Development of collection methods and comparison of
_in vivo_ biodegradation of urethane-modified and
bisGMA based resin-composites

By

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\textbf{ABSTRACT}

\textit{Background:} Human salivary esterases have been shown to degrade composite resin restoration \textit{in vivo}.

\textit{Objective:} To optimize \textit{in vivo} protocols to recover biodegradation products and to compare the biostability of urethane-modified-bisGMA- (ubis) and bisGMA-based (bis) commercial resin composites.

\textit{Methods:} Class V and III composite restorations were placed in patients using adhesive and composite resin. Gingival crevicular fluid (GCF), plaque and a 2-minute oral rinse with 20\% ethanol in saline (n=10) were collected immediately and 7-days after restoration placement. Samples were analyzed for biodegradation products using high performance liquid chromatography. The oral rinse protocol was then used to compare the bis and ubis composite resins (Z250, 3M; TPH, Dentsply) (n=58).

\textit{Results and conclusions:} The bisGMA composite matrix derived product, bishydroxypropoxyphenylpropane (BisHPPP) was only detected from oral rinse collected immediately after restoration placement. There was no statistical difference in the amount of bisHPPP collected from bis and ubis composite resins.

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Chapter 1

Literature Review
1.0 LITERATURE REVIEW

A composite is a material composed of two or more substances, with interatomic or molecular bonding between them, which together display properties greater than the sum of their individual parts (Dogon, 1990). Dental resin composites are made up of four main substances: an organic matrix, inorganic filler particles, coupling agents, (Tanaka et al, 1991; Gerzina and Hume, 1994) initiators and accelerators. A variety of other components also contribute to the mechanical and esthetic properties of the resin (Powers and Sakaguchi, 2006). The clinical performance of restorative resin composites is affected by the character and ratio of these principal components, allowing them to be customized for various uses, including coronal restorations, fissure sealants, core build-ups, and more (Asmussen, 1982).

1.1 RESIN COMPOSITE COMPONENTS

1.1.1 Resin Composite Organic Matrix

The relative percentages and different types of monomers influence the polymerization shrinkage, viscosity and water uptake of resin composites. The organic matrix in current resin composites is primarily composed of the monomer bisphenol A glycidyl dimethacrylate (bisGMA) with additional diluent monomers added to produce the desired properties for each specific resin (Davy et al, 1998). BisGMA has a higher molecular mass than traditional methylmethacrylate materials (512 vs. 87 dalton). In addition,
features in its chemical structure, such as reactive hydroxyl groups, which form hydrogen bonds, produce a stronger, stiffer resin with lower volatility, lower polymerization shrinkage, more rapid hardening, and higher viscosity (Smith, 1985). Excessive viscosity of the monomer system used to manufacture resin composite poses great difficulties during the stage of filler incorporation, as the resultant mixture is virtually impossible to process. This has lead to the addition of diluent monomers in today’s composite materials (Venhoven et al, 1993; Sandner et al, 1997). The specific properties that each diluent monomer provides depend on the monomer's structure, composition, polarity, and ratio relative to bisGMA (Smith, 1985).

\[
\begin{align*}
\text{CH}_2\text{C} & \text{CH}_3 \\
\text{C} & \text{O} \text{CH}_2 \text{CHCH}_2 \text{O} \\
\text{C} & \text{CH}_3 \\
\text{O} & \text{OH} \\
\text{O} & \text{CH}_3 \text{CH}_2 \text{O} \\
\text{O} & \text{OH} \\
\text{C} & \text{C} \text{CH}_3 \\
\text{O} & \text{CH}_2 \text{C} \text{HCH}_2 \text{O} \\
\text{C} & \text{C} \text{CH}_3
\end{align*}
\]

Figure 1.1: Structure of BisGMA monomer (Ruyter, 1981)

Triethylene glycol dimethacrylate (TEGDMA), for example, is a flexible, low molecular weight monomer commonly used as a diluent (Smith, 1985). Adding TEGDMA to bisGMA improves the working characteristics of the resin. However, it also increases polymerization shrinkage (Venhoven et al, 1993) and water uptake (Kalachandra and Turner, 1987). Thus the specific monomer composition of a resin composite material involves a balancing of these properties.
1.1.2 Resin Composite Filler Systems

Inorganic filler particles are added to improve various physical properties of resin composite. They provide the bulk of the material weight and volume of resin composites, improve the strength and modulus of elasticity, improve wear resistance, improve marginal adaptation and provide radiopacity to the material. Fillers may also decrease undesirable properties such as water sorption, polymerization contraction, heat of polymerization, and coefficient of thermal expansion (Cook et al, 1984; Ruyter & Øysæd, 1987; Dogon, 1990; Anusavice, 1996). Examples of filler particles are: quartz; colloidal silica; silica glasses with barium, strontium or zinc; zirconia and lithium-aluminum silicate (Söderholm, 1985; Dogon, 1990) with filler particles size range of 0.1 to 100µm (Anusavice and de Rijk, 1990). The most common classification system for resin composite is based on the size of filler particles. Microfilled resin composites contain fillers that range from 0.01µm to 0.04 µm. Hybrid resin composites contain fillers that range from 0.01µm to 6 µm. Z250, a micro-hybrid composite, contains fillers ranging from 0.01µm to 3.5µm with an average particle size of 0.6µm, whereas other hybrid
composites are a mixture of 0.1µm and 1µm particles. Smaller filler particles improve polishability and surface texture by allowing for closer compaction of resin (Santerre et al, 2001) while the larger particles in hybrid resins provide improved wear resistance.

1.1.3 Coupling Agents

Coupling agents, most commonly silanes, are used to facilitate chemical bonds between the monomer and filler particles (Anusavice and de Rijk, 1990). Silanes are organic silicons that contain vinyl groups capable of reacting with the resin matrix and methoxy groups that hydrolyze, forming silanol groups capable of reacting at the filler surface. Through these mechanisms, the coupling agents bond to each other as well as to the monomer and filler particles (Söderholm, 1985). This bonding decreases the water sorption and subsequent disintegration in water of the cured resin composite (Bowen, 1963).

1.2 RESIN COMPOSITE POLYMERIZATION

Resin composites are polymerized via free radical polymerization, in which un-reacted methacrylate monomers are added to a terminal free radical site on the molecule (Young and Lovell, 1991). Initiation of this reaction can be through a thermal, chemical or photochemical process, with photochemical polymerization (photopolymerization) being the most commonly used in dentistry (Cook et al., 1984). Organic peroxide is utilized to
initiate chemical-curing of resin composites. Camphoroquinone or phenyl propanedione is utilized to initiate photochemical-curing of resin composites. The addition of an organic amine, such as 2-dimethyl (aminoethyl) methacrylate (DMAEM), accelerates these reactions, while burylated hydroxytoluene helps prevent premature polymerization. UV light absorbers promote light curing and inorganic oxide pigments provide colour variations for shade matching (Powers and Sakaguchi, 2006).

During photopolymerization, a light source is used to expose the unreacted resin to 468nm wavelength visible light. The most common initiator, camphorquinone, is excited and accepts electrons from a coinitiator amine (ie. DMAEM), generating free radicals which then react with unsaturated double bonds on the monomers in an addition reaction (Kilian, 1979; Ruyter and Øysæd, 1987). The disadvantage of this system is that polymerization is not uniform either in rate or completeness throughout the entire mass of resin, with more rapid and complete reaction near the surface than deep within the restoration (Cook et al., 1984). Even at the resin surface, polymerization is incomplete, due to formation of an oxygen inhibition layer, in which the free radicals are oxidized to form stable peroxides which react very little with resin monomers (Gauthier et al, 2005). Throughout the resin composite, 25-60% of methacrylate groups remain un-reacted after the photopolymerization step (Anusavice and de Rijk, 1990).
Components within the resin composite also influence the degree of conversion. For example, increased bisGMA levels raise the viscosity of unpolymerized resin, which decreases the potential for reactive group interactions, thereby decreasing the degree of polymerization (Chung and Greener, 1990). Alternatively, increased TEGDMA decreases viscosity thereby increasing the degree of polymerization (Sanders and Schreiber, 1992). Initiators, such as camphoroquinone, also influence the degree of monomer conversion (Yoshida and Greener, 1993). The degree of conversion is important, as free reactive vinyl groups can be hydrolyzed from the resin matrix, and play a significant role in the toxicity of dental resin composites (Hanks et al, 1991).

1.2.1 Polymerization Shrinkage

When methacrylate based resin composites polymerize, they undergo 0.8- 5.68 % volume shrinkage (Goldman, 1983; Tarle et al., 1998). This shrinkage is due mainly to conversion from a less dense arrangement of van der Waals forces and single covalent bonds to more closely associated van der Waals forces and double bonds within the resin (Venhoven et al., 1993). Shrinkage during polymerization will result in tension and possibly a 5-30 µm gap between the composite and the tooth (Saltzberg et al, 1976; Bouillaguet, 2004). Bacteria, fluids, molecules and ions can enter this gap through a process termed microleakage, promoting secondary caries at the tooth-resin interface, pulpal inflammation and sensitivity (Kidd and Beighton, 1996; Bergenholtz, 2000). Dentin bonding agents are used to resist the forces of polymerization shrinkage and to form a seal between the tooth
1.3 Adhesive System

Adhesive systems provide the coupling between resin composites and tooth structure, compensating for resin composite polymerization shrinkage, enhancing retention of the restoration and sealing its margins. A secure bond to dentin, which prevents the ingress of fluids and bacteria (microleakage) towards the pulp, is critical to the prevention of post-operative sensitivity and recurrent decay, and to the maintenance of pulpal health and restoration longevity. Current adhesive systems must accomplish three steps either separately or in combination. These are etching, priming, and the placement of a resin adhesive layer.

