in the oral cavity [2].

Secondary caries start by a phenomenon known as micro-leakage, as mentioned briefly in section 1.1. Micro-leakage is defined as the "diffusion of the bacteria, oral fluids, ions and molecules into the tooth and the filling material interface" and as the "clinically undetectable passage of bacteria, fluids, molecules or ions between tooth and the restorative or filling material" [13].

Our oral cavity is home to the bacteria known as Streptococcus mutans (*S. mutans)*, gram-positive bacteria that constitute biofilms on the surface of teeth. These anaerobic organisms produce high levels of lactic acid and are resistant to the effects of low pH, properties which encourage further propagation of the caries disease [8]. *S. mutans* is known as the main etiological factor in the development of dental caries [14]. One of the main areas of dental materials research has focused on the evaluation of the effects of different antibacterial agents incorporated into dental adhesives. Zhang, *et al*. [15] reported that dimethylaminododecyl

methacrylate (DMADDM) adhesives slowed the pH drop and decreased the lactic acid production of *S. mutans* with a 10-30 fold difference compared to the control group.

In recent years, a profound paradigm shift in the treatment of caries has been observed from a traditional model to a medical approach [16]. Whereas the traditional caries model was focused on the treatment of the actual cavitation present in teeth (i.e. consequence of the disease), the contemporary medical model revolves around prevention of the disease through a thorough understanding of the factors contributing to its development.

1.3. RESIN-BASED COMPOSITE RESTORATIVE MATERIALS

With the development and more recent improvements in the properties of tooth-colored restorative materials of the last few decades, patients' treatment preferences have shifted from silver amalgams to tooth-colored resin based composite materials [17]. Both dentists and patients alike opt for resin-based composite restorative materials for a number of reasons including conservativeness and aesthetics. By definition, a composite is a material made of two or more materials, which are different in chemical composition and physical properties, and when combined are able to produce a material with greater overall properties [18]. Resin-based composites combine an organic matrix and an inorganic component and can be used for a number of applications including anterior and posterior restorations, pits and fissure sealants, for the bonding of ceramic veneer and other types of fixed prosthesis.

1.3.1. Composition

Resin-based composites consist of four major components: organic matrix, inorganic filler particles, coupling agent and an initiator-activator system. Each component is essential to the overall structure and function of these materials.

The organic matrix, the phase to which all other components are added, is typically a combination of high and low molecular weight monomers including Bis-GMA (bisphenol-A glyceril methacrylate), UDMA (urethane dimethacrylate), TEGDMA (triethylene glycol dimethacrylate) and hydroxyethyl methacrylate (HEMA). Functional monomers serve several purposes including the etching for demineralization and infiltration of the dental hard tissues, fluoride release and antibacterial effects among others. Commercial adhesives typically include carboxylic acid-based monomer and phosphate-based monomer functional groups [19]. Universal dental adhesives such as Single Bond (3M ESPE), used for the present thesis, contains Bis-GMA, HEMA, 10-methacryloyloxydecyl dihydrogen phosphate (MDP), and other undisclosed monomers. A study by Asmussen and Peutzfeldt (1998) demonstrated that variations of the BisGMA/TEGDMA/UEDMA ratio greatly affected the mechanical properties of the composite, suggesting that specific combinations should be developed based on the specific applications of the material [20]. Regardless of the ratio, because of its organic composition, the resin matrix is the weakest phase.

The inorganic filler particles are generally silica particles, quartz, and glass. The role of fillers is to provide strength to the composite while also improving their overall properties, decrease the amount of the matrix material and consequently the water sorption, coefficient of thermal expansion and polymerization shrinkage. Polymerization shrinkage occurs when monomer molecules are converted into a polymer network, by the number of covalent bonds formed. This phenomenon is unfavorable because of the resultant poor marginal seal that eventually leads to the formation of secondary caries [21].

Resin-based composite restoratives can be classified based on the amount and size of the filler particles. Currently, the two main types are hybrid and micro-fill resins, as depicted in

Figure 1. Hybrid resins are typically a blend of macro-filler particles and micro-fine silica particles. Hybrid resins are regarded as superior in mechanical properties; the increased filler loading enhances the stress transfer between particles in the composite [22]. Hybrid composites have reduced polymerization shrinkage compared to other resin based composite materials due to a reduced matrix content.

Micro-filled composites contain micro-fine filler particles of silica (SiO_2) [23]. Because of their lower filler content, micro-filled composites have weaker physical properties compared to hybrid composites [23]. Generally, they have greater polymerization shrinkage and water sorption than hybrids. In terms of the marginal integrity, color, and marginal discoloration, hybrid composites generally perform better [24].

The filler particles can be incorporated into the resin matrix by heterogeneous or homogeneous methods. The homogeneous method means that the micro-filler is added directly to the resin. The heterogeneous method requires compression of the micro-filler first and then addition of the fumed silica to a heated resin [25].

A coupling agent, silica, is responsible for providing adequate interfacial adaptation between the resin matrix and the filler particles [26] **(Figure 2)**. It prevents the leaching of the filler particles by preventing water from penetrating along the resin-filler interface [27]. Coupling agents can be chemically bonded by filler-particle resin matrix, which transfers stresses or by a bi-functional organosilane molecule [26]. The latter, siloxane end bonds to hydroxyl groups on the filler and the methacrylate end polymerized with the resin [26].

Dental restorations are fixed onto the tooth's surface by polymerization via a light curing unit. Photo-polymerization allows for the dissociation of initiator molecules into free radicals. The radical chain polymerization reaction is initiated by the camphorquinone-amine complexes

[28]. The reaction is activated by these complexes with a light curing unit, at a wavelength within a range of 400-500nm and a given intensity [29]. This results in a reaction with double bonds in the monomers or pre-polymers which break into single bonds and provide links between the monomers to cross-link into a polymeric network [29, 30].

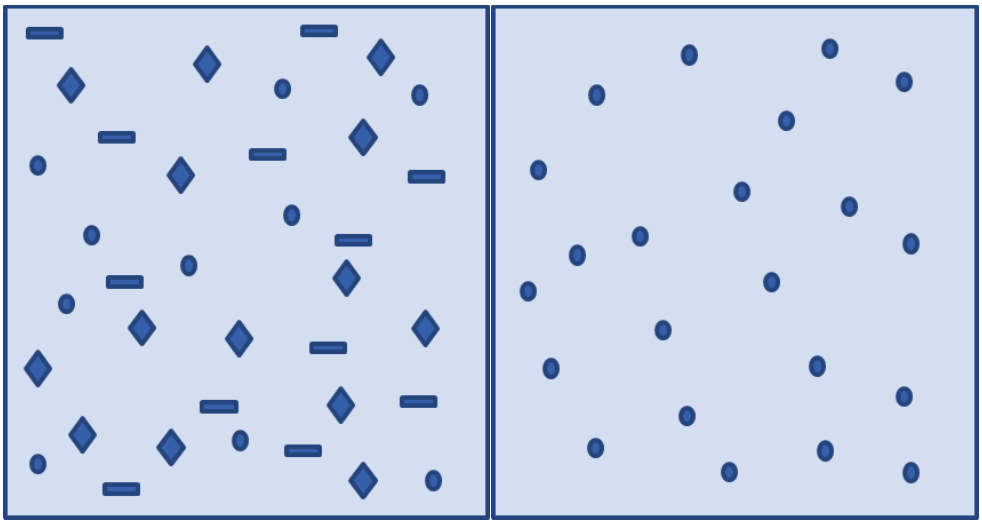

Figure 1. Hybrid (left) and micro-filled (right) resin-based composites.

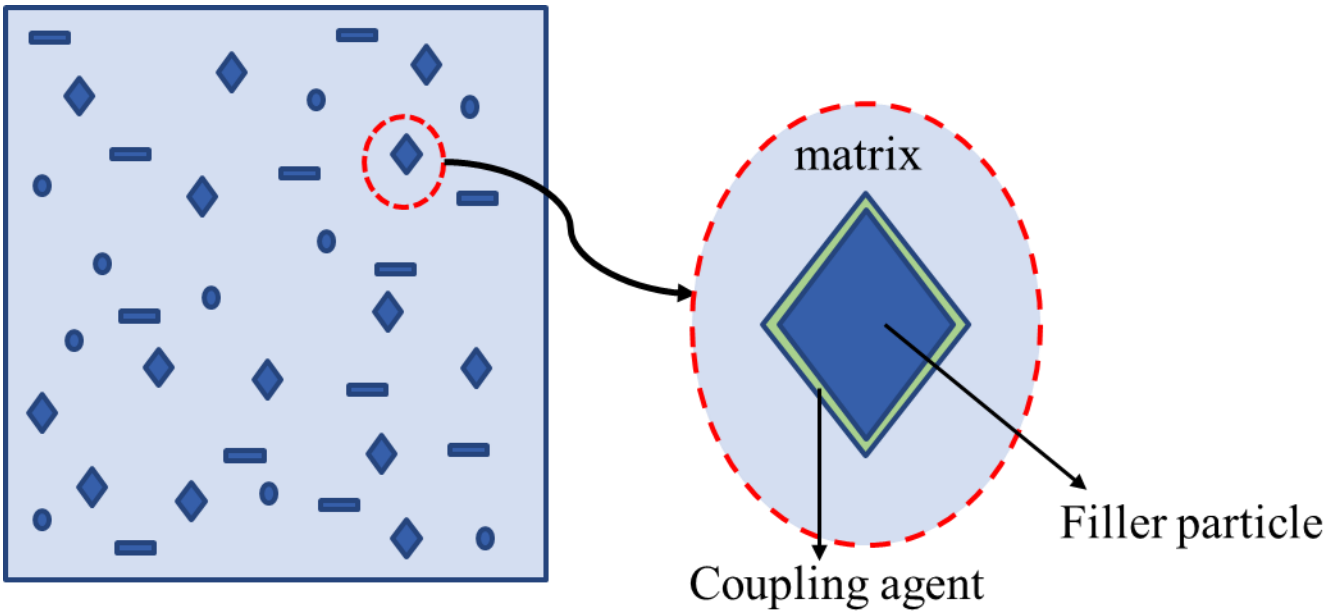

Figure 2. Bonding of fillers to resin matrix. The coupling agent provides interfacial adaptation between the matrix and filler particle.

1.4. MECHANISMS OF ADHESION

1.4.1. Micro-mechanical via hybrid layer formation

The bonding mechanism of adhesives can be compared to an exchange system that allows for the substitution of inorganic tooth material by resin monomers [31]. A result of the process of micro-mechanical interlocking is the formation of a hybrid layer [32]. After removal of the smear layer and inorganic content by acid etchants or self-etching primers, subsequent infiltration of the partially demineralized tooth substrates with resin monomers yields the so-called hybrid zone **(Figure 3)**. This layer consists of partially demineralized collagen, resin monomers, residual solvents and water.

Because the collagen network that is exposed after etching remains embedded in the hybrid layer, the layer is particularly susceptible to the effects of hydrolytic degradation [33]. The properties of the hybrid layer determine largely the overall quality and long-term integrity of

these restorations. Resin-enamel bonds are typically very stable and durable [34]; however, resin-dentin bonds can be unpredictable because of the substrate's variability for bonding [35].

1.4.2. Chemical adhesion

Adhesion through molecular interactions between the adhesive and substrate surface is known as chemical adhesion. A strong chemical adhesion bond results when an adsorbed molecule dissociates on contact with a surface and the atoms rearrange themselves to form covalent or ionic bonds [36]. Ionic bonds known to form between the carboxyl groups of the glass polyalkenoate cements and calcium ions in enamel and dentin is an example of chemical adhesion to tooth structure [37].

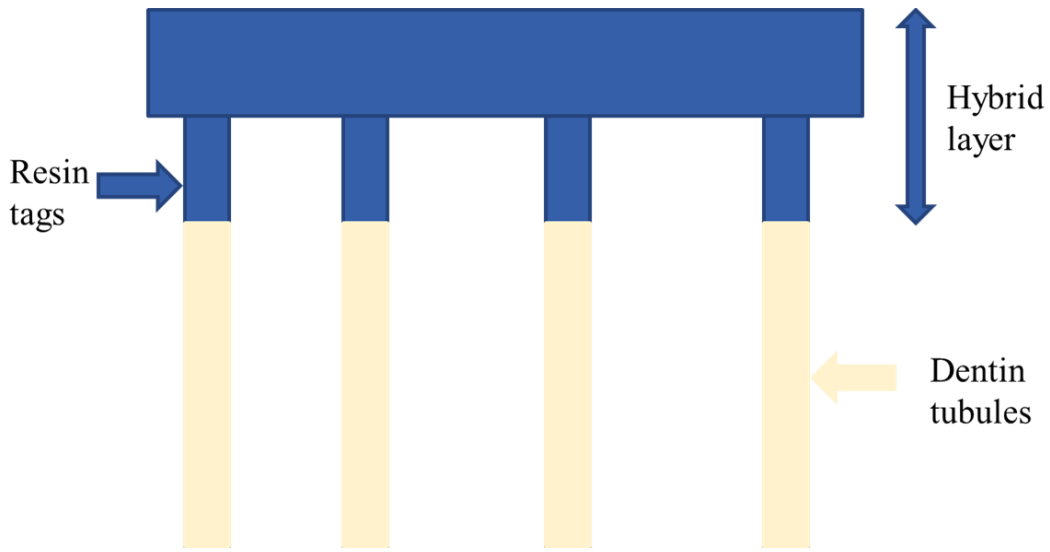

Figure 3. Schematic of dentin bonding, demonstrating the hybrid layer.

1.5. DENTAL ADHESIVE SYSTEMS

1.5.1. Composition

Resin based dental adhesives are unfilled or lightly filled resins. The same components as those described in the previous section for resin-based composites are present, an organic matrix,

a coupling agent (silane) and an initiating-activating system. The inorganic filler loading is either minimal, making the mixture less viscous, or non-existing.

1.5.2. Classification

Dental adhesive systems can be classified by generation, clinical application steps, and underlying adhesion strategy. These classifications are based on the three main steps involved in achieving micro-mechanical retention to tooth structure, etching, primer and adhesive. Acid etching involves the use of 35-40% phosphoric acid to remove the smear layer, a layer of inorganic debris that remains on the surface of the dentin after tooth preparation, and other inorganic phase from the tooth substrates opening the dentinal tubules for subsequent infiltration by resin monomer [38]. This rough layer provides greater surface area for bonding [39].

In 1955, Buonocore, pioneer of adhesive dentistry, spoke for the first time about the benefits of acid-etching [40]. After acid etching, the partially demineralized tooth structure is infiltrated with primers. Primers are hydrophilic monomers as hydroxyethylmethacrylate (HEMA) and solvent carriers as ethanol, acetone or water and their primary function is to raise collagen's surface energy facilitating subsequent infiltration with hydrophobic adhesive [41]. In other words, the primer allows for better adhesion by re-wetting the partially demineralized collagen fibers and preventing it from collapsing. The wetting process is critical to achieving molecular attraction. Last, the collagen network is infiltrated with a hydrophobic adhesive resin, which is then polymerized in situ providing bonding to the overlaying resin based composite restorative material. To achieve optimal wettability of the primed surface, the adhesive must display a low contact angle on dentin [41].

1.5.2.1. By generation

Classification by generation includes the first to the eighth generation. This is an older system which was used to signify the changes made by each generation in the products being put on the market. Generations four through seven are the bonding systems still used today in clinical dental practice [40].

The first generation bonding agents were designed for ionic bonding to hydroxyapatite or for covalent bonding (hydrogen bonding) to collagen [42]. The issue with this adhesive system, was its reduction in bonding after its immersion in water [40]. To overcome this issue, Bowen [43] used a coupling agent, N-phenylglycine and glycidyl methacrylate, or NPG-GMA. Nonetheless, the first generation bond strength was still relatively weak, producing bond strengths in the range of only 1-3 MPa [43]. The second generation incorporated polymerizable phosphates, which were added to Bis-GMA resins, but the low bond strength was still an issue [44]. The first and second generations did not remove the smear layer, resulting then in weak bond strengths. The third generation presented a significant improvement by partially removing, for the first time, the smear layer by acid etching and opening up the dentinal tubules [45]. However, the bond strength was still relatively weak because the unfilled resins did not penetrate the smear layer effectively [46].

The complete removal of the smear layer was achieved by the fourth generation [47]. This was accomplished by applying the etchant, primer, and bonding separately. The fourth generation accomplished a far greater bond strength, reaching about 20 MPa, as compared to the earlier generation systems [40]. This thus became the gold standard. The only downside to this generation was the multi-step process involving many bottles. The goal then became combining the multiple step process into fewer steps without a compromise to the bond strength. There was

a simplified process following in the fifth to eighth generations by combining some steps such as the primer and adhesive bonding [48].

1.5.2.2. By clinical application steps

Clinical application steps refer to whether adhesive systems are applied in one, two or three steps. The number of clinical application steps vary depending on the adhesion strategy as per description below. Etch-and rinse systems can be applied either in two or three steps, whereas self-etch systems can be applied in one or two steps.

1.5.2.3. By underlying adhesion strategy

"Etch-and-rinse" and "self-etch" are bonding strategies that differ in their approach and interaction with tooth substrates. Etch-and-rinse systems require the complete removal of the smear layer and a greater depth of demineralization of the dentin by phosphoric acid. Etch and rinse systems can be applied in two or three steps. First, the phosphoric acid is applied to demineralize the hard tissues for 15 sec, which is followed by vigorous rinsing for at least five sec to remove all the etchant from the surface. Following, the primer and adhesive are applied either as separate steps (Three-step etch-and-rinse) or combined into a single step (Two-step etch-and-rinse).

Currently, the most commonly used bonding technique for etch-and-rinse adhesives is the so called "water-wet-bonding" [49].

In the early 1990's, Kanca [50] showed that water-wet-bonding produced higher bond strengths than dry bonding. Gwinnett [51] demonstrated that the water wet-bonding technique was attributed to water induced expansion of shrunken, dried matrices. The disadvantage of wet-bonding is that the matrix is too soft and can easily shrink when the solvent is evaporated [52]. Although wet-bonding does not solubilize collagen, it has the advantage of breaking inter-

peptide hydrogen bonds that open up spaces between collagen fibrils for resin infiltration [53] . However, the monomers must be relatively soluble in water. The simplest way to ensure that solubility is to solvate them in at least 50% ethanol or acetone [52].

