AN ABSTRACT OF THE DISSERTATION OF

Dmytro Khvostenko for the degree of Doctor of Philosophy in Materials Science presented on July 11, 2014.

Title: Bioactive Glass Filled Resin Composites: Mechanical Properties and Resistance to Secondary Tooth Decay.

Abstract approved: _____

Jamie J. Kruzic

Dental resin composites are widely used in modern dentistry due to their aesthetic appearance. However, resin based restorations have inferior mechanical properties (fracture is the second reason for restorations replacement) in comparison to alloy based composites and their antimicrobial properties still remain poor, leading to secondary tooth decay development on tooth-filling interface (which is the primary reason for restorations failure). In this work the Bioactive glass (BAG) was used as an antimicrobial filler. Experimental composites were prepared with 0-15 wt% BAG filler and 72-57 wt% silane treated silica glass, keeping a total filler content constant at 72 wt%. BAG composite's mechanical properties were examined, using 3-point bending beams for flexural strength measurement, pre-cracked compact-tension samples, C(T), for fracture toughness and fatigue crack growth evaluation. All mechanical properties were tested for different soaking treatments of

the samples: 24 hours in DI water (all experiments) 2 months in sterile media (flexural test only) and 2 months in media with *Streptococcus mutans* bacteria (all experiemtns). All mechanical properties findings were compared to those ones of commercial Heliomolar composite. Main toughening mechanisms for BAG composites were evaluated post-test by SEM. The results showed that all mechanical properties for BAG composites were unaffected by increasing the filler content from 0 to 15 wt%. Moreover, all mechanical properties of BAG composites were significantly superior over Heliomolar composites. BAG composite's flexural strength properties were not affected by any of aging treatments and a slight decrease in fracture toughness and fatigue crack growth resistance was observed after 2 months soaking in media with bacteria. Main toughening mechanisms were related to BAG composite's increased filler, which resulted in more frequent crack deflection, and crack bridging at the crack tip and far away from it.

In next phase of the research, a novel bioreactor and new test specimen type were developed, which allowed investigating the synergistic effect of cyclic loading and bacterial exposure on secondary tooth decay. The bioreactor was able to provide environment suitable for bacteria, similar to lab incubators. Teeth samples were machined into the disk shape (3 mm tall, 9 mm diameter), mainly consisting of dentin and the middle of sample was drilled and restored with 0 wt% BAG (0BAG) composite (2 mm deep, 5 mm diameter), introducing semi-circumferential gap between dentin and restoration of 10-30 microns. All samples were initially disinfected with 1% chloramine solution. *Streptococcus mutans* biofilm was grown over the samples and then they were placed in bioreactors and subjected to 1.5 Hz loading cycling at maximum load of 25% from

breaking load and R=0.1 for 2 hours, followed by 4 hours resting at minimal load. The procedure was repeated for total of 2 weeks of an experiment, during which bacteria biofilm was constantly supplied with BHI media, carbon dioxide and 37^{o}C temperature. After 2 weeks test, samples' biofilm viability was evaluated with live/dead staining kit and then, after fixing the biofilm on the samples in 4% gluteraldehyde, all samples were sectioned across the gap and stained with Gram Crysrtal Violet CAT+ dye , followed by fluorescence microscopy to reveal the depth of bacterial penetration down the gap. The depth of bacterial penetration of loaded samples was compared to one of non-loaded samples and results revealed a significantly deeper bacterial penetration for cyclically loaded samples versus non-loaded, thus, it was concluded that cyclic loading and bacterial exposure together aid secondary tooth decay propagation.

In the last phase of this project the anti-microbial properties of BAG composites were evaluated. Using the same sample preparation technique and methods from the previous phase (except that now the composite was 15 wt% BAG, a.k.a 15BAG), we found out that bacterial penetration down the gap for 15BAG composite was significantly lower (almost 50% difference) in comparison to 0BAG composite, which made us confident to draw a conclusion that 15BAG composite possesses good antimicrobial properties and can be used for secondary tooth decay prevention.

Bioactive Glass Filled Resin Composites: Mechanical Properties and Resistance to Secondary Tooth Decay

by

Dmytro Khvostenko

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Dmytro Khvostenko, Author

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1. Background

1.1 Tooth and its structure

Teeth are defined as the “hard processes within the mouth, attached (usually in sockets) in a row to each jaw in most vertebrates except birds (but also in some extinct birds), having points, edges, or grinding surfaces, and serving primarily for biting, tearing, or trituration of solid food, and secondarily as weapons of attack or defense, and for other purposes; in singular, each of these individually “ [2].

The cross-section of a tooth is depicted in Fig. 1.

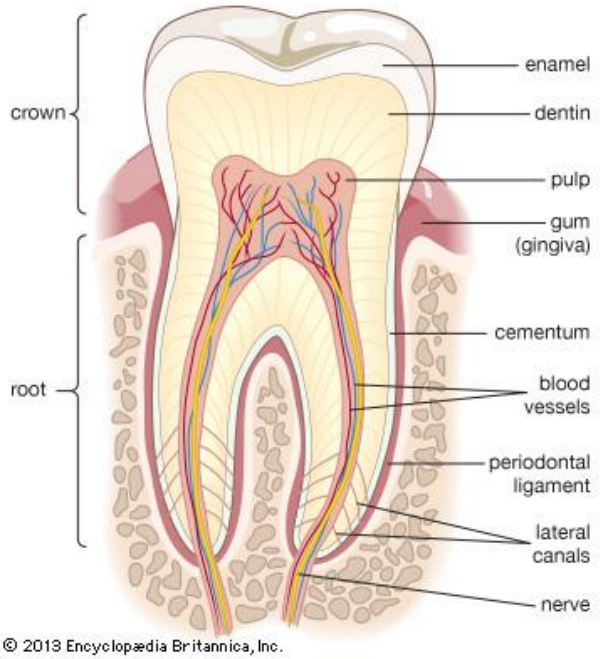

Figure 1. Tooth anatomy. By courtesy of Encyclopaedia Britannica, Inc., copyright 2013; used with permission.

A tooth consists of two major parts: Crown and one to four roots [3]. The crown is a part above the gums and bone, and a root is one, embedded into bone and gums.

The main components of tooth are:

- Enamel,
- Dentin,
- Cementum,
- Pulp.

1.1.1 Enamel

Enamel is the strongest part of human body, which is able to take stresses up to 2.5 GPa during mastification [4]. Enamel serves to protect the softer dentin and tender pulp against mechanical and/or chemical damage. Its thickness varies from 1 to 2.5 mm from root-crown junction to the surface of a crown [5]. Being mostly a mineralized tissue, enamel by weight consists of 96% inorganic crystalline phase – hydroxyapatite (HA) and 4% of organic phase – non-collagenous proteins [6]. A fundamental structural unit of enamel is the enamel rod, consisting of packed HA fibers, which are bonded by organic matrix [7]. Enamel rods are formed by process called amelogenesis in the direction perpendicular to enamel-dentin junction (DEJ) by ameloblasts. Ameloblasts only play a role during development of the enamel and die after completion, thus, enamel is a dead tissue at its maturity [8]. Enamel is avascular tissue and has no nerve supply, thus, can't be regenerated intrinsically, however, mineralization changes still can be observed [9].

1.1.2 Dentin

Dentin is not as hard as enamel and has the approximate composition of 70 wt% mineral HA, 20 wt% of organic matrix and 10 wt% of water. Dentin consists of four major structural types: dentin matrix, dentin tubules, mineral and dentinal fluid [10]. The dentin matrix surrounds dentin tubules and mineralizes upon dentin development and forms intertubular dentin. The main connective tissue cells, responsible for dentin production are called odontoblasts, originally residing in the pulp. Dentin tubules run from the pulp toward DEJ. Dentin protects the pulp and supports the more brittle enamel.

1.1.3 Cementum

Cementum is another hard tissue of a tooth that covers the entire surface of the root, serving as a interface between root dentin and periodontal ligament. Although it is considered a part of a tooth itself, it functionally belongs to the dental attachment apparatus. Besides attachment of the root to periodontal ligament, cementum also protects the entire root [11]. Similar to enamel, cementum is avascular and not innervated, however, unlike enamel, cementum is experiencing remodeling during the entire life, just like a regular bone [12]. Primary cells, responsible for cementum production are cementoblasts. The composition is 61 wt% inorganic mineral material, 27 wt% organic material and 12% water [13].

1.1.4 Pulp

Pulp is a very soft tissue, located in the middle of a tooth. It is highly innervated, very vascular and gives rise to odontoblasts [14]. As pulp is a source for odontoblasts, it is responsible for forming the dentin around itself. Also, pulp nourishes dentin and repairs it [15]. It was shown that pulp plays a role in tooth defense against bacterial attacks in comparison to teeth where the root has been removed endodontically [16].

1.2 Caries

Caries is considered to be one of the most common diseases in the world. It occurs when demineralization-remineralization equilibrium shifts to the demineralization side [17]. Such shift can be resulted from non-proper tooth development as well as acidification. As a result, the affected spot becomes softer and speeds up caries propagation even further. Dentin and cementum are more susceptible to caries, as their mineral content is lower. As a result, tooth decay propagates faster once it reaches dentin, making the pulp less isolated. As pulp becomes more exposed to the outside, nerves in the pulp will sense different pressures associated with hot and cold drinks, causing pain while consuming those drinks [18]. Caries is also a direct pathway for bacteria to the pulp, which often causes pulpitis – the pulp inflammation. Due to inflammation, the pressure builds up inside the pulp, causing significant pain. The most common treatment is mechanical pulp cleaning, commonly known as a root canal procedure [19].

1.2.1 Causes of caries

It has been shown that caries requires a triad of factors:

- Dietary carbohydrates,
- Dental plaque bacteria and
- Caries-susceptible teeth [20].

Although the oral cavity is a habitat for a very wide variety of bacteria, not all bacteria are harmful for teeth. Ones that are harmful are also commonly referred as acidifying bacteria. Dental plaque consists of bacteria themselves and the plaque matrix is permeable to sugars and ions (Ca and P from tooth) [21]. A variety of carbohydrates serves as the primary food sources for bacteria. The by-products are often organic acids, for example lactic acid [22]. The local drop in pH favors enamel and dentin demineralization. It was shown that sugar intake restriction indeed improves oral health as well as affects the bacterial ecology in an oral cavity [23].

The most common types of bacteria found to be responsible for caries formation belong to lactobacilli and mutans streptococci. Those bacteria were selected in evolution due to their high tolerance to acid and great ability to attach to a tooth, forming a plaque [24], [25].

1.2.2 Secondary caries

Secondary caries, also known as recurrent caries, is a caries that appears on a spot previously affected by caries. The usual spot for appearance is the filling-tooth margin [26] (see Fig. 2).

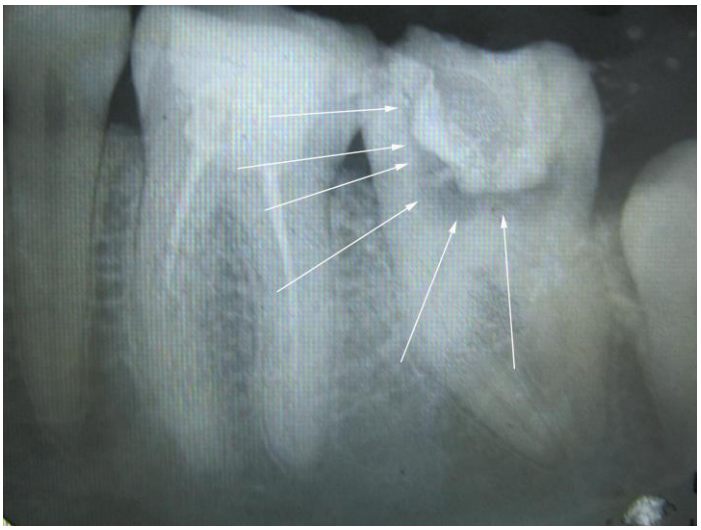

Figure 2. Secondary tooth decay at dentin-filling margin.

The reason for formation of secondary tooth decay is bacterial biofilm formation on the tooth-filling interface with subsequent demineralization of adjacent tooth tissue [27]. Microbiological analysis reveals that the most abundant bacterial species found under fillings in teeth with secondary decay symptoms are Streptococcus mutans, Actinomyces naeslundii and Lactobacillus casei [28]. Further analysis has shown that Streptococcus mutans is the most abundant species under composite restorations [29]. Colonization of the tooth-filling interface by bacteria is promoted by gap formation between filling and dentin.

1.3 Dental restorations overview

Unfortunately, oral microflora has found its way in evolution to coexist along with the human body's natural defense mechanisms. That can lead to potential infection of the tooth tissues, which requires their removal. As mentioned before, dentin should not stay unprotected, thus, artificial tissue needs to replace the infected ones. There are evidences of cavity site preparation and restorations used in ancient civilizations (Egypt, Greek, Maya, Roman etc). A bow drill was used in the very early civilizations. The most popular restoration materials were stone chips, resin, cork and gum [30]. Later on, metals and their alloys started to be popular after around the 1400s until beginning of the 1800s. Many historians show a use of tin, lead and gold in that period [31]. A bloom of dental restorative materials happened in the 19^{th} – 20^{th} centuries and still continues. In 1826, August Taveau introduced the first amalgam restoration, which consisted of 50 parts Hg, 45 parts Ag and5 parts of Cu in total of 100 parts. The original purpose was to restore a tooth in an effective and cheap way and amalgam restorations were later brought to USA by the Crawcour brothers in 1833 [32]. For the rest of the 1800s, the "amalgam war" was taking place in the dental research society as there were many opponents of it. However, despite this hot debate, amalgam still was the leading material [33]. In the early 1900s cements were introduced, which prepared by mixing phosphoric acid with powders of zinc oxide and magnesium oxide; however, they did not have very good longevity. However, because of not having anything better they were widely used for approximately half of a century. Later in 1972 the glass-ionomer cements were developed and they consisted of fluoroaluminosilicate glass mixed into liquid phase (polyacrylic acid itaconic acid and

water). In 1960s resin composites were developed that made a big impact in cosmetic and operative dentistry [34]. Nowadays, amalgams, glass ionomer cement compounds and resin based restorations are prevailing.

1.4 Classification of dental restorations

All current dental restorations can be put into two major categories:

- Indirect restorations, and
- Direct restorations.

Direct restoration is a technique where a soft material is put into a prepared tooth followed by setting to make the material hard. Direct restorations can be applied during the same day as tooth preparation. Commonly used materials for this technique are amalgams, resin composites and glass ionomer compounds. The disadvantage of direct restorations is that they cannot be used where lots of strength is needed [35]. In such case, indirect restorations can be used instead. Indirect restorations are prepared outside of a patient's mouth, commonly with a help of previously taken mold impressions. Procedures, involving indirect restorations, cannot usually be done during the same day and may require several visits. The examples of indirect restorations include inlays/onlays, bridges, veneers, crowns etc.

1.4.1 Indirect restorations

1.4.1.1 Inlays/Onlays

Inlays and onlays are used when a tooth is too damaged to fix it with a filling, but not damaged enough to require a crown or a surgical extraction. Usually it happens when a big portion of a tooth has decayed and placing a big piece of direct restoration material would create many contact interfaces. Such interfaces give a higher possibility of fracture for a large direct restoration. Thus, premade pieces of tooth are made and they are fit onto the prepared tooth surface. Considering that an inlay/onlay will take up a majority of a tooth, they will bear lots of stress. Mostly ceramics, metals and sometimes composites are used that have much better mechanical properties than direct restoration materials. Inlays usually are placed between cusps, while onlays take a space between cusps and expand on at least one cusp itself (Fig. 3).

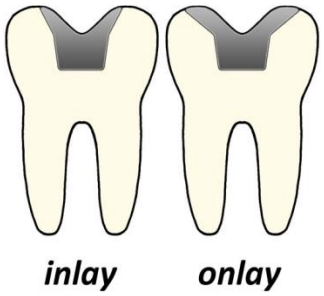

Figure 3. Dental Inalys and Onlays.

1.4.1.2 Crowns and bridges

When a tooth cannot be restored by changing of a part of it, or a tooth does not have a nerve anymore and becomes brittle, crowns are used. Crowns cover the portion of the tooth above the gum line. Usually significant tooth tissue removal is needed to fit a crown over the prepared tooth and it is cemented in place, usually using GIC (glass-ionomer cement). Caries reoccurrence is still a possibility under the crown, which often requires crown removal, caries filling and crown replacement [36]. Due to expected high stresses experienced by a crown, the materials used are ceramic, porcelain-fused to metal or just metal. Bridges are similar to crowns, except that there are several teeth (at least 3) that are interconnected. The first and the last tooth in this series are the ones that are crowns and are attached to real teeth. In between them, teeth are replacing the whole real teeth (Fig. 4).

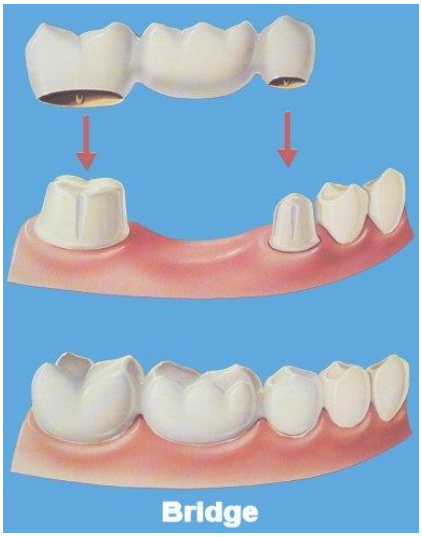

Figure 4. Dental bridge.

1.4.1.3 Veneers

Veneers usually have a purely cosmetic purpose. They are premade in a lab to fit over the anterior surface of human teeth, usually incisors (Fig. 5). They can cover teeth with bad color, cracked or misplaced teeth and teeth with diastema (large space between teeth). Veneers still require a little anterior tooth tissue removal to preserve the dimensions of the restored teeth.

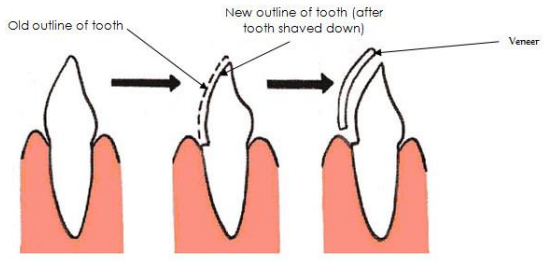

Figure 5. Dental veneer.

1.4.2 Direct restorations

1.4.2.1 Amalgam

Dental amalgam is a restoration that is prepared by mixing mercury with powdered metals (mostly tin, copper and silver). Right after mixing the restoration is in a paste-like state, which allows it to be placed and packed (condensed) into cavity preparation. After several minutes, the paste hardens as a result of cascade of chemical reactions, leading to a solid intermetallic compound [37]. Being a metal alloy, amalgam restorations are resistant to wear, have good load-bearing properties and can be placed under various conditions,

including wet cavity preparation [38]. It has also been reported that amalgams have a unique ability of sealing tooth-restoration margins due to corrosion under oral conditions, which can greatly reduce a chance of caries reoccurrence [39]. Although amalgams have good mechanical properties and are durable, there are several shortcomings associated with this type of restoration:

- Bad aesthetic properties, and
- Cytotoxicity (associated with mercury).

The dark appearance of amalgam makes it to be a bad choice for anterior restorations. As for mercury toxicity, it is still a debatable question. Some studies show no noticeable effect of mercury containing amalgam on health; however, on the another hand, some studies report a negative effect, like, for instance, an increase in mercury concentration in some tissues as a result of exposure to dental amalgams [40]. Despite the uncertainties about health-related issues, amalgams are still one of the most popular restorations due to their effectiveness and relatively cheap cost.

1.4.2.2 Glass-ionomer cements (GIC)

GIC is a filling material that resembles the color of a tooth and usually is used in places where high mechanical stresses are not expected due to GIC's low mechanical properties in comparison to amalgam or resin based composites [41]. However, GIC possesses some advantageous features, like self-adhesion, biocompatibility and caries-resisiting properties [42]. GIC begin as a powder-liquid system, which is mainly composed of acid-soluble glass

(powder phase) and polyacrylic acid (liquid phase) [43]. Acid –base reactions occur to set the GIC after mixing, giving a structure much like a dental composite. Glass ionomer cements can be used in many applications due to the ability to change their liquid/powder ratio, which gives a variation in curing times and viscosity [44]. GICs are commonly used on patients with higher risk of caries reoccurrence, as this material is able to leach out fluoride ions, providing local remineralization of affected tissues [45]. Due to poor longevity properties, GIC are also often used for pediatric restorations because the service time is expected to be short.