Etching the tooth-structure allows for micro-mechanical retention and is achieved in one of two ways: with an etch-and-rinse or self-etch system. In etch-and-rinse systems, 30-40% phosphoric acid is applied to the tooth structure to remove the smear layer and demineralize the dentin to an average depth of 5 µm before being rinsed off. Self-etching systems, on the other hand, do not require rinsing and do not remove the smear layer. Instead, they penetrate through the smear layer and incorporate it into the resin-dentin interface hybrid layer, a thin layer in which the resin monomers are intermixed with the dentin collagen (Van Meerbeek et al., 2003; Schwartz and Fransman, 2005).
The priming step utilizes a bifunctional molecule. Hydrophilic groups on one end of the primer penetrate and bind to the wet dentin, while hydrophobic methacrylate groups bind to the adhesive and composite. Adequate priming is dependent on the state of the dentin substrate prior to primer application and on full evaporation of the solvent within the primer solution after primer application.

The adhesive layer is used to fill dentinal tubules, seal the primed dentin surface and form a final hydrophobic layer on top of the dentin for bonding to the subsequently placed resin composite material. It ranges from 5-40 µm in thickness. Similar to resin composite materials, it contains bisGMA and various other monomers but generally does not contain filler particles.

1.4 HUMAN SALIVA

Human saliva is a complex mixture of muco-serous hypotonic fluids and particulates from major and minor salivary glands, gingival crevicular fluid, oral microorganisms, polymorphonuclear leukocytes, epithelial cells and food debris (Nakamura and Slots, 1983; Edgar, 1990; Humphrey and Williamson, 2001). Saliva is approximately 99% water, and contains proteins, including immunoglobulins and enzymes, electrolytes, mucins, and the nitrogenous products urea and ammonia (Lindqvist et al, 1977; Humphrey and Williamson, 2001). With a pH of 6-7, saliva is slightly acidic and
contributes to protection and lubrication of the oral cavity, buffering and clearance, promotion or prevention of plaque aggregation, de and remineralization of the enamel matrix, antibacterial activity, taste and digestion (Rudney, 2000; Humphrey and Williamson, 2001). The average daily flow of saliva in healthy individuals is 1-1.5L with 65% of unstimulated saliva released from the submandibular gland, 20% from the parotid gland and 8% from the sublingual gland. The remaining 10% comes from minor salivary glands located in the lip, tongue, palate, cheeks and pharynx (Humphrey and Williamson, 2001). During stimulated salivary flow, which accounts for approximately 2 hours of each day, the parotid gland produces greater than 50% of the total volume. The average unstimulated rate of flow is 0.3mL/min, though this value varies greatly, ranging from 0.1mL/min up to 7mL/min (Humphrey and Williamson, 2001), with recognized diurnal, annual and seasonal variations in flow (Shannon et al, 1972; Dawes, 1974; Culp et al, 1991).

There are more than 30 enzymes found in saliva and they can be divided into five main groups: carbohydrases, esterases, transferring enzymes, proteolytic enzymes, and others (Chauncey, 1961). Cholinesterases, specifically pseudocholine esterase (PCE), were isolated from human saliva. Their source is the GCF, microorganisms, etc (Ryhänen, 1983; Labow et al, 1998). Esterase activity is present in human saliva at levels capable of degrading cured dental composites. While cholesterol esterase (CE) is not found in human saliva, CE-like activity has been identified, allowing for the use of CE-like activity units for measurement and quantification of enzymatic activity (Santerre et al, 1999; Finer and Santerre, 2003).
Saliva is a useful study tool as it is easily obtained in a non-invasive manner, and is already widely used as a screening tool for hormone and drug levels, including polypeptides, steroids, antibodies, alcohol and various other substances (Humphrey and Williamson, 2001). It is also a useful diagnostic tool for caries risk assessment, periodontal disease parameters, systemic and salivary gland disease/dysfunction and fungal, bacterial and viral infections (Klinkhammer, 1968; Raeste & Aura, 1978; Liu et al, 1990). As the saliva continuously coats the oral cavity, including dental restorations, biodegradation products from resin composite restorations may be collected in saliva, as demonstrated previously by Jaffer (2005).

Mouth rinses are recognized as a useful tool in enhancing the collection of materials from the oral environment. Some authors have used rinses as diagnostic tools in periodontal disease (Klinkhammer, 1968; Raeste and Aura, 1978) and for collection of chemical compounds to be analyzed by High Performance Liquid (HPLC) (Fakhry-Smith et al, 1997). More recently, resin composite degradation products have been isolated from rinse samples in patients with resin composite restorations (Chiu, 2007; Jaffer, 2005).

1.5 GINGIVAL CREVICULAR FLUID

Gingival crevicular fluid (GCF) is a fluid found in the gingival sulcus, which is similar to blood serum exudate, with components originating from the subgingival bacterial biofilm and from cells of the gingival tissues (Champagne et al, 2003). GCF can be collected via capillary tubes, filter papers or paper points. Most GCF studies originally involved
volumetric quantification of the fluid as measured by weight, linear distance the fluid migrated on a paper, or via a commercial electronic measuring device (Griffiths et al, 1988). In addition, GCF has been used as a tool to recover inflammatory mediators in assessment of periodontal disease (Champagne et al, 2003). Like saliva, GCF collection is minimally invasive, straightforward and efficient, and has the added benefit of site specificity.

1.6 DENTAL PLAQUE

Dental plaque is a type of biofilm that forms on the supragingival and subgingival surfaces of teeth and is composed of an organized community of bacteria, which produce a protective and adhesive glycocalyx matrix. Dental plaque has the ability to take in components from the saliva and GCF, such as enzymes. The bacteria that have been shown to adhere to the resin composite surface and at the tooth-restoration interface also generate hydrolytic enzymes (Grieve et al, 1991). Thus plaque and plaque bacteria may play a direct role in local biodegradation as well as absorbing some breakdown product from the saliva. Plaque collection is also straightforward, efficient and minimally invasive and provides a potential reservoir for biodegradation products of resin composite restorations.
1.7 BIODEGRADATION OF DENTAL RESIN COMPOSITES

In the oral cavity, resin composites can be degraded through a variety of mechanisms, including physical processes such as wear by mastication, and chemical processes such as disintegration and dissolution from components in saliva, interactions with foods and drugs, and bacterial activity (Øilo, 1992). The majority of studies published have focused on physical breakdown of the resin composite; including softening, stress, fatigue, fracture and wear. These processes are enhanced by the biodegradation process. Biodegradation is defined as “the gradual breakdown of a material that is mediated by a specific biological activity” (Smith et al, 1987). Resin specimens incubated in buffers with no enzymes demonstrated increased wear and surface hardness as compared to specimens incubated in water alone (Chadwick et al, 1990); this was thought to be due to enzyme-mediated hydrolysis of the ester groups within the resin matrix. In addition, leaching of filler components produces cracks at the resin-filler interface, and may consequently weaken the resin and potentially increase the surface area of resin exposed to enzymatic degradation (Roulet and Walti, 1984; Söderholm et al, 1984).

1.7.1 Diffusion of Unreacted Resin Moieties

Unreacted resin components are leached from resin composites in a diffusion rate-dependent manner. The leaching depends on the resin type, the surface treatment of filler particles and type of solvent (Øilo, 1992; Ferracane, 1994). It was shown that immersion in water resulted in 50% elution of leachable components in 3 hours, while the addition of
ethanol to the solution increased elution to 75% of the leachable components (Ferracane and Condon, 1990). Within 24 hours all leachable components had been eluted into both solutions, suggesting that it is the rate, rather than the degree of elution that is affected by solvent type. The completion of nearly all eluted moieties by 24 hours suggests that elution would result in transitory rather than continuous exposure of the oral cavity to unreacted monomer. However this study was conducted in vitro, without mechanical wear, and therefore it is unclear whether continued wear and washing away of the surface composite layer would result in exposure of more unreacted monomer and their elution into the oral environment. Detection of leached unreacted monomers, TEGDMA and bisGMA from dental resin composites have been demonstrated by HPLC, gas–liquid chromatography and mass spectrometry (GC-MS) (Tanaka et al., 1991; Gerzina and Hume, 1994).

1.7.2 Hydrolysis of Resin Components

Resin composites are prone not only to leaching water of unreacted methacrylates, but also to hydrolytic degradation of polymerized methacrylates. Hydrolytic degradation involves the cleavage of the OC=O bond between the acyl group and the oxygen in the unprotected ester linkage, resulting in pores through which degradation products are released (Gopfrerich, 1996). This process is further catalyzed by enzymes, particularly esterases, as demonstrated by microhardness changes in several in vitro and in vivo studies (van Groeningen et al, 1986; Santerre et al, 1999 Munksgaard and Freund, 1990;
Larsen and Munksgaard, 1991). Esterase, such as acteylcholinesterase, porcine liver esterase, cholesterol esterase, pseudocholine esterase and pancreatic lipase are all capable of catalyzing the hydrolysis of resin monomers (Santerre et al, 2001; Yourtee et al, 2001).

Human saliva contains enzymes capable of degrading commercial resin composites. The effect of saliva on resin composites is important when considering the in vivo performance of resin composites. Esterase activities, CE- and PCE-like, have been detected in human saliva at levels comparable to those used in in vitro studies with CE and PCE (Larsen and Munksgaard, 1991; Finer and Santerre, 2003; Lin et al, 2005). When bisGMA/TEGDMA based resin composites were incubated with esterases, a greater decrease in mass was noted than when incubated in buffer alone (Munksgaard & Freund, 1990; Finer and Santerre, 2004a). Likewise, incubation with cholesterol esterase resulted in greater surface degradation than when incubated in buffer alone (Finer and Santerre, 2004a). Human salivary derived enzymes (HSDEs) are capable of degrading bisGMA and TEGDMA monomers within 24 hours (Jaffer et al, 2002). Likewise, incubation of dental monomers in human saliva resulted in conversion of bisphenolA dimethacrylate to bisphenol-A (BPA) in a period of 24 hours in both a pH and temperature dependent manner (Atkinson et al, 2002; Finer and Santerre, 2004; 2004b). When resin-composite bonded to dentin specimens were incubated with PCE-CE solution, in activity levels simulating intra-oral conditions, the specimens demonstrated both biochemical markers of degradation; with the release of a bisGMA derived biodegradation product,
bishydroxypropoxyphenylpropane (bisHPPP), as well as increased depth and spatial volume of bacterial cell penetration within the resin-dentin interface (Kermanshahi, 2009). The latter process is clearly a marker of reducing the clinical value of the restoration. Recent pilot clinical studies have demonstrated that bisHPPP and MA released from the commercial resin, Z250, can be isolated with HPLC from whole saliva samples and in saline rinse with or without ethanol (Jaffer, 2005; Chiu, 2007).