Self-etch systems, unlike etch-and-rinse, don't require a rinsing step. They use self-etching primers containing acidic methacrylate primers with phosphate or carboxylic functional monomers that can etch and prime simultaneously. The smear layer is partially removed, exposing a thin layer of demineralized collagen. The water in the primer is then evaporated with air and the adhesive is then applied to the surface. Self-etching adhesives can be applied in one or two steps. Two-step self-etch systems combine etching and primer in a first step called self-etching primer, which is then followed by the application of a hydrophobic adhesive resin as a second step. One-step self-etch adhesives combine all three steps, etching, primer and adhesive, into a single bottle. As expected and due to the diverse chemistry present in these single bottle adhesives, they are highly unstable leading to the most failures [54].

Both systems lead to the formation of a hybrid layer as the resins infiltrate enamel and dentin layers replacing the inorganic component removed by an acid etchant or self-etching primer. Unlike self-etching systems, etch-and-rinse systems have been traditionally regarded as the gold standard since phosphoric acid can create a more prominent etching pattern, thus resulting in a thicker hybrid zone [55]. Self-etching systems, on the other hand, may be the preferred technique for many as they are known to be less technique sensitive and thus are less prone to post-operative sensitivity issues [56].

1.6. ADHESION SUBSTRATES - ENAMEL AND DENTIN

Enamel and dentin serve as the main two adhesive substrates. Enamel is highly inorganic and composed of hydroxyapatite crystals. It contains 96% inorganic material and 4% organic and

water by weight [57]. Being the outermost part of the tooth makes it most vulnerable to wear and caries [58]. However, bond strengths to enamel are typically high and predictable due to its tightly packed hydroxyapatite crystals [59], and thus the durability of the enamel bonds is much greater than those of dentin.

Dentin, directly under enamel, is a more organic tissue consisting of 70% calcium phosphate mineral (hydroxyapatite), 20% organic material, and 10% water by weight [60]. This layer can act as a barrier to bacterial infiltration and bonding depends on the preparation technique used [32]. The resin-dentin bond can be achieved by micro-mechanical and chemical adhesion, as described in sections 1.4.1 and 1.4.2, respectively.

1.7. REASONS FOR FAILURE OF RESIN COMPOSITE RESTORATIONS

Resin composites may fail due to several factors. Composites may undergo incomplete resin infiltration, hydrolytic degradation of the adhesive interface, marginal micro-leakage and consequently, secondary caries.

1.7.1. Incomplete resin infiltration

Incomplete resin infiltration is the result of the insufficient impregnation of the exposed collagen space following the application of bonding resin [61]. Attempts to infiltrate resin monomers into etched dentin by etch-and-rinse adhesives has been demonstrated to be only partially effective [62]. Given the short clinical time, achieving the complete replacement of the rinsing water by adhesive monomers is difficult and always results in hybrid layers that contain voids [63]. These voids are usually located at the hybrid layer, just above the mineralized dentin. They are the result of spaces around collagen fibrils that were not encircled by the infiltrating resin, and have been referred to as nano-leakage [62]. The use of self-etching adhesives, which remove less mineral from the dentin surface while simultaneously replacing them with resin

monomers, is known to minimize the discrepancies between the depth of demineralization and the depth of resin infiltration [64]. Long-term bond strength requires intimate penetration of the adhesive into the demineralized dentin and the formation of a durable hybrid layer [61].

1.7.2. Hydrolytic degradation of adhesive interface

Because of the hydrophilic nature of dentin, hybrid layers are susceptible to hydrolytic degradation and thus resin leaching [65]. Water penetration into the hydrophilic domains of the adhesive encourages leaching and these domains lack monomer/polymer conversion because of adhesive phase separation [66]. The resulting poorly polymerized hydrophilic phase degrades in the aqueous environment. The collagen matrix is now exposed and susceptible to the attack by proteolytic enzymes [67]. Methacrylate adhesives containing hydrolytically susceptible groups such as ester and urethane, hydroxyl, carboxyl, and phosphate groups, may then be hydrolyzed by chemical and enzymatic degradation [68]. Hydrolysis contributes to the reduction of bond strength over time and is considered a primary reason for resin degradation within the hybrid layer [69].

1.7.3. Marginal micro-leakage and secondary caries

Micro-leakage is defined as “the penetration of bacteria, fluids, molecules, or ions into the spaces between the cavity walls and the restorative materials, resulting in sensitivity, recurrent (secondary) caries, discoloration of the restoration margins, irritation of the pulp, and restoration failure” [70]. In a study by Hansen [71], it was shown that the incremental technique, applying composites in increments, tends to improve marginal adaptation by resisting the resin composite shrinkage stress. Stresses applied on the restoration can disrupt the bonding and lead to the formation of gaps [72]. Thus, the proper bond of an adhesive to the dental tissue contributes to avoid marginal micro-leakage. Deficient margins eventually leads to the formation

of secondary caries [3]. This process usually relates to the failure of the bonding in the hybrid layer as discussed in section 1.7.1.

1.8. ANTIBACTERIAL MONOMER AND SURFACTANT CROSS-LINKER

Recently synthesized monomers and cross-linkers (details regarding chemical composition and synthesis are described in chapter 2) containing antibacterial properties incorporated in dental adhesives may help to reduce the colonization of the caries causing bacteria, *S. mutans* without compromising their longevity and tensile strengths. In this present study, the incorporation of a quaternized amine group allows for the positive charge on nitrogen to disturb the cell membrane of bacteria. An electrostatic interaction is present between NBr, where bromide is the counter ion and can be replaced by other counter ions in the media. As previously mentioned, Single Bond (3M ESPE) contains methacrylate groups. Methacrylates are used in resin adhesives because of their photopolymerization reactivity and superb mechanical properties. However, polymethacrylates themselves do not have antibacterial properties. Surfactant monomer and cross-linker have acrylate bonds for copolymerization with methacrylates in Single Bond to form polymer chains. Testing the monomer and cross-linker in various concentrations (0.05, 0.1, and 0.25 mg/mg) will provide us with critical information on their antibacterial effects on *S. mutans*, cytotoxicity, tensile strengths, and micro-hardness.

1.9. LABORATORY TESTING FOR ASSESSING BOND DEGRADATION AND TENSILE STRENGTH

Evaluating the bond degradation and tensile strengths of new adhesive systems is critical in determining its potential long-term efficacy. Ideally, *in vivo* long-term testing of these specific characteristics would provide the most optimum results. However, due to the fast-paced environment of adhesive production on the market, most newly developed adhesive systems

become quickly outdated. Therefore, the research and dental community accept the well-established *in-vitro* testing of these adhesive systems to predict its clinical usefulness [73].

1.9.1. Micro-tensile Bond Strength (MTBS)

The micro-tensile bond strength test was first introduced by Sano, *et al*. in 1994 [74]. The main characteristic of the micro-tensile test is the reduced specimen size, which provides a better assessment of the potential effects of hydrolytic degradation [75].

We can assess the physical properties and modulus of elasticity of the strength of adhesive bonds by utilizing the micro-tensile test. An advantage of this testing is the combination of evaluating both inter- and intra-tooth variabilities. Bonded molar teeth can be sectioned to about 1 mm thickness and stored for long-term testing. The test is ideal for small specimen and reduces the probability of the initiation of a crack, as is the case with some macro-testing methods.

1.9.2. Ultimate Tensile Strength (UTS)

Ultimate tensile strength test assesses the cohesive strength of the body of adhesive. Here, the specimens are the adhesives alone, e.g. not bonded to teeth as is the case with micro-tensile bond strength. Each specimen is fabricated into an hourglass shape with a fixed cross-sectional area. The specimen shape is critical for allowing the failure to occur in the mid part of the specimen to measure the changes in the cross sectional dimensions [76]. Thus, failure can be induced in the same section across all specimens for consistency and accuracy. For example, if a rectangular specimen is tested, failure may result anywhere in the sample. The ultimate tensile bond strength gives us useful information in determining the maximum stress a material can endure before its tension failure [77].

1.9.3. Micro-hardness

In 1951, Tabor [78] helped to settle the scientific basis for the indentation hardness testing. Micro-hardness tests are used to evaluate the hardness of a material and can be correlated to tensile strength [79]. This type of test is an indicator of wear resistance and ductility and is used specifically for smaller specimens where a macro-hardness test is not appropriate.

Common standard micro-hardness tests include Knoop (HK) and Vickers (HV)-indentation. The hardness is correlated with the depth of the indenter into the material, under a given load and within a specific period of time (sec). For both HK and HV, the test principles, procedures, and verification procedures are substantially identical [80]. By the use of an accurately controlled applied force, the testing machine produces indentations onto specimen surface.

The differences between these two tests are the indenter geometry types and method of calculation of the hardness numbers. The HK indenter produces an elongated diamond-shaped indentation. Likewise, the HV produces an indentation, but of a different geometry, a pyramid-shaped diamond. The size of the indentation is then measured using a light microscope equipped with a measuring device. It is critical that specimens are polished and have a smooth surface to appropriately identify and analyze the indentations.

HV may be used at higher force levels than HK. HK penetrates about half as deep as the HV, making it more ideal to test brittle materials and thin layers. Because the HK major diagonal is about three times longer than the HV, this reduces the error in readings—which is critical when producing small indents with low loads and hard materials. HK test is the preferred method for evaluation of polymeric materials, such as resin composites, because it decreases the effect of elastic recovery [81]. Higher filler content may be present in one area of a sample compared to

another similar area and thus may result in greater variation. However, a study by Poskus, *et al.* [82] and Chang, *et al.* [81] demonstrated that there were no statistical significance between the HV and HK data of resin composites.

1.10. LABORATORY TESTING FOR ASSESSING BIOCOMPATIBILITY

With the fabrication of new biomaterials, it is important to evaluate the ability of the material to perform their intended function without eliciting any undesirable effects. Biocompatibility testing is usually conducted following accepted published standards for medical devices [83] (ISO 10993). We can assess the antibacterial or cytotoxic effects of biomaterials *in-vitro.* This will provide us with useful information that can be applied clinically.

1.10.1. Cell viability

The MTT assay (Sigma-Aldrich, St Louis, MO, USA) is a common biological assay used to assess the cytotoxicity effects of biomaterials. The main principle of this assay is based upon cell metabolism. NAD(P)H-dependent cellular oxidoreductase enzymes should reflect the number of viable cells present. These enzymes reduce the tetrazolium yellow dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to its insoluble formazan. This can be viewed visually as the solution turns to a purple color, indicating cell viability [84]. For quantifying the results, this solution's absorbance can be measured via spectrophotometer at a wavelength between 500-600 nm.

1.10.2. Antibacterial

Because of the potential for secondary caries to develop under pre-existing restorations, it is critical, and it is the main objective of this thesis, to develop antibacterial additives that may be added to dental adhesives. Evaluating the effect of these potentially antibacterial adhesives against cariogenic bacteria *Streptococci mutans* and *Lactobacilli species* becomes crucial.

Currently, a number of dental materials possess antibacterial benefits. Their effects, however, are somewhat short-term, losing their antibacterial function overtime. Ideally, a material with slow, gradual and especially sustained release would be able to provide the intended long-term favorable effect [15].

There are multiple methods to test the antibacterial properties of biomaterials. Testing of dental materials include the agar disc-diffusion (qualitative) and colony forming unit (quantitative) methods [85]. The agar disc diffusion method measures the "zone of inhibition" of bacterial growth around the sample. This testing is less standardized than other antibacterial tests and its reproducibility should be within ±2mm [86]. It is an inexpensive commonly used method, especially for routine clinical diagnostic work [86]. Conversely, the colony forming unit (CFU) method allows us to interpret the antibacterial effectiveness of a biomaterial by counting the bacterial colonies resulting on the agar plate. This technique is more labor intensive as dilutions are tested and adjusted to analyze the number of colonies on a dilution plate showing between 30 and 300 colonies [87]. This range is considered statistically significant. Less than 30 colonies on the plate indicates small errors in dilution technique or the presence of a few contaminants. If there are more than 300 colonies on the plate, colonies will have grown together and as a result poorly isolated. The data for this assay should reflect the number of CFUs per milliliter (mL) of sample.

1.11. LABORATORY TESTING FOR ASSESSING MICROSTRUCTURE CHARACTERIZATION

Different tests are available to characterize, at a microscopic level, the micro-structure of new biomaterials. It is critical, in this context, that both surface and interfacial micro-structure

are evaluated since characteristics of the adhesive as well as its ability to infiltrate dentinal tubules are equally important.

1.11.1. Scanning Electron Microscopy (SEM)

Through the use of SEM, the micro-structure of these adhesive systems can be characterized. SEM produces images based on the interactions of the electron beam with atoms within a sample. The response of these interactions are then collected by a detector. Common detectors include; secondary electron (SE) and backscattered electron (BSE).

Secondary electrons originate from the surface of the sample. These electrons result due to the inelastic interactions between the primary electron beam and the sample [88]. Secondary electrons contain lower energy than the backscattered electrons. Typically, if the sample of interest is being analyzed for topographical purposes, then SE is the preferred method.

Backscattered electrons are a result of elastic collisions of electrons with atoms, causing a change in electrons' trajectory [88]. Higher signals are produced from larger atoms because they are stronger scatterers. BSE imaging is ideal for obtaining more information on a specimen's topography, crystallography, and magnetic field.

CHAPTER 2
MATERIALS AND METHODS

2.1. EXPERIMENTAL AND CONTROL TESTING MATERIALS

The following syntheses of all experimental materials in this section were conducted by Dr. Cheng's lab group, Furnas Hall, University at Buffalo.

2.1.1 Synthesis of cross-linker

First, a functional surfactant precursor for the cross-linker synthesis was prepared as follows: A 250-mL round-bottomed flask equipped with a magnetic stir bar was charged with 1-bromododecane (3.19 g, 12.8 mmol) and *N*-methyldiethanolamine (15.4 g, 128 mmol). The solvent used was acetonitrile (70 mL). The reaction mixture was stirred at 80 °C for 24 hrs. The solution was placed in an oil bath to maintain a consistent heat source. When the mixture was cooled to room temperature, the solvent was removed by rotary evaporation and the residue was washed with diethyl ether. After filtration, a white solid functional surfactant product precipitated (4.24 g, 11.5 mmol, yield: 90%). Verification of this product was performed by Proton Nuclear Magnetic Resonance (NMR) as shown in **Figure 4**.

The following was preformed to obtain the final cross-linker surfactant product:
A 100-mL round-bottomed flask equipped with a magnetic stir bar was charged with the functional surfactant precursor described above (1.29 g, 3.50 mmol), acrylic acid (0.756 g, 10.5 mmol), N,N'-dicyclohexylcarbodiimide (DCC; 2.163 g, 10.5 mmol),and 4-dimethylaminopyridine (DMAP; 0.085 g, 0.70 mmol). Dichloromethane was used as the solvent (35 mL). The reaction mixture was stirred at room temperature for 24 hrs. After this, the solvent was removed by rotary evaporation. Silica column chromatography was performed with methanol and dichloromethane (DCM; 1:10) to obtain the final yellow solid cross-linker product

(1.12 g, 2.35 mmol, yield: 67%). This product was verified by Proton Nuclear Magnetic Resonance, shown in **Figure 5**.

2.1.2 Synthesis of monomer

The following synthesis was performed to obtain the final monomer surfactant product: A 50-mL round-bottomed flask equipped with a magnetic stir bar was charged with 1-bromododecane (1.80 g, 7.2 mmol) and *2*-(dimethylamino) ethyl acrylate (0.86 g, 6.0 mmol). Because the monomer contains a double bond and has potential to polymerize during the heating, a trace amount of *2,2*-diphenyl-1-picrylhydrazyl was used to inhibit the potential premature-polymerization. The solvent used was acetonitrile (15 mL). The solution was then placed in an oil bath. The reaction mixture was stirred at 80°C for 24 hrs. After the reaction, the solvent was removed by rotary evaporation and the residue was washed with ether (50 mL × 3). After filtration, a yellow solid monomer product precipitated (2.51 g, 6.56 mmol, yield: 91%). **Figure 6** confirms this product by Proton Nuclear Magnetic Resonance.

All reactants and solvents described above were obtained from Sigma Aldrich, St. Louis, MO, USA.

2.1.3 Incorporation of monomer and cross-linker into the resin-based adhesive

After synthesis described in 2.1.1, monomer and cross-linker were weighed out *(mg)* using a standard AGCN 200 laboratory balance (Fulcrum Inc, Clifton, NJ, USA) and added to a certain amount of commercial Single Bond (SB) adhesive (3M ESPE) as per the synthesis in **Figure 7** to obtain the respective testing concentrations in **Table 1**. For example, to obtain a 0.05 mg/mg monomer: 15 mg of monomer was mixed with 285 mg of Single Bond. All samples were vortexed (Scientific Industries Inc, Bohemia, NY, USA) for two mins to ensure consistent blend.

Adhesives incorporating only monomer demonstrated greater viscosity than the rest of the blends.