1.4.2.3 Resin composites

Resin composites are the focus of this dissertation and will be discussed separately in the next chapter.

2. Resin Composites.

Resin composites were discovered in 1960s and became very popular in dentistry. Up to now they have been constantly improved by composition variations to obtain the best performance. Resin composites represent a mixture of organic polymer resin matrix and inorganic filler. The advantages of resin based dental composites include, but are not limited to, aesthetic appearance, self-adhesive properties, workability and versatility in properties depending on area of application. These days there are lots of different organic pigments that can be dissolved in polymer matrix of resin composites, giving them almost any shade to make the restorations almost indistinguishable from the tooth [46]. Some resin

composites are designed for high stress-bearing areas (premolars and molars), whereas others can be used in low stress-bearing areas (incisors). The disadvantages of resin composites are lower mechanical performance and polymerization shrinkage upon composite curing [47].

2.1 Classification of resin composites

Resin composites are described and distinguished by their filler size and filler volume fraction. Nowadays, there are five common types available: traditional, microfilled, hybrid, small-particle hybrid and nanocomposites. Table I gives a brief summary of those types [48], [49].

Table 1. Classification of resin composites

Property	**Traditional**	**Microfilled**	**Hybrid**	**Small-particle hybrid**	**Nano - composite**
Filler wt%	70-75	35-50	70-75	65-80	60-70
Filler size, µm	10-20	0.01-0.05	0.04-5	0.1-6	0.005-0.075
Type of restorations	Anterior and Posterior	Anterior	Posterior	Anterior and Posterior	Anterior and Posterior
Pros	Durability	Well polishable and stain resistant	Resistant to wear, strong	Good surface polish	Better tooth color, increased filler surface area
Cons	Poor surface finish, dull appearance	Poor mechanical performance	Poor surface polish	Poor wear resistance	Poor wear resistance and mechanical properties

2.2 Components of resin dental composites

Resin composites consist of:

- Resin matrix,
- Fillers,
- Coupling agents, and
- Cure catalyzers/inhibitors.

Each of the components will be reviewed in the following sub-sections.

2.2.1 Resin

Typical dental composite resins consist of a few types of mono-/oligomers. One of the most popular resins is bisphenol A glycidyl dimethacrylate (Bis-GMA), it is used in most of the resin composites. Other dimethacrylate resins are also used a lot, including triethyleneglycol dimethacrylate (TEGDMA), urethane dimethacrylate (UDMA), ethoxylated bisphenol-A-dimethacrylate (Bis-EMA), decanediol dimethacrylate (D3MA), bis(methacryloyloxymethyl) tricyclodecane, and urethane tetramethacrylate (UTMA) [50]. It was shown that Bis-GMA itself has a very high viscosity due to intermolecular hydrogen bonding interactions, making the overall composite to be less fluent; thus, there is a need to mix Bis-GMA and other less viscous resins to achieve the better workability [51]. TEGDMA is significantly less viscous and dilutes Bis-GMA. Previous studies claim that 1:1 ratio of Bis-GMA and TEGDMA give the best results in overall matrix viscosity and

polymerization [52]. However, the high degree of polymerization shrinkage of TEGDMA becomes a new problem. After this discovery, TEGDMA was partially or completely displaced by UTMA, as UTMA forms less double bonds with Bis-GMA per unit of molecular weight, thus less shrinkage occurs after curing [53]. Double bonds are shorter than single bonds, thus, having fewer double bonds gives less shrinkage.

All dimethacrylate based resins cure by radical driven polymerization reaction, creating a branched cross-linked network. Such polymerization reaction can be chemically initiated, light initiated or a combination of both. Due to the long time needed for chemically initiated curing, light curing is the most popular technique nowadays. Blue light with wavelength of 410-500 nm creates free radicals that start the overall reaction [31]. Ferracane at al. [54] proved that curing time and depth are the aspects that may change the properties of cured resin significantly. The longer a sample is cured, the less free monomers remain. Free monomers increase the plasticity of the sample, making it more ductile, thus, complete 100% polymerization is not desired as it embrittles the material. Due to the light absorption, scattering and reflection, light units cannot always cure the resin all the way through the restoration depth, thus, filling placement is done by layers. However, newly developed LED cure units have higher depth penetration potential [31].

2.2.2 Fillers

Initially fillers were added to the resin composites to replace the matrix, which is the most expensive component of the whole composite. Later on, various fillers became popular for reinforcement purposes. Higher filler fraction and larger particle size shows an

improvement in strength, stiffness, polymerization shrinkage and elastic modulus [55], [56]; however, polishing does not give a good finish shine as light scatters on coarse particles. This problem can be improved by adding smaller particle fillers instead. Thus, to reach the optimum, both small and coarse particles are added as filler.

Over the years, many different materials served as fillers. Commonly used are different glasses, silica, or zirconia based particles. Some of those, like strontium glass or zirconia particles, are added mostly for radiopacity [57]. This is done because in clinical practice caries looks darker on X-rays, thus, if the filling composite is more radiotranslucent, it becomes very difficult to distinguish it from caries. Moreover, material with good radiopacity properties can help to identify secondary tooth decay that starts under a filling, as the contrast between darker caries and brighter filling will be more noticeable [58]. Filler can also play a caries-prevention role. Dicalcium phosphate anhydrous (DCPA) and tetracalcium phosphate can release calcium and phosphate ions that help to restore and remineralize adjacent tooth tissue [59], [60]. Other caries preventing fillers can show antimicrobial properties. Recently developed bioactive glass (BAG) fillers for dental composites were shown to significantly reduce bacterial colony counts and mitigate biofilm formation [61]. In this dissertation it will also be shown that BAG composites also have same or improved mechanical properties over present-day commercial composites [62].

2.2.3 Coupling agents

Coupling agents are used to create a better bonding between polymer matrix and filler, reducing the incidence of particles debonding [63]. One of the most commonly used

coupling agents is silane. Silane contains C=C groups on one side of the molecule and Si-OH (silanol) groups on opposite side. C=C groups can create covalent bonds with carbon chains of dimethacrylate chains, whereas silanol groups form covalent bonds with silica glass particles (popular filler) in condensation-dehydration reactions [64]. Due to the role of silane as a "glue", the final properties of composites can be improved [65].

2.2.4 Cure catalyzers/inhibitors.

Resin curing initiation is a very endothermic process and requires catalyzers to initiate the polymerization reaction [66]. When light shines on the resin, light energy is distributed between resin monomer molecules which makes the effective energy per monomer too low to create free radicals. Thus, a molecule is needed to absorb the light energy and start radical chemistry (photo-initiator). Camphorquinone (CQ) is one of the most popular photo-initiators. Under light it loses an electron from each alpha-carbon and turns into a radical, which will attack the nearest and the least sterically hindered resin monomer molecule, leading to polymerization [67]. At the same time, resin composites need to be stored for prolonged periods of time under different conditions; thus, a cure inhibitor must be added to prevent a spontaneous polymerization reaction. Butylated hydroxytoluene (BHT) is commonly used as an inhibitor.

2.3 Mechanical properties of resin composites

Among the many characteristics of dental materials, mechanical properties take one of the central roles because dental restorations can be subjected to various loads and stresses. As mentioned previously, during mastication the local stress experienced by a tooth can rise up to 2.5 GPa. Direct occlusal restorations are subjected to shear, tensile, compressive and bending loadings. Moreover, the loadings are cyclic during mastication. Thus, dental composites must have adequate flexural strength, fracture toughness and fatigue resistance properties. Those properties will be discussed in the following chapters.

2.3.1 Flexural strength

According to ISO 4049, flexural strength is one of the important characteristics for dental material performance and it should be at least 50 MPa [68]. For dental materials, flexural strength is usually measured using three or four point bending tests. It was noticed that three point bending tests usually give higher values for flexural strength than four point bending tests. This happens because for four point tests there is a larger area that experiences the maximum applied stress. The larger area is more likely to contain various flaws (impurities, microcracks, voids etc) that serve as stress concentrators and/or fracture initiation sites, decreasing flexural strength results [69]. Flexural strength can be affected by many treatments.

Many works show the effect of filler content on flexural strength of resin dental composites. The trend is that composites with lower filler content generally have lower

flexural strength [70]. The matrix itself may influence flexural properties a lot. It was shown that mixing a very viscous Bis-GMA with less viscous UDMA significantly improves flexural properties [71]. On another hand, some works suggest no dependence of flexural strength of composites on viscosity of their matrices [72]. Degree of curing of matrix influences flexural strength too. A 100% polymerization leaves no unreacted monomers in matrix and, consequently, plasticity of the composite drops [73], [74].

2.3.2 Fracture toughness

Fracture toughness (Kc) measures a material's ability to resist fracture by crack propagation under loading. This property is very important for dental composites as the second most common reason for dental restoration replacement is fracture [75]. A higher Kc indicates better resistance of the material to fracture. Fracture toughness is equated as $K_c=Y\sigma(\pi a_0)^{0.5}$. If one knows a_0 – a critical size of a flaw and K_c and applied stress σ, the clinical performance of a given restoration can be estimated [76]. For most of dental resin composites the fracture toughness is in range of 1-2 $MPa(m)^{0.5}$ [77]. Fracture toughness tests are performed usually on pre-cracked samples. Many ways of measuring fracture toughness were developed: single edge notch (SEN), compact tension specimen, short rod, Chevron notch, notched and grooved plate, double torsion etc. Some work shows that the short rod test gives the highest value and the double torsion method gives the lowest value, and the double torsion method was also reported as the most difficult to conduct (50% success rate) [78]. The compact tension specimen method is one of the most commonly

used due to its ease. Like in the case of flexural strength, fracture toughness can increase with increasing filler content. That is related to energy dissipation as crack hits a particle due to crack pinning by particle, crack deflection etc. [79]. Wang at al showed that energy dissipation is maximized when crack hits a particle at its center [80]. Thus, if filler content increases, the probability of crack hitting a particle increases as well, resulting in higher fracture toughness. However, the filler content effect has its limit and at filler fractions more than 55 vol% no further improvement is observed [81]. Filler-matrix interface has also been shown to have an influence on the overall fracture toughness of composites. Modeling suggests that interfacial toughness between 2 and 3 times of matrix toughness gives a positive effect on the toughness of composites [82]. It may also be expected that the size of filler particles should have a significant effect on the fracture toughness of composites [83]; however, some studies do not support this claim [84], [85].

2.3.3 Fatigue of resin composites.

Teeth and restorative materials on teeth are constantly subjected to cyclic loadings during mastication with average chewing frequency of 1.45-1.68 Hz [86]. Humans dedicate around 3 hours per day for mastication, which is around $5.8x10^6$-$6.6x10^6$ cycles of mastication loadings per year. Because of the fatigue loadings on teeth, static methods used to describe and determine mechanical properties of dental composites are not enough. Studies concluded that static strengths do not correlate with fatigue values [87]. Many studies showed that lots of different dental composites are highly susceptible to fatigue damage [88], [89]. Fatigue damage arises from multiple external and internal cracks that almost

always exist in the material. Those cracks can grow during cycling at subcritical loads (loads predicted by static fracture toughness tests), leading eventually to failure of the material.

There are several ways to measure fatigue properties of dental restorations. The staircase method is one of them. In this method the cyclic stress level and number of cycles to failure are estimated before the test. If the sample does not break at the end of the test, then it will be cycled at a higher stress level. If sample breaks before the predicted number of cycles, then another sample will be tested consequently at lower stress level, until experimental results are approaching the predicted parameters [90]. This method gives very little information to be used for fatigue behavior prediction, on another hand, this method allows to do a quick comparison among materials. A more effective method is stress-life (S/N) approach [91]. In this method only a stress level is pre-selected and then the sample is fatigue cycled until fracture occurs. When that happens, the total number of fatigue cycles is recorded. Such tests can be repeated at different stress levels, resulting in S/N plots, where stress amplitude is plotted against number of cycle to failure. S/N method is widely used for lifetime estimation of various materials, including dental composites. A decrease in stress to failure was shown to be 35% and 75% for S/N approach and staircase method, respectively, for commercially available composite Filtek Z250 [92], [93].

It was suggested that it is more convenient to study fatigue behavior of dental composites with slow-crack growth approach [94]. This method is based on the Paris law $\frac{da}{dN} = C(\Delta K)^m$, where da/dN is the crack growth rate, m and C are scaling constants and $\Delta K = K_{max} - K_{min}$ (stress intensity range) [95]. Scaling constant m is related to sensitivity of

crack growth rate to change in stress intensity and determined as slope of the Paris region on the plot (Fig. 6). For the latter method, when crack growth rate is plotted against stress intensity range (both on Log scale), the plot has three identifiable regions (Fig. 6).

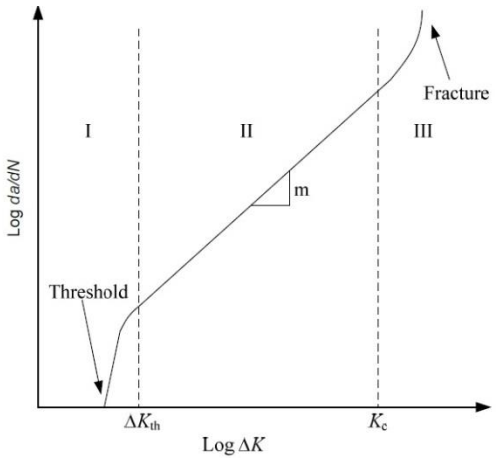

Figure 6. Fatigue plot, showing tree different regions, with Region II to be Paris region [95].

Region I shows a very rapid crack growth acceleration with stress intensity range increase, same as Region III, which leads to fracture very quickly. Both Regions I and III are difficult to describe and difficult to study, as they are relatively short. However, Region II (Paris region) is a steady-state crack growth period that can be approximated as a power law dependence of crack growth rate on applied stress intensity range. This test method also can preserve the cracked samples for further examinations. Filler content was shown to have an effect on crack growth resistance [96]. The higher the filler content, the better resistance to fatigue crack growth due to crack arrest, crack deflection etc., that will be discussed in further chapters.

2.4 Toughening mechanisms in resin dental composites

Toughening mechanisms can be put into two categories:

- Intrinsic toughening mechanisms, and
- Extrinsic toughening mechanisms.

Crack growth, whether it is critical or subcritical, is mostly controlled by competition between damage inducing (intrinsic) and damage preventing (extrinsic) mechanisms. The examples of toughening mechanisms can be seen in Fig. 7.

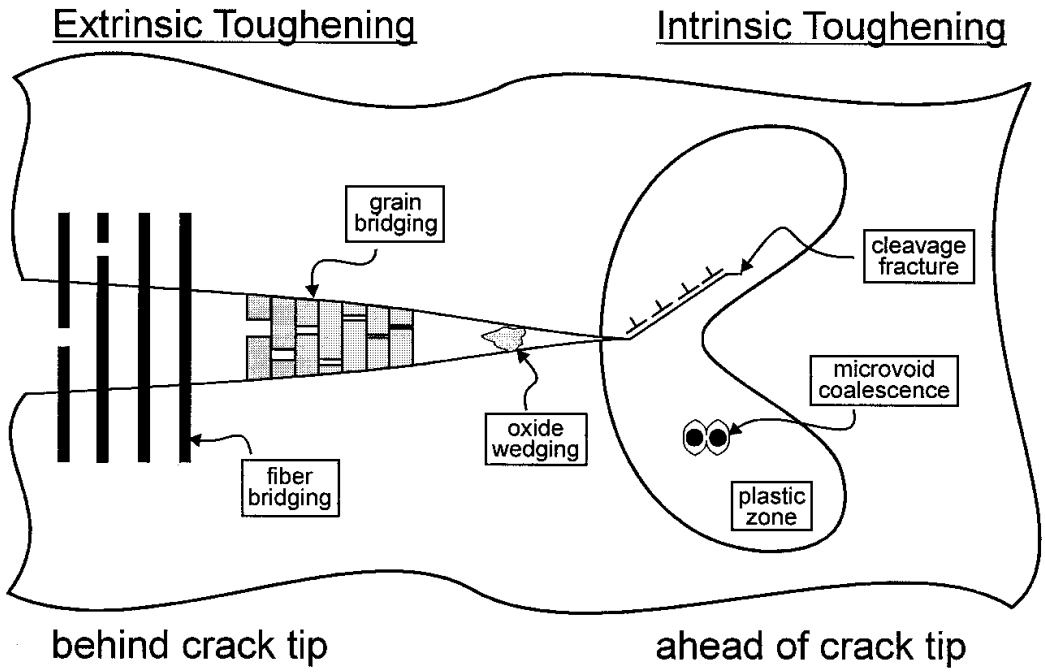

Figure 7. Examples of intrinsic and extrinsic toughening mechanisms [1].
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2.4.1 Intrinsic mechanisms

Intrinsic mechanisms usually occur ahead of crack tip. In ductile materials, e.g. metals, the mechanism involves crack blunting and re-sharpening under cyclic loading conditions [97]. Under static loading conditions, intrinsic damage mechanism involves creating microcracks and voids in front of crack tip, where the stress is very concentrated. That, eventually, leads to failure by microvoids coalescence, intergranular cracking or cleavage. Being an inherited property of materials, intrinsic mechanisms do not depend on crack initial shape and size [1]. Intrinsic toughening mechanisms are dominant in most of the ductile materials and are associated with crack tip plasticity, which involves dislocation motion. Thus, for brittle materials, usage of intrinsic toughening mechanisms by promotion of crack tip plasticity is not possible.

2.4.2 Extrinsic toughening mechanisms

Brittle materials cannot dissipate elastic strain energy very well, thus, extrinsic toughening mechanisms become the dominant ones used to improve a material's resistance to fracture. Extrinsic toughening mechanisms usually occur before the crack tip or in crack's wake by shielding the crack tip and reducing stress intensity, leading to $K<K_c$, thus, higher stress intensities (higher loads) are needed to cause a crack to propagate. That generates a so-called resistance curve (R-curve). Extrinsic toughening mechanisms often occur due to the formation of a bridging or process zone. Process zone mechanisms include transformation

toughening, microcracking or twin formation. Bridging zones occur due to different reinforcing particles that are capable of resisting crack opening under the load and bearing this stress partially, leading to decrease in stress intensity at the crack tip: $K_{tip}=K_{applied} - K_{shielding}$ [98]. It has been shown that for many biological hard tissues extrinsic toughening mechanisms are dominant [99], [100], [101], as well as for bio-inspired artificial materials, including resin dental composites [102], [103], [62]. In experiments, determination of the type of extrinsic toughening mechanism usually involves microscopic examination of crack-structure interaction as well as fracture surface examination. The next section will discuss some toughening mechanisms that are important for this work.

2.4.2.1 Crack deflection

Under various loading conditions in non-reinforced materials the crack will propagate in the direction that would maximize elastic energy release [104], [105]. For instance, for compact tension samples under mode I loading conditions the crack propagates in the direction perpendicular to applied load. However, in composite materials the crack can meet an obstacle which may deflect the crack from its preferred mode of propagation. The crack tip of a deflected crack will experience a smaller stress intensity than a non-deflected crack. Thus, more energy will be required to keep the crack propagating and the material will be toughened [106]. Particle shape and size play a significant role in the effectiveness of crack deflection. For instance, rod shaped fillers in composites can induce higher crack deflection angles than spherical particles due to the increased aspect ratios of rods [107].

2.4.2.2 Crack bridging

We all know that two pieces will fall apart if they are not being hold together by something. A very similar idea exists in the concept of crack bridging. Crack bridging is a very important extrinsic toughening mechanism in composite materials. It has been shown that the absence of crack bridging leads to almost no toughening in composites and such materials fracture at the crack initiation stress intensity [108]. Crack bridges are called bridges because they, indeed, do connect two fracture surfaces together (Fig. 8).

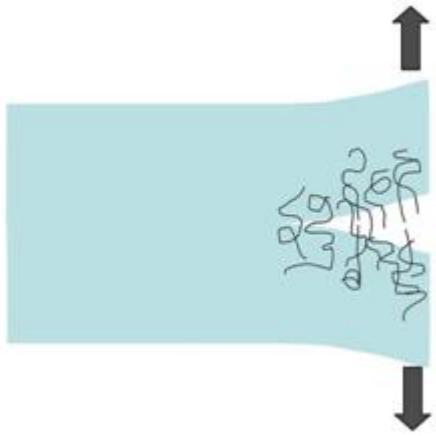

Figure 8. Scheme of crack bridging.