![Chemical structure of bisGMA degradation pathways](image)

**Figure 3**  Degradation pathways of bisGMA: hydrolysis of unreacted bisGMA monomer (Finer, 2000)

Kinetic studies of CE and PCE demonstrated affinities for degrading certain monomers over others when exposed to a variety of composites. For example, CE degrades bisGMA
at a faster rate than TEGDMA, while PCE degrades TEGDMA faster (Finer et al, 2004). The combination of CE and PCE produced a synergistic degradation reaction on both bisGMA/TEGDMA and urethane modified bisGMA/TEGDMA/bisEMA monomer based composites (Finer et al, 2004). This may have been due to increased stability of CE in the presence of PCE, and may indicate that the presence of multiple enzymes in the saliva may enhance their stability and biodegradative activities.

1.7.3 Isolation and Identification of Biodegradation By-Products

Hydrolysis of bisGMA and TEGDMA based resin composites, such as Z250, produces methacrylic acid (MA), bishydroxypropoxyphenylpropane (bisHPPP) and triethylene glycol methacrylate (TEGMA). Urethane modified bisGMA and bisphenol A polyethylene glycol diether dimethacrylate (bisEMA) based composites, such as Spectrum TPH, produce the similar degradation byproducts, but have an additional byproduct, ethoxylated bisphenol A (EBP-A) (Leung et al, 1997; Finer and Santerre, 2004a). Each of these products has varying utility as a marker for biodegradation. TEGMA, for example, is derived from the cleavage of pendant TEGDMA molecules and can be further hydrolysed to triethylene glycol (TEG) and one molecule of MA. Unreacted TEGMA also demonstrates a high level of leaching, without any matrix degradation. Therefore, it is not possible to determine whether TEGMA or TEG in a sample came from matrix degradation or from leaching of TEGMA (Finer and Santerre, 2007). Therefore, TEGMA has limited value as a marker for matrix biodegradation. MA is a common degradation end product for all unreacted and partially reacted methacrylate
based monomers. One or two MA molecules are produced from the degradation of, respectively, pendant bisGMA or unreacted bisGMA. An in vitro study, however, showed that the amounts of isolated bisHPPP did not correspond with the amount of MA as outlined above (Finer and Santerre, 2004a). Therefore, the authors concluded that bisHPPP must have originated in part from bisGMA molecules that were incorporated into the resin matrix and that MA does not reflect a direct correlation to the degree of resin composite matrix biodegradation.

In methodologies that use freeze-drying as part of the processing technique prior to product analysis by high performance liquid chromatography (HPLC), 90% loss of MA during freeze drying has been reported (Finer and Santerre, 2004a; Chiu, 2007) adding to the unsuitability of MA as a useful marker of biodegradation. BisGMA has a high molecular weight, rigid hydrophobic phenyl rings and hydrogen bonding capacity. All of these features contribute to limited diffusion of bisGMA out of polymerized resin. Therefore, bisHPPP that is detected in biodegradation studies can be attributed primarily to degradation of pendant bisGMA or repeat segments within the matrix (Finer and Santerre, 2004a; 2004b). Also, bisHPPP is a degradation end product and the amount of bisHPPP in solution is therefore a good marker of amount of resin matrix degradation, and the one used in this study.
1.7.4 Effect of Resin Formulation on Biodegradation

The amount of product released from different resin-composites depends on the composite’s specific formulation. For example, Finer and Santerre (Finer and Santerre, 2004a) demonstrated a 2.6 to 86 fold increased release of degradation products from a bisGMA/TEGDMA based resin composite (Z100) than a urethane modified bisGMA/TEGDMA composite (TPH) in vitro. The authors attributed this difference in degradation to increased stability of the urethane-modified bisGMA molecules in the presence of esterases (Finer and Santerre, 2004a).

1.8 RESIN COMPOSITE BIOCOMPATIBILITY

It has been suggested that resin composite monomers and biodegradation products released into the oral cavity could have a systemic and/or local effect on host and bacterial cells. TEG and MA have been shown to induce apoptosis in human gingival fibroblasts (Engelmann et al, 2004), while TEGDMA increases reactive oxygen species and decreases glutathione (Engelmann et al, 2005). Elevated reactive oxygen species may cause detrimental alterations of cell membranes, DNA and other cellular structures. All three of these monomers have been shown to affect bacteria, with an increase in s. sobrinus and l. acidophilus, significant for their role in the development of dental caries (Hansel et al, 1998; Takahashi et al, 2004). The presence of TEG causes changes in gene expression in S. mutans with resultant biofilm formation and transcription regulation changes, and log phase bacterial growth was inhibited when cells were grown in the presence of bisGMA
and UDMA (Khalichi et al, 2009).

BisGMA has also been reported as cytotoxic (Guertsen et al, 1998) leading to a reduction in glutathione, which is important for self-protection of cells against oxidative stress and toxic xenobiotics; as well as contributing to increased gingival fibroblast apoptosis (Engelmann et al, 2004). In addition, inhibited wound healing in the presence of bisGMA has been reported (Hanks et al, 1991). Flowable composites were also shown to be more cytotoxic than traditional resin composites, perhaps due to a higher amount of monomer and less filler content. The authors reported that levels of TEGDMA were above the effective dose (ED₅₀), the dose required to have an effect in 50% of the population for permanent 3T3 fibroblasts in the flowable, but not the traditional, composites (Al-Hiyasat et al, 2005).

Bisphenol-A (BPA) has received a lot of recent media coverage for its reported estrogenic activity and other links to toxic effects, for example in increased cell proliferation. This has led to a call to ban the use of BPA in baby bottles and there is a growing trend to avoid the use of BPA in food storage containers. It is important to reassure patients and the dental community that resin composite restorations are not associated with BPA exposure. Virtually all commercial resin composites do not contain significant amounts of BPA, but the risk of trace amounts cannot be totally eliminated. Concise™ (3M, ESPE), an older generation resin-composite material, is the only commercial restorative resin
composite containing BPA as a listed ingredient. BPA is also present in Delton fissure sealer material, and has been reported as a degradation product from resin sealants. However, the product was released into saliva over a very short time period (3 hours), at levels significantly lower than those shown to cause local oral toxicity (Olea et al., 1996; Arenholt-Bindslev et al, 1999; Fung et al., 2000; Tyl et al., 2002). BPA was not detected in the bloodstream of patients with even the highest reported local levels of BPA (Olea et al, 1996), which indicates that the systemic exposure to BPA from dental sealants is very low, and undetectable. Bisphenol A dimethacrylate, which can hydrolyze to produce BPA and may be responsible for the low levels of BPA detected from resin sealants, has a distinctly different chemical structure and stability than bisGMA (Atkinson et al, 2002).

It is not expected that the biodegradation of commonly used resin composites will produce BPA. BisGMA is a major component in dental resin-composites and produces bisHPPP, which was selected as the marker of biodegradation used in this study. While bisHPPP may resemble BPA due to the existence of phenol rings, it is a distinctly separate chemical moiety and it will not degrade further into BPA.
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STATEMENT OF THE PROBLEM

A characterization of resin composite biodegradation *in vivo* is necessary in order to understand the material's clinical behaviour in terms of longevity and potential toxicity. Based on previous studies, resin composite biodegradation products are measurable via HPLC analysis from a rinse protocol, and a 2-minute mouth rinse with 20% ethanol in saline extracted the most product while remaining acceptable to the patient, as compared to a 4- or 8-minute rinse. However, all rinse protocols failed to identify any breakdown product 7 days after restoration placement. It is hypothesized that if biodegradation products are still present in the oral cavity 7 days after restoration placement, they may be contained in detectable amounts concentrated within sheltered areas such as the gingival crevice or within adherent materials such as plaque.

Previous studies also demonstrated that a bisGMA/TEGDMA based resin composite (Z100) showed a greater degree of biodegradation than a urethane modified bisGMA/TEGDMA composite (TPH) *in vitro*. It is important to determine if there are differences in biodegradation activity among various resin composite materials *in vivo*. This would have impact on the direction of material formulation and on material selection. Therefore, it is also hypothesized that different formulations of resin will experience varying levels of biodegradation, detectable by measurement of the amount of breakdown products.

This study initially focuses on establishing methodology protocols to measure composite resin biodegradation products released *in vivo*. Using the optimized measurement
protocol, we later compare the biodegradation products released from two different commercial composites, a urethane-modified-bisGMA based and a bisGMA based resin composite material in vivo.

**OBJECTIVES**

The following body of the thesis will be divided into two parts prepared in article format for journal submission.

The objectives of the first study (chapter two) are to establish and optimize methods for reproducibly measuring composite-resin biodegradation products released in vivo.