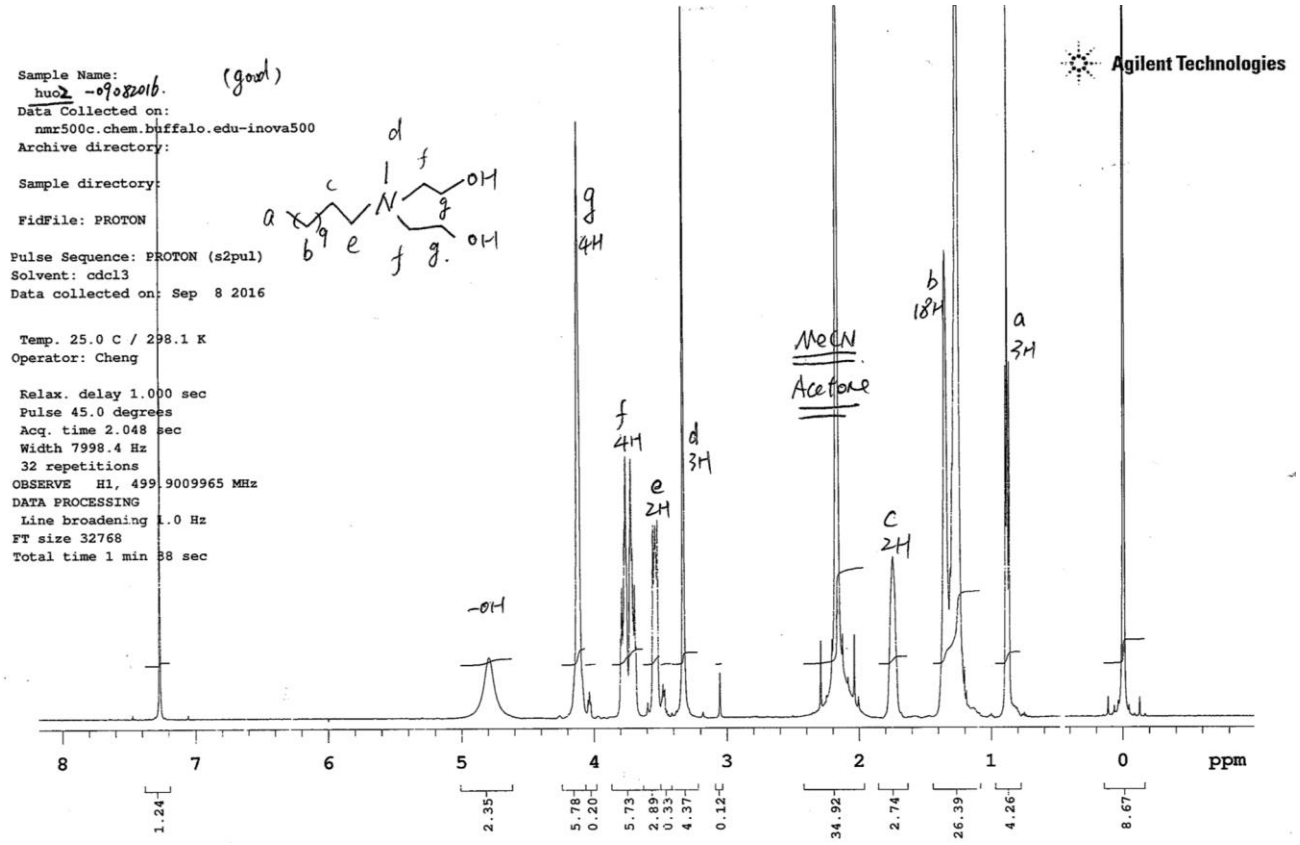

Figure 4. Proton Nuclear Magnetic Resonance spectrum (500 MHz, $CDCl_3$) of the functional surfactant precursor used to synthesize the experimental cross-linker. The letters indicate the location of the protons in the structure.

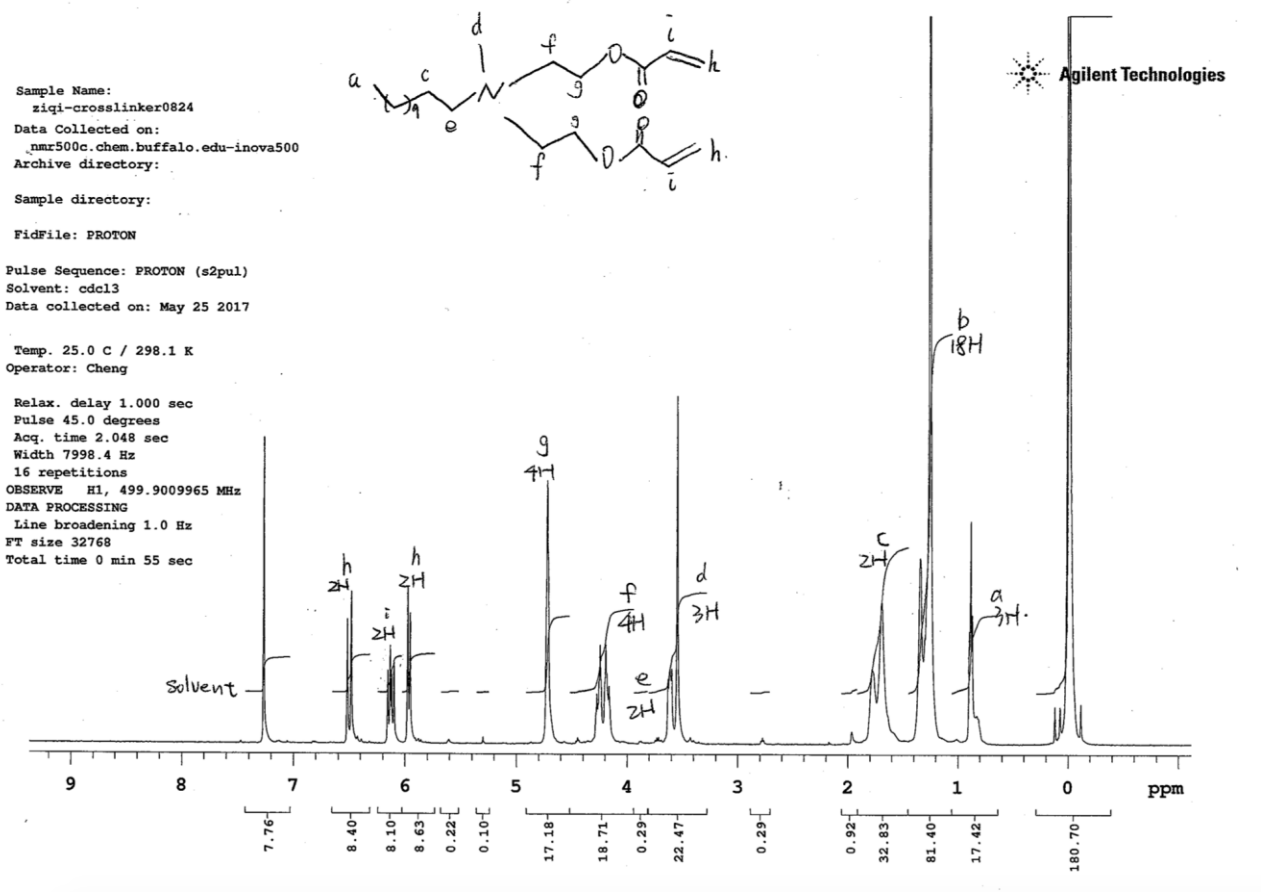

Figure 5. Proton Nuclear Magnetic Resonance spectrum (500 MHz, $CDCl_3$) of the cross-linker surfactant. The letters indicate the location of the protons in the structure.

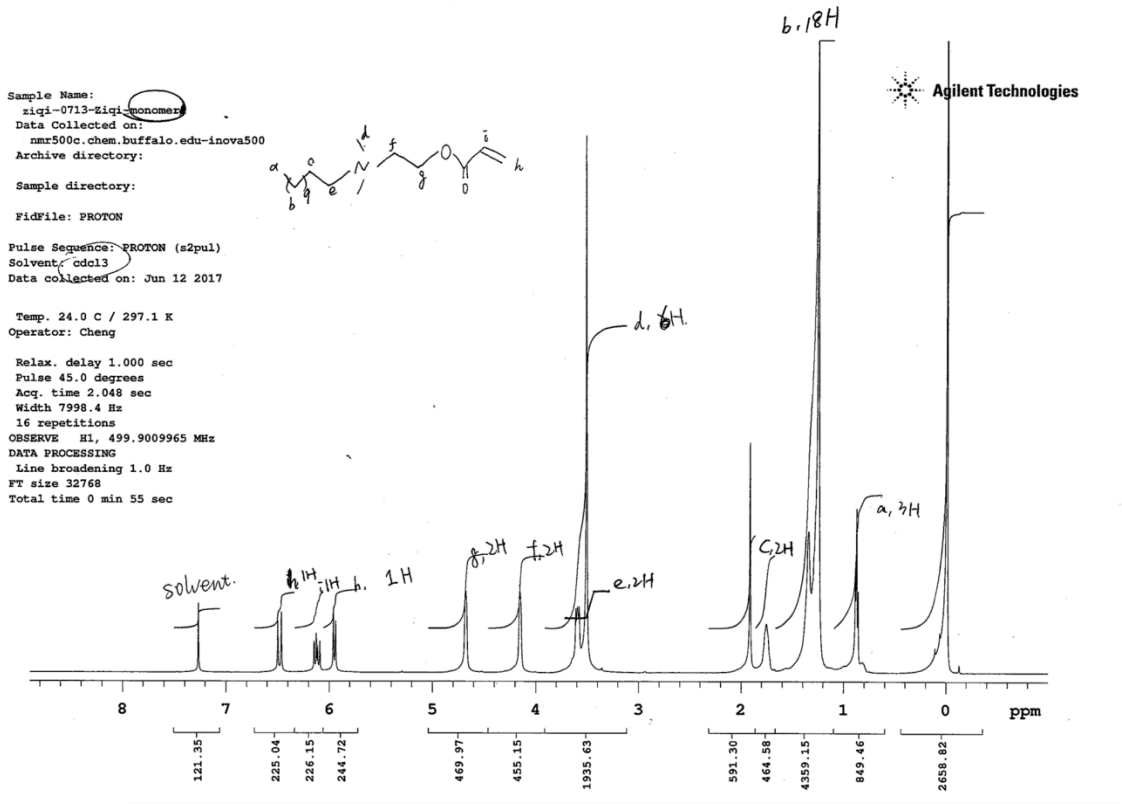

Figure 6. Proton Nuclear Magnetic Resonance spectrum (500 MHz, $CDCl_3$) of the monomer surfactant. The letters indicate the location of the protons in the structure.

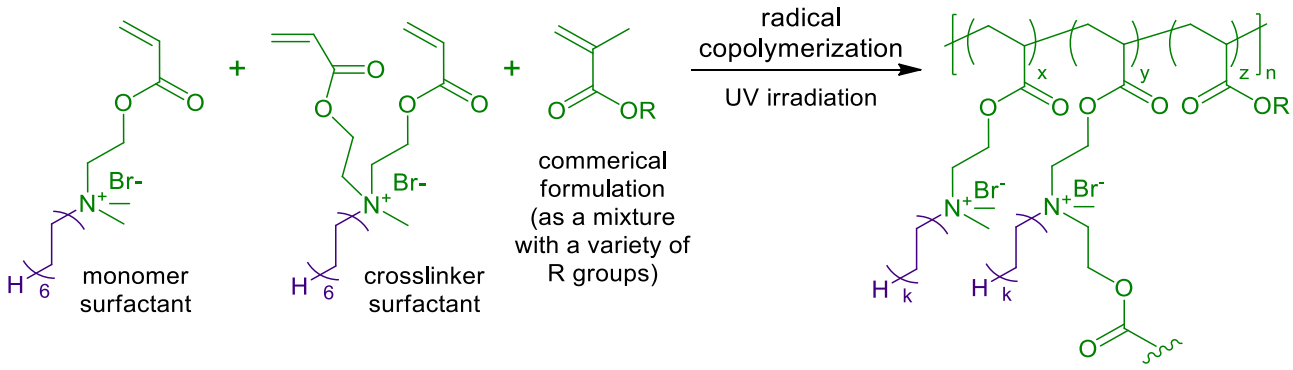

Figure 7. Schematic of incorporation of monomer and cross-linker into the commercial formulation SB. Here, a monomer and cross-linker containing quaternized amine group gives the antibacterial characteristic of the functional surfactant. Blue light irradiation was applied for 20 sec to induce radical polymerization. In the resulting copolymer product, concentration of antibacterial quaternized amine group is determined by the sum of x and y; crosslinking density is determined by y; the fraction of base dental materials gives z.

Table 1. The nine experimental adhesives tested in this study. Each group is listed with its respective concentration, description, and abbreviation or code.

Group	Concentration (mg/mg)	Description (per 1 mg of total mass)	Code
1	0.05	Single Bond + 0.05 mg monomer	0.05 M
2	0.1	Single Bond + 0.1 mg monomer	0.1 M
3	0.25	Single Bond + 0.25 mg monomer	0.25 M
4	0.05	Single Bond + 0.05 mg cross-linker	0.05 C
5	0.1	Single Bond + 0.1 mg cross-linker	0.1 C
6	0.25	Single Bond + 0.25 mg cross-linker	0.25 C
7	0.05	Single Bond + 0.05 mg monomer + 0.05 mg cross-linker	0.05 MC
8	0.1	Single Bond + 0.1 mg monomer + 0.1 mg cross-linker	0.1 MC
9	---	Single Bond	SB

2.2. MICRO-TENSILE BOND STRENGTH (MTBS)

2.2.1 Aims and hypotheses

The purpose of this test was to evaluate both the bond strength at different time periods, as well as the bond degradation patterns of the experimental adhesives relative to the unmodified control Single Bond. Because the samples were prepared using different ratios of cross-linker and monomer, observing the effects this may have on the bond strength were of interest. The null hypotheses evaluated in this part of the study were:

1. There would be no effect of the different concentrations of monomer or cross-linker on the bond strength when evaluated either at 24 hrs or 6 mos.

2. There would be no effect of the different concentrations of monomer or cross-linker on the bond degradation pattern after 6 mos.

2.2.2. Specimen preparation

Dentin substrate was obtained from thirty-six recently extracted, healthy human molars, under a protocol approved by the State University of New York's institutional review board (IRB ID No. 00000133). The teeth were equally and randomly assigned to the nine study groups outlined in the above section with four teeth per group.

Occlusal enamel was ground and superficial occlusal dentin revealed using a water-cooled lab trimmer (Whip Mix, Louisville, KY, USA). A standardized smear layer was created with progressively finer silicon carbide abrasive papers of 320-, 400-, 600-, and 800-grit (SiC paper, Buehler, Lake Bluff, IL, USA). All adhesives were applied and polymerized with LED light-curing unit (VALO, Ultradent, South Jordan, UT, USA) with a power density of 1,400 mW/cm^2 following manufacturer's recommendations for Single Bond as per **Table 2**. The tooth structure was conditioned by applying 35% phosphoric acid etchant (Ultra-Etch, Ultradent, South Jordan, UT, USA) for 15 sec, rinsed for 5 sec and blot dried leaving dentin slightly moist. Single Bond adhesive was then applied in 2-3 coats with gentle agitation of the surface for 15 sec. The surface was then air dried for 5 sec to evaporate the solvents and ensure a thin even layer of adhesive and polymerized for 10 sec. Resin composite (Filtek Z100, 3M ESPE, Saint Paul, MN, USA) was applied to the bonded surface in increments less than 2 mm and polymerized for 40 sec (**Table 2**). All teeth were incubated for at least 24 hrs before sectioning by the non-trimming technique (**Figures 8 and 9**) into beams with a cross-sectional area of 0.9±0.1mm [89]. Forty beams were obtained for each study group, twenty of which were randomly allocated for micro-tensile bond strength evaluation at 24 hrs and 6 mos respectively (n=20).

2.2.3. Micro-tensile bond strength test

For micro-tensile bond strength test (MTBS) evaluation, individual beams were mounted on a stabilizing jig with cyanoacrylate (Zapit, Dental Ventures of America, Corona, CA, USA) as per **Figure 10** and stressed to failure with a universal testing machine (Micro-tester, Bisco, Schaumburg, IL, USA) at a cross-head speed of 1 mm/min. The load required to fracture the specimen was expressed in megapascals (MPa) by dividing the obtained load in Newtons (N) by the cross-sectional area of the bonded specimen (mm^2).

2.2.4. Statistical analyses

The data met the requirements of normal distribution (Shapiro-Wilk test, $p>0.05$) and equal variance (Brown-Forsythe test, $p>0.05$). A two-way (ANOVA) was used to analyze the effect of variables 'group' 'time' and the interaction "group × time" on MTBS values. Post-hoc Tukey's tests were used for pairwise multiple comparisons among group means. A significance level of $p<0.05$ was used for all tests. All statistical analyses were performed with SigmaStat version.3.5 (San José, CA, USA).

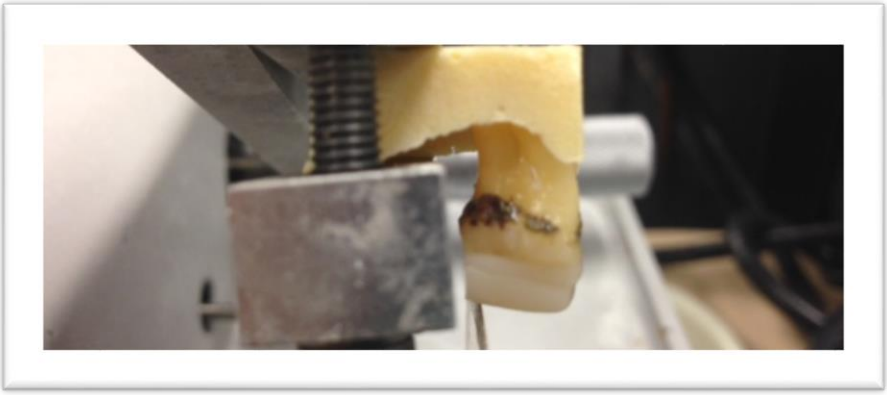

Figure 8. Lateral view of a resin-bonded tooth specimen on a mounting jig being prepared for sectioning with Buehler diamond saw blade.

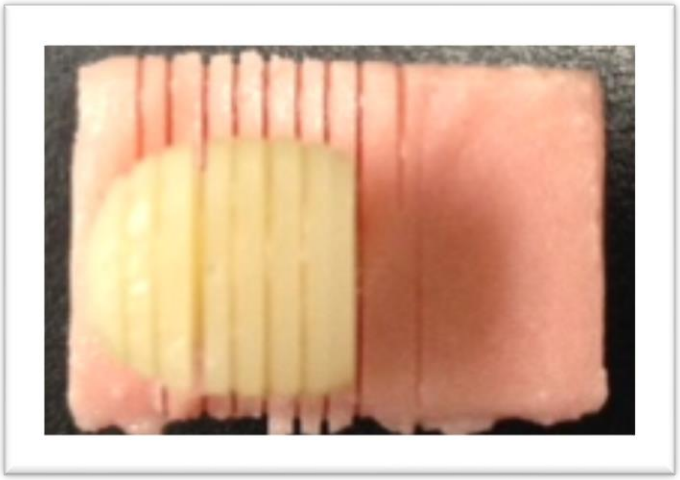

Figure 9. Occlusal view of resulting sectioning of 1mm slabs. The same sectioning was performed in the horizontal direction to obtain 0.9 ± 0.1 mm^2 beams.