By holding two pieces of materials together at the crack wake, those bridges resist crack opening and sustain some amount of the applied load – all of that shields the crack, decreasing stress intensity at the crack tip. In composites, filler particles of uncracked matrix can serve as bridges. In ductile polymer materials the polymer chains can orient perpendicularly to applied load and become bridges [109]. In case of reinforcing filler,

bridge formation depends on the strength of the interface between the particles and the matrix. As the crack wake opens, complete particle debonding can occur in the case of a weak interface, which creates friction between matrix and particle while the last one is being pulled out [110]. In case of a strong interface, the crack can be arrested at a particle and that can lead to the nucleation of a secondary crack ahead of the primary crack tip due to the stress concentration ahead of the primary crack tip. The same can happen within the matrix itself if there are areas with residual compressing stresses that could arrest a crack [111]. That leads to uncracked bridges that hold on some stress and does not let the crack open as wide as there were no bridges [112].

2.5 Effect of aging mechanical properties of resin composites

Dental restorations, when placed in teeth, are subjected to various conditions that exist in the oral cavity (saliva, chemicals from food/drinks, bacteria, saliva proteins etc.) [113]. Thus, restoration stability also depends on their ability to resist degradation in oral environment. While food/drink chemicals have a relatively short time in the mouth, saliva is present most of the time bringing importance to the study of aging properties of dental composites in aqueous media.

Rodrigues et al. reports no significant difference in flexural strength of hybrid and nanocomposites after 24 hours soaking in DI water, however, a significant reduction was observed after 12 months of hydration [69]. The authors concluded that strength is highly

dependent on the morphology and size of filler. Those observations were supported by works of other researchers [114]. In the case of fracture toughness, some studies show a lowering of Kc, while others show an increase in fracture toughness after aging or no difference at all. Ferracane and Marker found that soaking samples for 14 months had no effect on Kc of filled composites or the unfilled matrix [115]. However, soaking samples in ethanol for same amount of time produced a significant reduction in fracture toughness. Another study has shown that long-term aging samples in water (up to 2 years) slightly, but significantly, decreases Kc of all the resin composites, but had no effect on flexure strength [116]. It was shown that mechanical performance does not always decrease after hydration. In fact, hydration is initially improving fracture toughness, flexural strength and fatigue behavior because absorption of water by polymer matrix makes resin more flexible and increases the plastic zone ahead of crack tips [117]. However, over the time degradation of cross-linking in resin worsens mechanical properties [118]. Fatigue behavior was shown to worsen after prolonged hydration as well due to matrix degradation and softening [62]. Takeshige at al report similar observations, showing that hydration is beneficial for fatigue resistance at first; however, further exposure to water leads to hydrolysis of the filler-matrix interface which worsens the mechanical properties [119]. Actually, water absorption mostly happens at filler-polymer interface and the rate of the absorption increases under cycling conditions due to pumping effect (opening and closing an interfacial crack), promoting faster hydrolysis [120], that explains water absorption increase with increased filler content. Hydrolysis occurs because of water attacking ester bonds in polymer, breaking the bonds [121]. Another explanation for worsening of mechanical performance after long-term soaking in water and other solvents suggests that unreacted (uncured) resin monomers are,

indeed, leaching out to the water through diffusion, and never come back [122]. As it was mentioned before, unreacted monomers play a role in plasticizing the matrix and the composite overall, thus, as they leave the composite, the matrix embrittles [123]. Moreover, the monomer's place between polymer chains is taken by water, which causes swelling and separates chains, weakening their interaction [124].

2.6 Demineralization studies

As mentioned before, primary decay is one of the most common diseases, and secondary tooth decay is the primary reason for tooth restoration replacement. Many efforts have been made toward developing new dental products to prevent caries development and its reoccurrence (anti-microbial materials, various dietary supplies, fluoride toothpastes, etc.). However, the effectiveness of some products is still questionable [125], [126]. Data obtained from clinical observational studies is very limited due to high number of variables. Thus, there is a need for experimental studies of demineralization in order to understand its mechanism and ways to prevent it. There are two major groups for tooth demineralization studies:

- In vivo, and
- In vitro.

In both methods, demineralization is observed as softening of the tooth tissue. Thus, hardness indentation was one of the first methods for revealing of demineralization. Hardness can be quantified, thus, one of the first quantifications of demineralization was related to microhardness [127]. As fluorescent staining techniques were developed, they became extensively used in demineralization studies. During tooth decay, mineral leaves the tissue and more collagen is exposed. Some stains have a high affinity to collagen and are used to identify tooth decay [128]. Quantification becomes more challenging with staining techniques; however, decay can be described in terms of fluorescent intensity (the more collagen exposed the more stain binds) [129].

2.6.1 In-vivo studies

There were many attempts to study tooth decay in vivo, however, most of them were observational studies and not controlled experiments [130]. There is a very large number of reasons for decay nucleation and development [131]; thus, such studies lead to difficulty in establishment of causation-correlation relationships. Controlled experiments in vivo often involve animal studies and ethical issues can be encountered. Demineralization studies in vivo are also very time consuming as the kinetics of tooth decay is relatively slow [132]. Nevertheless, there is quite a bit data available from in vivo studies. O'Reilly et al [133] reports 15% demineralization at 50 μm depth of premolar teeth that were treated with acidic orthodontic etch. They also report remineralization of those areas by the mean of fluoride wash and weekly fluoride topical treatment. Xylitol (artificial sweetener) in vivo studies

have become very popular. Many of them report an inhibitory effect of xylitol on demineralization process by suppressing acid production by dental plaque bacteria [134], [130]. On the another hand, Takahashi et al [135] showed no effect of xylitol on acid production by bacteria and degree of subsequent demineralization; however, they proved that fluoride is more effective way of demineralization inhibition. Similar results about the positive effect of fluoride were reported by other studies [136].

2.6.2 In vitro studies

In vitro studies are performed outside of the living object and they require some sort of bioreactor that imitates oral conditions and is well controlled. The field is not well developed, but will be addressed in this current project. In vitro studies of demineralization processes in teeth are more popular nowadays. The advantages of in vitro studies include, but not limited to, a more precisely controlled experiments, reduced amounts of variables that could affect the interpretation of results, better chance of causation-correlation relationship establishment, etc. [137]. However, the oral environment is very complex. That makes studies in vitro very challenging due to the fact that imitation of various oral conditions is very challenging. Nevertheless, in vitro studies allow us to separate variables that affect tooth decay. Moreover, since it has been shown that bacteria are predominantly responsible for acid-induced tooth demineralization [20], in vitro methods became very

popular for studying microflora effects on dental lesion formation, which would be a more challenging case for in vivo studies.

Similar to results from in vivo studies, in vitro studies also report a positive effect of fluoride treatment on enamel and dentin remineralization[138], [139], [140]. With the in vitro studies, Ten Cate et al [141] showed that fluoride treatment gives 15-50% remineralization after artificially inducing the lesions. Food chemicals' effects on demineralization kinetic can be assessed in controlled experiments in vitro also. Bibby et al [142] studied snack food effects on lesion formation and concluded that artificially flavored candies result in the highest degree of demineralization. Such experiments would be very inhumane if they were done in vivo. A similar deteriorating effect is attributed to soft soda drinks, where whole tooth erosion was observed as the result of soda acidity [142].

As new resin composites are developed, in vitro studies can allow studies of their antimicrobial effect, caries prevention effect and remineralization effect on the adjacent dentin. It was shown that nanocomposites with amorphous calcium phosphate give up to 22% remineralization of previously demineralized tissue, which was significantly higher than the effect of fluoride containing nanocomposites (6.5%) [143]. Many biocompatibility and antimicrobial studies have been done in vitro to assess the potential of newly developed dental resin composites, and they will be discussed in the following section.

2.7 Antimicrobial properties of dental resin composites

Bacterial biofilm formation is a number one reason for secondary caries nucleation and development at the dentin-filling margin. Another problem is that no existing sealant can

prevent further microleakage between tooth and filling due to interface failure, which may occur because of interface fatigue and/or tooth-filling gap formation due to polymerization shrinkage of resins [144]. Moreover, it was claimed that resin composites accumulate more biofilm plaque both in vivo and in vitro than any other type of restoration composites (amalgams and GIC) [145], [146]. While mechanical performance of dental composites has been greatly improved, their antimicrobial properties are still very much limited [147]. Thus, there is a strong need for a dental restorative composite to possess antimicrobial properties in order to suppress microflora penetration and colonization under a filling. An antibacterial effect can come from the composite matrix or filler. For instance, it was shown that matrix monomers, such as Bis-GMA and UDMA have a negative effect on bacterial colonies, suppressing their ability to adhere and reproduce [148]. Imazato et al report that the newly developed monomer methacryloyloxydodecylpyridinium bromide (MDPB) was proven to suppress oral streptococci; moreover, this monomer still retains its antimicrobial properties even after it becomes a part of the cured composite [149]. However, selection of resin monomers is more limited than selection of filler particles. Thus, many scientists have focused on antimicrobial properties of the fillers. Many studies discuss the introduction of antibacterial agents into resin composites like antibiotics, silver ions, iodine and ammonium [150]. The problem arises that those agents need to be released slowly in a controlled way, otherwise they may become toxic for a human [151]. Thus, concentration of the antimicrobial agents and kinetics of their release are important aspects to consider. Ammonium polyethelenimine agent was shown to exhibit antimicrobial effect as early as 24 hours after incubation on agar with Streptococcus mutans by disrupting streptococcal chains (biofilm) [152]. Antibacterial properties of silver have been known to humanity

since ancient times, where a silver coin in a pitcher could make water safe to drink [153]. It is no wonder that silver containing fillers were extensively studied. Silver infused SiO_2 particles showed a significant reduction in oral streptococci count after consequent 2, 6 and 12 hours of incubation, in comparison to no significant antibacterial effect given by just SiO_2 particles [154]. Besides silver, copper and zinc ions also seem to have an antimicrobial effect. However, metal ion agents have a big disadvantage: upon exposure to saliva, metal ion containing fillers are responsible for overall discoloration of filling [155]. Fluoride releasing fillers are still the most popular antimicrobial agents nowadays. However, fluoride related health issues are still questioned, as fluoride ions are very reactive and possess potential to damage eukaryotic cells [156].

In recent years, the scientific community started focusing on finding an agent that would also have remineralization properties in addition to being antibacterial. Such materials would require less of damaged tooth tissue removal, as well as decreasing the chance of secondary caries reoccurrence due to mistakenly leaving some decaying dentin before filling placement. Human saliva has a great anti-caries effect as it contains a high quantity of calcium and phosphate ions. That shifts the tooth hydroxyapatite equilibrium toward remineralization. Thus, such fillers like calcium phosphate were developed and it was shown to have both antimicrobial and regeneration effects on teeth [157].

Another kind of biological fillers are ones that contain bioactive glass (BAG). There are various formulations of BAG and it possesses a high scientific interest as a potentially great antimicrobial/remineralizating agent. More details will be given about BAG in the next section.

2.8 Bioactive glass

BAG originally was developed in 1969 and very rapidly became a very widely used material for hard and soft tissues surgeries [158]. The initial proposal for BAG development was based on the following hypothesis: "The human body rejects metallic and synthetic polymeric materials by forming scar tissue because living tissues are not composed of such materials. Bone contains a hydrated calcium phosphate component, hydroxyapatite [HA] and therefore if a material is able to form a HA layer in vivo it may not be rejected by the body" [158]. The original material was prepared with the composition (in wt%) of 45% SiO_2, 24.5% Na_2O, 24.5% CaO and 6% P_2O_5 by sol-gel process. The phase diagram can be seen on Fig. 9 [159].

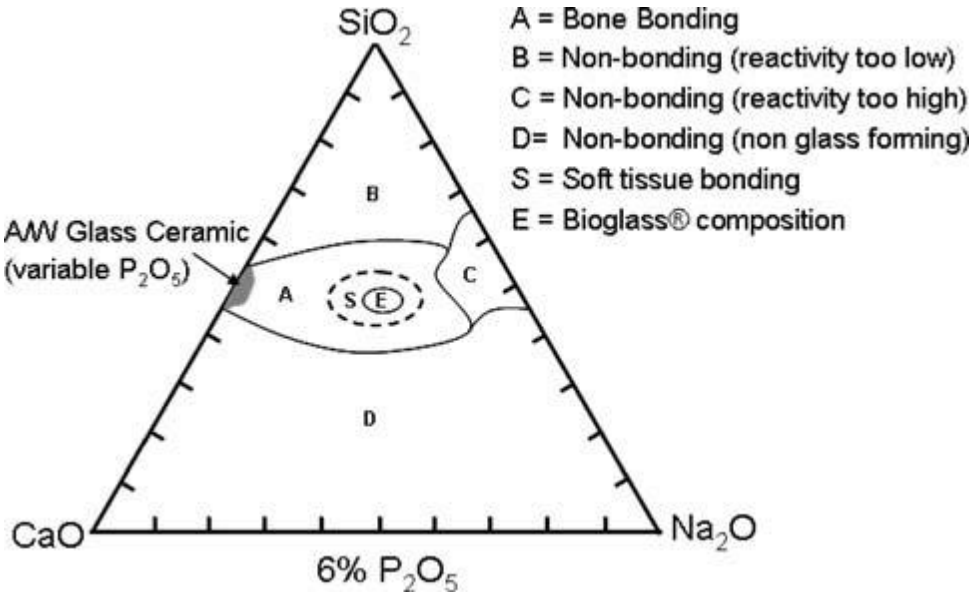

Figure 9. Compositional diagram of original BAG. *Figure reprinted with permission from Springer*

Multiple studies have proven that upon exposure to aqueous media BAG goes through a series of reactions and forms hydroxyapatite [160]. That greatly increases the rate of hard

tissue regeneration and remineralization as well as adhesion and biocompatibility [161]. Moreover, BAG seems to increase the expression of several genes that are responsible for bone regeneration [162]. Beside being beneficial to hard tissues, BAG was proven to improve the regeneration of soft tissues as bioactive glass particles influence the secretion of vascular endothelial growth factor (VEGF), leading to higher rate of vascularization of newly growing tissues [163], [164].

Usage of BAG for dental restorative purposes started relatively recently – in the early 2000s. High interest arose from potential antibacterial and regenerative properties of BAG, which could make BAG composites a great fit for secondary tooth decay prevention. The release of Ca^{2+} ions from the BAG and simultaneous incorporation of protons into the corroding material lead to a pH increase surrounding the BAG, which is not well tolerated by most of the oral bacteria [165]. Silica ions are also linked to an antimicrobial effect [166]; moreover, silica also induces remineralization of adjacent dentin [167]. Although antibacterial properties of BAG is still inferior to calcium hydroxide and silver, decreasing BAG filler size was shown to increase ions leaching and improve antimicrobial action [168]. Another attempt to increase ion leaching was to use non-silanized BAG filler (no coupling agent). The concern was that it could affect mechanical performance of such composite; however, several works show that mechanical properties do not change significantly [62], [169]. Current studies of the antimicrobial effects of BAG fillers claim that oral pathogens loose viability as early as 10 minutes after spreading bacterial inoculate over prepared BAG samples and incubation under natural conditions for those bacteria [170]. Up to 99% of colonies are dead after 100 minutes for a similar test [171].

The actual molecular mechanism of antimicrobial action of BAG is not very well understood [172]. However, there are some hypotheses in the current scientific literature. For instance, it is known that most sugars are taken up by bacteria using a proton pump driven symport [173], which can be seen in Fig. 10. According to this model, protons are being pumped out of a bacterial cell by one of the pumps in the electron transport chain (ETC). That creates a proton gradient. Electrochemical potential of this gradient can be used to bring various sugars to the inside of the bacteria though the membrane channel that can be generally called "symport" (lactose permease in this example), as it takes protons and lactose (in this example) back to the cell. If the gradient is disrupted by increased pH coming from BAG, as the base absorbs protons sugar transport becomes negatively impacted; that, eventually, may lead to cell starvation and death [174].

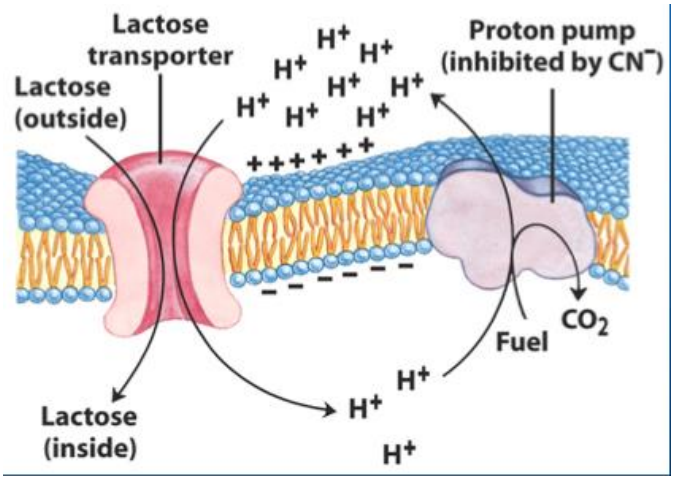

Figure 10. Lactose permease symport, powered by proton gradient. *Copyright 2009 by W.H. FREEEMAN AND COMPANY. Used with permission of the publisher.*

Based on the review provided, it can be concluded that up to nowadays secondary caries is still an unresolved problem worldwide, as no single dental sealant can prevent microleakage at tooth-filling interface. Moreover, the effect of chewing on secondary tooth decay propagation rate has not been identified yet. Antimicrobial properties of various dental composites still far from ideal and very little effort was done to implement BAG in resin dental composites.

In the current work, we intend to: 1) investigate mechanical performance of BAG resin composites; 2) Before investigating BAG composites' resistance to secondary tooth decay, we are interested to see whether cyclic loading (chewing) is important to secondary caries development at the gap between tooth and non-antimicrobial filling with the help of new test method and new bioreactor system that could accommodate such tests; and 3) see the effect of antimicrobial BAG on secondary tooth decay in terms of biofilm penetration depth with presence of chewing, if the last turns out to be an important factor for secondary caries.

3. MANUSCRIPT I: MECHANICAL PERFORMANCE OF NOVEL BIOACTIVE GLASS CONTAINING DENTAL RESTORATIVE COMPOSITES

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Abstract

Objectives. Bioactive glass (BAG) is known to possess antimicrobial properties and release ions needed for remineralization of tooth tissue, and therefore may be a strategic additive for dental restorative materials. The objective of this study was to develop BAG containing dental restorative composites with adequate mechanical properties comparable to successful commercially available composites, and to confirm the stability of these materials when exposed to a biologically challenging environment.

Methods. Composites with 72 wt.% total filler content were prepared while substituting 0 – 15% of the filler with ground BAG. Flexural strength, fracture toughness, and fatigue crack growth tests were performed after several different soaking treatments: 24 hours in DI water (all experiments), two months in brain-heart infusion (BHI) media+*S. mutans* bacteria (all experiments) and two months in BHI media (only for flexural strength). Mechanical properties of new BAG composites were compared along with the commercial composite Heliomolar by two-way ANOVA and Tukey's multiple comparison test ($p \leq 0.05$).

Results. Flexural strength, fracture toughness, and fatigue crack growth resistance for the BAG containing composites were unaffected by increasing BAG content up to 15% and

were superior to Heliomolar after all post cure treatments. The flexural strength of the BAG composites was unaffected by two months exposure to aqueous media and a bacterial challenge, while some decreases in fracture toughness and fatigue resistance were observed. The favorable mechanical properties compared to Heliomolar were attributed to higher filler content and a microstructure morphology that better promoted the toughening mechanisms of crack deflection and bridging.

Significance. Overall, the BAG containing composites developed in this study demonstrated adequate and stable mechanical properties relative to successful commercial composites.