The second study (chapter three) aims to compare the biostability of a urethane modified and a traditional bisGMA based commercial composite; TPH and Z250, by measuring the amount of released bisHPPP in vivo. A secondary objective is to determine if any BPA is released from either resin composite material.
Chapter 2

Development of methods to measure *in vivo* biodegradation of resin composite: a pilot study
ABSTRACT

Objective: to establish methods for reproducibly measuring resin-composite biodegradation products released in vivo. Class V composite restorations were placed in adult patients (University of Toronto Research Ethics Reference #15482) using a three-step adhesive (Scotchbond MP™, 3M) and resin-composite (Z250, 3M). Three collection methods were compared in 10 patients: collection of rinse solution from a two-minute rinse protocol using saline mouth rinse with 20% ethanol, collection of plaque and collection of gingival crevicular fluid. Samples were collected prior to, immediately after, and 7 days after restoration placement. The samples were analyzed for the presence of resin degradation products using high performance liquid chromatography. The bisphenyl glycidyl dimethacrylate (bis-GMA) breakdown product, bishydroxypropoxyphenylpropane (bisHPPP), was detected only in the rinse media obtained from the oral cavity immediately after resin composite placement. Conclusion: a two-minute rinse protocol with 20% ethanol in saline is well-suited for in vivo analysis of resin composite biodegradation products.

INTRODUCTION

The average dental patient today has an increased expectation for aesthetic restorations and, despite the lack of supporting evidence, public concern over the release of mercury from amalgam restorations is a continuing problem (Mjor et al, 1999). The mechanical properties of current resin composite materials have improved, yielding resin restorations that are stronger, more versatile and more workable. As a result, the use of resin restorations is increasing (Leinfelder, 1993), and the need for improved understanding of the in vivo stability of composite materials is vital.

A composite is composed of two or more substances, with interatomic or molecular bonding between them, which together display properties greater than the sum of the individual parts (Dogon, 1990). Dental resin composites are made up of four main substances: an organic matrix, inorganic filler particles, coupling agents, and initiators or
accelerators along with a variety of other materials that contribute to the mechanical and esthetic properties of the resin (Craig, 2001). The organic matrices bisphenyl glycidyl dimethacrylate (bis-GMA) and triethylene glycol dimethacrylate (TEGDMA) are common in resin composite systems. These monomers contain ester linkages, which are susceptible to hydrolysis, a process that can be further catalyzed by salivary enzymes. Hydrolysis is the scission of condensation type bonds (i.e. esters, ethers, amides, etc.) by reaction with water (Figures 1 and 2). Enzymatic degradation, or biodegradation, products of resin composite include methacrylic acid (MA), bishydroxypropoxyphenylpropane (bisHPPP), triethylene glycol methacrylate (TEGMA), and triethylene glycol (TEG) (Santerre et al, 2001).

Figure 1: Schematic drawing of hydrolysis reactions for triethylene glycol dimethacrylate (TEGDMA): (A) TEGDMA monomer undergoing hydrolysis; and (B) partially cured TEGDMA undergoing hydrolysis.
Figure 2: Schematic drawing of degradation pathways of bisGMA: (A) hydrolysis of unreacted bisGMA monomer (B) producing bisHPPP as the end product (Finer, 2000)

The majority of studies on the breakdown of resin composites have focused on clinical function of the restorations, with few investigations specifically relating to biochemical degradation. Biodegradation of resin composite restorations leads to softening of the surface, which results in increased mechanical wear and marginal leakage leading to restoration failure. A number of studies suggest that biodegradation products could also have systemic and local effects on the host and bacterial cells. (Olea et al, 1996; Hansel et al, 1998; Arenholt-Bindslev et al, 1999; Mazzaoui et al, 2002).

Human saliva is a complex mix composed of fluids and particulates from salivary glands, gingival crevicular fluid, oral microorganisms, polymorphonuclear leukocytes, epithelial cells and food debris (Roth and Calmes, 1981; Nakamura and Slots, 1983; Edgar, 1992).
Saliva is approximately 99% water, with proteins such as immunoglobulins and enzymes (Edgar, 1990; Lindqvist et al, 1977. Cholinesterases are a class of esterase enzymes found in saliva that hydrolyze choline esters at a higher rate than other esters, and levels may increase in the presence of a biomaterial (Ryhanen et al, 1983; Labow et al, 1998). Cholesterol esterase- (CE) and pseudocholine esterase- (PCE) like activities are present in human saliva at levels capable of degrading polymerized dental composites (Finer and Santerre, 2003; Shokati et al, 2010). Saliva is a useful study tool as it is obtained easily and non-invasively, and it is already widely used as a screening and diagnostic tool for hormone and drug levels, caries risk assessment, periodontal disease parameters, salivary disease and infections (Mandell, 1993; Hofman, 2001; Liu and Delgado, 1999). As the saliva continuously coats the oral cavity, including dental restorations, biodegradation products from resin composite restorations can be collected in saliva samples.

Mouth rinses are a useful tool in collecting information from the oral environment, and are less invasive than blood or urine samples, which can be used to detect degradation products systemically (Fung, 2000). Some authors have used rinses as diagnostic tools in periodontal disease, and for collection of chemical compounds to be analyzed by High Performance Liquid Chromatography (HPLC) (Olea, 1996; Fakhry-Smith et al, 1997). More recently, resin composite degradation products have been isolated for HPLC analysis using saline alone and saline with ethanol rinses (Jaffer, 2005; Chiu, 2007).
Gingival crevicular fluid (GCF) is a fluid found in the gingival sulcus and is similar to serum exudate, with components from the subgingival bacterial biofilm and from cells of the gingival tissues (Toneti, 1998; Champagne et al, 2003). GCF can be collected via capillary tubes, filter papers or paper points. Most GCF studies originally involved volumetric quantification of the fluid as measured by weight, linear distance the fluid migrated on a paper, or via a commercial electronic measuring device (Griffiths et al, 1998). In addition, GCF has been used as a tool to recover inflammatory mediators in assessment of periodontal disease (Champagne et al, 2003). GCF collection is minimally invasive, quick and easy, with the added benefit of site specificity.

Dental plaque is a biofilm that forms on the supragingival and subgingival surfaces of teeth and is composed of an organized community of bacteria, which produce a protective and adhesive glycocalyx matrix (Ten Cate, 2006). Plaque incorporates components from the saliva and GCF, such as enzymes. Bacteria also generate hydrolytic enzymes (Fotos et al, 1990), and have been shown to adhere to the resin composite surface and tooth-restoration interface (Grieve et al, 1991). Thus plaque and plaque bacteria may play a direct role in local biodegradation, and Kawai and Tsuchitani (2000) reported that leachable monomers and degradative products accumulate in plaque. Plaque collection is also quick, easy, minimally invasive and provides a potential reservoir for biodegradation products of resin composite restorations.
Due to the difficulty in reproducing the complex oral environment, *in vivo* studies are needed to provide the most accurate information regarding the biodegradation of dental resin composites. Pilot studies have demonstrated that composite degradation products are measurable via high performance liquid chromatography (HPLC) analysis from several rinse protocols (Jaffer, 2005; Chiu, 2007). A 2-minute mouth rinse protocol with 20% ethanol in saline extracted the most biodegradation product while remaining acceptable to the patient (Chiu, 2007). However, all rinse protocols in both studies failed to identify any breakdown product 7 days after restoration placement (Jaffer, 2005; Chiu, 2007). It was hypothesized that if product is still present in the oral cavity, it may be contained in detectable amounts within sheltered areas such as the gingival crevice or within adherent materials adjacent to the restoration such as plaque. The goal of the current study was to establish methods for reproducibly measuring resin composite biodegradation products released *in vivo* at various time points after restoration. Three collection media were compared: oral rinse solution, GCF and plaque. HPLC, UV spectroscopy and mass spectrometry were used to analyze for the resin composite biodegradation products from the three collection media.

**NULL HYPOTHESIS**

There is no difference in the effectiveness of oral rinse, gingival crevicular fluid or plaque degradation collection methods to detect the resin composite biodegradation product, bisHPPP *in vivo*.

**MATERIALS & METHODS**
Resin composite material (Filtek™ Z250, 3M, London ON) was used with three-step adhesive (Scotchbond™ Multi-purpose Plus dental adhesive system, 3M, London, ON) for restorative procedures. The restorative materials were photo-polymerized using light curing systems (The Spectrum 800 Curing Unit, Dentsply Caulk, Canada; Demetron LC™, Kerr Corporation, Orange, CA or SmartLite LED Curing Light Model 1000, Dentsply).

Human ethics approval was granted by the University of Toronto Health Sciences Research Ethics Board (Protocol Reference #15482). Prior to entering the study, patients were provided with both verbal and written information, were given the opportunity to ask questions and if amenable to inclusion in the study, signed a consent form.

At the beginning of the initial clinic visit, the investigator (MM) established patient acceptability for inclusion in the study and obtained informed consent. Patient demographics (age, gender) and baseline information (Decayed missing and filled teeth index [DMFT], periodontal health, oral hygiene status and date of most recent composite restoration) were recorded. Patients included in the study were adult volunteers (>18 years, not pregnant or lactating) who were treatment planned for at least one Class V or III resin composite restoration with no occlusal contact. The majority of patients (55/58) were patients of the University of Toronto, Faculty of Dentistry, while the remainder (3/58) were patients from private practice. Patients accepted into the study must not have had a composite restoration placed in the preceding seven days, were willing and able to participate in the study including returning for a seven-day follow-up appointment, and
had a non-contributory medical and dental history (i.e. no xerostomia, bruxism or periodontal disease). Indications for restoration placement included caries, abrasion or abfraction lesions, and restoration replacement due to recurrent decay or colour changes of lost restorations.