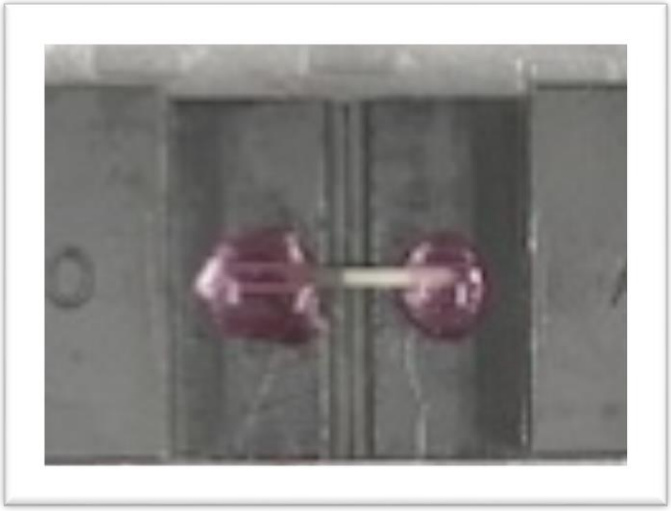

Figure 10. Top view of a composite-dentin beam mounted on a stabilizing jig with cyanoacrylate. Sample is ready to be stressed to failure with a universal testing machine at a crosshead speed of 1 mm/min.

Table 2. Study Materials included in this study

Material	Composition	Application protocol as per manufacturer
Filtek Z100 Composite (3M ESPE)	Matrix: Bis-GMA and TEGDMA Filler: Zirconia and Silica 84.5% (0.6 um)	• Place and light cure restorative in 2mm increments for 40 sec • Use a condensing instrument for packing • Cure with exposure to LED light with a minimum intensity of 400 mW/cm^2 in the 400-500nm range
Single Bond (3M ESPE) Etch-and-rinse	HEMA, Bis-GMA, dimethacrylates, ethanol, water, photoinitiator system, methacrylate functional copolymer of polyacrylic and polyitaconic acids, and silica nano-filler 10% wt. (5nm)	• Etch with 35% phosphoric acid (15 sec) • Rinse (10 sec) and blot dry • Apply 2-3 coats of adhesive with gentle agitation (15 sec) • Air thin (5 sec) • Polymerize (10 sec)

2.3. ULTIMATE TENSILE STRENGTH (UTS)

2.3.1. Aims and hypotheses

The purpose of this experiment was to evaluate whether incorporation of the cross-linker and monomer into Single Bond adhesive had an effect on the ultimate tensile of the unmodified adhesive blend. The null hypothesis was that the incorporation of monomer or cross-linker into the adhesive blend would have no effect on their ultimate tensile strength.

2.3.2. Specimen preparation

Specimens corresponding to the different study groups were fabricated using a rubber hourglass-shaped mold (10 mm long × 2 mm wide at neck × 1mm deep). After coating the mold with a thin layer of petroleum jelly to allow easier specimen retrieval, the mold was placed over a polyester strip, which was sat on top of a microscope slide. The mold was carefully filled with the corresponding adhesive blend and covered by another polyester strip and microscope slide to ensure producing flat specimens free of internal voids. The adhesives were then polymerized through the glass slabs using LED light-curing unit (VALO) for 40 sec with a power density of 1,400 mW/cm^2, according to manufacturer instructions. Ten specimens were fabricated per group (n=10), which were polished using a series of SiC abrasive grit papers of progressively finer grit (320-800). All specimens were then incubated in DW at 37°C and evaluated for UTS after 24 hrs, 1 wk or 6 mos.

2.3.3. Ultimate tensile strength test

For UTS evaluation, the specimens were mounted on a stabilizing jig with cyanoacrylate (Zapit) as per **Figure 11** and stressed to failure by applying tensional force with a universal testing machine at a cross-head speed of 1 mm/min (Micro-tester). The load required to fracture the specimen was recorded and expressed in megapascals (MPa).

2.3.4. Statistical analyses

The data met the requirements of normal distribution (Shapiro-Wilk test, p=0.717) and equal variance (Brown-Forsythe test, p=0.087). A two-way (ANOVA) was used to analyze the effect of variables ‘group’ ‘time’ and the interaction “group × time” on MTBS values. Post-hoc Tukey’s tests were used for pairwise multiple comparisons among group means. A significance

level of $p<0.05$ was used for all tests. All statistical analyses were performed with SigmaStat version.3.5 (San José, CA, USA).

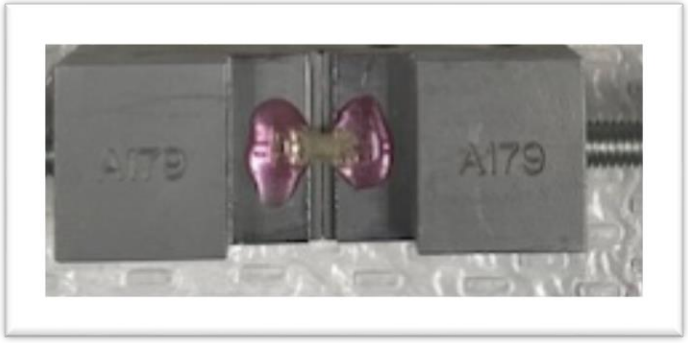

Figure 11. Top view of an adhesive beam mounted on a stabilizing jig with cyanoacrylate. Sample is ready to be stressed to failure with a universal testing machine at a crosshead speed of 1 mm/min.

2.4. MICROHARDNESS

2.4.1. Aims and hypotheses

The goal of this part of the experiment was to evaluate the surface micro-hardness of each of the adhesive blends evaluated in this study using the Knoop hardness test. The null hypothesis was that the incorporation of monomer or cross-linker would have no effect on the surface micro-hardness values of the adhesive blends.

2.4.2. Specimen preparation

To evaluate adhesive degradation in solvent, five specimens of 5 × 1mm for each experimental adhesive (n= 5) were made by polymerizing the adhesive in a polytetrafluoroethylene (PTFF) mold between two glass slabs. After polymerization, the samples were incubated for 24 hrs at 37°C and 100% humidity to allow post-cure polymerization. After 24 hrs, the specimens were embedded in acrylic resin and polished with wet SiC abrasive discs of progressively finer grits, 240, 320, 400, 600, 800 and 1200 as depicted in **Figure 12**.

2.4.3. Micro-hardness test

The specimens were allowed to dry at 37 °C for 24 hrs, and then subjected to a micro-hardness test in which five indentations (50 g/15 sec), one central and four radial approximately 100 μm apart, were recorded using a digital micro-hardness tester (HMV 2, Shimadzu, Tokyo, Japan). The instrument reports the hardness value using the following information: KHN= $14228 \times c/d^2$, where 14228 is a constant, *c* is the load in grams and *d* is the length of the longer diagonal in μm. For a given specimen, the five hardness values for each surface were averaged and reported as a single value. For each specimen, the initial Knoop hardness number (KHN_1) was registered as the average of the five hardness values. The specimens were then subjected to softening in absolute ethanol for 4 hrs at 37 °C as a form of accelerated aging [90]. The hardness test was repeated, and the post-conditioning hardness value was recorded (KHN_2). The percentage difference of KHN_1 and KHN_2 was calculated.

2.4.4. Statistical analyses

Since the data met the requirements of normal distribution (Shapiro-Wilk test, $p>0.05$) and equal variance (Brown-Forsythe test, $p>0.05$), individual Student's t-tests were conducted to evaluate the differences in micro-hardness values between baseline and 4 hrs of incubation in ethanol. A significance level of $p<0.05$ was used for all tests. All statistical analyses were performed with SigmaStat version.3.5 (San José, CA, USA).

Figure 12. Preparation of specimens for micro-hardness testing. Group 2 (0.1 M) shown above. Five 5 × 2mm adhesive discs were embedded to an acrylic mold for testing in the micro-hardness tester.

2.5. CELL VIABILITY

2.5.1. Aims and hypotheses

Since the monomer and cross-linker added to the resin based adhesive have an unknown effect on the host, this part of the study was set out to investigate whether incorporation of these additives would have an effect on the cell viability of gingival fibroblastic cells as compared to the control adhesive without any additives. Evaluation of the effect of these materials on fibroblastic cells provides valuable information in determining if these materials are biocompatible and safe for *in-vivo* use. The null hypothesis investigated in this experiment was that there would be no differences in cell viability between the adhesive blends modified with the different concentrations of monomer or cross-linker and the corresponding unmodified control adhesive.

2.5.2. Specimen preparation

Three adhesive discs per group (n=3), 5 mm in diameter and 2 mm in thickness, were fabricated and incubated in 350 uL media as per International Organization for Standardization (ISO) for the testing of biomaterials describing the required ratio of specimen surface area to incubation solution volume [83]. Fresh adhesive blends corresponding to each of the study groups were used to fill a 5 × 2 mm mold, which sat between two microscope glass slabs to produce flat resin adhesive specimens of uniform dimensions. Following manufacturer recommendations, discs were polymerized for 20 sec with LED light curing unit (VALO, Ultradent Products) with a power density of 1,400 mW/cm^2. After 24 hrs of incubation in distilled water at 37°C, discs were then sterilized in 70% ethanol and dried for a minimum of 48 hrs before testing. A total of ten groups were evaluated for this experiment; nine groups corresponding to the adhesive blends mentioned in section 2.1. and a cell control group with gingival fibroblast growth medium (alpha-MEM + 10% FBS + 1% antimycotic, pH 7.4, Gibco, Life technologies) and no disc present.

2.5.3. Human gingival fibroblast (HGF) isolation and culture

Discarded healthy gingival tissue was collected from subjects undergoing surgical treatment at the University at Buffalo School of Dental Medicine clinics (IRB protocol 663292-1). Human gingival fibroblasts (HGFs) were isolated from these tissues and washed in minimum essential media (MEM) containing 10× antibiotics followed by 3 washes of media containing 1× antibiotics. Tissues were then minced into small pieces and attached to wells of a 6-well tissue culture plate for 30 mins. 1 mL of fresh MEM media + 10% FBS with antibiotics was then

added. Explants were then incubated at 37°C, 5% CO_2 until the migration of cells from the tissue explant could be observed. Media was replaced every three days.

To maintain routine culture, HGFs were grown to 80% confluence in MEM+10% FBS and passaged using trypsin/EDTA. This confluence was used to ensure there will be enough cells for the assay.

2.5.4. MTT cell viability assay

Human gingival fibroblast cells were cultured in a 96-well plate. Each well contained 10,000-20,000 cells. The media from the cells was aspirated and 100 uL of serum-free growth media was added. After 24 hrs, the serum-free media was removed from the cells and 100 uL of disc-conditioned media was added per well. This was then incubated for 48 hrs. To prepare disc-conditioned media, 3 discs per group were incubated in 350 uL of gingival fibroblast growth media for 24 hrs.

The disc-conditioned media was then aspirated post 48 hrs and 100 uL of clear MEM media and 10 uL of 12 mM MTT (Invitrogen, Thermo Fischer Scientific, Eugene, OR, USA) stock was added to each well. This was incubated for 4 hrs, after which 75 uL of medium was removed from the wells. 50 uL of DMSO was then added to each well and mixed by pipetting. After incubation for 10 mins, the purple formazan appeared and the plate was measured at an absorbance of 540 nm (Flexstation 3, Molecular Devices, Sunnyvale, CA, USA). Groups were plated in triplicate (n=3).

2.5.5 Statistical analyses

The data met the requirements of normal distribution (Shapiro-Wilk test, p=0.291) and equal variance (Brown-Forsythe test, p=0.949). After normalizing the data, a one-way (ANOVA) was used to analyze the effect of the group variable on cell viability values. If differences were

present, an all-pairwise multiple comparison procedure Tukey's test was used to identify these differences. A significance level of $p<0.05$ was used for all tests. All statistical analyses were performed with SigmaStat version.3.5 (San José, CA, USA).

2.6. ANTIBACTERIAL

2.6.1. Aims and hypotheses

Both the monomer and the cross-linker are expected to possess antibacterial properties and consequently their addition to an adhesive resin material may render the material antibacterial. The aim of this part of the study was to identify which concentrations of monomer, cross-linker or both would exert effective antibacterial properties without a detrimental effect to the mechanical and physical properties of the polymerized resin adhesive material. The null hypothesis was that there would be no differences in antibacterial affect between the different concentrations of monomer and cross-linker.

2.6.2. Specimen preparation

Discs for each of the nine study groups were prepared as described in section 2.4.2. with the only difference being the dimensions of the mold used to fabricate the specimens (8 × 1 mm in thickness). Following the methods by Imazato, *et al.*[91] and Renne, *et al.*[92], this experiment was carried out as per the ISO standards for the testing of biomedical devices. The average colony forming units (CFU) of three replicate experiments (n=3) were plated in duplicate.

After polymerization, each group was put into its labeled well with distilled water and incubated at 37°C for a minimum of 24 hrs. Discs were sterilized after this time for 10 mins in 70% ethanol at room temperature. Each disc was then aseptically removed from the ethanol and

dried in a 24-well plate at room temperature that would then be used for the experiment after 48 hrs.

2.6.3. Antibacterial assay

Streptococcus mutans (*S. mutans*) were gifted by Dr. Stefan Ruhl from the Oral Biology Department at University at Buffalo, SUNY (ATCC 25175). *S. mutans* was streaked on a Brain Heart Infusion (BHI) (Sigma-Aldrich, St Louis, MO, USA) agar plate and incubated in an anaerobic chamber at 37°C. 5 mL of BHI broth was inoculated with *S. mutans* and left overnight in the anaerobic chamber at 37°C.

The following morning, *S. mutans* were sub-cultured by inoculating 5 mL of fresh BHI media with 0.5 mL of overnight culture and incubated for 4-6 hrs until the culture reached an OD_{600} reading of 0.3. 100 uL of the culture was pipetted directly onto the surface of each disc in a sterile 24-well plate. 1 mL of sterile distilled water was added next to each well containing a disc to provide a hydrated environment. The discs were incubated in an anaerobic chamber for 18 hrs at 37°C.

15-mL conical tubes were filled with 9.9-mL of anaerobic BHI broth per group. The conical tubes and 24-well plate containing the discs were then removed from the anaerobic chamber and onto a bench. Each disc was aseptically removed from the well and placed in its respective 15-mL conical tube. Each tube was vortexed for 3 mins to disperse any bacteria left on the surface of the disc into the media and serially diluted in 1:10 dilutions for countable CFU data. 100 uL of each dilution was plated onto BHI plates in duplicate. All plates were then incubated in the anaerobic chamber at 37°C for 24 hrs after which data was retrieved.

2.6.4. Statistical analyses

Since the data did not meet the requirements of normal distribution (Shapiro-Wilk test, $p<0.05$) and equal variance (Brown-Forsythe test, $p<0.05$), a Kruskal-Wallis one-way ANOVA on ranks was used to analyze the effect of the group variable on absorbance values. If differences were present, an all-pairwise multiple comparison procedure Dunn's test was used to identify these differences. A significance level of $p<0.05$ was used for all tests. All statistical analyses were performed with SigmaStat version.3.5 (San José, CA, USA).

2.7. SCANNING ELECTRON MICROSCOPY (SEM)

2.7.1. Aims

The purpose of this part of the investigation was to observe the differences in hybridization patterns that may be achieved with the different adhesive blends.

2.7.2. Specimen preparation

During the sectioning of the bonded teeth in the MTBS study (section 2.2.2), 1 mm-thick slabs with the resin-dentin interface were set aside for SEM analysis, **Figure 13**. A thickness of 1 mm was used for the slabs to ensure complete and rapid dehydration of the specimens in the SEM vacuum. One slab per group was polished using the following grits of SiC abrasive paper, 320, 400, 600, and 800. The slabs were then left to dry for 24 hrs before analysis in the SEM.

2.7.3. SEM procedure

After a drying period of 24 hrs, slabs were placed on aluminum stubs with conductive tape and coated with carbon. Carbon coating helps to eliminate the charging that may occur which can obscure the images of the specimens in SEM. Each slab was placed in a vacuum evaporator (Denton DV-502, Douglasville, PA, USA) for 20 mins. Different areas of one slab were analyzed at a time at 20kV and SEM images generated (SU-70, Hitachi, Tokyo, Japan).

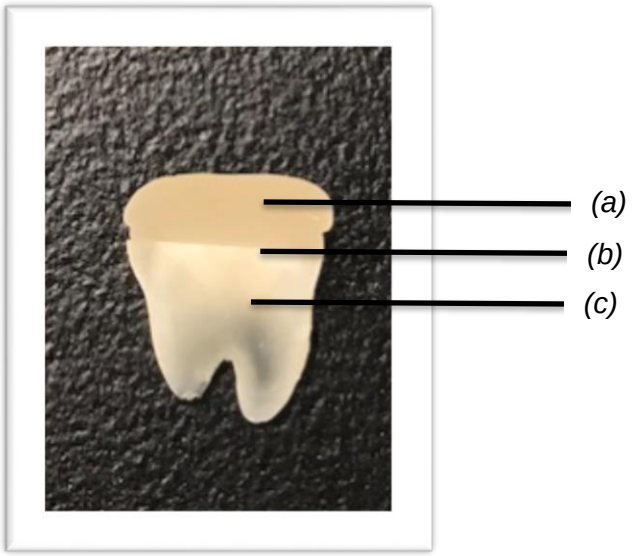

Figure 13. Cross section view of a third molar tooth. The following layers where prepared for SEM imaging as pictured above before drying and applying carbon coating: *(a)* composite layer, *(b)* adhesive layer, and *(c)* dentin.

CHAPTER 3
RESULTS

3.1. MICRO-TENSILE BOND STRENGTH (MTBS)

Two-way ANOVA demonstrated a significant effect of the group ($p<0.001$) and the interaction group × time ($p<0.001$), but no effect of the time on mean MTBS values.