Keywords: Resin Composite; Bioactive Glass; Strength; Fracture Toughness; Fatigue; Bacteria; Hydration

3.1 Introduction

While the use of dental restorative composites has increased dramatically for posterior teeth, annual failure rates up to 15% have been reported depending on restoration class [175], and a review of the literature has suggested the average lifetime of posterior dental composites is only six years [176]. Secondary caries at margins has been considered for over twenty years the most common reason for restoration replacement [177-181]. The second most common reason is partial or complete fracture of the composite restoration, while other significant causes are erosion and discoloration [182, 183]. It has been reported that the replacement of posterior composites is primarily due to fracture of the restoration within the first five years, but as a response to secondary caries thereafter [184], although

this has not been observed in all clinical studies [185]. A review of the numerous causes identified for restoration replacements based on multiple surveys may be found in Deligeorgi et al, 2001 [186].

One of the most common reasons for secondary caries is biofilm (plaque) formation on the margin of the tooth and restoration. Bacteria in the plaque (e.g., *Streptococcus mutans*) metabolize sucrose to lactic acid which can demineralize tooth tissue [187, 188]. Resin based composites may ideally provide good sealing of the cavity with no marginal gaps; however, polymerization shrinkage during placement, combined with cyclic mechanical loading during function, may lead to local interface failure and gap development. These marginal gaps can serve as suitable anchorage sites for bacterial colonies [189]. A minimum gap size exceeding 0.4 mm has been suggested for significant bacterial colonization of dental amalgam [190], but a similar relationship has not been discerned for composites. Moreover, increased roughness of the restoration increases the ability of bacteria to colonize a given area, by affecting pellicle formation and causing a favorable environment, often resulting in secondary caries formation [191, 192]. Microfloral analysis of marginal biofilms revealed that anaerobic bacteria are dominant with *Streptococcus mutans*, *Actinomyces naeslundii* and *Lactobacillus casei* being the most abundant bacterial species [193]. Svanberg et al. found significantly larger *Streptococcus mutans* colony counts at the tooth interface with composite restorations compared to interfaces with amalgam [194].

One possible approach to increasing the resistance of restorations to secondary caries formation is to add agents that 1) negatively influence the micro-organisms and/or 2)

promote remineralization of tooth structure after damage has occurred. In this regard, there is a substantial amount of published literature demonstrating the antibacterial qualities of various bioactive glass (BAG) compositions against many different bacterial species [195-207]. However, to date there have been no published studies of dental restorative composites containing bioactive glasses. There are several concerns regarding the development of a successful bioactive glass dental restorative composite. First, there is a concern that BAG fillers not well adhered to the composite matrix will result in unsuitably low mechanical properties. Second, because the composite will leach ions there is a concern about the stability of the mechanical properties over time. Finally, it must be confirmed that sufficient antimicrobial and/or remineralization activity can be achieved in BAG containing composites to slow secondary caries at the marginal gaps of tooth restorations. The goal of this study is to address the former two issues, while the latter will be addressed in the future by ongoing studies. Accordingly, the objective of this paper was to test the hypotheses that new BAG-containing dental restorative composites can be developed with mechanical properties comparable to successful commercially available composites, and that the properties will remain adequately stable after aging in a bacterial environment.

3.2 Materials and methods

3.2.1 Materials

The bioactive glass (BAG) used in this study had the composition 65% SiO_2, 31% CaO and 4% P_2O_5 (mol%) and was produced by a sol-gel process, as previously described [208].

In brief, the BAG was produced from high-purity metal alkoxides including tetraethyl orthosilicate (TEOS, $Si(OC_2H_5)_4$), calcium methoxyethoxide (CMOE, $C_6H_{14}O_4Ca$), and triethyl phosphate (TEP, $(C_2H_5)_3PO_4$). All reagents were purchased (Sigma Aldrich), except the CMOE was synthesized from pure Ca metal and methoxyethanol, to produce a 20% solution in methoxyethanol, and this alcohol served as a mutual solvent for all of the alkoxides as well as being the water source used to initiate hydrolysis and glass formation. The solutions were prepared in a dry nitrogen-environment glovebox, aged in distilled water, air-dried and stabilized in a dedicated furnace at 600°C to completely remove residual alcohols and alkoxide components, while retaining high surface area (between 200 to 300 m^2/g). After rapidly cooling, the glass was ball milled in ethanol and sieved to a gross particle dimension of less than 38 μm. The particles were then further processed to a fine particle size (0.04-3.0 μm) using a Micronizer jet mill. (Sturtevant Inc., Hanover, MA).

Three BAG-containing composites were produced by mixing the glass into a 50:50 mixture of bisphenol A glycidyl methacrylate (BisGMA):triethylene glycoldimethacrylate (TEGDMA) monomers with 0.4 wt% of camphorquinone (CQ), 0.8 wt% of 4-dimethylaminobenzoic acid ethyl ether (EDMAB), and 0.05 wt% of 3, 5-di-tertbutyl-4-hydroxytoluene (BHT). Samples denoted as 5BAG, 10BAG, and 15BAG were produced by combining the resin with 3.0 μm average size silanated strontium glass (Bisco Inc.) and 5, 10, or 15 wt% unsilanated bioactive glass, respectively, to a total filler of 72 wt% and mixed in a DAC-150 speed mixer (FlackTek Inc., Landrum, SC) at 3000 rpm for 2 minutes. Control samples (denoted 0BAG) had the same formulation as 5BAG with 5 wt% silane treated aerosol-silica filler (OX-50, Degussa) substituted for the BAG.

Mechanical property values are compared to published literature values and also the full set of mechanical property experiments were conducted on the commercial composite Heliomolar (Ivoclar Vivadent AG, batch # 4432). Heliomolar has a composition of 19 wt% Bis-GMA + urethane dimethacrylate, 3 wt% decandiol dimethacrylate, 66.7 wt% total filler content (highly dispersed silicon dioxide + ytterbium trifluoride) along with prepolymer and <1wt% stabilizers, catalysts and pigments. Heliomolar is classified as an inhomogeneous microfilled composite (< 1μm filler size) and was chosen for this study because it is a clinically successful example of a composite for anterior and posterior restorations [209-211].

3.2.2 Specimen preparation for mechanical testing

For flexural strength testing, three-point bend beams (N=10 for each composition) were prepared by dispensing the composite paste into 25 mm long quartz tubes (square 2 mm × 2 mm cross-section) followed by curing for 40 s on two opposite sides in a visible light curing unit (Triad II, Dentsply International, York Division, PA, USA). Compact-tension, C(T), specimens were made for fracture toughness (N=5 for each composition) and fatigue crack growth (N=3 for each composition) experiments. The composites were dispensed into a stainless-steel rectangular split mold, pressed flat, and cured for 40 s on each side as described above. The cured rectangular blanks were then machined into C(T) specimens as shown in Fig.10a. To enable observation and measurement of cracks, samples were ground and polished using progressively finer SiC grinding papers and alumina oxide polishing

compounds down to 0.05 μm and finally finished with MasterPolish (Buehler, Lake Bluff, IL, USA). A sharp pre-crack (Fig.11b) was introduced by manually extending the starter notch using a razor blade until a pre-crack formed from the notch.

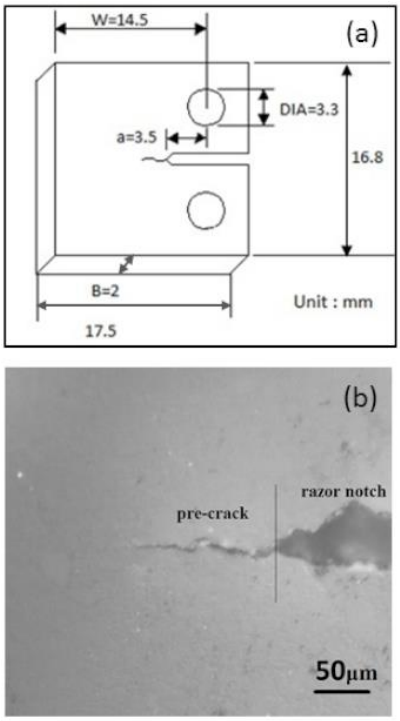

Figure 11. (a) Schematic and dimensions of C(T) specimen and (b) optical micrograph of a typical pre-crack profile.

3.2.3 Post-cure treatments

Specimens for all experiments were treated in two different ways: 24 hours aging in deionized (DI) water and 58 ± 3 days at 37°C in brain-heart infusion (BHI) media with *Streptococcus mutans* (strain ATCC25175) cultures growing in logarithmic phase with the media changed every other day. The longer of the two aging times was chosen since

previous studies have estimated that is the amount of time needed for the specimens to become fully (>98%) saturated with water [212]. Lyophilized bacterial cultures were obtained from the American Type Culture Collection (ATTC, Manassas, VA, USA). All DI water aged samples were tested immediately after removal from the water. After the aging period in BHI media with *Streptococcus mutans*, composite samples were immersed in 50% bleach for 5 min and then rinsed with BHI three times. The specimens were then soaked in BHI without sucrose and stored in a refrigerator until mechanical testing. With the exception of the fatigue tests, all mechanical testing was performed within a day of removal from the test aging solution. Due to the time required for fatigue testing, some samples needed to be stored for days or weeks while waiting for testing, so these samples were continually stored in sterile BHI media at ~4^{o}C. Finally, bending beams made from the experimental composites were also soaked in sterile BHI media without bacteria for ~60 days and used for strength testing.

3.2.4 Mechanical Testing

Flexure strength-was tested in 3-point bending (20 mm span) on a universal testing machine at a cross-head speed of 0.254 mm/min, in general accord with ISO 4049 [213]. The steel supports had rollers of 2 mm diameter and the loading piston was a steel ball of 2 mm diameter. The flexure strength was determined using the maximum load.

Fracture toughness tests were conducted on wet samples immediately after removal from the storage solution using a computer controlled hydraulic testing machine (Instron

8872, Canton, MA, USA). Tests were conducted in load control with a 1.1 N/s loading rate until fracture occurred. K_{IC} was calculated from the peak load at fracture according to the standard stress intensity factor equation for the C(T) sample geometry [214].

Fatigue crack growth testing was done in general accordance with ASTM standard E647 [215], using computer controlled hydraulic testing machine (Instron 8872, Canton, MA, USA) and a sine waveform with frequency v = 1.5 Hz, which corresponds to a typical human chewing frequency [216]. A constant load ratio $R=P_{min}/P_{max}=0.1$ was used, where P_{max} and P_{min} are the maximum and minimum loads experienced during the loading cycle, respectively. Fatigue crack growth rates, *da/dN*, were characterized as a function of the stress intensity range, $\Delta K = K_{max} - K_{min}$, where K_{max} and K_{min} are the stress intensity values calculated from P_{max} and P_{min}, respectively. After initial establishment of a high crack growth rate of 10^{-7}-10^{-6} m/cycle, the test was conducted in decreasing ΔK control using a normalized K-gradient ($1/\Delta K[d\Delta K/da]$) of -0.08 mm^{-1}. Crack length was determined by measuring the load point compliance using a capacitance displacement gage (HPT150, Capacitec, Inc., Ayer, MA) attached to the clevises of the testing machine. The sample compliance was converted to crack length using published calibrations [217]. Data points were collected roughly every 10^{-5} m of crack extension. Samples were kept wet during the entire test using a sponge to surround the sample and a custom drip system to keep it wet. After the test, the final crack length was measured optically. When the final compliance and optically measured crack lengths differed, the crack length data was corrected by assuming the error accumulated linearly with crack extension. From the crack length data, the crack

growth rates (*da/dN*) were determined as a function of Δ*K* by fitting over ranges of ~100 μm of crack length change.

For statistical comparisons of data, ANOVA followed by Tukey's multiple comparison test was used with $\alpha < 0.05$ considered statistically significant.

After fatigue crack growth and fracture toughness experiments, both crack profiles and fracture surfaces, respectively, were examined using a scanning electron microscope (Quanta 600 FEG SEM, FEI Company, Hillsboro, OR) in order to discern crack-microstructure interactions and toughening mechanisms.

3.3 Results

3.3.1 Flexural strength results

Flexural strength and statistical test results are shown in Table 2.

Table 2. Mean flexural strengths with standard deviations in parentheses

	24h DI Water	**2 months bacteria**	**2 months media**
Material	**Flexural Strength (MPa)**	**Flexural Strength (MPa)**	**Flexural Strength (MPa)**
Heliomolar	73.2 (4.4)[a]	61.7 (7.9)[a]	-
0% BAG	123.5 (16.2)	114.9 (12.3)	107.4 (12.8)
5% BAG	112.8 (12.9)	108.4 (12.2)	107.4 (11.2)
10% BAG	116.4 (14.2)	112.6 (13.0)	95.7 (16.2)
15% BAG	116.9 (10.7)	105.6 (17.7)	101.2 (10.8)

[a] denotes value has statistically significant difference from rest of column

BAG composites did not show a significant difference in flexural strength as a function of the various soaking treatments. However, Heliomolar composites did show a significant reduction in flexural strength between 24h water and 2 months in bacteria. The experimental BAG composites all had superior flexural strength when compared to Heliomolar.

3.3.2 Fracture toughness results

Fracture toughness data and statistical test results for both Heliomolar and BAG composites is shown in Table 3.

Table 3. Mean fracture toughness results with standard deviations in parentheses

	24h DI water	2 months bacteria	
Material	K_{IC} (MPa√m)	K_{IC} (MPa√m)	% decrease
Heliomolar	0.98 (0.17)[a]	0.77 (0.02)[a]	-21%[b]
0% BAG	1.45 (0.13)	1.25 (0.15)	-13%
5%BAG	1.52 (0.17)	1.12 (0.13)	-26%[b]
10% BAG	1.54 (0.17)	1.40 (0.01)[a]	-9%
15% BAG	1.31 (0.14)	1.10 (0.05)	-16%[b]

[a] denotes value has statistically significant difference from rest of column
[b] denotes statistically significant difference between the two aging conditions

Statistical analyses showed that the experimental BAG composites all have significantly higher fracture toughness compared to Heliomolar at 24 hours and after 2 months aging in bacteria (Table 3). Furthermore, the degradation in toughness of the experimental BAG

composites after two months of aging was comparable to, or less severe (i.e., not significant) than Heliomolar (Table 3).

3.3.3 Fatigue crack growth results

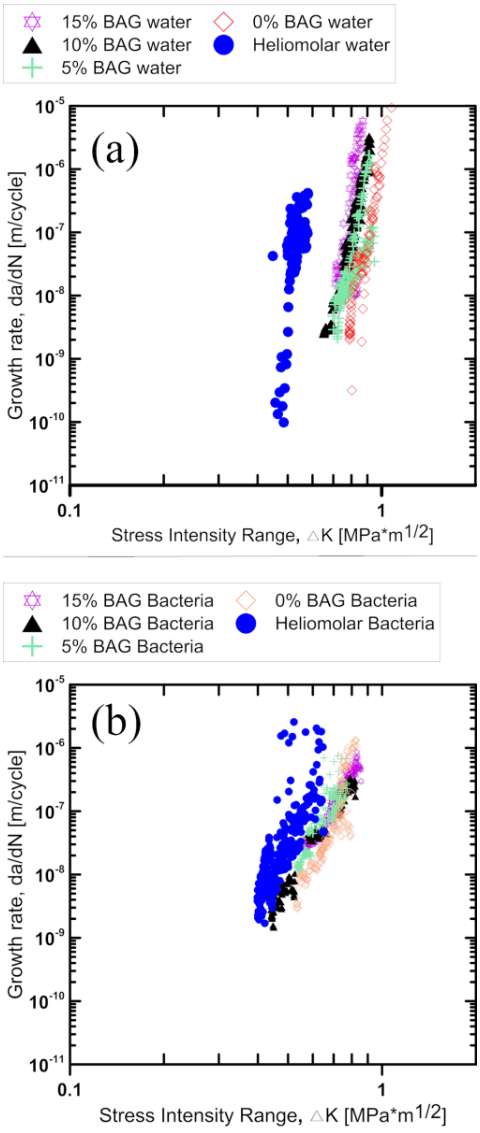

Figure 12. Fatigue crack growth rate data for (a) 24h water aging and (b) 2 months bacteria aging treatments.

Fatigue crack growth rate, *da/dN*, results are shown in Fig. 12 plotted as a function of the stress intensity range, ΔK. Generally, the data for the 0 – 15BAG composites produced in this study overlapped considerably. Furthermore, the commercial composite Heliomolar showed inferior fatigue crack growth resistance for both aging conditions with the curves shifted to higher growth rates at a given stress intensity range. The difference between Heliomolar and the 0 – 15BAG composites was more pronounced for the 24 h water aging condition.

Table 4. ΔK_{TH} values and Paris exponents

	24h in DI water		60 days in bacteria		
	ΔK_{TH}	m	ΔK_{TH}	m	% decrease in m
Heliomolar	0.40	23.0	0.53	9.3	-60%
0%BAG	0.79	24.7	0.54	11.5	-53%
5%BAG	0.70	14.3	0.54	8.3	-42%
10%BAG	0.65	22.0	0.46	7.8	-65%
15%BAG	0.71	25.8	0.56	8.2	-68%

Each curve in Fig. 12 was produced from a composite of $N = 3$ samples measured over different, overlapping, ranges of growth rates; thus, fatigue crack growth data for each material was pooled and fit to the Paris law [218]:

$$\frac{da}{dN} = C(\Delta K)^m \qquad [1]$$

and mean values for the Paris exponent, *m*, are given in Table 4. All of the composites showed decrease in *m* after two months aging in bacteria. The fatigue thresholds, ΔK_{TH}, below which cracks are presumed not to propagate was also assessed for each sample in Table 3. Due to practical time limitations of testing, ΔK_{TH} was defined where growth slowed to a rate

approaching 10^{-9} m/cycle. Since each curve in Fig. 12 was produced from multiple samples tested with different growth rates, the ΔK_{TH} given in Table 4 were taken from the sample tested at the slowest growth rates.

3.3.4 Crack Paths and Fractography

Due to similarities of the observed microstructures and crack-microstructure interactions for all of the BAG composites, only micrographs for the 0BAG and 15BAG composites will be shown as representative. Crack path observations were made on the fatigue crack growth specimens, while fracture surface observations were made on both the fracture toughness and fatigue samples.

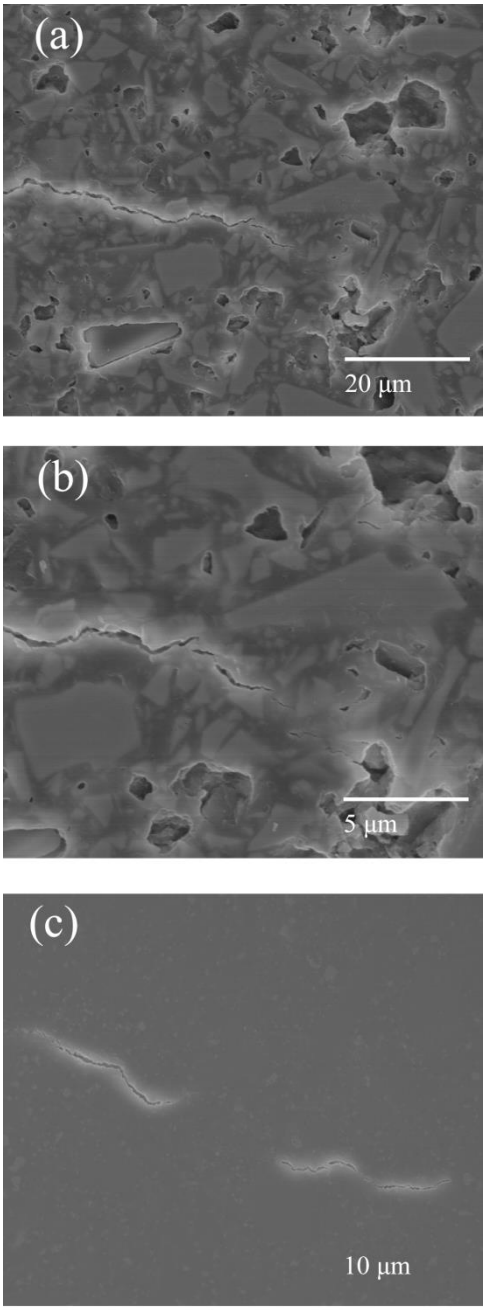

Figure 13. Micrographs of crack tips (2 months bacteria treatment). a-b) Crack deflection and bridging at a fatigue crack tip in the 15BAG composite. c) Crack bridging at a fatigue crack tip in the Heliomolar composite. Direction of crack propagation was left to

Figs. 13a & 13b show multiple crack deflections and crack bridging near the fatigue crack tip of a 15BAG composite. For the Heliomolar composite crack bridges were also observed at the crack tip (Fig. 13c). For all of the 0 – 15 BAG composites, but not Heliomolar, crack bridges were also found in the crack wake far behind the crack tip (Fig. 14a).