Initial media samples included a 3-5 mL whole saliva sample for baseline CE-like enzyme activity analysis as described below (Lin et al, 2005). The patient sat upright in the dental chair while rolling the tongue to allow saliva to pool and then expectorated the saliva into the provided polyethylene tube. This was continued until 3-5mL volume was accumulated. A plaque sample was then collected from the buccal surface of the tooth to be restored with a sterile metal instrument (spoon excavator or periodontal probe), which was then transferred to a sterile paper point and placed in a sterile tube (Eppendorf™, Mississauga, Ontario). Following this, a GCF sample was collected. The tooth to be restored was air dried for five seconds and a sterile paper point was inserted 1-2mm into the gingival crevice for 30 seconds. The paper point was then placed into another sterile tube (Eppendorf™, Mississauga, Ontario). For the rinse collection, patients rinsed with 5 mL of 20% ethanol in 0.9% saline for 30 seconds and expectorated into a sterile 50 mL centrifuge tube. The procedure was repeated for a total of four rinses, providing 20 mL rinse solution in total. All samples were immediately placed in a -22°C freezer pending processing.

Class V restoration(s) were then prepared under local anaesthetic by the student and checked by a clinic demonstrator. Rubber dam or cotton roll isolation was used as
determined by the student and demonstrator. A photograph of the tooth preparation was obtained with a periodontal probe providing reference for surface area determination. The approximate surface area of each preparation was calculated using standardized digital photography and a computer software program, SigmaScan (SPSS Inc., Chicago, IL) using the periodontal probe as the standard for software calibration.

Adhesive was placed in the preparation according to manufacturer’s instructions. The preparation was then incrementally packed and photo-polymerized for 40 seconds with the restorative composite. The intensity of the photo-polymerization devices was routinely checked to verify that they exceeded the minimum intensity recommended by the International Standards Organizations (ISO) (Fan et al 2002).

The restoration was finished and polished with high speed multi-fluted burs and low speed aluminum oxide discs. Once the completed restoration was approved by the supervising clinical demonstrator, a second plaque, GCF and rinse sample were collected within 10 minutes.

Patients returned seven days following initial restoration placement, at which time a third round of plaque, GCF and rinse samples were collected and the patient was provided with $30 compensation. All samples were stored at -22°C until processing.

**Laboratory Procedures**

Whole saliva samples were analyzed for CE-like activity within one week of collection.
They were thawed, homogenized, and centrifuged at 2400rpm for 20 minutes at 4°C. Supernatant was filtered with a 0.8/0.2 µm syringe filter (Supor® Arcodisc®, 32mm syringe filter unit, Cat No. 4658, PALL Gelman Sciences, Ann Arbour, MI) and then analyzed with a UV spectrophotometer (Ultrospec II, LKB Biochrom Cambridge, England) using a CE activity protocol with para-nitrophenol butyrate (pNPB) as the enzyme substrate for measurement of baseline human salivary derived esterase activity (HSDEA) (Finer and Santerre, 2003; 2007; Shokati et al, 2010). One unit of esterase activity was defined as the amount of CE capable of generating 1 nmol of paranitrophenol per minute at 401nm, pH 7.0 and 25°C. Each saliva sample was measured for CE-like activity three times.

GCF samples were extracted with a sequential rinse cycle of sterile filtered water and sterile filtered methanol for a total of 3 water and 3 methanol rinses (0.5 mL each time). The sequential rinse solutions were pooled, then homogenized and centrifuged at 2400rpm for 20 minutes at 4°C. The supernatant was removed and filtered with a 0.8/0.2 µm syringe filter. All processed GCF collections were freeze-dried (Vertis Benchtop Freeze Dryer) and stored in the -80°C freezer until HPLC analysis.

Plaque samples were processed in a similar manner, with sequential extraction; repeated until the plaque had gone through a total of 3 water and 3 methanol rinses. The sequential rinse solutions were pooled, then homogenized and centrifuged at 2400rpm for 20 minutes at 4°C. The supernatant was removed and filtered with a 0.8/0.2 µm syringe
filter. All processed plaque collections were freeze-dried and stored in the -80°C freezer until HPLC analysis.

Rinse samples were homogenized then centrifuged at 2400rpm for 20 minutes at 4°C. The supernatant was removed and filtered with a 0.8/0.2 µm syringe filter. Excess ethanol was evaporated with nitrogen gas and the sample was placed in the -80°C freezer for two days, freeze dried, and stored in the -80 freezer until HPLC analysis.

The freeze-dried samples were reconstituted using 1mL of the mobile phase (0.4 mL HPLC grade methanol and 0.6 mL 2mM ammonium acetate buffer (pH 3.0)), filtered with a Millipore centrifuge filter at 4000rpm at 4°C and analyzed using reverse phase high performance liquid chromatography (HPLC) in combination with ultraviolet spectroscopy. A thirty minute gradient method established by Finer and Santerre was used on a Waters™ HPLC system (Finer and Santerre, 2004; 2003), using a Phenomenex Luna 5µm C18 column with HPLC grade methanol and buffered 2mM ammonium acetate (pH 3.0). BisHPPP was identified by comparing retention time of the peak and the UV spectra from the samples with those of bisHPPP standards and previous studies (Jaffer, 2005; Chiu, 2007). Final verification of product identity was performed by mass spectrometry (Perkin-Elmer/Sciex (Concord, ON, Canada) API-III triple-quadrupole mass spectrometer, (LC/MS/MS); Carbohydrate Research Center, University of Toronto, Ontario, Canada).
Statistical analysis was performed using SPSS software (SPSS version 16.0, SPSS Inc. Chicago, IL, USA). Amounts of bisHPPP were normalized using the surface area of restoration(s) placed, and compared using the Kruskall-Wallis test. The Pearson correlation analysis was conducted to investigate possible correlation between HSDE activity and amount of bisHPPP detected (both total and normalized values). P values of <0.05 were considered significant.

RESULTS

A total of 10 patients were enrolled in the study; nine were treated by 3rd or 4th year dental students at the University of Toronto undergraduate dental clinic, and one treated in private practice. Patients were labeled numerically from 1-10. The mean age of patients was 61 years with 50% male:female ratio. The mean DMFT (excluding third molars) was 21.9. All patients had fair to excellent oral hygiene and met the inclusion criteria.

Indications for restorations placed during the study were caries (7/10), and abfraction/abrasion (3/10). The number of restorations placed per patient ranged from 1-5 with a mean of 1.6. The mean (±SE) surface area for all patients was calculated to be 101.6 (± 58.2) mm².

The whole saliva samples collected at baseline were analyzed for HSDE activity. The mean HSDE activity for all patients was 10.3 (+/- 2.73) units/mL with a range of 1.83 to 31.0 units/mL, as shown in Figure 3.
Figure 3: HSDE activity (units/mL) in saliva for each patient and the mean (n=10).

One unit of esterase activity was defined as the amount of cholesterol esterase-like (CE-like) activity capable of generating 1nmol of pNPA per minute at 401nm, pH 7.0 and 25°C. Each saliva sample was measured for CE-like activity three times.

All of the three initial sample types (GCF, plaque, and rinse) were analyzed for the presence of bisHPPP. BisHPPP was not detected in any of these baseline samples.

Mean ±SE amount of bisHPPP levels (normalized for surface area) for rinse samples collected immediately after (within 10 minutes) restoration placement in all patients was 0.56 (± 0.24) µg/mm² (Figure 4). BisHPPP was not detected in any of the plaque or GCF
samples. BisHPPP was not detected in any of the samples from the 7 day follow-up visit. There was no significant correlation between HSDE activity and the normalized amount of bisHPPP detected from rinse samples obtained after restoration placement (p > 0.05).

Figure 4: Amount of bisHPPP (µg/mm²) isolated from rinse samples obtained immediately after restoration placement in each patient and the mean (n=10)

DISCUSSION

Esterase Activity in Human Saliva and Biodegradation

Levels of HSDE activity in baseline saliva samples were measured to explore the observed in vitro interaction between HSDEs and resin composites in the in vivo setting. In vitro
studies have firmly established that human salivary derived enzymes (HSDEs), containing both CE- and PCE-like activities are capable of degrading both monomers and polymerized resin composites (Finer and Santerre, 2003; 2007). CE degrades the commercial resin composite, Z250, more than PCE (Lin et al, 2005). The HSDE CE-like activity levels in the current study were in the ranges close to those found in previous studies (Jaffer, 2005; Chiu, 2007) and similarly showed a wide range of activity levels (Figure 3). There is natural variation in salivary flow and composition that occurs throughout the day. Salivary flow decreases with increasing age and is typically higher in men than in women (Rudney, 1995). Differences in salivary flow and composition potentially affect salivary esterase levels and subsequently, the exposure of resin composite restorations to esterase in the oral cavity.

There is evidence that salivary enzyme levels increase in the presence of periodontal disease, due to inflammatory and bacteria modulation of enzyme levels. (Nakamura and Slots, 1983; Zambon et al, 1985). All patients accepted into the study had at least fair oral hygiene and no evidence of active periodontal disease. Likewise, cariogenic bacteria such as S. mutans may increase enzyme levels (Mahler et al, 1957). As both periodontal disease and caries are common, the ability of bacteria to modulate enzyme activity should be better understood in vivo. This may have clinical relevance in the placement of resin composites in patients with varying levels of specific bacterial colonization. To date there is no evidence that periodontal disease or high caries rate influences the degree of biodegradation of resin composite restorations.
As with previous *in vivo* studies, there was no correlation between HSDE activity and amount of bisHPPP detected (Jaffer, 2005; Chiu; 2007). Finer and Santerre (2003) noted a dose dependant biodegradation response to enzyme activity *in vitro*. However, there was a saturation limit of 0.1 units/mL of CE, beyond which no further increase in degree of degradation was noted. All patients in the study had HSDE activity levels greater than the saturation level reported above. This explains the lack of dose response noted and suggests that despite the wide variation in HSDE activities, it was well above the saturation levels and therefore not corresponding to a similar variation the bisHPPP production.

The effect of inclusion of ethanol into the mouth rinse on esterase activity in this study was considered. An *in vitro* study by Finer (2000) demonstrated that the addition of methanol reduces CE activity. To investigate the effects of methanol *in vivo*, Chiu (2007) measured HSDE activity *in vivo* before and after the rinsing protocol in this study. No specific trend in change to HSDE activity was noted, and changes were attributed to natural circadian variation (Chiu, 2007).