Figure 14 summarizes the MTBS values at 24 hrs and 6 mos of incubation. When the 24 hrs data was evaluated, none of the groups demonstrated significantly different bond strength values from the control SB. When the different study groups were evaluated at 6 mos, only 0.25 C demonstrated significantly lower bond strength values than control SB ($p<0.001$).

A pairwise multiple comparison Tukey test revealed a significant increase in mean MTBS values after 6 mos for groups 0.25 M ($p=0.010$) and 0.1 MC ($p=0.033$), and a significant decrease in mean MTBS values for groups 0.05 C ($p=0.009$), 0.1 C ($p=0.011$) and 0.25 C ($p=0.027$) relative to their 24 hrs values. The remaining groups demonstrated no significant variations in bond strength values after 6 mos of incubation relative to their 24 hrs values.

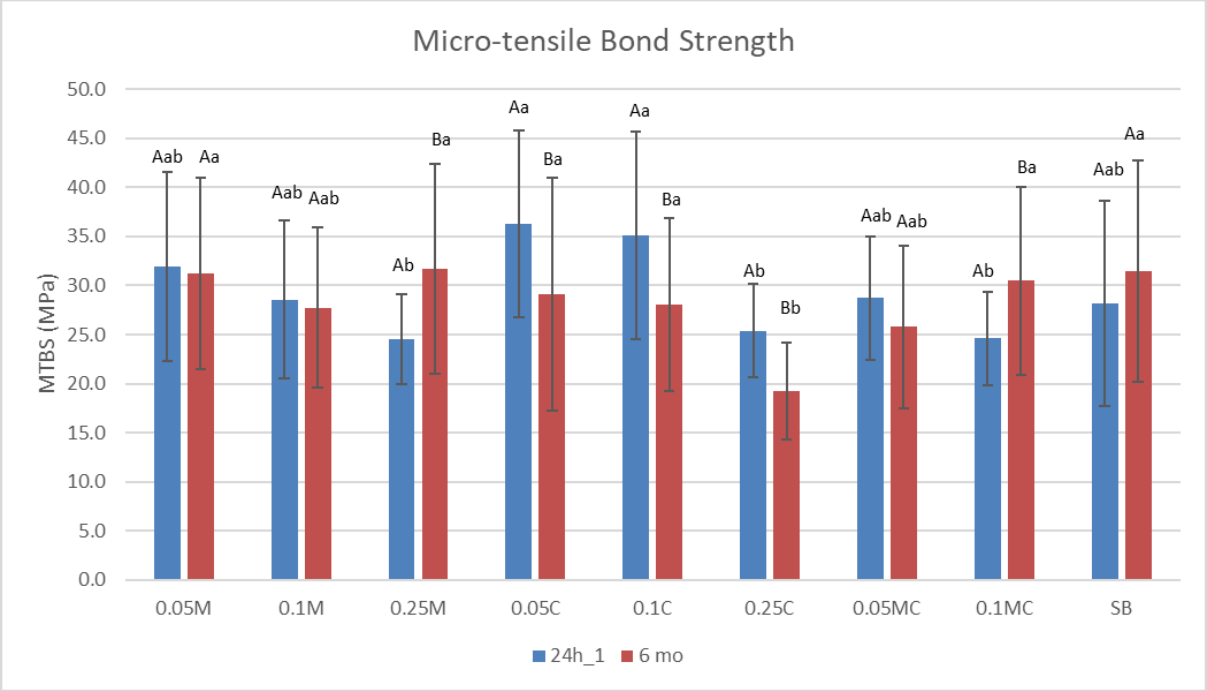

Figure 14. Mean micro-tensile bond strength values for all study groups at 24 h and 6 mo of incubation. Bars represent mean values; brackets indicate SD values. Groups identified by different letters are significantly different (Tukey's test, $p<0.05$). n=20. Upper case denotes differences between 24 hrs and 6 mos for each group. Lower case denotes differences among groups for each testing time.

3.2. ULTIMATE TENSILE STRENGTH (UTS)

Two-way ANOVA demonstrated a significant effect of the group ($p=0.032$), time($p<0.001$) and the interaction group × time ($p<0.001$), on mean UTS values.

Figure 15 summarizes the UTS data. No significant differences were found among the groups when they were evaluated at 24 hrs or 1 wk. When the groups were compared at 6 mos, both 0.25 M and 0.25 C demonstrated significantly higher UTS values than the control SB ($p=0.005$ and $p<0.001$ respectively). None of the other groups demonstrated statistically significantly differences from the control SB.

When the UTS values for each group were individually evaluated overtime, they all remained stable with no decrease in UTS values overtime. The only exceptions were 0.25 M and 0.25 C, which in fact demonstrated increased UTS values after 6 mos of incubation.

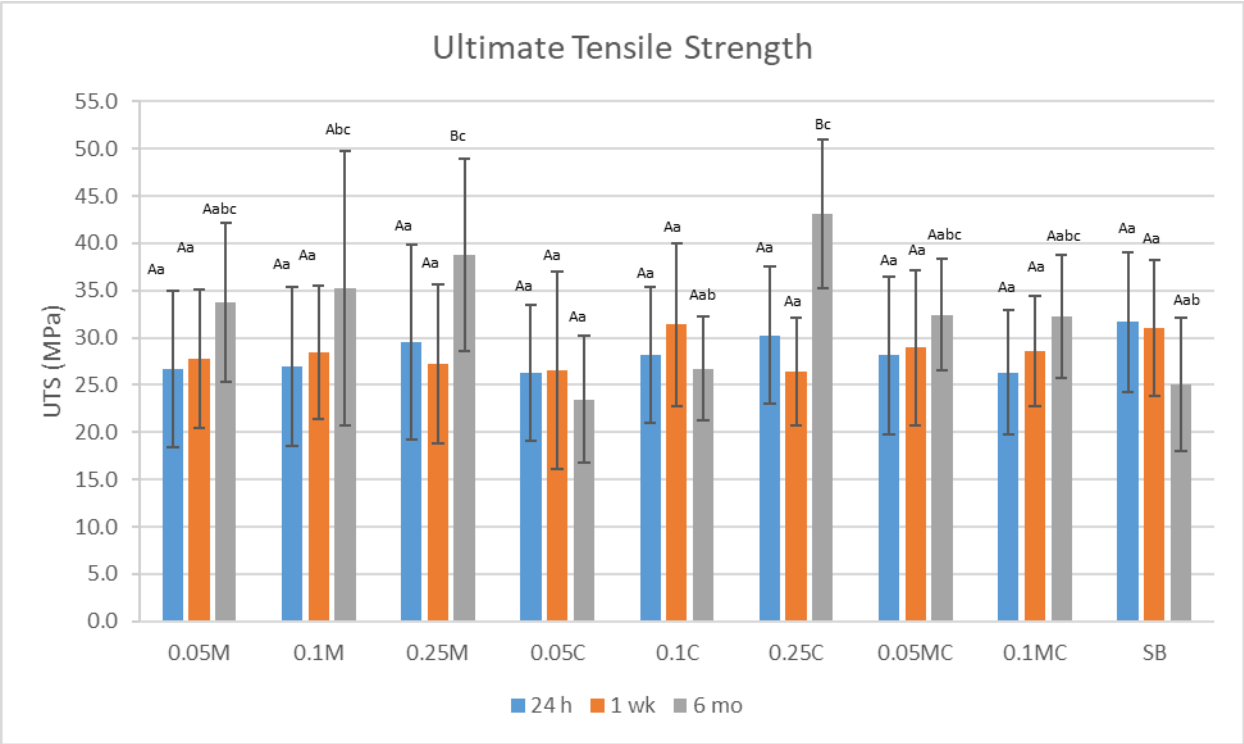

Figure 15. Mean ultimate tensile bond strength values for all study groups at 24 hrs, 1 wk and 6 mos of incubation. Bars represent mean values; brackets indicate SD values. Different letters indicate significant differences between groups (Tukey's test, $p<0.05$). n=10. Upper case denotes differences among testing times for each study group. Lower case denotes differences among study groups for each testing time.

3.3. MICRO-HARDNESS

Individual student's t tests demonstrated a statistically significant reduction in surface micro-hardness values after incubation in ethanol for 4 hrs for all groups. P values for all groups are provided in **Table 3.** The only exceptions were 0.05 C, and 0.1 C. While 0.05 C demonstrated no significant variation in micro-hardness values after incubation in ethanol for 4 hrs, 0.1 C demonstrated a statistically significant increase in these values. **Figure 16** summarizes

the surface micro-hardness values for all study groups. **Table 4** summarizes the percent decrease or increase in hardness for all groups. All groups demonstrated a percent decrease, with the exception of 0.1 C and 0.25 C.

Table 3. P-values for the surface micro-hardness values at baseline and 4 hrs of incubation in ethanol.

0.05 M	0.1 M	0.25 M	0.05 C	0.1 C	0.25 C	0.05 MC	0.1 MC	SB
<0.001	<0.001	<0.001	0.082	<0.001	0.044	0.002	<0.001	<0.001

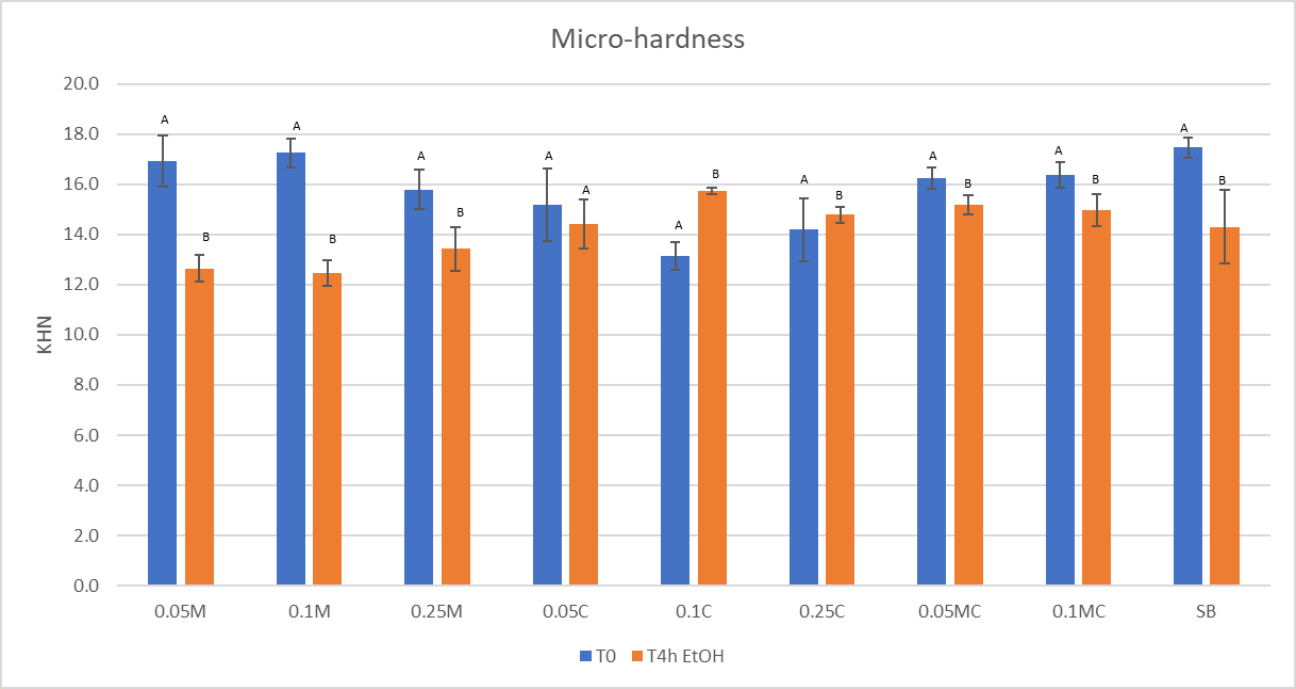

Figure 16. Surface micro-hardness values for all study groups. Bars represent mean values; brackets indicate SD values. Different letters indicate significant differences between testing periods for each of the study groups (Student's t-test, $p<0.05$).

Table 4. Percent decrease/increase in micro-hardness after 4 hrs ethanol

0.05 M	0.1 M	0.25 M	0.05 C	0.1 C	0.25 C	0.05 MC	0.1 MC	SB
-25%	-28%	-15%	-5%	17%	4%	-7%	-9%	-18%

3.4. CELL VIABILITY

The one-way ANOVA demonstrated no significant difference in absorbance percentage values among the different study groups, specifically, there were no significant differences between the experimental adhesives and the control unmodified Single Bond adhesive as shown in **Figure 17**. Group 0.05 M demonstrated a slightly higher cell viability (76%) than all other groups, although according to statistical analyses it is non-significant.

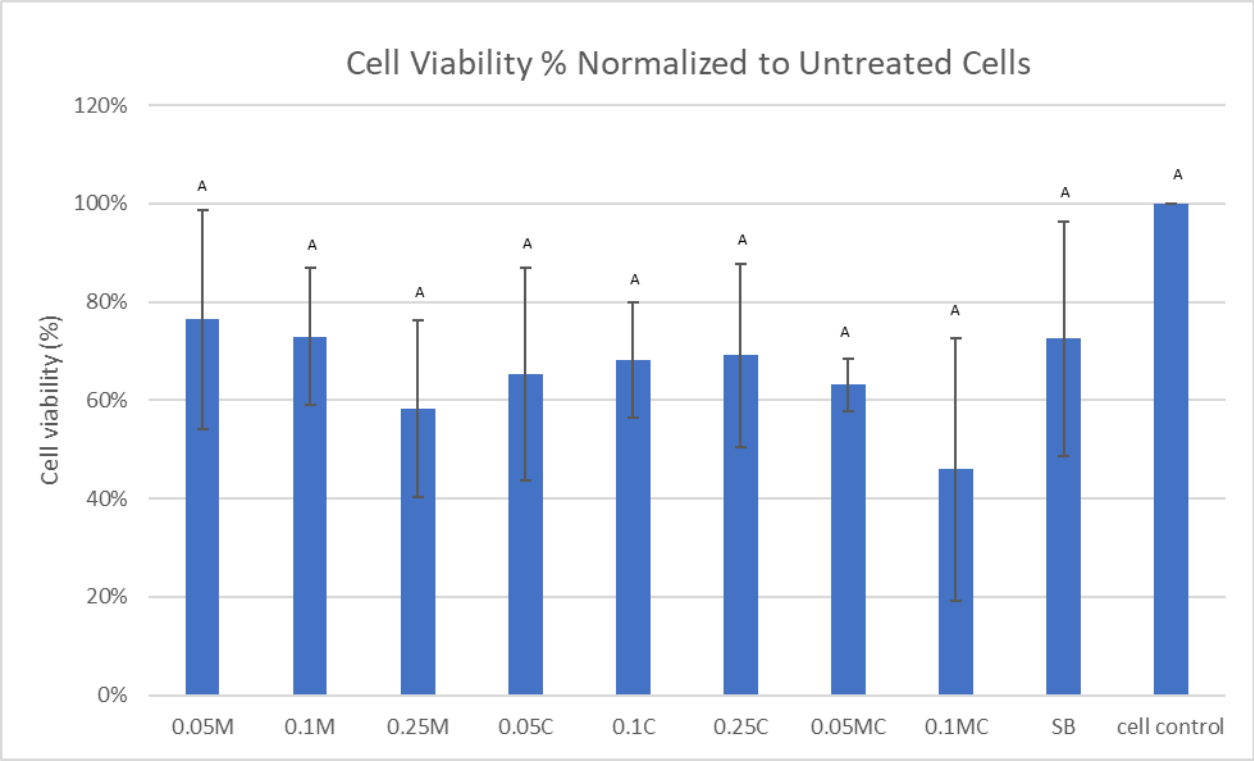

Figure 17. Cell viability values against human gingival fibroblastic cells for all study groups. Bars represent mean absorbance values; brackets indicate SD values. Groups identified by different letters are significantly different (One-way ANOVA, $p<0.05$). n= 3.

3.5. ANTIBACTERIAL

Kruskal-Wallis one-way ANOVA on ranks revealed a significant effect of the treatment group on the observed antibacterial properties ($p<0.001$). Pairwise multiple comparisons with

Dunn's test demonstrated that the only groups with significantly reduced bacterial counts relative to the control group SB were 0.1 M and 0.25 M. **Figure 18** summarizes the antibacterial findings.

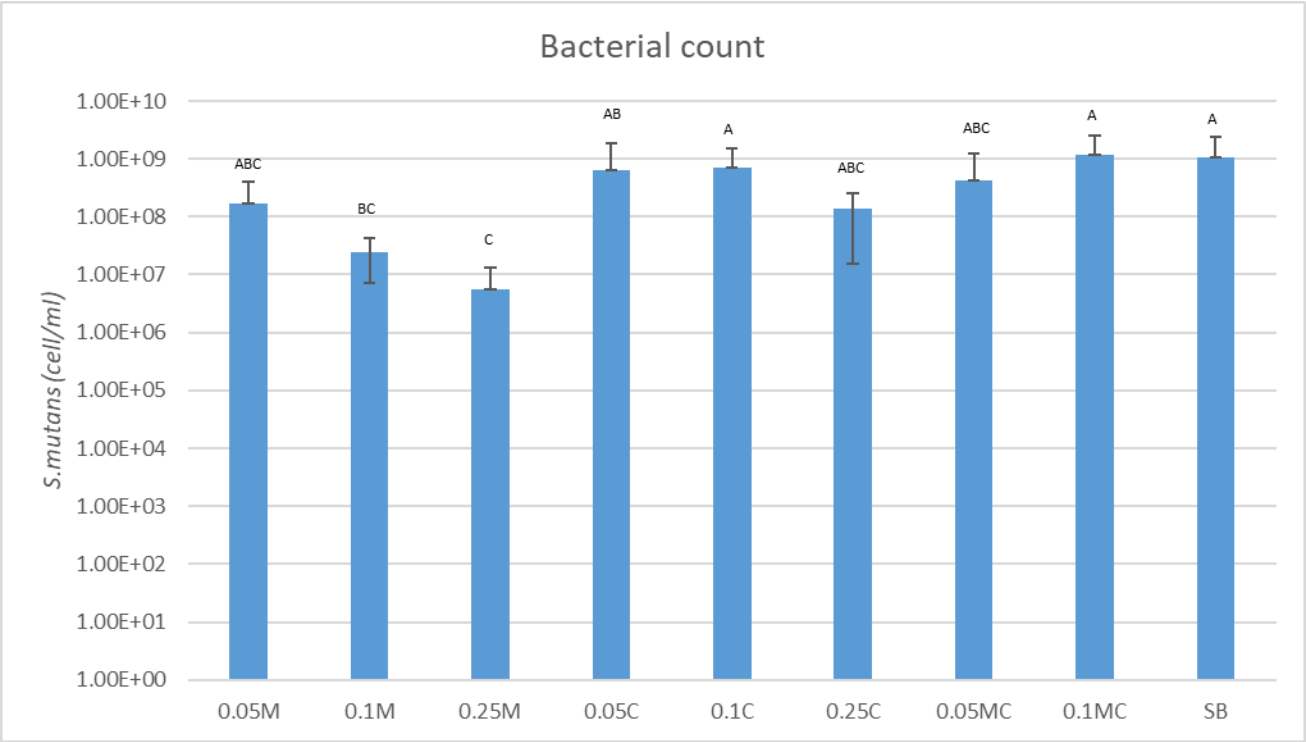

Figure 18. Antibacterial values against *S. mutans* for all study groups. Bars represent mean absorbance values; brackets indicate SD values. Groups identified by different letters are significantly different (Dunn's test, $p<0.05$). n= 3.