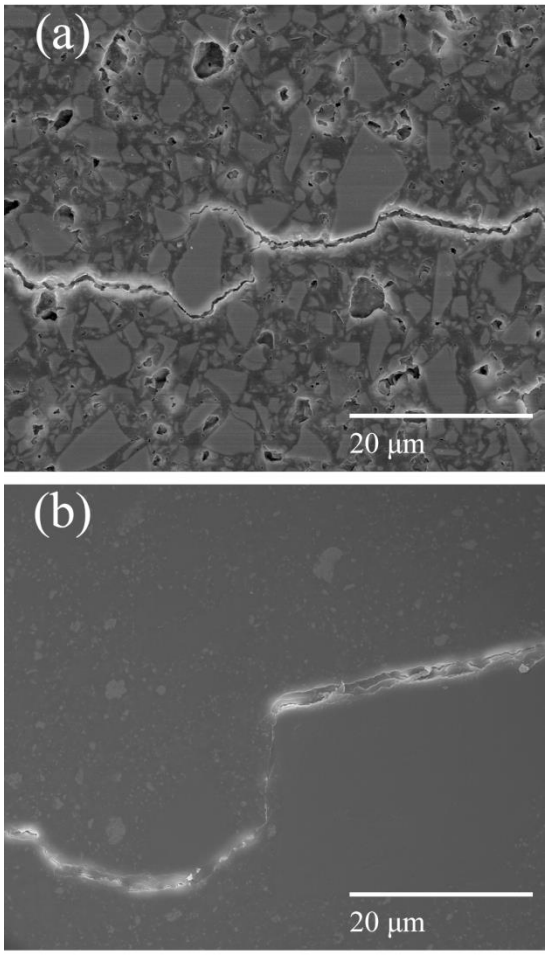

Figure 14. Micrographs of toughening mechanisms (2 months bacteria treatment). a) Crack bridge created by filler particle in the 15BAG composite. This bridge was located 0.052 mm behind the fatigue crack tip. b) A rare high angle crack deflection by a large pre

Significant crack deflections were rarely observed in the Heliomolar composite and were located only at the widely spaced, large prepolymerized agglomerates of composite that were

often ~20 μm or larger in size (Fig. 14b). Fig.15 shows an equal magnification comparison of the crack paths for the 15BAG and Heliomolar composites over a larger amount of crack extension. On the microscale, the crack in the 15BAG composite appears more tortuous due to the high frequency of deflection by particles (Fig. 15a), while for the Heliomolar composite there is some crack meandering over large extensions, but generally a less tortuous path on the microscale (Fig. 15b). Also, in Figs 12-14 there is evidence of BAG particle dissolution from the polished surfaces due to the 2 month aging treatment for the 15BAG composite (Fig. 13a, 13b, 14a, 15a) which was absent for the 0BAG samples (Fig. 15c).

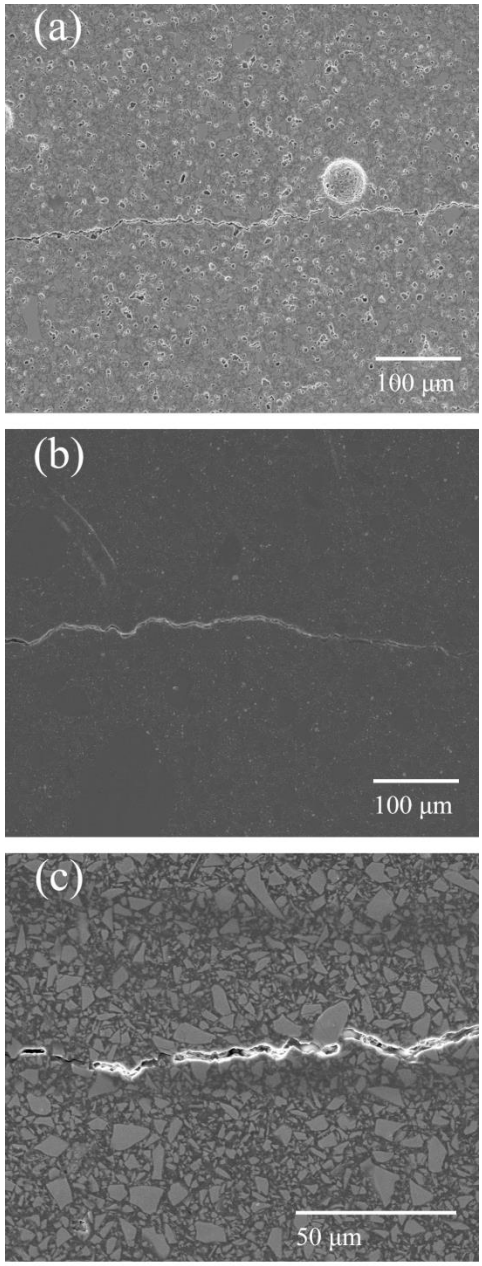

Figure 15. Micrographs of fatigue crack paths (2 months bacteria treatment). Panel a) shows the 15BAG composite, b) the Heliomolar composite and c) the 0BAG composite. Direction of crack propagation was left to right.

Overall, the 0 – 15BAG composites show a rougher fracture surface while the fracture surface of Heliomolar is smoother and entirely covered with resin (Fig. 16). It is seen in Figs.

16a & 16c that some of the particles of the 15BAG and 0BAG composites debonded cleanly from the matrix, giving a mixed matrix/interface crack path. In contrast, for Heliomolar (Fig. 16e) the crack always moved through the resin matrix leaving a polymer coating on the fracture surface. The resin coated fracture surface is seen more clearly in Fig. 16f at higher magnification where, in contrast, the smooth surface of debonded glass particles can be seen for the 0BAG and 15BAG composites at higher magnifications (Fig. 16b & 16d). Figs. 16a & 16c also show that the 0BAG composite has fewer debonded particles than the 15BAG composite. Approximately 94 debonded particles/mm^2 and 159 debonded particles/mm^2 were observed on the fracture surfaces for 0BAG and 15BAG respectively.

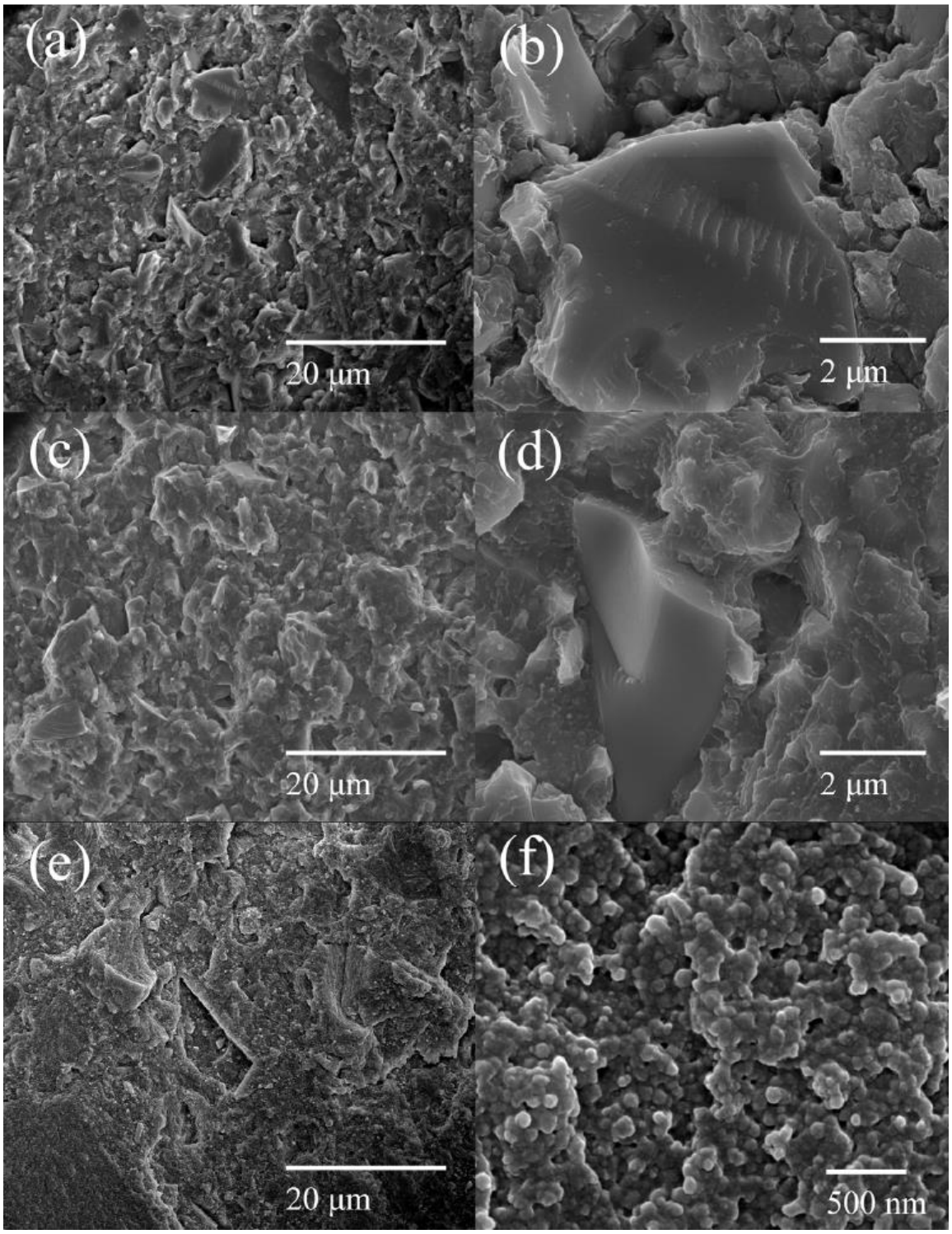

Figure 16. Fracture surfaces of fracture toughness samples for (a, b) 15BAG, (c, d) 0BAG and (e, f) Heliomolar composites after 2 months bacteria treatment.

3.4 Discussion

3.4.1 Mechanical behavior

Overall, BAG composites exhibited significantly better mechanical properties than Heliomolar both after aging in DI water for 24 h as well as after two months soaking in bacteria containing media. These differences in mechanical properties may be attributed primarily to differences in the material composition and microstructures, as will be discussed below. Also, Table 4 shows mechanical properties for two other commercial composites after 60 days soaking in H_2O. Generally, the strength, fracture toughness, and fatigue threshold properties of the BAG containing composites after soaking for two months in bacteria containing aqueous media are comparable to, or better than, these two popular commercial composites.

Table 5. Published mechanical properties of some commercial composites with standard deviations in parentheses [103, 219]

	Flexural Strength (MPa)	Fracture Toughness* (MPa√m)	Fatigue Threshold (MPa√m)
Filtek Z250 (60 days H_2O)	91.4 (14.8)	1.26 (0.05)	0.54
Filtek Supreme Plus (60 days H_2O)	52.7 (12.9)	0.81 (0.06)	0.41

*These toughness values are the maximum point of the fracture resistance curve, which gives an upper bound for the critical value of toughness, K_{IC}.

3.4.2 Flexural strength

The first notable difference between the microstructures is that the 0 – 15BAG composites have significantly higher filler particle concentration than Heliomolar (72 wt% versus 66.7 wt%, respectively). The higher measured strength values for the BAG composites is thus consistent

with published observations for resin based dental composites that increased filler content leads to increased flexural strength [220, 221] and that filler content is the dominant factor even with different filler morphologies [222].

It is interesting to note that the BAG composites showed no degradation in strength after soaking approximately two months in both sterile and bacteria containing media. Many commercial dental restorative composites demonstrate a loss in strength after similar long term aging in aqueous media [212, 223-226]. This degradation in strength is attributed to water uptake causing plasticization of the resin matrix and/or a degradation of the matrix-filler interface [212, 223-226]. Since the BAG-containing composites are designed to have ions leaching out of the BAG filler, there is a legitimate concern that such ion leaching could lead to degradation of the filler, and thus decreased mechanical properties. While the SEM images show evidence for particle dissolution from the composite surface (Figs. 13-15), the mechanical testing results reflect that the flexural strengths of the present BAG composites are quite stable after aging two months in aqueous media.

3.4.3 Fracture toughness and fatigue crack growth resistance

While some correlations have been observed over a specific groups of composites [227], the fracture toughness and fatigue properties of resin based dental restorative composites often do not scale simply with factors like filler volume fraction or filler size and, rather, are highly dependent on the morphology of the composite microstructure [84, 212, 220, 228-235]. Previous studies have suggested that microstructures that maintain good matrix/particle adhesion while promoting toughening mechanisms such as crack deflection and crack bridging are advantageous for achieving good fracture and fatigue properties [212, 228-230, 233-235].

Generally, it was observed that crack deflection and bridging was more pronounced in the BAG composites than in Heliomolar. Heliomolar is categorized as a microfill composite with filler size <1 μm; however, the microstructure is quite inhomogeneous and contains widely spaced large agglomerates composed of prepolymerized resin fillers added to increase overall filler volume and reduce curing shrinkage. Based on crack path (Fig. 14b) and fracture surface (Figs. 16e-16f) observations, cracks moved through the homogeneous microfilled regions with few deflections, with significant high angle deflections only rarely observed at the occasional large agglomerate (Fig. 14b). Furthermore, the only detectable crack bridges were very small and only observed very near the crack tip (Fig. 13c). In contrast, the BAG composites contained significantly more medium to large sized particles capable of 1) deflecting the crack at the microscale and 2) creating large crack bridges that sustain load far behind the crack tip (Fig. 13-15).

Crack deflection and bridging are two toughening mechanisms that often act in concert; indeed, crack deflection commonly leads to crack bridging. Furthermore, natural tooth enamel and dentin are also toughened by those same mechanisms [236-239]. Both mechanisms increase the toughness by lowering the mode I stress intensity at the crack tip. Since both the fracture toughness and fatigue crack growth resistance are determined by the mode I stress intensity, both are generally improved by these mechanisms. With crack deflection, the microstructure forces the crack to deviate from the mode I path, causing a decrease in the mode I stress intensity at the crack tip. Crack bridging often results where the crack locally arrests or is deflected by microstructural inhomogeneities. As a result, load bearing bridges may be left in the crack wake due to either imperfect connection of the three-dimensional crack front, or new microcracks

forming ahead of the crack tip. Overall, these mechanisms were observed to be more active in the BAG composites resulting in higher fracture toughness and fatigue crack growth resistance.

Some studies have shown particle/matrix debonding can be detrimental to the fracture and fatigue performance of resin based dental composites [212, 229]. In the present BAG composites, some fraction of the particles were observed to cleanly debond from the matrix (Fig. 16); however, overall adequate properties were achieved nonetheless when compared to the commercially successful Heliomolar. Also, the fraction of debonded particles was higher for the 15BAG composition than the 0BAG composition, suggesting the lack of silane treatment of the BAG particles led to less particle-matrix adhesion. However, overall it may be concluded that since the majority of particles were well bonded even for the 15BAG case (Fig. 16a) adequate mechanical properties were achieved.

Although the fracture toughness and fatigue crack growth resistance for the BAG composites degraded somewhat after long term aging in aqueous media, the properties remained better than Heliomolar in all cases. A degradation in fracture toughness is not unusual for other commercial resin based dental composites after long term exposure to water [212]. Moreover, the fracture toughness and fatigue threshold properties of the BAG containing composites are comparable to, or better than, the popular commercial composites (Table 5) that were tested after two months soaking in water [212, 229]. Overall, it is concluded that the presence of the BAG particles does not raise immediate concerns regarding long term stability of the mechanical reliability of these composites.

3.5 Limitations of this study

Although crack bridging was identified as a toughening mechanism for the BAG composites, in the present study the contribution of crack bridging was not quantified. Such quantification would require the measurement of the fracture resistance curves (*R*-curves) for the materials to separate out the contribution of bridging [212, 228, 235], and such studies are left for future work. Another limitation is the aging time was limited to roughly two months. Although previous work suggests this is long enough to reach nearly full water saturation of the composite [212], it is unclear if degradation may occur at longer time scales and such studies are also left for future work.

3.6 Conclusions

Based on a study of the mechanical properties of a series of bioactive glass (BAG) containing composites, the following conclusions can be made:

1. All BAG containing composites exhibited superior mechanical properties over the commercial Heliomolar composite both after 24 hours in water and after 2 months in a bacteria containing aqueous media. Properties were also found to be comparable to, or better than, published values for two other commercial composites, Filtek Z250 and Filtek Supreme Plus.
2. The superior mechanical properties of the BAG composites compared to Heliomolar were attributed to: 1) a higher filler content; and 2) a microstructure morphology that better promoted the toughening mechanisms of crack deflection and bridging.

3. Overall, the BAG containing composites developed in this study demonstrated adequate mechanical properties relative to successful commercial composites.

Acknowledgements

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4. MANUSCRIPT II: THE EFFECT OF CYCLIC MECHANICAL LOADING ON BACTERIAL ATTACK OF COMPOSITE TOOTH RESTORATIONS

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Abstract

Objectives: Secondary tooth decay is the primary reason for restoration replacement and usually forms between dentin and the filling. The goal of the current work was to investigate the combined effect of cyclic loading and bacterial exposure on secondary caries at the dentin-filling margin using, a novel bioreactor system that simulates oral conditions as well as new test specimen design to accommodate the test.

Methods: Human molars were machined to 3 mm thick disks and 2 mm deep composite restorations were placed into each sample. A 10-30 micrometer wide (small) or 250 micrometer wide (large) dentin-filling gap was introduced along half of the interface between the dentin and filling. *Streptococcus mutans* biofilms were grown on each sample prior to testing in a bioreactor both with and without cyclic loading. Both groups of samples were tested for 2 weeks and post-test biofilm viability was confirmed with a live-dead assay. Samples were fixed and cross-sectioned to reveal the gaps and observe bacterial penetration.

Results: It was shown that for large gap samples the bacterial biofilm has no problems penetrating the gap independent of loading or non-loading conditions. The results for all cyclically loaded small gap samples show a consistently deep bacterial penetration into the gap (~100%) while only 50% of the non-loaded samples demonstrated such deep penetration.

Significance: A new bioreactor was developed that allows combining mechanical loading and bacterial exposure for demineralization studies. Cyclic loading was shown to possibly promote secondary caries by aiding bacterial penetration into narrow marginal gaps.

4.1 Introduction

The use of resin based dental restorative composites has increased dramatically for posterior teeth as a replacement for dental amalgam. Key driving forces behind the increased use of composite materials are 1) their tooth-like appearance [240] and 2) their lack of the potentially toxic element mercury that is present in amalgam [241, 242]. Additional clinical advantages include minimal removal of native tooth structure [243], the ability to be bonded to enamel and dentin surfaces [244], convenient handling, and the ready availability of a wide range of tooth shades. However, annual failure rates up to 15% have been reported for composite restorations depending on restoration class [175], and a review of the literature has suggested the average lifetime of posterior dental composites is only six years [176].

The most common reason for restoration replacement is secondary tooth decay (caries) at a dentin-filling interface [177, 245-248], while the second most observed reason is restoration failure caused by occlusal loading combined with chemical and physical degradation of the restoration material in the oral environment [249]. Furthermore, it is known that secondary caries appearance is associated with bacterial biofilm formation on the dentin-filling interface. Indeed, the biofilm byproduct lactic acid promotes demineralization of the adjacent dentin which can lead to tooth decay [188, 250].

Although bacterial biofilm formation is considered a necessary ingredient, the presence of the biofilm alone does not guarantee secondary tooth decay [251]. Numerous studies have identified the presence of bacteria within marginal gaps between the restoration and the dentin [252-254], and the presence of such gaps is likely an important factor as well. No clinical correlation between marginal gap size and bacterial colonization has been found for resin based composite restorations [255]; however, clinical data is limited and recent in vitro evidence from

microbial caries models shows demineralization along the cavity walls of composite restorations increasing in magnitude with larger gap sizes [256, 257]. Overall, there is a need for further studies to better understand the factors that control secondary caries formation at the marginal interface between composite restorations and tooth dentin.