**Biodegradation Products**

BisHPPP was used to measure resin biodegradation in this study. Resin composite biodegradation products include MA, bisHPPP, TEGMA, TEG. They are each of varying utility as markers of degree of biodegradation. TEGMA is derived from the cleavage of pendant TEGDMA molecules and can be further hydrolysed to TEG (Freund
and Munksgaard, 1990; Larsen et al, 1992; Munksgaard and Freund, 1990) (Figures 1-2). However, when resin composite samples were incubated in buffer alone, a high amount of leached TEGDMA, without any matrix degradation, was reported (Finer and Santerre, 2006). Therefore, TEGDMA and its degradation product TEGMA are not good representatives of enzymatic degradation of the resin matrix, as they relate to both leaching and degradation.

MA is a common end product for all unreacted or partially reacted methacrylate based monomers. Reacted bisGMA does not produce MA as a degradation product. One to two MA molecules are produced from the degradation of, respectively, pendant bisGMA or chain bisGMA (Figure 2) (Finer and Santerre, 2006). Freeze-drying is an integral part of the processing technique used in the current study and 90% loss of MA during freeze drying has been reported (Chiu, 2007). For these reasons, MA is not a useful marker for biodegradation using the methods of this study.

BisGMA has a high molecular weight, rigid, hydrophobic phenyl rings and hydrogen bonding capacity. All of these features contribute to a limited diffusion of unreacted BisGMA molecules out of the polymerized resin matrix. Therefore, negligible amounts of bisGMA are detected when resin composite samples are incubated in buffer solutions. Initial isolated bisHPPP is primarily attributed to unreacted and partially reacted bisHPPP within the resin matrix, while long term production of bisHPPP that is detected in vitro is primarily from degradation of pendant bisGMA or repeat segments within the
matrix (Finer and Santerre, 2004b, 2004a). BisHPPP is an end product; there is no further degradation once it reaches this point. Therefore the amount of bisHPPP in solution is a good marker of amount of resin matrix degradation.

**Biodegradation Kinetics**

*In vitro* studies suggested that there was a higher degree of degradation during initial time periods compared with later time periods in resin composite incubated with CE or HSDEs. The highest amount of bisHPPP was produced in the first 24 hours, with ~ 85% of bisGMA hydrolyzed in the first eight hours of incubation with HSDE (Jaffer *et al.*, 2002; Jaffer, 2005). Degradation continued at lower levels beyond 16 days up to 180 days (Lin *et al.*, 2004; Finer and Santerre, 2006; Shokati *et al.*, 2010). Kinetic studies demonstrated a higher amount of bisHPPP produced from Z250 in the initial 15 days, with a significant reduction by day nine, that decreased to a constant rate of degradation which continued in vitro for 30 days (Jaffer, 2005). It was hypothesized that the initial bisHPPP was largely derived from hydrolysis of the more accessible pendant groups and any free monomer. Due to their size and limited diffusion through the polymeric matrix, esterases have more difficulty accessing deeper sites. After the matrix surface is degraded, there is a subsequent drop in bisHPPP production, to a baseline of 2.9 +/- 0.5 ug/cm² per day. In the *in vivo* setting, continuous production of saliva and ingestion of fluids may continuously dilute any low levels of bisHPPP that may be present so that the bisHPPP levels were below the detection threshold of the HPLC system at later time periods. This may explain why bisHPPP was detected in the immediately post-restoration samples, but not in the seven-day post-restoration samples.
In this study, the bisHPPP results were normalized to the restoration surface area. This normalization was not done in original pilot study, but it was done for the subsequent pilot studies (Chiu, 2007). Finer and Santerre (2004a) observed that the rate of degradation products was dependent on the restoration surface area \textit{in vitro}.

BisHPPP was detected in the ppm range from rinse samples collected immediately after restoration. 0.56 ± 0.24 µg/mm² of bisHPPP/ restoration surface area was detected, which is comparable to levels detected in previous pilot studies (Jaffer, 2005; Chiu, 2007). The high degree of variation may be due to \textit{in vivo} differences among individual patients and differences in the timing of collection and freezing of the samples. HSDE is stable and once samples were collected, remaining activities could continue to degrade any monomer or mid-products present in the solution. All samples were frozen within 15 minutes of collection, however the time varied from 2-15 minutes. Some dental students completed the finishing and polishing procedure more quickly than others, and while samples were collected within 10 minutes of approval by an instructor, the overall time from initial polymerization to freezing of the sample may have ranged from 10-45 minutes.

In addition, the adhesive used in the current study contained bisGMA. A recent study demonstrated the susceptibility of the adhesive subject to enzymatic degradation, with 1.8 times more bisHPPP released from adhesive than from resin composite (Z250) (Shokati et al, 2010). There were a wide variety of operators, the vast majority of whom were
dental students who may have differed in the amount of adhesive initially placed and accuracy with which the adhesive was confined to the cavity preparation or excess removed. Any remaining excess adhesive would likely be poorly polymerized and available for immediate hydrolysis, thus affecting the immediate post-restoration samples. By seven days, the adhesive would be completely consumed and no longer contribute to any bisHPPP detected.

**Biodegradation Collection Methods**

It was hypothesized that bisHPPP may be concentrated in the plaque or GCF immediately adjacent to the restoration site, which would allow for a more sensitive test to detect bisHPPP than the rinse sample. However, bisHPPP was not detected in either plaque or GCF samples immediately after or seven days after restoration placement. GCF is turned over 40 times every minute, so while degradation products such as bisHPPP may be concentrated close to the restoration surface, it is likely that the lack of bisHPPP detected in GCF samples was due to the very small volume collected, as well as continued outflow of GCF to the oral cavity where it mixed with and became a component of the saliva.

Plaque collection presented a unique challenge in that all patients had varying levels of plaque present for sampling, and tended to be a very small volume. BisHPPP was not detected in plaque samples collected immediately after restoration placement, which was likely due to two additional reasons. First, plaque immediately adjacent to the restoration site was removed during the finishing and polishing process during placement. Therefore
immediate post-restoration plaque samples were collected from teeth adjacent to the tooth that was restored, rather than the tooth with the resin composite restoration. Second, very little time (approximately 10 minutes) passed from restoration completion to sample collection. This provided little time for bisHPPP to infiltrate and incorporate within the plaque that was present.

BisHPPP was not detected in any of the baseline GCF, plaque or rinse samples in any patients. Thus, any bisHPPP detected was considered to have come from the current restoration and not from previously placed restorations. These results suggest that GCF and plaque are not as effective in detecting degradation products from resin restoration and therefore the null hypothesis, that “There is no difference in the effectiveness of oral rinse, gingival crevicular fluid or plaque degradation collection methods to detect the resin composite biodegradation product, bisHPPP in vivo” is rejected.

BisHPPP was not detected in rinse samples obtained seven days after restoration placement. These findings are consistent with findings from other in vivo studies. Pilot studies on Z250 restorations detected bisHPPP in a proportion of patients immediately after restoration placement, but none seven days later (Jaffer, 2005; Chiu, 2007). Degradation products from resin sealant placement were detected up to one to three hours after restoration placement, with no product detected at a later time period (Olea et al, 1996: Arenholt-bindslev, 1999; Fung et al, 1999).
Bacteria can contribute to biodegradation of resin composite. Therefore it can be hypothesized that plaque could increase the rate of composite degradation, as well as act as a reservoir for bisHPPP. This would result in seven-day post-restoration samples that might have more bisHPPP than initial samples. However, bisHPPP was not detected in the seven-day samples. Regardless of the amount of plaque present at seven days, any bisHPPP, if present, was below the detection limits of the system. Therefore, the postulated capacity for plaque to act as a reservoir for bisHPPP was not demonstrated in this study. Oral hygiene procedures and the reduced production levels of bisHPPP over time are possible explanation for this.

**CONCLUSIONS:**

1. BisHPPP, derived from the enzymatic degradation of matrix components of resin composite restorations by salivary enzymes, was identified in all patients at levels of µg /mm² of restoration surface area immediately after restoration placement, using a 20% ethanol in saline rinse for 2 minutes.

2. In contrast, no biodegradation products were detected in baseline or 7-day post-restoration samples, or in any GCF or plaque samples from the same patients.

3. There was no correlation between amount of degradation product and baseline HSDE CE-like activity.
4. Future studies will aim to investigate the comparative levels of degradation product from different commercial resin composite systems \textit{in vivo}. The 20\% ethanol in saline 2-minute rinse protocol will be chosen for future studies because of excellent collection capabilities and acceptance by patients.

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Chapter 3

*In vivo* biodegradation of urethane-modified and bisGMA based resin-composites
ABSTRACT

Aim: to compare levels of degradation of a urethane-modified and bisphenyl glycidyl dimethacrylate (bisGMA)-based resin-composites in vivo. Methods: Class V or III composite restorations without occlusal contacts were placed in adult patients (University of Toronto Research Ethics Reference #15482) using 3-step adhesive (Scotchbond MP® 3M) and one of two resin-composites; a bisGMA-based (Z250, 3M)(n=28) or a urethane modified bisGMA-based composite (Spectrum TPH, DENTSPLY), (n=30). Patients followed a 2-minute rinse (saline containing 20% ethanol) protocol before, immediately after, and 7 days after restoration placement. The rinse samples were analyzed for the presence of (bisGMA) breakdown product, bishydroxypropoxyphenylpropane (bisHPPP), and bisphenol-A (BPA), using high performance liquid chromatography. Results: BisHPPP was detected from both composites only in rinse samples immediately after resin composite placement (0.59±0.16 and 0.68±0.16 for Z250 and TPH respectively, p=0.767). BPA was not detected in any rinse samples. Resin type had no significant effect on the biostability of the composite.