3.6 SCANNING ELECTRON MICROSCOPY (SEM)

Figures 19-27 depict the different patterns of resin infiltration of dentin tubules by the resin adhesive groups evaluated in this study. The three layers corresponding to the partially demineralized tooth substrate, dentin, the adhesive layer and the overlaying resin are shown in these images. From bottom to top, the partially demineralized dentin appears porous due to the presence of the dentinal tubules. The different shapes of the dentinal tubules depicted in the various images correspond to slight variations in cross-sectional cuts of the different specimens.

Immediately above, a darker thin layer corresponds to the resin adhesive layer, different varieties of which were tested in the present thesis. The layer appears dark as the adhesive is lightly filled resin **(Table 2)**. Immediately between the layer of adhesive and the underlying dentin, dark projections (i.e. resin tags) corresponding to the areas of resin infiltration can be observed. The last layer on top corresponds to the overlaying resin composite restorative material. The restorative material is a highly filled resin composite **(Table 2)**, very light and round in shape, are the filler particles.

No differences in the patterns of hybridization were shown for the different study groups.

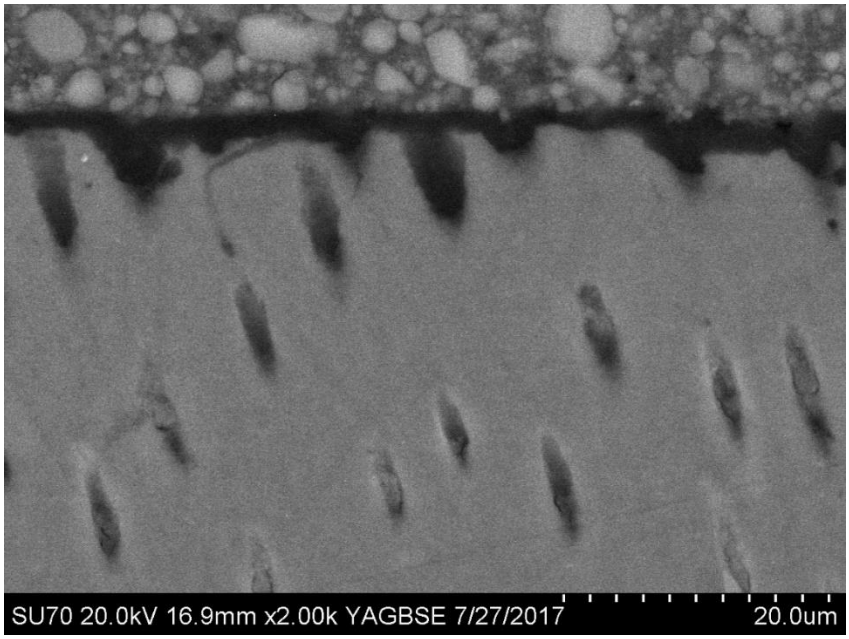

Figure 19. Backscattered SEM image of 0.05 M showing resin infiltration in dentin tubules.

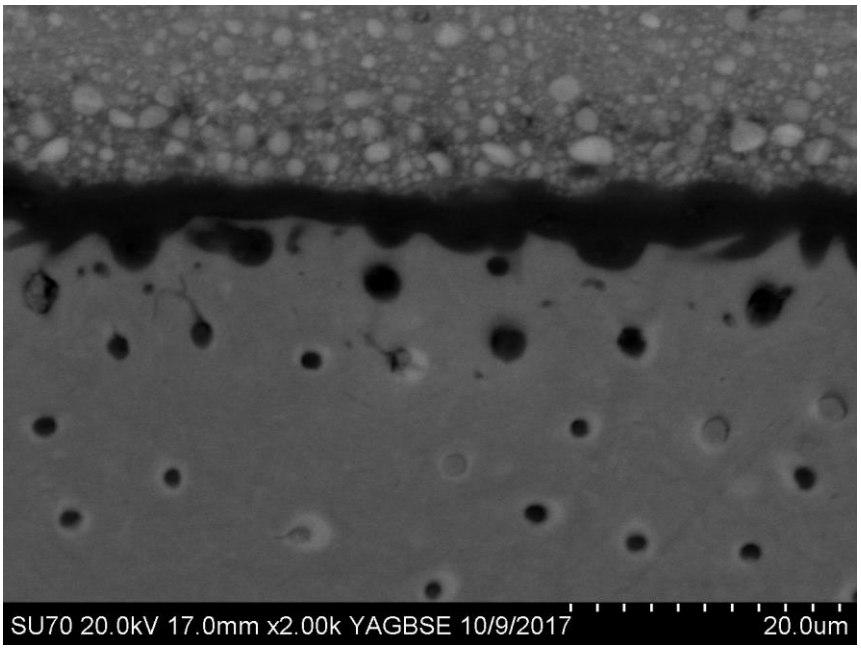

Figure 20. Backscattered SEM image of 0.1 M showing resin infiltration in dentin tubules.

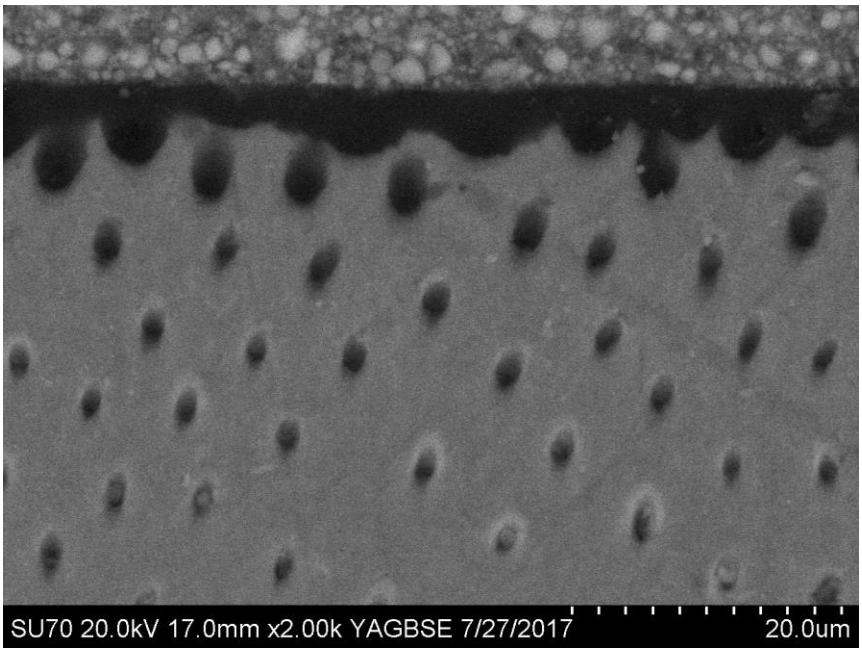

Figure 21. Backscattered SEM image of 0.25 M showing resin infiltration in dentin tubules.

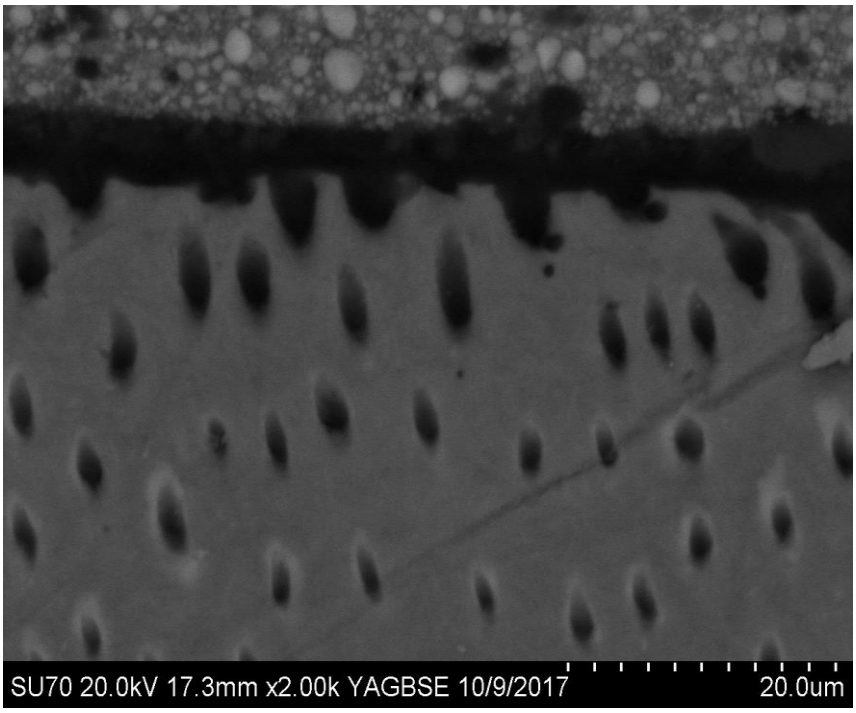

Figure 22. Backscattered SEM image of 0.05 C showing resin infiltration in dentin tubules.

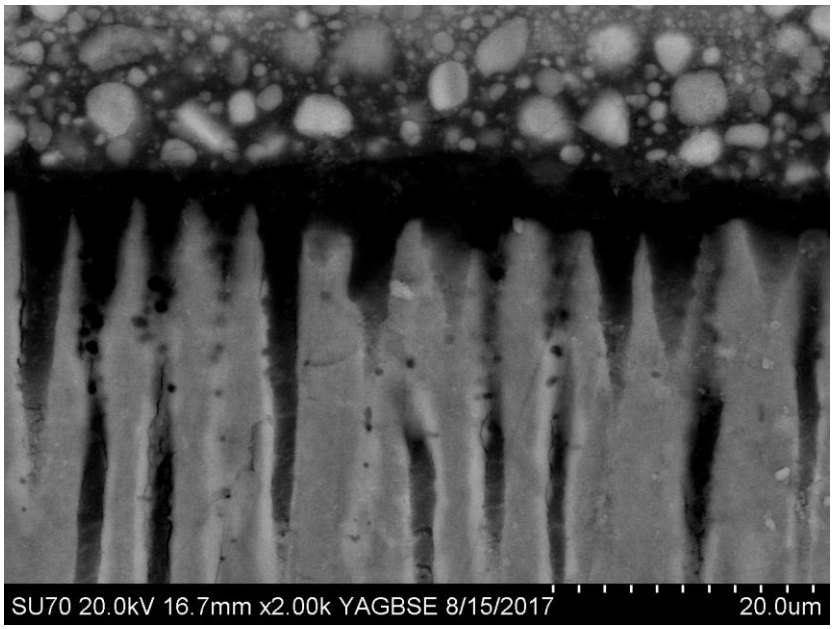

Figure 23. Backscattered SEM image of 0.1 C showing resin infiltration in dentin tubules.

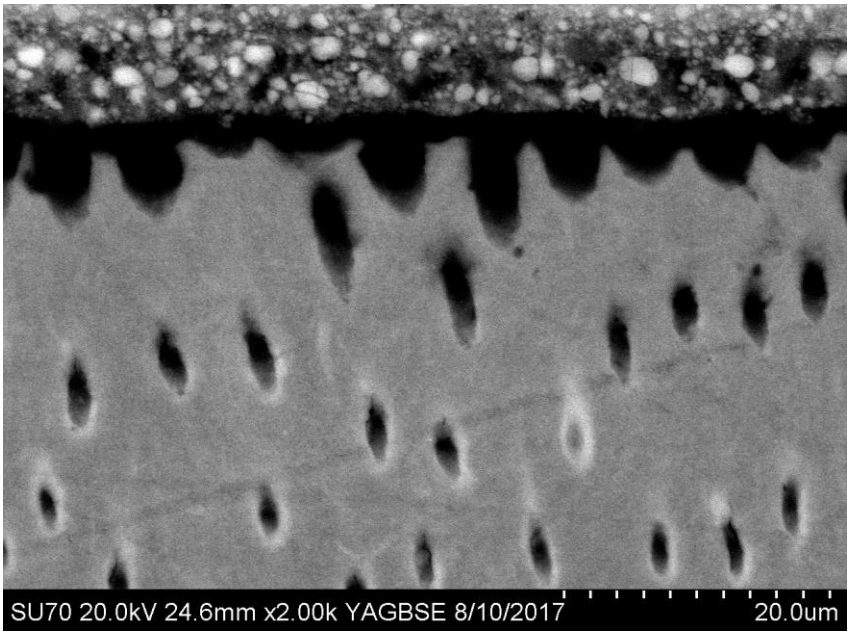

Figure 24. Backscattered SEM image of 0.25 C showing resin infiltration in dentin tubules.

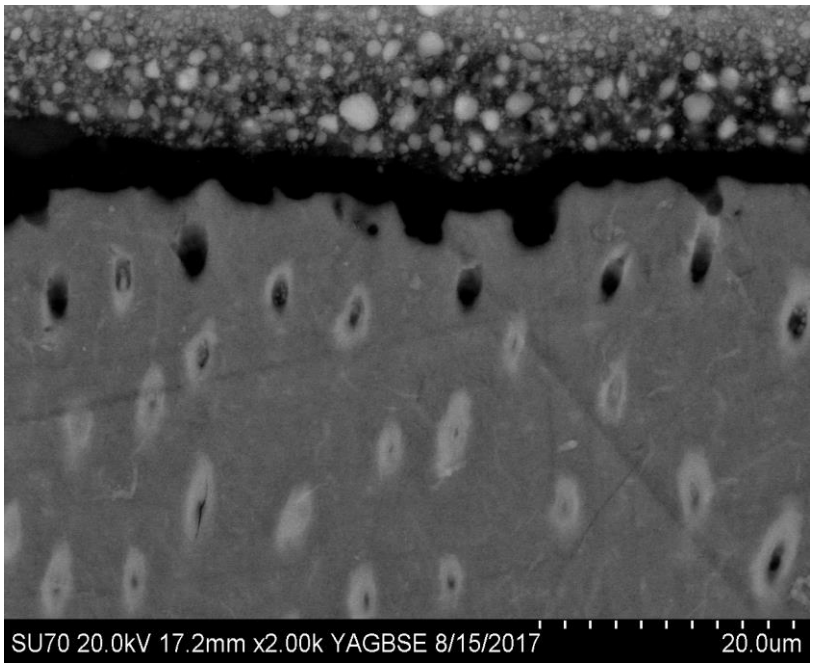

Figure 25. Backscattered SEM image of 0.05 MC showing resin infiltration in dentin tubules.

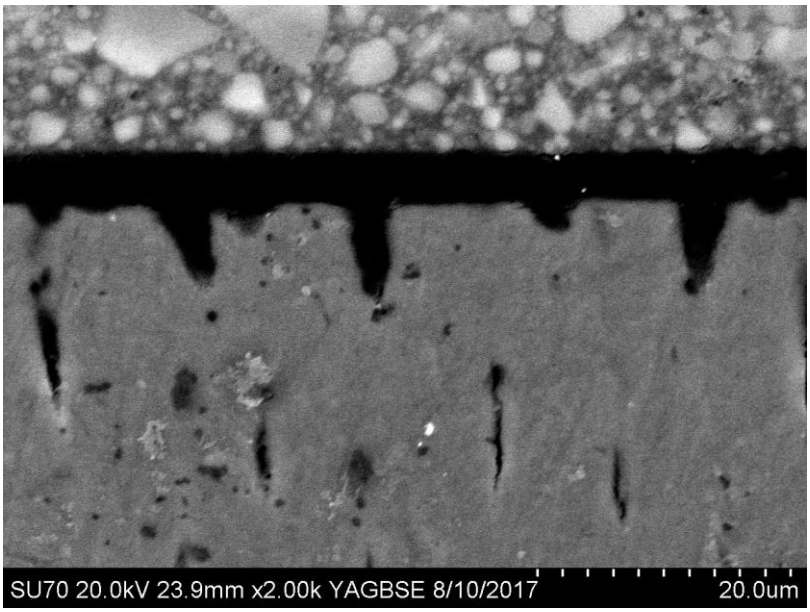

Figure 26. Backscattered SEM image of 0.1 MC showing resin infiltration in dentin tubules.