Another complication is that teeth are also subjected to cyclic loading during mastication, bruxism (grinding), etc. Cyclic loading may promote marginal gap formation and growth; indeed, some studies have shown the degradation of restoration margins during cyclic mechanical loading [258-262]. Furthermore, it has been demonstrated that the presence of bacteria and a marginal gap does not guarantee further secondary caries formation [188, 263, 264]. Thus, there may be an additional role of cyclic mechanical loading beyond simply creating a marginal gap or growing it above a critical size to allow bacterial penetration. Based on a survey of the available literature, understanding the mechanism of recurrent decay at the margins of dental composite restorations likely requires the evaluation of the simultaneous effects of bacterial biofilm presence, marginal gaps, and cyclic loading. To date, no such studies have been reported.

Accordingly, the objectives of this paper are the following: 1) to describe a bioreactor based test method (and new test specimen design) that has been developed to allow cyclic mechanical loading of simulated tooth restorations while a controlled oral biofilm environment is allowed to grow; 2) to study the synergistic effects associated with bacteria and cyclic loading on the mechanisms of secondary caries formation at dental restorations.

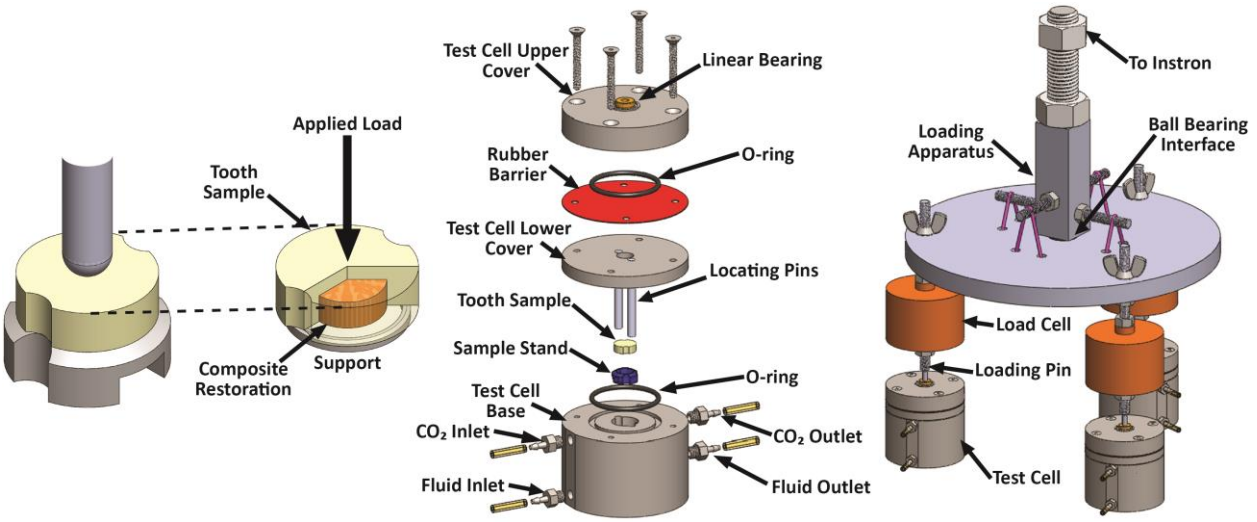
4.2 Materials and Methods

4.2.1 Bioreactor Fatigue Test System

While bioreactor systems are commonly used for the in vitro study of oral biofilms in controlled laboratory settings [265-270], to date no such systems allow the simultaneous application of cyclic loading to the test sample. However, this is thought to be important for the study of secondary caries because it has been demonstrated that the presence of bacteria and a marginal gap does not guarantee further secondary caries formation [188, 263, 264]. Accordingly, it is reasonable to suspect that there is a synergistic effect of mechanical loading in combination with the presence of bacteria and a marginal gap. In order to develop a system to study dental restorations in a simulated oral environment the main considerations include:

1) Developing a simulated tooth restoration sample design that is practical to reproducibly manufacture and test.
2) Growing oral bacterial biofilms on the samples in a sealed environment free of external contamination.
3) Providing nutrients for the bacteria without introducing external contaminants.
4) Applying cyclic stress without introducing external contaminants.
5) Maintaining a test temperature of 37°C.

For the sample design, the physiological size of human teeth dictates the maximum possible dimensions. Based on a survey of typical human teeth it was determined 9 mm diameter disks could be readily machined from the crowns of human molars. Such disks allow the placement of a 5 mm diameter composite restoration on one side (Fig. 17a).



(a) (b) (c)

Figure 17. (a) Schematic of the simulated tooth filling sample and the loading configuration; at right, the dentin is made transparent to observe the composite disk. (b) Exploded view of a bioreactor. (c) Schematic of the load distribution system for loading three test cells simultaneously.

A simple radial symmetric biaxial bending and shear loading geometry was selected to allow even loading on the composite/dentin interface for a well bonded restoration with no gaps (Fig. 17a). Loading is applied in the center of the sample by a loading pin, while the sample sits on a ring shaped support. Finally, matching grooves were machined in the sides of the specimens to allow each sample to be centered on the ring shaped sample stand by locating pins (Figs. 17a, c).

Next, providing a sealed system for testing was achieved through the use of individual bioreactor for each simulated tooth restoration, as seen in Fig. 17b. The bioreactors were fabricated out of stainless steel to provide a durable, stiff platform for loading the specimens while also minimizing corrosion of the system and allowing for sterilization by autoclaving. Bioreactors consisted of five interlocking elements denoted as the upper cover, lower cover,

rubber barrier, base and sample stand. The two cover elements and base were manufactured from 316 stainless steel and make up the exterior of the bioreactor. Viton O-rings were used between parts to ensure a sealed system connected with four 316 stainless steel bolts. In order to create an active area for loading, the sample stand (shown in Figs. 17a, b) was designed with a 3.5 mm radius rounded edge ring that supported the sample. To achieve high strength for loading the specimen, the sample stand was machined from 17-4 stainless steel that was heat treated at 482°C for 60 minutes after machining to achieve peak strength. Locating pins were integrated into the lower cover and allowed for the sample to be both located and constrained within the bioreactor and centered over the sample stand for symmetric loading. These locating pins are designed to mesh with the bioreactor base, sample stand and sample itself. Sample position within the bioreactor was tested and it was shown that the center of the sample was held within <0.25 mm when agitated.

Required nutrients and pH buffering were provided via brain-heart infusion (BHI) liquid media and 5% CO_2 in air flowed into and out of each bioreactor. CO_2 enriched air was flowed through the upper fluid inlet/outlet set seen in Fig. 17b and flow was controlled using a pressure regulator. BHI media was flowed through the lower fluid inlet/outlet set seen in Fig. 17b. The BHI media flow was designed with the inlet set below the sample and the outlet set above the sample to ensure that liquid covers the entire sample throughout testing and fresh media continuously flows past the bottom, or stressed, side of the sample. Liquid flow was controlled using low flow peristaltic pumps (Model FB 70381, Thermo Fisher Scientific Inc., Waltham, MA, USA), with one pump per bioreactor inlet and an additional pump on each bioreactor outlet. The tube dimensions were 3.175 mm inside diameter and 6.35 mm outside diameter (Fischer Scientific, Pittsburgh, PA, USA).

The act of applying fatigue stress was controlled using an computer controlled, servohydraulic fatigue test system (Model 8872, Instron Corporation, Norwood, MA, USA). A ball bearing was used to evenly distribute the load to three loading rods for three separate bioreactors (Fig. 17c). The load distribution system was monitored over a two week period of cyclic loading and the maximum difference between bioreactors never exceeded 5%. Loading rods were machined from 316 stainless steel with semi-spherical loading points and were outfitted with thumb screws to manually adjust rod heights as needed during test setup. These rods were guided into the center of the bioreactors using linear bearings that were housed in the bioreactor upper covers as seen in Fig. 17b. The accuracy of loading position was tested and it was shown that when fully assembled the loading rod was always within <0.25 mm of the specimen center. To avoid external contamination, the loading rods passed through a flexible rubber barrier. The hole placed in the barrier was undersized relative to the rod diameter to ensure a seal was maintained throughout testing.

Temperature was maintained throughout testing with the use of a temperature controlled water bath. The bioreactors and inlet fluid tubing were immersed in deionized (DI) water and the temperature was controlled using a Cole-Parmer Polystat digital immersion circulator (Cole-Parmer, Vernon Hills, IL, USA). Within the bath, the temperature at the bioreactor locations was found to be maintained at 37 ± 0.2 ºC. The sealing of the entire circulation system was verified by pumping water dyed with red food coloring through entire system for two weeks and observing that no color change occurred in the bath water.

Applied loads and bath temperatures were collected with universal fatigue load cells (Model LCF300, FUTEK Advanced Sensor Technology Inc., Irvine, CA, USA) and immersion thermocouples. Signals were collected with a commercial data acquisition system (Model

NI9237 24-Bit Bridge with NI9211 and NI9932 modules, National Instruments Corporation, Austin, TX, USA). All data was collected and analyzed utilizing a custom LabView software program.

4.2.2 Sample preparation

Simulated restoration samples were produced from recently extracted human molar teeth chosen with adequate dimensions to machine the specimens shown in Fig. 17a. The teeth were mounted in dental stone to the cementoenamel junction, and then horizontally sectioned on a diamond saw to remove the cusps. The teeth were then further horizontally sectioned to just above the pulp horns, leaving a slab of tooth 3 mm thick and 9 mm diameter with a flat surface of predominantly dentin with some peripheral enamel. Then 5 mm diameter, 2 mm deep cylindrical cavity preparations were prepared in the flat dentin surface using a computer controlled milling system (CNC specimen former, U. of Iowa). The composite used in this study had a resin composed of a 50:50 mixture of bisphenol A glycidyl methacrylate (BisGMA):triethylene glycoldimethacrylate (TEGDMA) monomers with 0.4 wt% of camphorquinone (CQ), 0.8 wt% of 4-dimethylaminobenzoic acid ethyl ether (EDMAB), and 0.05 wt% of 3, 5-di-tertbutyl-4-hydroxytoluene (BHT). The composite was produced by combining the resin with 67 wt% silanated strontium glass (3.0 μm average size, Bisco Inc., Schaumburg, IL, USA) and 5 wt% aerosol-silica filler (OX-50, Degussa).

The cavity was filled with composite by first applying a dentin bonding agent to the floor and one half of the walls of the cavity (if producing a gap specimen – if not, the entire circumference of the cavity was bonded). A self-etch, two-step adhesives (Clearfil SE Bond) was used. The self-etching primer was applied for 20 seconds with light scrubbing of the dentin walls and floor

and then lightly air dried. Then the adhesive resin was applied to the cavity and light cured for 10 seconds. In order to keep the adhesive off of the surface not meant to be bonded, a flexible shim made of polyvinylsiloxane impression material was molded to the wall to protect it. To accelerate secondary caries formation, roughly half of the circumference of the filling was produced with an interfacial gap. Whether the composite was placed into the cavity with a lubricated metal shim for a large gap ~300 μm, or no shim was used and the composite was allowed to shrink away from the non-bonded wall, producing small gaps of approximately 15-30 μm. The composite was cured with a single exposure from the curing light (20 seconds – total radiant exposure ~12J/cm^2; Demi Light, Kerr, Orange, CA, USA). Then the shim, if present, was removed. The sides and bottom of the tooth were then coated with a dental adhesive (Optibond FL, Kerr, Orange, CA, USA) in order to seal them from the acidic environment and prevent demineralization of these surfaces. Finally, the surface of the cavity margins were exposed by polishing with coarse grit silicon carbide disks (Sof-lex, 3M ESPE, St. Paul, MN, USA) in a slow-speed handpiece. The specimens were then stored in sterile water before being further sterilized in ethanol as described.

4.2.3 Sample Sterilization

Two sample sterilization techniques were evaluated, one using a sodium hypochlorite (bleach) solution and one using 1% chloramine T. For the bleach procedure, freshly prepared tooth cavities were sterilized by 1) soaking in 50% household bleach solution for 1 h in an ultrasonic water bath, 2) soaking for 10 min in 5% sodium thiosulfate solution in water ultrasonic bath to remove the chlorine, and 3) washing three times in autoclave sterilized deionized (DI) water for 5 minutes in water in an ultrasonic bath. Sterilized teeth were filled with composites and then put into 15 mL autoclaved brain heart infusion (BHI) media and incubated at 37°C, 5% CO_2 and 95% relative humidity (BBD 6220 incubator, Thermo, Asheville, NC, USA) to ensure sterility. To verify this sterilization procedure, samples were observed over a >2 week period to ensure the media stayed clear and no biofilm appeared on the surface indicating the samples were sterile.

For the chloramine T procedure, the teeth with prepared cavities were stored for one week in 1% chloramine T at 22°C before being filled with composite. After filling, the specimens were soaked for one hour in 70% ethanol, and then washed three times with sterile water before being placed in to BHI media as for the bleach sterilization specimens.

4.2.4 Biofilm growth procedure

For samples tested with a living biofilm, after the sterilization procedure the samples were placed into small sterile Petri dishes with 15 ml of sterile BHI media+3% sucrose. Then 1 ml of *Streptococcus mutans* bacteria culture was added and the samples were incubated at previously

described conditions for about 4 days, changing media each day, until a visible biofilm was observed.

4.2.5 Test Procedure

Two weeks was chosen as the length of test based on typical demineralization studies found in the literature [271, 272]. Indeed, results within this study showed that two weeks is long enough to see a robust, measurable demineralization. The cyclic loading schedule was chosen as alternating blocks of two hours cyclic loading at 1.5 Hz and four hours held at the minimum load. While the two hours cyclic loading time is not clinically relevant, it provides a compromise to attain a large number of cycles within the total test period. Considering that a person may chew at about 1.5 Hz [216], and that the actual chewing time per day may be conservatively estimated as about 20 minutes, this would equate to 1800 chews/day or 657,000 chews/year. An intermittent cycling phase of two hours followed by four hours without cycling gives a total of 43,200 cycles per day, or approximately 605,000 cycles in two weeks. Thus, the total number of cycles is equivalent to nearly one year of normal human exposure. The four hour resting time was chosen to represent a normal time between meals and also should provide ample opportunity for the biofilm to grow and potentially double [273] before being subjected to loading again. Finally, the minimum cyclic load and holding load was always set as 10% of the maximum load.

Table 6 shows the four types of experiments were conducted to assess both the viability of the bioreactor fatigue testing system and synergistic effects of mechanical loading and bacteria (Table 6). Many of the procedures were common among experiments while differences are noted in Table 6 and the following section.

Table 6. Summary of experiments

Experiment Type	Cell Environment	Loading	Outcomes
I	sterile BHI media	none	1) Ensure system remains sterile 2) Monitor samples for degradation
II	*Streptococcus mutans* in BHI media	none	1) Monitor gap for changes due to only bacterial attack
III	sterile BHI media	cyclic	1) Ensure system remains sterile 2) Monitor samples for degradation
IV	*Streptococcus mutans* in BHI media	cyclic	1) Monitor gap for synergistic effects of loading and bacteria

For type I & III experiments the bleach sterilization procedure was used since it enabled the maintenance of a 100% sterile environment through the course of the two week test, ensuring the system design is adequately sealed from external contaminants. However, it was also found that this procedure deteriorates the dentin. This deterioration was observed as a whitening of the tissue after the test and a loss of mechanical integrity that caused the dentin to erode under the loading rod during type III experiments (Fig. 18). Thus, for type IV experiments the chloramine T procedure was used. While chloramine T was found not to degrade the dentin, it also was found not to give 100% sterilization. However, it was observed that this sanitation procedure was adequate to allow S. mutans biofilm growth without excessive competition from other organisms found in the tooth tissue.

Dehydrated BHI media (Thermo Fisher Scientific Inc., Waltham, MA, USA) was prepared according to manufacturer instructions. A separate 2L Erlenmeyer glass flask for used as the media supply for each bioreactor. Each flask was sealed by a rubber stopper with a glass tube (7mm dia.) penetrating it and reaching the bottom of the flask. When the fluid was pumped out of the flask, air was allowed to enter the flask through a stainless steel subdermal syringe needle penetrating the rubber stopper. A 0.2 μm syringe filter (Acrodisc$^{@}$ Syringe filter 0.2 μm, Pall

Corporation, Port Washington, NY USA) was inserted on the needle in order to avoid contamination of the media from the air. The flow of media was maintained at approximately 0.5 ml/min per bioreactor using the peristaltic pumps.

Each 2L of media was autoclaved along with the flask, glass tube, cork, tubing, bioreactors, and loading rods for 45 minutes. All components were then allowed to cool to room temperature under sterile conditions in a BioSafety Cabinet (Baker SterilGARD III, The Baker Company, Sanford, ME, USA) prior to assembling the bioreactor with the sterile sample inside. The media was replenished approximately every three days by replacing the flasks with freshly prepared media. A 5% CO_2-air mixture was flowed through a 0.2 μm syringe filter into each bioreactor at the low flow rate to just maintain visible bubbles at the outlet. The enhanced CO_2 helps provide pH buffering for the media.

During each test, the bioreactors were submerged ~3 cm into the DI water bath. The temperature of the water bath was continuously monitored. To protect the bath water from mold/bacterial contamination, 5 ml of SterilityAqua-clean (Thermo Fisher Scientific Inc., Waltham, MA, USA) was added per each liter of water.

Media leaving the bioreactors was collected into a waste container and used to check for contamination during the type I & III experiments. Every three days one drop of waste media from each bioreactor's exit tubing was applied onto BHI agar plate (Anaerobe systems, Morgan Hill, CA, USA) and incubated for 24-48 hours.

After completion of the type I & III experiments, the bioreactors were opened in a biosafety cabinet and samples were put into 10 ml sterile BHI media and again incubated using the previously described conditions to ensure the absence of bacterial activity. After the completion

of type II & IV experiments, samples were subjected to live/dead staining procedure (Invitrogen live/dead assay kit) and the biofilm was evaluated under confocal fluorescent microscopy to ensure the biofilm was still viabile at the end of the experiments. Once sterility (type III) or biofilm viability (type IV) were confirmed the samples were fixed in 10 ml 4-gluteraldehyde and left at T=-4^{o}C overnight.

Specimens were retrieved, stained with gram positive stain, and then allowed to desiccate for 48 hours in air. The biofilm was carefully removed from the surface with a swab, avoiding the gap area (if there was one on the specimen), and the surface was impressed with a dental vinyl polysiloxane impression material (Aquasil Ultra, Dentsply). The impression was later poured in epoxy to make a replica of the surface and the margin for examination in the SEM to determine if debonding or further gap formation occurred as a result of the loading in the bioreactor. The specimens were then mounted in epoxy resin, sectioned on a slow speed diamond saw (Streuers), and examined under the stereomicrosope for the presence of dentin demineralization and the extent of penetration of the stained bacterial biofilm.
Bacterial penetration was quantified as a fraction of the gap depth.

4.3 Results and Discussion

Most bioreactor studies are conducted in a well controlled biosafety cabinet environment where potential sources of external contamination (bacteria, fungi, etc.) can be easily controlled. In the present study, that was done for type I and II experiments. In contrast, a significant challenge for the current system was to develop a bioreactor and test protocol sufficiently robust to integrate into a standard servo-hydraulic fatigue testing system found in a typical laboratory environment. Type I and III experiments both served to ensure external contaminants did not

enter the bioreactors during the two week test period and that the samples did not degrade in the absence of bacteria. Results of both type I and III experiments using the above procedures typically showed no external contamination of the system during the testing period, though this was somewhat difficult to control and in some experiments, some media clouding was noted. However, observations of bleach sterilized samples from type I and III tests indicated structural degradation of the samples (Fig. 18) that motivated the use of chloramine T as an alternative sample sterilizing agent.