INTRODUCTION

Dental resin composites are made up of four main substances: an organic matrix, inorganic filler particles, coupling agents, and initiators or accelerators along with a variety of other materials that contribute to the properties of the resin (Craig, 2001). Resin-composites can be degraded through a variety of mechanisms, including disintegration and dissolution from materials in saliva, wear and erosion by mastication, interactions with foods and drugs, and bacterial activity (Olio, 1992). The majority of studies published have focused on physical breakdown of the resin-composite; including softening, stress or fatigue fracture and wear. These processes are enhanced by chemical degradation, known also as biodegradation. However, there is relatively little information in the literature regarding the chemical stability of dental resin-composites (Santerre et al., 2001).

Resin-composites are prone to both leaching in water and hydrolytic degradation. Hydrolytic degradation involves the cleavage of the C-O bond between the acyl group


and the oxygen in the unprotected ester linkage (Figure 1), resulting in pores through which degradation products are released (Jaffer et al., 2001; Gopferich, 1996).

Figure 1: Schematic drawing of degradation pathways of bisGMA: (A) hydrolysis of unreacted bisGMA monomer (B) producing bisHPPP as the end product (Finer, 2000)

This process is further catalyzed by enzymes, particularly esterases. Incubation of bisphenyl glycidyl dimethacrylate (bisGMA)-based resin-composites with enzymes increases both loss of mass and surface degradation as compared to incubation in buffer alone (Munksgaard and Freund, 1990; Finer and Santerre, 2004b). Concerns have been raised by studies suggesting that biodegradation products could also have systemic and local effects on the host and bacterial cells. (Olea et al., 1996; Hansel et al., 1998; Arenholt-Bindslev et al., 1999; Mazzaoui et al., 2002; Khalichi et al., 2009).
Bis-GMA and triethylene glycol dimethacrylate (TEGDMA) are commonly used monomers in resin-composite systems (Santerre, 2001). Filtek Z250 (3M) is a bisGMA and TEGDMA based resin-composite. The hydrolysis of these monomers produces methacrylic acid (MA), bishdroxypropoxyphenylpropane (bisHPPP) and triethylene glycol methacrylate (TEGMA) (Finer and Santerre, 2004, 2007). Hydrolysis of Spectrum TPH (DENTSPLY), which is a urethane modified bisGMA and bisphenol A polyethylene glycol diether dimethacrylate (bisEMA) based composite, yields the same breakdown products, as well as ethoxylated bisphenol A (EBP-A) (Shajii and Santerre, 1999; Finer and Santerre, 2004b). It is not expected that the biodegradation of commonly used resin composites will produce BPA. BisGMA is a major component in dental resin-composites and produces bisHPPP, which was selected as the marker of biodegradation used in this study. While bisHPPP may resemble BPA due to the existence of phenol rings, it is a distinctly separate chemical moiety and it will not degrade further into BPA.

The above studies found that the level of biodegradation was influenced by the resin's formulation. For example, Finer and Santerre (2004b) demonstrated a 2.6 to 86 fold increased release of degradation products from bisGMA/TEGDMA based resin-composites than urethane modified bisGMA/TEGDMA composites in vitro. The authors attributed this difference in degradation to increased stability of the urethane-modified bisGMA to cholesterol esterase (CE) and human salivary derived esterase (HSDE) hydrolysis (Finer and Santerre, 2004b).
HSDEs are capable of degrading bisGMA and TEGDMA monomers within 24 hours (Jaffer et al., 2002). Enzyme activity has been specifically identified in human saliva, at levels capable of degrading bisGMA and TEGDMA monomers, and the commercial resin-composites Z250 and TPH (Lin et al., 2005). Kinetic studies have demonstrated that the combination of CE and PCE produced a synergistic degradation reaction on both formulations (Finer et al., 2004c). This may have been due to increased stability of CE in the presence of PCE.

Previous studies showed that resin-composite degradation products are released into the oral cavity after restoration placement using a 2-minute mouth rinse protocol with 20% ethanol in saline (Jaffer, 2005; Chiu, 2007). This sampling protocol was shown to be more effective than other methods (saline rinses without ethanol, plaque and gingival crevicular fluid collections) and can be used to assess the biochemical stability of different resin formulation in vivo, providing useful information regarding material selection and product development. The objective of this study was to compare the biostability of two different commercial composites, Z250 and TPH, by measuring the amount of released bisHPPP in vivo, and to determine if any BPA is released from either product.

**NULL HYPOTHESIS**

The in vivo biostability of resin composites, as measured by the levels of biodegradation products, is not affected by their formulations.
MATERIALS & METHODS

Restorative materials

A bisGMA based (Filtek™ Z250, 3M Canada Inc, London ON)(Z250) and a urethane modified bisGMA based (Spectrum TPH, Dentsply Caulk, Canada)(TPH) composite material were used with a three-step adhesive system (Scotchbond™ Multi-purpose Plus dental adhesive system, 3M Canada Inc, London, ON) for restorative procedures. The Spectrum 800 Curing Unit, SmartLite LED Curing Light Model 1000 (Dentsply Caulk, Canada), and Demetron LC™ (Kerr Corporation, Orange, CA) light curing units were used for photo-polymerization.

Clinical Protocol

Human ethics approval was granted by the University of Toronto Health Sciences Research Ethics Board (Protocol Reference #15482). Prior to entering the study, patients were provided with both verbal and written information, were given the opportunity to ask questions and if amenable to inclusion in the study, signed a consent form.

At the beginning of the initial clinic visit, the investigator (MM) established patient acceptability for inclusion in the study, and obtained informed consent. Patient demographics (age, gender) and baseline information (Decayed missing and filled teeth index [DMFT], periodontal health, oral hygiene status and date of most recent composite restoration) were recorded. Patients included in the study were adult volunteers (>18
years, not pregnant or lactating) who were treatment planned for at least one Class V or III resin composite restoration with no occlusal contact. The majority of patients (55/58) were patients of the University of Toronto, Faculty of Dentistry, while the remainders (3/58) were patients from private practice. Patients accepted into the study must not have had a composite restoration place in the preceding seven days, were willing and able to participate in the study including returning for a seven-day follow-up appointment, and had a non-contributory medical and dental history (i.e. no xerostomia, bruxism or periodontal disease). Indications for restoration placement included caries, abrasion or abfraction lesions, and restoration replacement due to recurrent decay or colour changes of lost restorations.

Initial saliva samples included a 3-5 mL whole saliva sample for baseline enzyme activity analysis. The patient sat upright in the dental chair while rolling the tongue to allow saliva to pool and then expectorated into the provided polyethylene tube. This was continued until the 3-5 mL of saliva was accumulated. Next, 20 mL of rinse solution was collected. Patients rinsed with 5 mL of 20% ethanol in 0.9% saline for 30 seconds, expectorated into a sterile centrifuge tube, and repeated this for a total of four rinses. All saliva and rinse samples were immediately placed in a -22 °C freezer pending processing.

The patient’s regularly assigned 3rd or 4th year dental student performed the restorative procedure in the undergraduate clinics at the University of Toronto Faculty of Dentistry under the supervision of a clinic demonstrator. Rubber dam or cotton roll isolation was used as determined by the student and demonstrator. Once the tooth was prepared, a
photograph was obtained with a periodontal probe providing reference for surface area determination. The surface area of each preparation was calculated using standardized digital photography and a computer software program, SigmaScan (SPSS Inc., Chicago, IL), using the periodontal probe as the standard for software calibration.

Adhesive was placed in each preparation as per manufacturer instructions. The tooth preparation was incrementally restored using the assigned composite material. Patients 1-10 were restored with Z250, patients 11-20 were restored with TPH and remaining patients were randomly assigned a resin composite type using a random numbers table. Each composite increment was photo-polymerized for 40 seconds. The intensity of the photo-polymerization devices was routinely checked to verify a minimum intensity as recommended by the International Standards Organizations (ISO) (Fan et al 2002).

The restoration was then finished and polished with high-speed multi-fluted burs and low speed aluminum oxide discs. Once the supervising dentist approved the completed restoration, a second rinse sample, using the protocol described above, was collected within 10 minutes.

Patients returned seven days following initial restoration placement, at which time another rinse sample was collected and the patient was provided with $30 compensation. All samples were stored at -22°C until processing.
Saliva and Rinse Sample Analyses

Baseline saliva samples were analyzed within one week of collection. They were thawed, homogenized then centrifuged at 2400 RPM for 20 minutes at 4°C. Supernatant was filtered with a 0.8/0.2 µm syringe filter (Supor® Arcodisc®, 32mm syringe filter unit, Cat No. 4658, PALL Gelman Sciences, Ann Arbour, MI) and then analyzed with a spectrophotometer (Ultrospec II, LKB Biochrom Cambridge, England) using a CE activity protocol with para-nitrophenol butyrate (pNPB) as the enzyme substrate for measurement of baseline human salivary derived esterase (HSDE) activity (Lin, 2005). One unit of esterase activity was defined as capable of generating 1 nmol of p-nitrophenol per minute, as measured at 410nm, pH 7.0 and 25°C.

Rinse samples were thawed, homogenized, and centrifuged at 2400 RPM for 20 minutes at 4°C. The supernatant was removed and filtered with a 0.8/0.2 µm syringe filter, excess ethanol was evaporated with nitrogen gas and the sample was then placed in the -80°C freezer for two days, freeze dried (Vertis Benchtop Freeze Dryer) and returned to the -80°C freezer until processing.