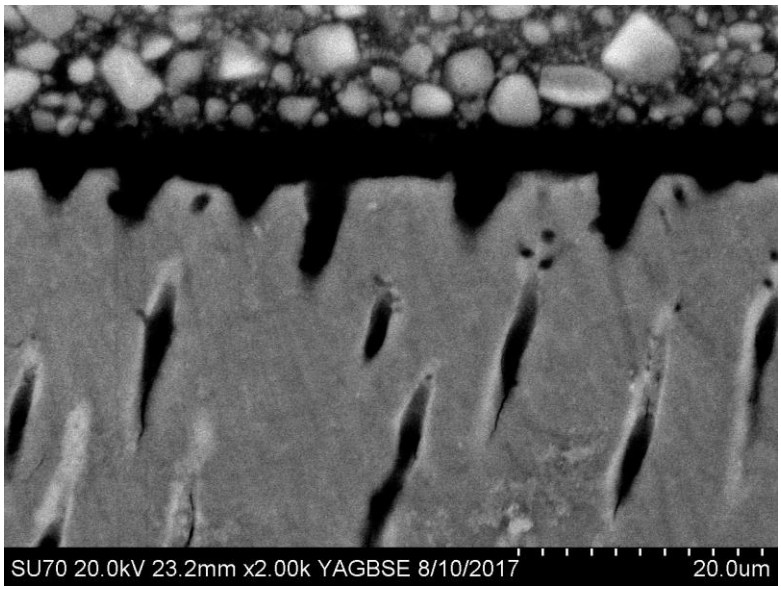

Figure 27. Backscattered SEM image of Single Bond showing resin infiltration in dentin tubules.

CHAPTER 4
DISCUSSION

4.1 MICRO-TENSILE STRENGTH (MTBS)

The dentin micro-tensile bond strength of experimental and control dental bond adhesives were measured at 24 hrs and 6 mos of water storage. Distilled water was selected as the storage media to avoid the undue influence of minerals and enzymes present in artificial saliva solutions.

The null hypothesis that there would be no effect of the different concentrations of monomer or cross-linker on the bond strength at 24 hrs or 6 mos was rejected. The initial bond strength values ranged from 25 MPa to 45 MPa, well above the accepted threshold for acceptable bond strength (20 MPa). While differences in mean bond strength values at 24 hrs were observed between experimental and control adhesives, these differences remained non-significant. This indicates that the incorporation of monomers and cross-linkers does not compromise the bond strength of SB.

After 6 mos, there was a significant increase in mean MTBS values for 0.25 M and 0.1 MC and a significant decrease in mean MTBS values for 0.05 C, 0.1 C, 0.25 C. The remaining groups demonstrated no significant variations in bond strength values after 6 mos relative to their 24 hrs values. It appears that increasing the concentration of monomer increases the bond strength, as they are able to demineralize tooth structure when hydrolyzed.

No definite trend was present for the monomer containing adhesives. While 0.05 M and 0.1 M demonstrated stable bond strengths after 6 mos, 0.25 M showed a higher bond strength after 6 mos relative to its 24 hrs values. Similarly, the groups containing both monomer and cross-linker demonstrated either stable (0.05 MC) or increased (0.1 MC) bond strength after 6 mos.

All the cross-linking groups, on the other hand, demonstrated a significant decrease in bond strength after 6 mos of incubation indicating that perhaps when combined with the resin adhesive components, makes them more susceptible to hydrolysis. We speculate that the increased concentration of cross-linker could have resulted in an inferior initial infiltration of the adhesive resin into the demineralized dentin perhaps compromising the bond strength values overtime.

Single Bond (SB) is an etch-and-rinse adhesive that contains groups such as BisGMA, HEMA, dimethacrylates, ethanol, water, novel photo-initiator system, and a methacrylate functional copolymer of polyacrylic and polyitaconic acids [93]. Etch-and-rinse systems are known to have more hydrophilic monomers that can lead to water sorption and thus a decrease in micromechanical strength properties [94].

Although 6 mos is typically not accepted as sufficient time to assess bond degradation of the adhesives, some adhesives may undergo early degradation so useful information can be obtained from 6 month water storage [95].

The MTBS was used as the method of testing tensile strength because a more uniform stress distribution is created resulting in lower cohesive failures and higher bond strengths as compared to shear bond strength testing [96]. Furthermore, this method is appropriate for simulating the clinical situation since failures typically occur from tensile rather than shear forces [96]. MTBS allows for small bonded area testing and intra-/inter- tooth variability that can be evaluated at different time periods from the same specimen tooth.

4.2. ULTIMATE TENSILE STRENGTH (UTS)

The adhesive ultimate tensile strength was evaluated at 24 hrs, 1 wk, 6 mos. The null hypothesis that the incorporation of monomer or cross-linker into the adhesive would have no

effect on their ultimate tensile strength was rejected as changes were observed for some of the groups, especially after 6 mos of incubation. While no significant differences were observed after 1 wk, most of the groups demonstrated greater UTS after 6 mos, especially those containing monomer. This may be explained by a prolonged degree of post-cure set for those groups containing monomer, which in turn may be attributed to a higher reaction extent if curing was initially incomplete [96]. Surfactant monomer and cross-linker have acrylate bonds for copolymerization with methacrylates in SB to form polymer chains. Through copolymerization, cross-linking leads to networks and thus potentially greater tensile strengths.

As stated in chapter 2, monomer containing adhesives appeared more viscous than the rest of the groups. This viscosity in turn may be partially responsible for a decrease in UTS values by reducing the degree of conversion of thick adhesive as the reactivity of major monomer could have been restricted [97]. The UTS values obtained in our study are within the range of what the literature has previously reported for SB. In a study by Loguercio et al., the authors reported a mean UTS value for SB of 41.5 MPa at 24 hrs and 28.0 MPa at 6 mos. These values are slightly higher than what we obtained in our research, 31.7 MPa and 25.1 MPa respectively.

4.3 MICRO-HARDNESS

A statistically significant reduction in surface micro-hardness values was shown for all groups after incubation in ethanol for 4 hrs with the exception of 0.05 C, 0.1 C, and 0.25 C. Hence, the null hypothesis was rejected. Groups 0.1 C and 0.25 C demonstrated an increase in micro-hardness values and 0.05 C was the only group that remained unchanged. We speculate that a greater degree of monomer cross-linking in the cross-linking groups may have been responsible for the more stable surface micro-hardness after incubation in ethanol. Previous

authors have shown that a more mechanically stable polymer would indeed result in increased hardness values [98].

Following the protocol by Leitune *et al*., specimens were softened in ethanol [90]. The diffusion of solvent through the polymer chains results in elution of components and the plasticization of the composite [99]. This process initially affects the surface properties, such as the hardness and wear resistance, and therefore the longevity of the restorative treatment [100]. In the present study, we opted to incubate adhesive resin specimens in ethanol as a form of accelerated aging. In addition to this, ethanol is commonly used to replace water within the collagen network and it is commonly thought to be as a better solvent for co-monomers than water, after solvent evaporation ethanol can also maintain the collagen fibrils in an expanded position for better resin infiltration [101, 102].

4.4 CELL VIABILITY

Cell viability is commonly dependent on dosage. The higher the concentration, the lower the cell survival rate [103]. A higher fibroblastic cell absorbance indicates higher cell survival. Ideally, a higher cell survival indicates a less cytotoxic material. *In vitro* studies investigating the cytotoxicity of dental adhesive systems are more convenient and reproducible than *in vivo* tests [104].

None of the groups in this study demonstrated statistically significant differences from the control unmodified adhesive SB. Therefore, the null hypothesis was accepted.

Although no statistical significance was present among the groups, 0.25 M and 0.1 MC demonstrated the least percent cell viability (58%, 46% respectively). It is possible that cytotoxic effects may result if adhesives are incompletely polymerized [105]. A limitation to this study could have been due to the absence of a dentin barrier since its presence reduces the adhesive's

diffusion and consequent toxicity [106]. For the cell viability assay, discs of adhesives were exclusively tested. Perhaps testing the adhesives' cytotoxic affects while bonded to dentin may have produced more clinically favorable results.

The incubation period of 24 hrs was used in our since the most cytotoxic effects are thought to occur during the first 24 hrs since the resin is still undergoing polymerization [107]. The main goal of this assay was to ensure that the additives would not make the SB any more cytotoxic than what it already is. It has been reported in literature that a biomaterial is considered to be non-cytotoxic at a threshold cut off of 70% [108]. Our SB mean percent cell viability is reported as 72%, and all groups demonstrated very close values, with 0.05 M yielding the highest cell viability (76%). 0.05 M was the lowest monomer concentration tested which may explain the high cell viability percentage. It has been reported that monomers in the resin matrix of resin composites, such as BisGMA, UDMA, TEGDMA and HEMA can be cytotoxic [109, 110]. However, a study by Pupo *et. al*. [111], reported SB cell viability percent as 73%, similar to our results. In another study by Elias *et. al.* [106], the authors reported a cell death average of 33-51%, "demonstrating a moderate cytotoxic effect of the tested systems."

4.5 ANTIBACTERIAL

In this part of the study, the increased colonization of *S. mutans* corresponds to less antibacterial properties of the material.

Only two groups, 0.1 M and 0.25 M, demonstrated significantly reduced bacterial counts relative to the control group, therefore the null hypothesis that there would be no differences in antibacterial properties between the different concentrations of monomer and cross-linker was rejected. It is important to note that the results may be due to the easily accessible cationic group in the monomer surfactant [112]. Because the cross-linker is a longer chain than the monomer,

the NBr is more tightly bonded in the core of the cross-linker, making it difficult to for access to its antibacterial properties.

The incorporation of the quaternary ammonium methacrylate by these monomer and cross-linkers to the adhesive as described in chapter 2, allows for the copolymerization with the resin by forming a covalent bond with the polymer network [113]. This allows for a durable antibacterial activity for the resin adhesive [113]. As demonstrated by a study, the incorporation of quaternary ammonium dimethacrylates into dental resins yielded effective antibacterial results [114]. Quaternary ammonium agents are known to potentially cause bacteria lysis by binding to the cell membrane [115]. After the positively charged (N^+) sites of the quaternary ammonium contacts the negatively charged bacterial cell, the electric balance of the cell membrane could be disturbed, causing the bacterium to lyse under its own osmotic pressure [112].

Because the carbon chain needs to be long enough to penetrate the cell membrane, length of the monomer is important when evaluating the antibacterial effectiveness. It was observed in a study by Zhou *et. al*, that increasing the chain length from 6 to 12 carbons increased the antibacterial activity [113]. However, from our results it is demonstrated that only groups 0.1 and 0.25 monomer showed statistical significance compared to the SB control. These groups contained the highest concentration of monomer. Unlike the study mentioned above, in our case the monomer is a shorter chain (19 carbons) than the cross-linker (23 carbons). It seems that with increasing monomer concentration there is an increase in antibacterial properties. This trend is also observed with the cross-linker.

4.6. SCANNING ELECTRON MICROSCOPY (SEM)

The purpose of the qualitative observations was to visualize if there were differences in the infiltration pattern of the different resin adhesives into the partially demineralized dentin. It

was not appropriate to attempt to compare the adhesive layer across all groups because monomer and cross-linker individual components are not visible through SEM. The infiltration pattern of the dentinal tubules was analyzed instead. All groups demonstrated similar infiltration pattern of the dentinal tubules. In the SEM images, some tubules appear deeper or longer than other groups, this is due to the slightly different angles of sectioning of each of the individual teeth and is not appropriate to attempt to quantify number of tubules that appear successfully infiltrated and attempt to establish comparisons among the groups. Tubules that are infiltrated appear dark, like the adhesive layer. While tubules that are empty appear a brighter gray.

4.7 COMPREHENSIVE ANALYSIS OF THE FINDINGS

One of the main objectives of adhesive dentistry is to provide the optimal adhesive system with superb antibacterial and micromechanical properties. Increased longevity and long-term antibacterial benefits are of special interest.

Overall, the highest concentrations of monomer (0.1 mg/mg and 0.25 mg/mg) demonstrated the most effective antibacterial properties, with the least amount of *S. mutans* colonies forming. However, because the highest concentration of monomer (0.25 mg/mg) produced a relatively low percent cell viability (58%), 0.1 M appears to provide the optimal balance between antibacterial properties with no cytotoxic effect or detrimental effect to the mechanical properties.

In the present thesis, monomer and cross-linker were only incorporated and tested using one commercial dental adhesive, SB. This presents a limitation to the study, perhaps it may be of benefit to test the incorporation of these materials in additional adhesives to observe the effect of different chemistries in the biocompatibility and micromechanical properties of the material.

Another limitation to this study was the long-term testing. The longest test period performed was 6 mos, testing for 1-2 yrs may provide useful information on bond degradation and strength over time. Additionally, thermocycling was not used for accelerated aging which subjects a restoration and tooth to temperature limits similar to those in the oral cavity. This type of testing may affect bond strength, thus providing further information on the effects of incorporating monomer and cross-linker into SB.

Testing the degree of conversion would have provided supplemental information for the cytotoxicity results, since dental adhesives are known not polymerize to 100%, which can consequently lead to a cytotoxic effect.

Further investigation with longer-term testing periods for micro-hardness, antibacterial, and cytotoxicity may provide more in depth information, expanding on what was explored in the scope of this thesis project. Based on the present results, future directions may include focusing on the testing of monomer groups since this appeared to provide the best antibacterial results and favorable micro-tensile bond strength, ultimate tensile bond strength, cell viability, and micro-hardness. Additionally, if there is a method to attach the antibacterial quaternary amine group onto the outside of the cross-linker as opposed to the inner portion of this chain, it may allow for optimal antibacterial results for enhanced accessibility for cells.

CHAPTER 5

CONCLUSIONS

Within the limitations of this *in vitro* study, the following can be concluded:

- Incorporation of monomer and cross-linker into the Single Bond (SB) adhesive did not affect their bond strength at 24 hrs.
- Incorporation of monomer and cross-linker into the SB adhesive did not affect their bond strength at 6 mos, except for 0.25 C, which demonstrated significantly lower bond strength than control SB.
- While 0.25 M and 0.1 MC demonstrated a significant increase in mean MTBS values after 6 mos relative to their 24 hrs values, 0.05 C, 0.1 C, 0.25 C demonstrated a significant decrease in mean MTBS values after 6 mo.
- While incorporation of monomer and cross-linker into the SB adhesive did not affect their ultimate tensile strength when evaluated at 24 hrs or 1 wk, when evaluated at 6 mos, groups 0.25 M and 0.25 C demonstrated significantly higher UTS values.
- Incubation in ethanol for 4 hrs yielded a significant reduction in surface micro-hardness for all groups except for 0.05 C and 0.1 C.
- Incorporation of monomer and cross-linker, in any of the concentrations tested, into SB adhesive demonstrated no additional cytotoxic effect relative to control SB.
- Only groups 0.25 M and 0.1 MC demonstrated a relatively lower mean percentage of viable cells, compared to SB

- Only 0.1 M and 0.25 M demonstrated antibacterial properties against *S. mutans*. No other groups did.
- SEM observations seems to indicate that the type of hybridization that can be obtained after incorporation of monomer and cross-linker is no different from the hybridization pattern obtained with control SB.
- It appears that a concentration of 0.1 mg/mg monomer provides the optimal balance for biocompatibility and micromechanical properties.

REFERENCES

1. Raible, R. *ADA Statement: New CDC Statistics Show Need for Increased Access to Dental Care, with a Greater Emphasis on Preventing Disease*. 2015 [cited 2018; Available from: https://www.ada.org/en/press-room/news-releases/2015-archive/may/new-cdc-data-on-adult-cavities.
2. Bohaty, B.S., et al., *Posterior composite restoration update: focus on factors influencing form and function.* Clinical, Cosmetic and Investigational Dentistry, 2013. **5**(1): p. 33-42.
3. Fabianelli, A., et al., *The relevance of micro-leakage studies.* International Dentistry SA, 2007. **9**(3): p. 64-74.
4. Kubo, S., *Longevity of resin composite restorations.* Japenese Dental Science Review 2011. **47**(1): p. 43-55.
5. Gordan, V.V., et al., *Repair or replacement of restorations: a prospective cohort study by dentists in The National Dental PBRN.* Journal of American Dental Association, 2015. **146**(12): p. 895-903.
6. Hiraishi, N., et al., *Effect of chlorhexidine incorporation into a self-etching primer on dentine bond strength of a luting cement.* Journal of Dentistry, 2010. **38**(6): p. 496-502.
7. Andre, C.B., et al., *Dentine bond strength and antimicrobial activity evaluation of adhesive systems.* Journal of Dentistry, 2015. **43**(4): p. 466-475.
8. Kianoush, N., et al., *Bacterial profile of dentine caries and the impact of pH on bacterial population diversity.* PLoS ONE, 2014. **9**(3): p. 92-102.
9. Blum, I.R., Lynch, C.D., and Wilson, N.H., *Factors influencing repair of dental restorations with resin composite.* Clinical, Cosmetic and Investigational Dentistry, 2014. **6**(1): p. 81-87.
10. Selwitz, R.H., Ismail, A.I., and Pitts, N.B., *Dental Caries.* The Lancet, 2007. **369**(9555): p. 51-59.
11. Wolff, M.S. and Larson, C., *The cariogenic dental biofilm: good, bad, or just something to control?* Brazilian Oral Research, 2009. **23**(1): p. 31-38

12. Kidd, E.A.M., *Diagnosis of Secondary Caries.* Journal of Dental Education, 2001. **65**(10): p. 997-1000.

13. Muliyar, S., et al., *Microleakage in endodontics.* Journal of International Oral Health, 2014. **6**(6): p. 99-104.

14. Ahrari, F., et al., *The antimicrobial sensitivity of Streptococcus mutans and Streptococcus sangius to colloidal solutions of different nanoparticles applied as mouthwashes.* Dental Research Journal, 2015. **12**(1): p. 44-49.

15. Zhang, K., et al., *Effect of antibacterial dental adhesive on multispecies biofilms formation.* Journal of Dental Research, 2015. **94**(4): p. 622-629.

16. Steinberg, S., *A paradigm shift in the treatment of caries.* General Dentistry, 2002. **50**(4): p. 333-338.

17. Gupta, S.K., et al., *Release and toxicity of dental resin composite.* Toxicology International, 2012. **19**(3): p. 225-234.

18. Sachdeva, S., et al., *Nano-composite dental resins: an overview.* Annals of Dental Specialty 2015. **3**(2): p. 52-55.

19. Lee, H.-Y., et al., *Bonding of the silane containing multi-mode universal adhesive for lithium disilicate ceramics.* Restorative Dentistry and Endodontics, 2017. **42**(2): p. 95-104.