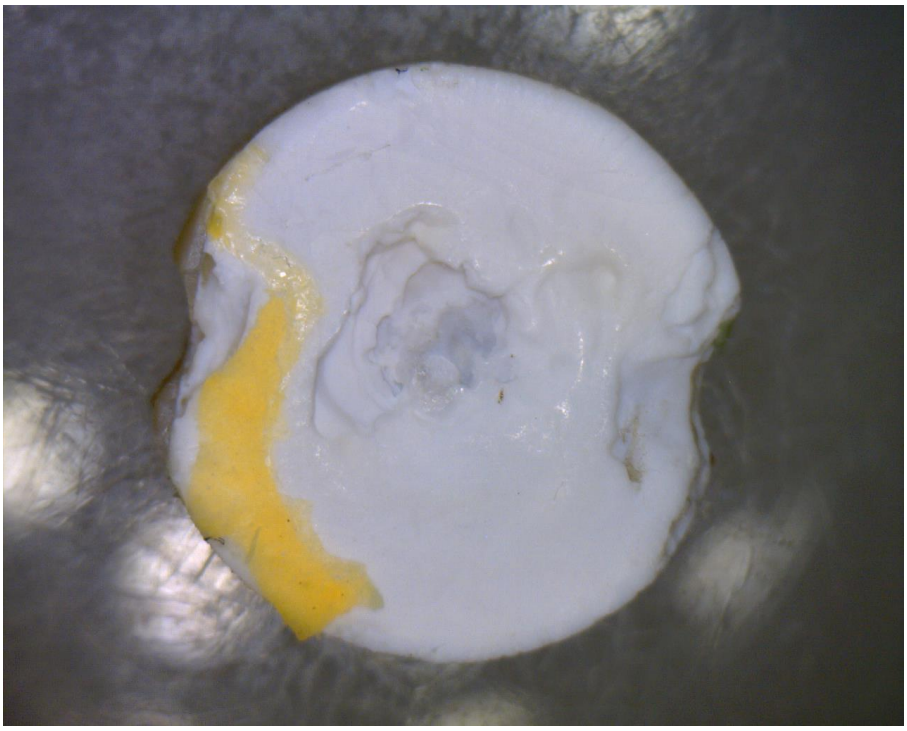


Figure 18. Image of the discoloration and dentin erosion under the loading pin that occurred for type III experiments using the bleach sterilization method.

Less of a challenge was creating a controlled temperature environment for the biofilm growth; indeed, controlled temperature tests are commonplace for fatigue testing of materials in

aqueous and gaseous environments. For types II and IV experiments, live/dead staining technique showed that biofilms were viable on all samples. Further gram positive staining and cross sectioning revealed biofilm presence on the surface and in the gaps of the samples, often penetrating to the depth of the gap for the large gap samples. As Fig. 19 shows, observations of the marginal gaps revealed that for large gap (~300 μm) samples, there was considerable bacterial penetration and demineralization for samples both with and without loading (Type II and Type IV experiments). Once it was found that bacteria penetrated easily into the large gaps both with and without loading (Fig. 19), experiments were instead focused primarily on the small gap samples.

Figure 19. Evaluation of marginal gaps of large gap sample (a)- with loading (type IV) and (b) without loading (type II). Purple gram positive staining shows the bacterial penetration into the gaps while darkening of the dentin indicates demineralization (left of the gaps).

Samples with small gaps showed differences in bacterial penetration depending on whether the sample was cyclically loaded (Fig. 20) or not loaded (Fig. 21). With fatigue loading (Fig. 20) the bacteria penetrated deeply to the floor of the cavity preparation for all samples (6/6). With no

loading, the half (2/4) of small gap samples had poor (~ 60% depth) penetration (Fig. 21a, d) and the other half had deep or nearly deep penetration (Fig. 21b, c), (Table 6).

Table 6. Percentage of biofilm penetration down the gap for loaded and non-loaded 0BAG samples

	Bacterial penetration (%)	
Sample	Loaded	Unloaded
1	94	20
2	100 †	100 †
3	100 †	100
4	100 †	21
5	100 †	
6	100	
7		
† denotes a significant visible bacterial penetration into the floor of the sample cavity, under the filling		

Unlike consistent results for cyclically loaded samples, data for non-loaded samples is very broad (20% - 100%). Statistical test has shown that penetration for loaded and non-loaded samples is statistically different ($p<0.05$). Although there is an extreme broadening in non-loaded samples data, it can be clearly seen that the half of non-loaded samples had a penetration of about 20% of the gap depth, which shows that cyclic loading is most probably aiding bacterial penetration due to a pumping effect, when the gap closes and opens under the cyclic loading, bringing fresh media into and removing the bacterial waste out of the gap.

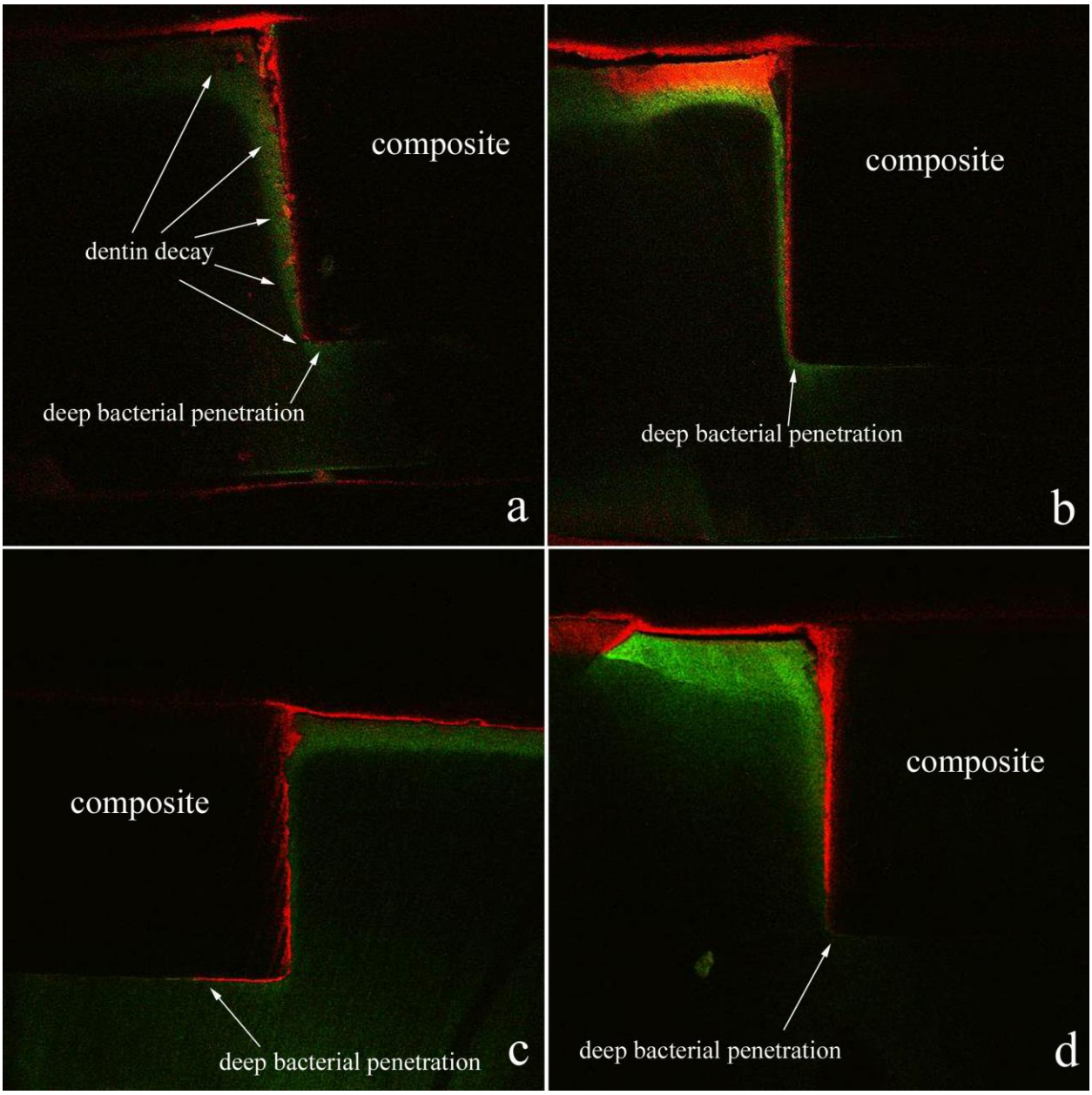


Figure 20. Evaluation of marginal gaps of loaded small gap sample. Red shows the bacterial penetration.

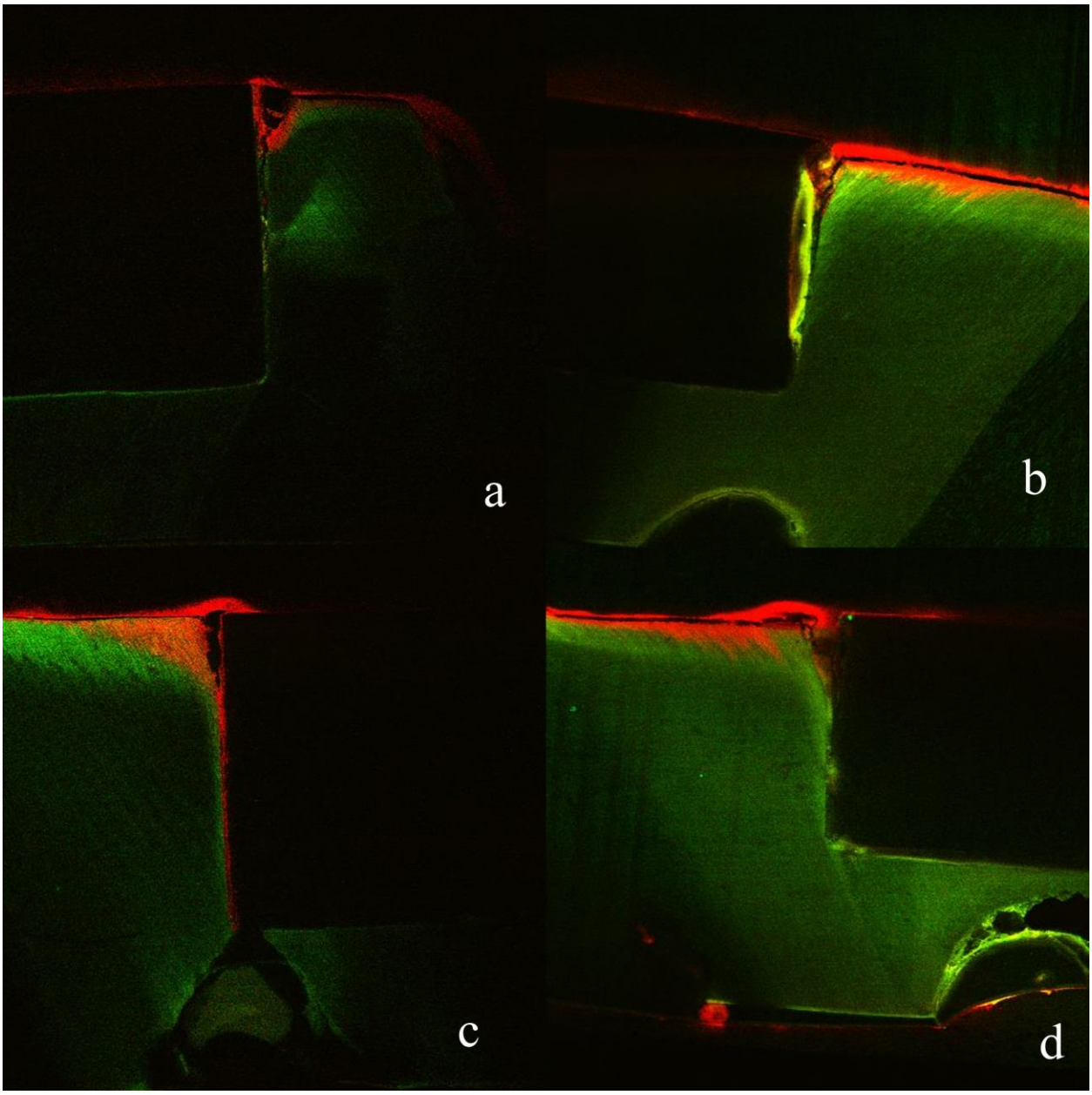


Figure 21. Evaluation of marginal gaps of non-loaded small gap samples. Red shows the bacterial penetration.

Finally, the bonded margins were evaluated after loading (type IV) experiments and results reveled bacterial demineralization to be accelerated along the bonded interface relative to the bulk dentin (Fig. 22). In this case, further bacterial exposure plus cyclic loading may lead to the complete interface failure, deep bacterial penetration, and demineralization.

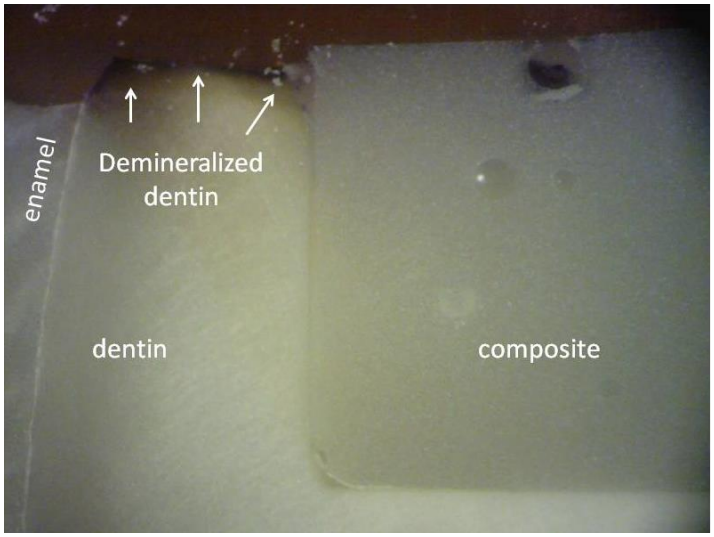


Figure 22. Bonded margin evaluation of a cyclically loaded sample showing preferred demineralization at a well bonded composite-dentin interface relative to the bulk dentin.

Based on Fig. 19, it may be concluded that when the gaps are large (~300 μm wide) the bacteria have free access to colonize the gaps and demineralize the dentin along the marginal interface. Accordingly, one may expect the effect of cyclic loading for large gap samples to be minimal. In contrast, for small gap samples (~15 – 30 μm wide) access of the bacteria deep into the gap appears to be more difficult, especially with no cyclic loading (Fig. 21). Accordingly, it is likely that the synergistic effects of bacterial exposure and loading are most important early in the process of gap formation and secondary caries development. Currently the mechanistic role of cyclic loading is unclear. For example, it is possible that a pumping effect due to cyclic loading

allows media to flow more easily in the small gaps, removing harmful waste and providing nutrients for the bacteria. Alternatively, the bacteria may sense the stress on the surfaces which somehow aids the attachment and colonization process. In any case, the bioreactor fatigue test system presented here represents a reasonable methodology for the study of the role of cyclic loading on biofilm colonization, proving that the combination of cyclic loading and bacterial exposure assists secondary caries propagation. Furthermore, the bioreactor test method presented here will be useful for evaluating new restorative materials (e.g., such as those described in [274]) that are intended to slow or prevent secondary tooth decay.

4.4 Conclusions

Based on an experimental study to develop a bioreactor system and test methodology to study the synergistic effects of cyclic loading and bacterial exposure on simulated tooth fillings *in vitro*, the following conclusions can be made:

- The new bioreactor and new simulated tooth filling test methodology are suitably robust to maintain biofilm during two week long experiments using a conventional servo-hydraulic fatigue testing machine. To the authors' knowledge, this system, this test specimen design and test method is the first ever developed for combined biofilm and cyclic fatigue loading studies.
- Complete sterilization of the simulated tooth filling samples proved problematic. While it was found this may be achieved using a sodium hypochlorite based procedure, significant mechanical degradation of the tooth tissue occurred making the samples unsuitable for mechanical testing. In contrast, the chloramine T based procedure did not

achieve perfect sterilization; however, the mechanical integrity of the sample remained intact.

- Based on results using this *in situ* bioreactor fatigue system, loading did not affect biofilm penetration into large gaps (~ 300 μm wide) since bacteria appear to have easy access even in the absence of cyclic loading. In contrast, non-loaded experiments using small gap (~ 15 – 30 μm wide) samples demonstrated deep penetration of bacteria into the gaps for only 2 out of 4 samples while cyclic loading caused deep bacterial penetration 100% of the time.
- We conclude that there is an evidence of a synergetic effect of loading and bacteria exposure that aides bacterial biofilm penetration at the dentin-filling margin, possibly leading to faster secondary tooth caries development.

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5. MANUSCRIPT III: NEGATIVE EFFECT OF BAG ON SECONDARY TOOTH PROPAGATION

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Abstract

Objectives: Bioactive glass (BAG) is known to possess antimicrobial properties. However, the use of BAG as a filler for dental composite restorations to prevent recurrent caries has not been studied. The goal of this experiment is to investigate the effect of 15 wt% BAG additions to a resin composite on secondary tooth decay at the dentin-filling margin.

Methods: Human molars were machined to 3 mm thick disks and 2 mm deep composite restorations were placed into each sample. A narrow 10-30 micrometer wide dentin-filling gap was introduced along half of the interface between the dentin and filling by not applying dental adhesive to that region. Two different composites were used, one with 15 wt% BAG additions (15BAG) and one without BAG additions (0BAG – control). Samples of both groups had *Streptococcus mutans* biofilms grown on the surface and then were tested inside a bioreactor for two weeks while subjected to periods of cyclic loading. After post-test biofilm viability was confirmed, each sample was cross-sectioned to reveal the gap profile. Depth of biofilm penetration for 0BAG and 15BAG was quantified as the fraction of gap depth. The data was compared using a student's t-test.

Results: The average bacterial penetration into the marginal gaps for the 15BAG samples was significantly smaller (~ 55%) in comparison to 0BAG, where nearly 100% penetration was observed for all sample with the biofilm penetrating underneath of the restoration in some cases.

Significance: BAG containing resin dental composites slow biofilm penetration into marginal gaps of simulated tooth restorations. This suggests BAG containing composites have the potential to slow the development and propagation of secondary tooth decay at restoration margins.

5.1 Introduction

The U.S. National Institutes of Health estimates that >122 million composite tooth restorations are placed in the United States annually; however, annual failure rates up to 15% have been reported [275] and a review of the literature suggests the average lifetime of posterior dental composites is only six years [276]. Furthermore, it is estimated that roughly 60% of restorations are conducted to replace failed tooth fillings. The most common reason for the replacement of restorations is secondary caries occurring at the margins of the restoration [277-280]. Secondary caries is caused by bacterial microflora [281, 282] and the formation of a biofilm (plaque) at the restoration-dentin margin [283]. While bacteria may gain access to the composite-dentin interface when some survive the cavity preparation, more often secondary caries is related to the tooth-filling interface failing and a gap forming between the restoration and tooth that allows bacterial colonization [284]. Margin failure and gap propagation may occur due to the cyclic loading experienced by restorations during mastication [285-288]. While resin composites have relatively good adhesive/sealing properties, polymerization shrinkage of the resin during curing stresses the interface which increases the chance of interface failure when combined with cyclic occlusal loading [284]. After successful gap colonization, bacteria consume saccharides and produce lactic acid as the byproduct [279, 289]. Few studies have been done to understand the factors influencing biofilm development and propagation into interfacial gaps; however, while bacterial biofilm formation is considered a necessary ingredient, a biofilm alone does not guarantee secondary tooth decay [251]. Furthermore, a recent study suggests that cyclic loading plays an important role in aiding biofilm penetration deep down into marginal gaps [Manuscript 2]. The decreased pH due to acid production then shifts the equilibrium dissolution reaction of hydroxyapatite towards demineralization with calcium and phosphate ions leaching out of the

tissue, causing caries propagation [290-292]. Streptococcus mutans strains have been identified as the most abundant bacterial species under the restoration in cases of secondary caries [24, 293]. Such findings suggest a strong need for restorative composites with antimicrobial and/or remineralization properties.

Many materials have antimicrobial properties: copper, zinc, silver, various silica based glasses etc. [155, 294]. Bioactive glass (BAG) has been shown to have both a significant antimicrobial effect on oral bacteria and the ability to remineralize adjacent mineralized tissues [171, 295], [296]. The antimicrobial effect of BAG is attributed in part to the release of ions (e.g., calcium and phosphate) and incorporation of protons into the BAG material, leading to a local increase in pH that is not well tolerated by many oral bacteria [165], as well as silicon ions antimicrobial properties. Although BAG was developed more than 40 years ago, exploration into its potential for use in resin based dental composites has only very recently begun. It has been shown that composites containing up to 15% volume weakly bonded BAG filler can have mechanical properties comparable to, or superior to, many commercial composites [62]. Furthermore, despite BAG ions leaching out of the composite the degradation of mechanical properties with ageing is no worse, or better than, many commercial composites [62]. While it appears clear that BAG containing composites can meet the mechanical property requirements for dental restorations, there is a need to study the secondary caries resistance of BAG composites. Accordingly, the goal of the current paper is to examine the effect of BAG containing composites on secondary tooth decay nucleation and propagation using a previously developed in-vitro testing model that applies cyclic loading while in a living oral bacteria environment [297]. It is hypothesized that BAG containing composites will have a negative effect on biofilm

formation and the ability for bacteria to penetrate deep into gaps at restoration margins due to the antimicrobial and/or remineralization effects of BAG.