20. Asmussen, E. and Peutzfeldt, A., *Influence of UEDMA, BisGMA and TEGDMA on selected mechanical properties of experimental resin composites.* Dental Materials, 1998. **14**(1): p. 51-56.

21. Carvalho, R.M., et al., *Durability of bonds and clinical success of adhesive restorations.* Dental Materials 2012. **28**(1): p.72-86.

22. Blackham, J., Vandewalle, K., and Lien, W., *Properties of hybrid resin composite systems containing prepolymerized filler particles.* Operative Dentistry, 2009. **34**(6): 697-702.

23. Ritter, A.V., *Direct resin-based composites: current recommendations for optimal clinical results.* Compendium of Continuing Education in Dentistry, 2005. **26**(7): p. 481-492.

24. Geitel, B., Kwiatkowski, R., and Zimmer, S., *Clinically controlled study on the quality of class III, IV and V composite restorations after two years.* Journal of Adhesive Dentistry, 2004. **6**(3): p. 247-253.

25. Drummond, J.L., *Degradation, fatigue and failure of resin dental composite materials.* Journal of Dental Research, 2009. **87**(8): p. 710-719.

26. Antonucci, J.M., et al., *Chemistry of Silanes: Interfaces in Dental Polymers and Composites.* Journal of Research of the National Institute of Standards and Technology, 2005. **110**(5): p. 541-558.

27. Cruvinel, D.R., et al., *Evaluation of radiopacity and microhardness of composites submitted to artificial aging.* Materials Research, 2007. **10**(3): p. 325-329.

28. Cramer, N.B., Stansbury, J.W., and Bowman, C.N., *Recent Advances and Developments in Composite Dental Restorative Materials.* Journal of Dental Research, 2011. **90**(4): p. 402-416.

29. Mateo, J.L., Bosch, P., and Lozano, A.E., *Reactivity of Radicals Derived from Dimethylanilines in Acrylic Photopolymerization* Macromolecules, 1994. **27**(1): p. 7794-7799.

30. Jakubiaka, J., et al., *Camphorquinone–amines photoinitating systems for the initiation of free radical polymerization.* Polymer, 2003. **44**(1): p. 5219-5226.

31. Miyazaki, M., et al., *Factors affecting the in vitro performance of dentin-bonding systems.* Japanese Dental Science Review, 2012. **48**(1): p. 53-60.

32. Turp, V., et al., *Adhesion of 10-MDP containing resin cements to dentin with and without the etch-and-rinse technique.* Journal of Advances Prosthodontics, 2013. **5**(3): p. 226-233.

33. Hasimoto, M., et al., *Ten-years degradation of resin–dentin bonds.* European Journal of Oral Sciences, 2010. **118**(1): p. 404-410.

34. Cardoso, M., et al., *Current aspects on bonding effectiveness and stability in adhesive dentistry.* Australian Dental Journal, 2011. **56**(1): p. 31-44.

35. Zhang, Z., et al., *On the durability of resin-dentin bonds: identifying the weakest links.* Dental Materials, 2015. **31**(9): p. 1109-1118.

36. Barlow, S.M. and Raval, R., *Complex organic molecules at metal surfaces: bonding, organisation and chirality.* Surface Science Reports, 2003. **50**(1): p. 201-341.

37. Khoroushi, M. and Keshani, F., *A review of glass-ionomers: From conventional glass-ionomer to bioactive glass-ionomer.* Dental Research Journal, 2013. **10**(4): p. 411-420.

38. Neel, E.A.A., et al., *Demineralization–remineralization dynamics in teeth and bone.* International Journal of Nanomedicine, 2016. **11**(1): p. 4743-4763.

39. Fraunhofer, J.A., *Adhesion and Cohesion.* International Journal of Dentistry, 2012. **20**(1): p. 1-8.

40. Sofan, E., et al., *Classification review of dental adhesive systems: from the IV generation to the universal type.* Annali Di Stomatologia, 2017. **8**(1): p. 1-17.

41. Aguilar-Mendoza, J.A., et al., *Effect of Acid Etching on Dentin Wettability and Roughness:Self-Etching Primers Versus Phosphoric Acid.* Journal of Biomedical Material Research, 2007. **84**(1): p. 277-285.

42. Gupta, N., *Bonding: Foundation of Dentistry.* Journal of Innovative Dentistry, 2011. **1**(3): p. 60-66.

43. Bowen, R.L., *Adhesive Bonding of Various Materials to Hard Tooth Tissues. II. Bonding to Dentin Promoted by a Surface-active Comonomer* Journal of Dental Research, 1965. **44**(5): p. 895-902.

44. Nandini, S., *Indirect resin composites.* Journal of Conservative Dentistry, 2010. **13**(4): 184-194.

45. Jayaprakash, T., Srinivasan, M.R., and Indira, R., *Evaluation of the effect of surface moisture on dentinal tensile bond strength to dentine adhesive: An invitro study.* Journal of Conservative Dentistry, 2010. **13**(3): p. 116-118.

46. Tao, L., Pashley, D.H., and Boyd, L., *Effect of different types of smear layers on dentin and enamel shear bond strengths.* Dental Materials, 1988. **4**(4): p. 208-216.

47. Mai, S., et al., *Evaluation of the true self-etching potential of a fourth generation self-adhesive methacrylate resin-based sealer.* Journal of Endodontics, 2009. **35**(6): p. 870-874.

48. Kugel, G. and Ferrari, M., *The science of bonding: from the first to sixth generation.* The Journal of the American Dental Association, 2000. **131**(1): p. 20-25.

49. Sharma, P., et al., *Bonding efficacy of etch-and-rinse adhesives after dentin biomodification using ethanol saturation and collagen cross-linker pretreatment.* Journal of Conservative Dentistry, 2015. **18**(4): p. 331-336.

50. Kanca, J., *Improved bond strength through acid etching of dentin and bonding to wet dentin surfaces.* Journal of American Dental Association, 1992. **123**(1): p. 35-43.

51. Gwinnett, A., *Moist versus dry dentin: Its effect on shear bond strength.* American Journal of Dentistry, 1992. **5**(1): p. 127-129.

52. Ayar, M.K., *A review of ethanol wet-bonding: Principles and techniques.* European Journal of Dentistry, 2016. **10**(1): 155-159.

53. Pashley, D.H., et al., *From dry bonding to water-wet bonding to ethanol-wet bonding: A review of the interactions between dentin matrix and solvated resins using a macromodel of the hybrid layer* American Journal of Dentistry, 2007. **20**(1): p. 7-21.

54. Malacarne, J., *Water sorption/solubility of dental adhesive resins.* Dental Materials, 2006. **22**(10): p. 973-980.

55. Manfroi, F.B., *Bond strength of a novel one bottle multi-mode adhesive to human dentin after six months of storage.* The Open Dentistry Journal, 2016. **10**(1): 268-277.

56. Ozer, F. and Blatz, M.B., *Self-Etch and Etch-and-Rinse Adhesive Systems in Chnical Dentistry.* Compendium of Continuing Education in Dentistry, 2013. **34**(1): p. 12-20.

57. Lakomaa, E.L. and Rytomaa, I., *Mineral composition of enamel and dentin of primary and permanent teeth in Finland.* European Journal of Oral Sciences, 1977. **85**(2): p. 89-95.

58. Jayasudha, et al., *Enamel Regeneration- current progress and challenges.* Journal of Clinical Diagnosis Research, 2014. **8**(9): p. 6-9.

59. Imthiaz, N., et al., *Comparison of hydroxyapatite and dental enamel for testing shear bond strengths.* Australian Orthodontic Journal, 2008. **24**(1): p. 15-20.

60. Goldberg, M., et al., *Dentin: Structure, Composition and Mineralization: The role of dentin ECM in dentin formation and mineralization,* F.i. Bioscience, Editor. 2011. **3**(1): p. 711-735.

61. Hashimoto, M., et al., *A review: Biodegradation of resin–dentin bonds.* Japanese Dental Science Review, 2011. **47**(1): p. 5-12.

62. Carvalho, R.M., et al., *A challenge to the conventional wisdom that simultaneous etching and resin infiltration always occurs in self-etch adhesives.* Biomaterials, 2005. **26**(9): p. 1035-1042.

63. Wang, Y. and Spencer, P., *Hybridization efficiency of the adhesive/dentin interface with wet bonding.* Journal of Dental Research, 2003. **82**(1): p. 141-145.

64. Pegado, R., et al., *Effect of Different Bonding Strategies on Adhesion to Deep and Superficial Permanent Dentin.* European Journal of Dentistry, 2010. **4**(2): p. 110-117.

65. Priyalakshmi, S. and Ranjan, M., *A Review on Marginal Deterioration of Composite Restoration.* Journal of Dental and Medical Sciences, 2014. **13**(1): p. 6-9.

66. Spencer, P. and Y. Wang, *Adhesive phase separation at the dentin interface under wet bonding conditions.* Journal of Biomedical Material Research, 2002. **62**(1): p. 447-456.

67. Armstrong, S.R., et al., *Resin-dentin interfacial ultrastructure and microtensile dentin bond strength after five-year water storage.* Operative Dentistry, 2004. **29**(1): p. 705-712.

68. Brzeska, J., et al., *Chemical and Enzymatic Hydrolysis of Polyurethane/Polylactide Blends.* International Journal of Polymer Science, 2015. **15**(1): p. 1-8.

69. Frassetto, A., et al., *Mechanisms of degradation of the hybrid layer in adhesive dentistry and therapeutic agents to improve bond durability—A literature review.* Dental Materials, 2016. **32**(2): p. 41-53.

70. Khoroushi, M. and Ehteshami, A., *Marginal microleakage of cervical composite resin restorations bonded using etch-and-rinse and self-etch adhesives: two dimensional vs. three dimensional methods.* Restorative Dentistry and Endodontics, 2016. **41**(2): 83-90.

71. Hansen, E.K., *Effect of Cavity Depth and Application Technique on Marginal Adaptation of Resins in Dentin Cavities.* Journal of Dental Research, 1986. **65**(11): p. 1319-1321.

72. Going, R., *Microleakage around dental restorations: a summarizing review.* Journal of the American Dental Association, 1972. **84**(6): p. 1349-1357.

73. Heintze, S. and Zimmerli, B., *Relevance of in vitro tests of adhesive and composite dental materials, a review in 3 parts. Part 1: Approval requirements and standardized testing of composite materials according to ISO specifications.* Research and Science, 2011. **121**(9): p. 804-816.

74. Sano, H., et al., *Tensile properties of mineralized and demineralized human and bovine dentin.* Journal of Dental Research, 1994. **73**(6): p. 1205-1211.

75. Andrade, A.M., et al., *Evaluating resin-enamel bonds by microshear and microtensile bond strength tests: effects of composite resin.* Journal of Applied Oral Sciences, 2010. **18**(6): p. 591-598.

76. Bezerra, U.T., et al., *Hourglass-shaped specimen: compressive strength of concrete and mortar (numerical and experimental analyses).* Ibracon Structures and Materials Journal, 2016. **9**(4): p. 510-524.

77. Gianninia, M., Soares, C.J., and Carvalho, R.M., *Ultimate tensile strength of tooth structures.* Dental Materials, 2004. **20**(1): p. 322-329.

78. Tabor, D., *Hardness of Metals.* Oxford Clarendon Press, 1951.

79. Pala, K., *Flexural strength and microhardness of anterior composites after accelerated aging.* Journal of Clinical and Experimental Dentistry, 2017. **9**(3): p. 424-430.

80. Broitman, E., *Indentation Hardness Measurements at Macro-, Micro-, and Nanoscale: A Critical Overview.* Tribology Letters, 2017. **65**(23): p. 1-18.

81. Chang, M., Dennison, J., and Yaman, P., *Physical Property Evaluation of Four Composite Materials.* Operative Dentistry, 2013. **38**(5): p. 144-153.

82. Poskus, L.T., Placido, E., and Cardoso, P.E.C., *Influence of placement techniques on Vickers and Knoop hardness of class II composite resin restorations.* Dental Materials, 2004. **20**(1): p. 726-732.

83. International Organization for Standardization 10993-5, *Biological evaluation of medical devices —Part 5: Tests for in vitro cytotoxicity.* 2009.

84. Posimo, J.M., et al., *Viability Assays for Cells in Culture.* Journal of Visualized Experiments, 2014. **83**(1): p. 1-14.

85. Imazato, S., et al., *Antibacterial activity and bonding characteristics of an adhesive resin containing antibacterial monomer MDPB.* Dental Materials, 2003. **19**(4): p. 313-319.

86. Gould, I.M., *Towards a common susceptibility testing method?* Journal of Antimicrobial Chemotherapy, 2000. **45**(6): p. 757-762.

87. Porteous, N., Sun, Y., and Schoolfield, J., *Evaluation of 3 dental unit waterline contamination testing methods*. General Dentistry, 2015. **63**(1): p. 41-47.

88. Paradella, T.C. and M.A. Bottino, *Scanning Electron Microscopy in modern dentistry research.* Brazilian Dental Science, 2012. **15**(2): p. 43-48.

89. Shonol, Y., et al., *Regional Measurement of Resin-Dentin Bonding as an Array.* Journal of Dental Research, 1999. **78**(2): p. 699-705.

90. Leitune, V., et al., *The addition of nanostructured hydroxyapatite to an experimental adhesive resin.* Journal of Dental Research, 2013. **41**(1): p. 321-327.

91. Imazato, S., et al., *Antibacterial activity and bonding characteristics of an adhesive resin containing antibacterial monomer MDPB.* Dental Materials, 2003. **19**(4): p. 313-319.

92. Renné, W.G., et al., *Antibacterial properties of copper iodide-doped glass ionomer-based materials and effect of copper iodide nanoparticles on collagen degradation.* Clinical and Oral Investigation, 2017. **21**(1): p. 369-379.

93. Sampaio, P., et al., *Comparative bonding ability to dentin of a universal adhesive system and monomer conversion as functions of extended light curing times and storage.* Journal of the Mechanical Behavior of Biomedical Materials, 2017. **75**(1): p. 41-49.

94. Nishitani, Y., et al., *Effects of Resin Hydrophilicity on Dentin Bond Strength.* Journal of Dental Research, 2006. **85**(11): p. 1016-1021.

95. Rêgo, H.M.C., et al., *Can long-term dentine bonding created in real life be forecasted by parameters established in the laboratory?* Scientific Reports, 2016. **6**(1): p. 1-8.

96. Sarr, M., *Microtensile bond strength and interfacial characterization of 11 contemporary adhesives bonded to bur-cut dentin.* Operative Dentistry, 2010. **35**(1): p. 94-104.

97. Loguercio, A., et al., *Effect of solvent removal on adhesive properties of simplified etch-and-rinse systems and on bond strengths to dry and wet dentin.* Journal of Adhesive Dentistry, 2009. **11**(3): p. 213-219.

98. Xu, C. and Wang, Y., *Cross-linked demineralized dentin maintains its mechanical stability when challenged by bacterial collagenase.* Journal of Biomedical Material Research, 2011. **96**(2): p. 242-248.

99. Ferracane, J., *Hygroscopic and hydrolytic effects in dental polymer networks.* Dental Materials, 2006. **22**(1): p. 211-222.

100. Rodrigues, S., Ferracane, J., and Della, B.A., *Influence of surface treatments on the bond strength of repaired resin composite restorative materials.* Dental Materials, 2009. **25**(4): p. 442-451.

101. Tay, F., et al., *Bonding BisGMA to dentin--a proof of concept for hydrophobic dentin bonding.* Journal of Dental Research, 2007. **86**(11): p. 1034-1039.

102. Hosaka, K., et al., *Durability of Resin-Dentin Bonds to Water- vs. Ethanol-saturated Dentin.* Journal of Dental Research, 2009. **88**(2): p. 146-151.

103. Koulaouzidou, E.A., et al., *Effects of a dental adhesive on cell cycle regulatory proteins.* Dental Materials, 2013. **32**(6): p. 986-991.

104. Williams, D.F., *On the mechanisms of biocompatibility.* Biomaterials, 2008. **29**(20): p. 2941-2953.

105. Koulaouzidou, E.A., et al., *Cytotoxicity of dental adhesives in vitro.* European Journal of Dentistry, 2009. **3**(1): p. 3-9.

106. Elias, S.T., et al., *Cytotoxicity of Universal, Self-Etching and Etch-and-Rinse Adhesive Systems According to the Polymerization Time.* Brazilian Dental Journal, 2015. **26**(2): p. 160-168.

107. Moharamzadeh, K., et al., *Cytotoxicity of resin monomers on human gingival fibroblasts and HaCaT keratinocytes.* Dental Materials, 2007. **23**(1): p. 40-44.

108. Gsib, O., et al., *Evaluation of Fibrin-Based Interpenetrating Polymer Networks as Potential Biomaterials for Tissue Engineering.* Nanomaterials, 2017. **7**(12): p. 1-21.

109. Geurtsen, W., *Cytotoxicity of 35 dental resin composite monomers/additives in permanent 3T3 and three human primary fibroblast cultures.* Journal of Biomedical Material Research, 1998. **41**(3): p. 474-480.

110. Geurtsen, W., *Aqueous extracts from dentin adhesives contain cytotoxic chemicals.* Journal of Biomedical Material Research, 1999. **48**(6): p. 772-777.

111. Pupo, Y., et al., *Cytotoxicity of Etch-and-Rinse, Self-Etch, and Universal Dental Adhesive Systems in Fibroblast Cell Line 3T3.* Hindawi, 2017. **17**(7): p. 1-7.

112. Namba, N., et al., *Antibacterial effect of bactericide immobilized in resin matrix.* Journal of Dental Materials, 2009. **25**(4): p. 424-430.

113. Zhou, C., et al., *Synthesis of new antibacterial quaternary ammonium monomer for incorporation into CaP nanocomposite.* Journal of Dental Materials, 2013. **29**(8): p. 859-870.

114. Cheng, L., et al., *Antibacterial nanocomposite with calcium phosphate and quaternary ammonium.* Journal of Dental Research, 2012. **91**(5): p.460-466.

115. McBain, A.J., et al., *Effects of quaternary-ammoniumbased formulations on bacterial community dynamics and antimicrobial susceptibility.* Applied and Environmental Microbiology, 2004. **70**(6): p. 3449-3456.