5.2 Materials and methods

5.2.1 Bioactive glass composite preparation.

BAG filler was produced by sol-gel process, which was reported previously in [208]. Breifly, pure alkoxides (tetraethyl orthosilicate (TEOS, $Si(OC_2H_5)_4$), calcium methoxyethoxide (CMOE, $C_6H_{14}O_4Ca$), and triethyl phosphate (TEP, $(C_2H_5)_3PO_4$)) were used for BAG synthesis. CMOE was synthesized from Ca metal and methoxyethanol, but all other reagents were obtained from Sigma Aldrich. Solutions of the alkoxides in methoxyethanol was prepared in inert dry nitrogen atmosphere glovebox.

The solution was aged, air dried and then dried at elevated temperatures to evaporate the solvent completely. The obtained product was first ball milled and then processed in a Micronizer jet-mill (Sturtevant Inc., Hanover, MA) to a final fine particle size of 0.04-3 μm. The final composition of the BAG filler used was 65% SiO_2, 31% CaO and 4% P_2O_5 (mol%).

The composite was prepared by combining 57 wt% silanated strontium glass (Bisco Inc.) and 15 wt% BAG with resin matrix (50:50 mixture of bisphenol A glycidyl methacrylate (BisGMA):triethylene glycoldimethacrylate (TEGDMA) monomers with 0.4 wt% of camphorquinone (CQ), 0.8 wt% of 4-dimethylaminobenzoic acid ethyl ether (EDMAB), and 0.05 wt% of 3, 5-di-tertbutyl-4-hydroxytoluene (BHT)). For control composite (0BAG), the filler was 5 wt% aerosol silica (OX-50, Degussa) and 62 wt% silanated strontium glass. Full details on the composite preparation and mechanical properties may be found in [208].

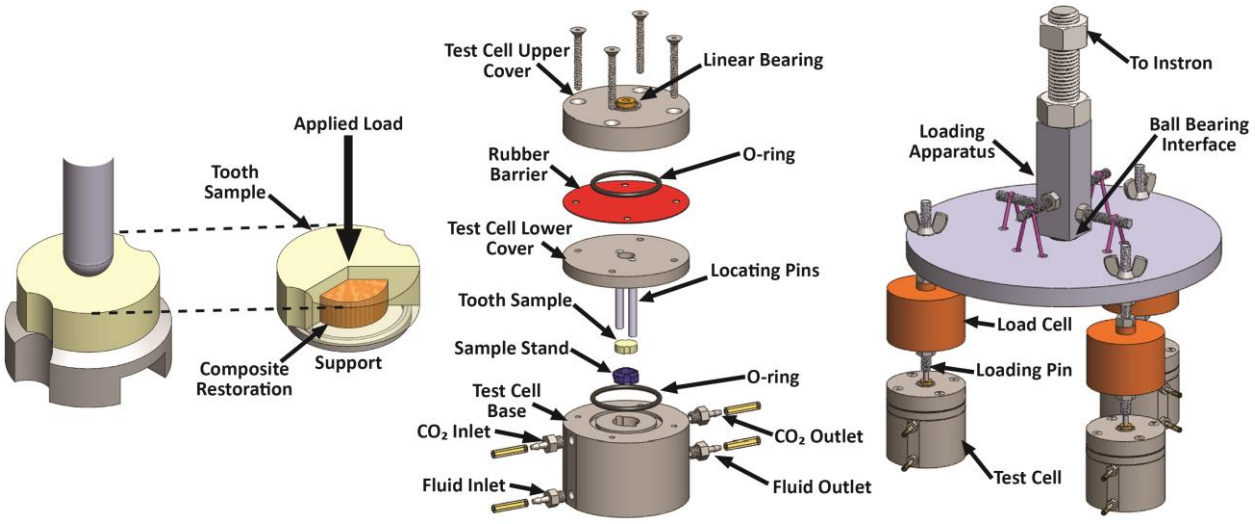
5.2.2 Sample preparation.

Recently extracted human molars were used to produce simulated tooth restoration samples for this study. The complete procedure for sample preparation was described previously in detail in [297]. In brief, the cusps of each molar were sectioned off, and then another section was made across the tooth right above pulp. This sectioning resulted in a disk-shaped slab of tooth ~3 mm thick and 5 mm diameter, composed mostly of dentin with some enamel on circumference. In the middle of sample the cavity was machined to be 2 mm deep and 5 mm in diameter using computer controlled milling system (CNC specimen former, U. of Iowa). Right after machining, the samples were soaked in 1% chloramine T for one week for sterilization. After initial sterilization, the bottom of the cavity and half of its circumference were first treated with a dentin bonding agent (Cleardil SE Bond) and then the composite was placed into the cavity and light cured. Due to polymerization shrinkage, a ~15-30 μm gap was formed around approximately half of the circumference where the dentin bonding agent was not originally applied. The composite surface of the sample was polished to reveal the gap. The samples were sterilized in 70% ethanol and then placed in sterile BHI media to ensure successful sterilization and for storage purposes. See Fig. 23a for a schematic of the sample. Six (N =6) 15% BAG and six (N = 6) 0BAG samples were tested.

Biofilms were grown on the samples by incubating them at 37ºC in 5% CO_2 and 95% relative humidity (BBD 6220 incubator, Thermo, Asheville, NC, USA) in separate small petri dishes with approximately 15 ml of initially sterilized BHI media+3% sucrose with 1 ml of *Streptococcus mutans* bacteria culture added to each dish. Incubation lasted approximately 4 days and the media was refreshed each day, until the biofilm could be observed with a naked eye and then the samples were placed in bioreactors for further testing.

5.2.3 Test method

A novel bioreactor capable of in-situ cyclic loading was used for the demineralization studies with full details reported in [297]. A schematic of the bioreactor may be seen in Fig. 23b, three samples were tested at a time as shown in Fig. 23c. Samples set in the bioreactor composite side down on top of a ring shaped stand giving active diameter of the sample of 3.5 mm. A combined biaxial bending and shear loading was applied by a semi-spherical ended loading rod at the center of the sample which was aligned and fixed in the horizontal plane by linear bearing in the top cover (Fig. 23a, b). The entire bioreactor when assembled was completely sealed from outside using rubber seals and was previously tested and proved to provide no external contamination over the entire time of an experiment [297]. During the experiment, incubator conditions (see above) were reproduced inside each bioreactor. Fresh sterilized BHI media was pumped in with 3.175 mm ID tubes (Fischer Scientific, Pittsburg, PA, USA) into the lower pipe fitting (Fluid inlet in Fig. 23b) of the bioreactor at approximately 1 ml/min flow rate and pumped out of the test cell at the level just above the sample through the outlet pipe fitting to ensure the constant level of media that would cover the entire sample. Pumping was done by peristaltic pumps (Model FB 70381, Thermo Fischer Scientific Inc., Waltham, MA, USA) at both the inlet and outlet. Each bioreactor was submerged up roughly half of its height in a 37^{o}C water bath controlled by a digital immersion circulator (Cole-Palmer, Vernon Hills, IL, USA).



(a) (b) (c)

Figure 23. (a) – restored sample scheme and relative position of sample, sample stand and loading rod. (b) – detailed assembly of bioreactor. (c) – The way to distribute load equally in order to test 3 samples at time.

The applied cyclic loading was applied using a computer controlled servo-hydraulic test system (Model 8872, Instron Corporation, Norwood, MA, USA). Fig. 23c shows how the load was equally distributed between 3 bioreactors using a ball bearing interface, the load for each sample was collected using an individual load cell (Model LCF300, FUTEK Advanced Sensor Technology Inc., Irvine, CA, USA) and the difference between each cell load was always <5%. Applied loads and the bath temperature at two locations were recorded using a data acquisition system (Model NI9237 24-Bit Bridge with NI911 and NI9932 modules, National Instruments Corporation, Austin, TX, USA) and custom LabView software program.

Sample loading was done by alternating blocks of cyclic loading and resting periods. Cyclic loading blocks were 2 hours of cycling with the maximum load of ~113 N and a minimum load of ~11. N which corresponds to 25% and 2.5% of the mean breaking force (450 N), respectively. During the resting periods samples were kept at the minimum (11.25 N) load for 4 hours. The

two block sequence was repeated 56 times to give a total of time of ~2 weeks for each experiment. Two weeks was chosen for the total test time because demineralization studies without cyclic loading have shown that length to be necessary to achieve reliably measurable demineralization [271, 272]. An average person chews at1.5 Hz [216], and that the actual chewing time per day may be conservatively estimated as about 20 minutes, this would equate to 1800 chews/day or 657,000 chews/year. An intermittent cycling phase of two hours followed by four hours without cycling gives a total of 43,200 cycles per day, or approximately 605,000 cycles in two weeks. Thus, the total number of cycles is equivalent to nearly one year of normal human exposure. The four hour resting time was chosen to represent a normal time between meals and also should provide ample opportunity for the biofilm to reproduce and potentially penetrate deeper into the gap [297].

After each experiment, the samples were taken out of the bioreactors and stained with Invitrogen live/dead assay kit for fluorescent laser scanning confocal microscopy evaluation of the biofilm viability. Samples were fixed in 4% gluteraldehyde. Then samples were sectioned in half with the cut line passing through the middle of the gap, revealing the gap profile on each side. Those halves were then stained to observe the depth of bacterial penetration in the gap. Depth of penetration was quantified in terms of fraction of restoration height. Bacterial penetration for the 15BAG and 0BAG samples were compared with a student's t-test (one-sided, alpha=0.95).

5.3 Results

Post-test evaluation using the live/dead assay and fluorescent confocal laser scanning microscopy confirmed a live biofilm on the surface of each sample. Thus, the conditions needed for successful biofilm growth (nutrition supply, carbon dioxide level and temperature) was maintained throughout the entire length of the experiments. Fluorescent microscopy images of the sample cross sections are shown in Fig. 24 and 25 for the 0BAG and 15BAG samples, respectively. The presence of bacteria in the gap can be seen as red in the images.

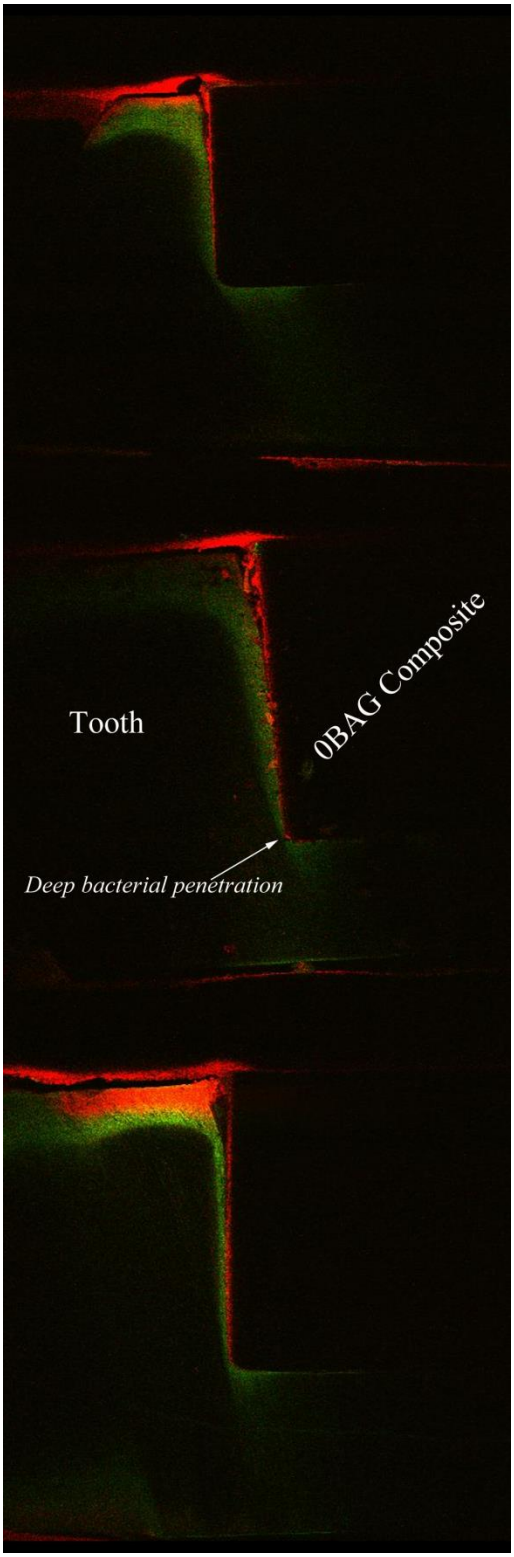


Figure 25. Bacterial penetration profile in the gap (Red), for 0BAG.

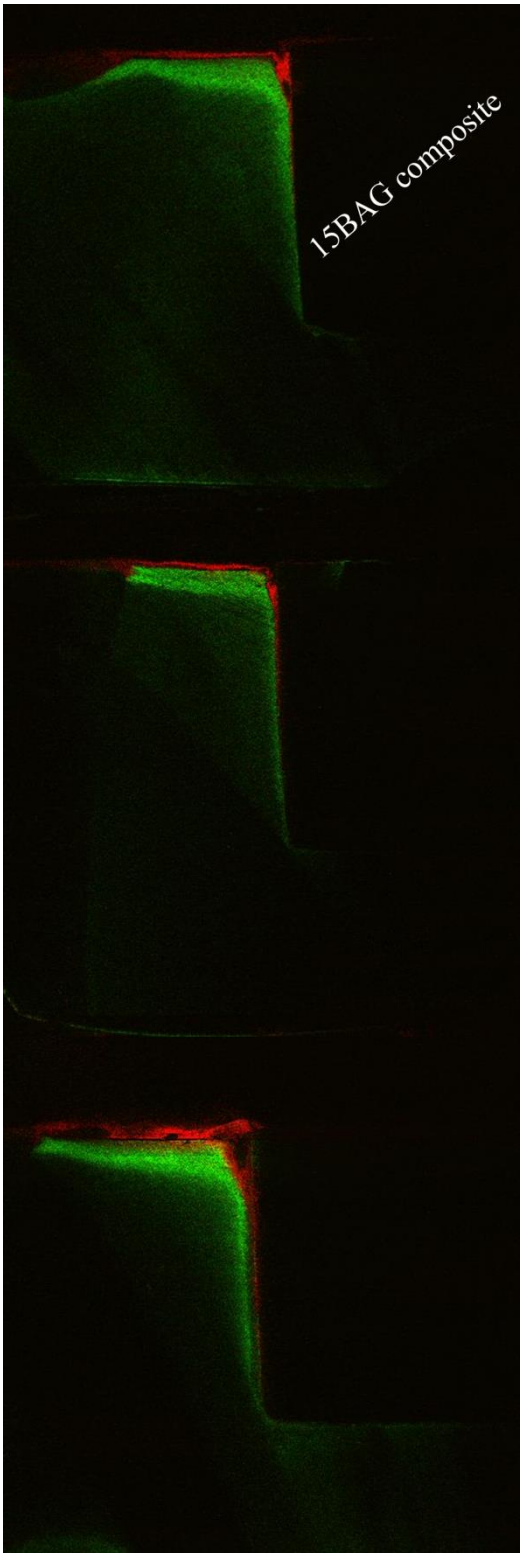


Figure 24. Bacterial penetration profile in the gap (Red), for 15BAG.

Visually it is seen that bacterial penetration is consistently much deeper for the 0BAG composite than for 15BAG. Moreover, bacterial penetration for 0BAG composite samples was generally to the full depth of the restoration, sometimes propagating along the floor of the cavity under the restoration. A quantitative comparison of bacterial penetration is shown in Table 7 in terms of the percentage of the gap colonized by bacteria.

Table 7. Bacterial penetration in the gap for 0BAG and 15BAG composites.

	Bacterial penetration (%)	
Sample	0BAG	15BAG
1	94	48.5
2	100 †	69
3	100 †	50
4	100 †	69
5	100 †	14.3
6	100 †	5
† denotes a significant visible bacterial penetration into the floor of the sample cavity, under the filling		

The students t-test showed that there is a statistically significant difference in the bacterial colonization of the gaps for the two composites, with the 15BAG composite showing significantly less colonization than for 0BAG at 95% confidence level (p-value <0.05).

5.4 Discussions

For the 0BAG control composite, the degree of bacterial penetration into the gaps was consistently very deep, usually reaching the bottom of the cavity. It is important to note that the floor of the prepared cavity was initially treated with dentin bond agent and, thus, originally

there was no gap between the floor of the cavity and restoration. However, in some cases bacterial penetration underneath the 0BAG restoration was observed (Fig. 25) suggesting the synergistic effect of cyclic mechanical loading allowed gap propagation and further bacterial penetration in some samples. Indeed, the important role of cyclic loading in aiding bacterial penetration into interfacial gaps between the dentin and composite has been reported previously [297].

A significantly lower degree of bacterial penetration into the gap was found for samples that were restored with 15% BAG resin composite. On average, for 15BAG composite the penetration was about 40-50% of the gap depth with consistently no penetration underneath the filling. This suggests that the release of BAG ions into the gap can help control the local gap chemistry and create an antimicrobial environment that slows biofilm development and propagation. As mentioned above, the exact mechanisms of the antimicrobial effect of BAG remain unclear, but it may be related to a local rise in pH in the gap [165], one or more of the ions affecting the bacteria (Si leaching out), or a combination of factors. While more studies will be needed to understand the exact antimicrobial mechanisms associated with BAG, the results of the present study suggest that BAG containing composites show potential to slow down the rate of secondary tooth decay saving teeth from the repetitive trauma associated with caries removal.

5.5 Conclusions

Based on an in vitro study of simulated tooth filling samples cyclically loaded in a custom bioreactor system, it was found that 15 wt% additions of bioactive glass (BAG) fillers to a resin based dental composite demonstrated a significant antimicrobial effect in slowing the rate of

bacterial biofilm penetration into pre-existing marginal gaps. These results suggest that BAG containing composites have potential to also slow the rate of secondary tooth decay in vivo.

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6. Summary and conclusions

The mechanical testing of BAG containing resin dental composites showed that these experimental composites:

- Exhibit mechanical properties (flexural strength, fracture toughness and fatigue crack growth resistance) comparable to, or better than, commercially available resin composites.
- Have no significant change in mechanical properties with increase of BAG filler content from 0 to 15 wt%.
- Show slight decrease in fracture toughness and fatigue crack growth resistance, and no significant change in flexural strength, after aging in bacterial media for 2 months, despite BAG particle debonding.
- The main toughening mechanisms were multiple crack deflection due to increased filler concentration and crack bridging.

A novel bioreactor was built and new type of test samples was designed, which made possible the study of secondary tooth decay under combined cyclic loading and bacterial attack. Using of this bioreactor produced the following results:

- Chewing (cyclic loading) was shown to have an effect on secondary tooth decay propagation, aiding biofilm penetration down narrow gaps at the tooth-filling margin.
- Composites with 15 wt% BAG additions possess a significant antimicrobial effect, slowing down the rate of bacterial penetration into gaps at the restoration margins (~55% bacterial penetration) when compared to 0 wt% BAG control composites (~100% penetration).

Overall, BAG containing composites are promising new dental materials that may help extend the lifetime of modern dental restorations by slowing secondary tooth decay and resisting restoration fractures.

In the future several steps would need to be taken and several improvements to be done in order to provide more evidence that BAG composite can be a better material for dental applications:

- In vivo studies, to see that in real mouth environment BAG composite's mechanical properties (flexural strength, fracture toughness, fatigue crack growth resistance) and secondary tooth decay resistance properties remain comparable to or above the ones of up to date commercial composites;
- Long term studies on material's aging behavior, as in this project 2 months was the longest aging time, however, a filling is expected to remain chemically and mechanically stable for much longer period of time, preferably longer than current average lifetime (6-10 years) without premature failure;
- Improvements on BAG composite's matrix needs to be done to reduce cytotoxicity of one of its components - Bisphenol;
- Remineralization studies of BAG composites are needed, because due to BAG's ability to restore mineralized tissues of a human, BAG composite has a potention to serve as a dental restorative material with regenerative power at filling-dentin interface.

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