The freeze-dried samples were reconstituted using 1 mL of the mobile phase (0.4 mL HPLC grade methanol and 0.6 mL 2mM ammonium acetate buffer (pH 3.0)), filtered with a Millipore centrifuge filter at 4000 RPM at 4°C and analyzed using reverse phase high performance liquid chromatography (HPLC) in combination with ultraviolet spectroscopy. A 30 minute gradient method was used on a Waters™ HPLC system
(Finer and Santerre, 2004), using a Phenomenex Luna 5µm C_{18} column with HPLC grade methanol and pH 3 buffered 2mM ammonium acetate. Based on preliminary HPLC analyses using bisHPPP and BPA standards, bisHPPP was identified at a retention time of 17.1 minutes, and BPA at 24.7 minutes. Final verification of product identity was performed by mass spectrometry; HPLC fractions of interest were collected and then analyzed via a Perkin-Elmer/Sciex (Concord, ON, Canada) API-III triple-quadrupole mass spectrometer, (LC/MS/MS) located in the Carbohydrate Research Center, University of Toronto, Ontario, Canada.

Statistical analysis was performed using SPSS software (SPSS version16.0, SPSS Inc. Chicago, IL, USA). Chi-square and independent t-tests were used to check for differences in patient demographic, the Mann-Whitney non-parametric test was used to compare levels of bisHPPP detected for each resin-composite, and Pearson correlation test was used to analyze for correlation between esterase activity and amount of bisHPPP. P values of p<0.05 were considered significant.

**RESULTS**

In total, 58 patients were enrolled in the study. Thirty patients had restorations placed using Z250, while 28 were placed with Spectrum TPH.

The oral hygiene of all patients was deemed to be at least fair, and no patient demonstrated signs of xerostomia or periodontal disease. Indications for restoration
placement were caries (19/28 Z250, 19/30 TPH), abfraction or abrasion (8/28 Z250, 8/30 TPH) and esthetics (1/28 Z250, 3/30 TPH). Patient demographics are summarized in Table 1. There were no significant differences in age (p = 0.76, t-test), DMFT (p = 0.06, t-test), gender (p = 0.80, Chi-square), or incidence in use of rubber dam (p = 0.22, Chi-square) between the two groups.

Table 1: Descriptive statistics and analysis of differences between demographic variables of the 2 experimental groups

<table>
<thead>
<tr>
<th></th>
<th>Z250</th>
<th>Spectrum TPH</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sample Size (n)</strong></td>
<td>28</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td><strong>Gender (% male)</strong></td>
<td>43% male</td>
<td>47% male</td>
<td>*0.798</td>
</tr>
<tr>
<td><strong>Age (mean +/- se)</strong></td>
<td>54.79 (2.41)</td>
<td>53.60 (2.98)</td>
<td><strong>0.760</strong></td>
</tr>
<tr>
<td><strong>DMFT (mean +/- se)</strong></td>
<td>19.32 (1.09)</td>
<td>16.17 (1.19)</td>
<td><strong>0.056</strong></td>
</tr>
<tr>
<td><strong>Rubber Dam (% yes)</strong></td>
<td>32% with RD</td>
<td>17% with RD</td>
<td><em>0.224</em></td>
</tr>
</tbody>
</table>

* Chi-square test
**independent t-test

Mean esterase activity in the saliva of the participants is depicted in Figure 2. The overall mean (+/-SE) esterase activity was 23.4 (+/- 1.9) units/mL, 22.6 (+/- 2.8) units/mL in the Z250 group, and 24.1 (+/- 2.1) units/mL in the TPH group (statistically not significantly different, p = 0.69, t-test). There was no statistically significant correlation between
esterase activity and amount of degradation product obtained (Pearson correlation test, 
$r=0.103$, $p=0.413$).

Figure 2: HSDE activity (units/mL) in saliva for all patients in each restorative material 
group (n=58, mean =23.4 +/- 1.87 units/mL). One unit of esterase activity was defined 
as the amount of cholesterol esterase-like (CE-like) activity capable of generating 1nmol of 
pNPA per minute at 401nm, pH 7.0 and 25ºC

The results of the HPLC analyses for the detection of composite degradation products are 
presented in Figures 3 and 4. BisHPPP was isolated only immediately after restoration 
placement in 26/28 and 27/30 of Z250 and TPH groups respectively. The bisHPPP 
collected from the Z250 group (0.59 +/- 0.16 µg/mm²) was not significantly different 
from the TPH group (0.68 +/- 0.16 µg /mm², $p=0.767$). The mean (+/- SE) amount of
bisHPPP normalized to surface area of restorative material detected from all patients was 0.64 +/- 0.11 µg/mm². BisHPPP was not isolated from baseline or seven-day samples.
Figure 3: Amount of bisHPPP (µg/mm²) isolated from rinse samples obtained immediately after restoration placement using (A) Z250 or (B) TPH.
BPA was not detected in the collected rinse solution at any time point for any patient and material.

**DISCUSSION**

**Esterase activity in Human saliva**

Human saliva contains esterase activities at levels that are capable of catalyzing the degradation of commercial resin composites (Santerre *et al*, 1999; Finer and Santerre, 2003). Esterase activity in the current study was found to be within the ranges demonstrated in previous studies (Jaffer, 2005; Chiu, 2007). As with the previous
studies, these ranges were found to be broad. This high degree of variability is likely due to normal variation within the study population. For example, natural variations in salivary flow rates occur throughout the day. Salivary flow rates decrease with increasing age and are typically higher in men than in women (Rudney, 1995). These differences potentially affect the exposure levels of resin composite restorations to esterase in the oral cavity. It was therefore important that there were no significant differences in age or gender between the two study groups.

There is evidence that salivary esterase levels increase in the presence of periodontal disease, due to inflammatory and bacterial modulation of enzyme levels. (Nakamura and Slots, 1983; Zambon et al, 1985). In addition, cariogenic bacteria such as S. mutans may increase salivary enzyme levels (Mahler et al, 1957). All patients accepted into the study had at least fair oral hygiene and no evidence of active periodontal disease. There was no significant difference in the incidence of caries as the indication for restoration placement between the two study groups. To date there is no evidence that indication for restoration influences the degree of biodegradation. As both periodontal disease and caries are common clinical conditions, the ability of bacteria to modulate enzyme activity should be better understood in vivo.

**Biodegradation**

As with previous in vivo studies, there was no correlation between HSDE activity and detected bisHPPP levels (Jaffer, 2005; Chiu; 2007). Finer and Santerre (2003) noted a
dose dependent biodegradation response to enzyme activity \textit{in vitro}. However, there was a CE saturation limit of 0.1 units/mL, beyond which no further increase in degree of degradation was noted. All patients in the study had HSDE activity levels greater than the saturation level, explaining the lack of a dose response relationship, and suggesting that the wide variation in HSDE activity had no significant effect on the variation in bisHPPP detected, as long as it is above the saturation levels of the enzyme.

Resin composite biodegradation products include MA, bisHPPP, TEGMA, and TEG. They are each of varying utility as markers for the degree of biodegradation. TEGMA is derived from the cleavage of pendant TEGDMA molecules and can be further hydrolysed to TEG and MA (Larsen et al, 1992; Munksgard and Freund, 1990). When resin composite samples were incubated in buffer alone, a high amount of leached TEGDMA, without any matrix degradation was reported (Finer and Santerre, 2006). MA is a common degradation end product for all methacrylate based monomers, and different forms of unreacted or partially reacted bisGMA produce different amounts of MA as a result of degradation. Reacted bisGMA does not produce MA as a degradation product, while one to two MA molecules are produced from the degradation of, respectively, pendant or chain bisGMA (Finer and Santerre, 2006). Freeze-drying is an integral part of the processing technique and 90% loss of MA during freeze drying this procedure has been reported (Chiu, 2007). For these reasons, MA is not a useful marker for biodegradation using the methods of this study.
BisGMA has a high molecular weight, rigid hydrophobic phenyl rings and hydrogen bonding capacity. All of these features contribute to limited diffusion of bisGMA out of polymerized resin. Therefore, negligible amounts of bisGMA are detected when resin composite samples have been incubated in buffer solutions. Initial isolated bisHPPP is primarily attributed to unreacted and partially reacted bisHPPP within the resin matrix, while long term production of bisHPPP that is detected in vitro is primarily from degradation of pendant bisGMA or repeat segments within the matrix (Finer and Santerre, 2004b, 2004a). Since bisHPPP is a degradation end product of bisGMA, the amount of bisHPPP in solution is a good marker of the total amount of resin matrix degradation that has occurred.

The HPLC protocol developed by Finer and Santerre was used to measure the amount of bisHPPP in all samples. Baseline chromatograms from the HPLC demonstrated multiple peaks similar to those present in the pilot studies (Jaffer, 2005; Chiu, 2007). The retention times and UV spectra forms of these peaks did not correlate with those of bisHPPP. The pattern observed resembled the chromatogram for HSDE alone, with the authors of the pilot studies concluding that the peaks were likely due to organic contaminants and saliva components extracted from the rinse solution (Jaffer, 2005; Chiu, 2007).

The mean retention time for bisHPPP in this study was 17.1 minutes. This fell between the times of the previous in vivo pilot studies at 16.9 minutes and 19.5 minutes (Jaffer,
Differences in retention time were believed to be due to minor differences in the individual HPLC columns used and their aging.

**Biodegradation Kinetics**

BisHPPP was not detected in initial rinse samples for any patient. Thus, any bisHPPP detected was considered to have come from the newly placed restoration.

BisHPPP was not detected in any sample obtained seven days after restoration placement, which is consistent with findings from other *in vivo* studies. Pilot studies on Z250 restorations detected bisHPPP in a proportion of patients immediately after restoration placement, but none 7 days later (Jaffer, 2005; Chiu, 2007). Degradation products from resin sealant placement have been detected up to 3 hours after restoration placement, with no product detected at a later time period (Olea *et al.*, 1996; Arenholt-Bindslev, 1999; Fung *et al.*, 1999).

Previous *in vitro* studies suggested that there was a higher degree of degradation in resin composite incubated with CE or HSDEs during the earlier time periods. The highest amount of bisHPPP was produced in the first 24 hours, with ~ 85% of the bisGMA that is hydrolyzed in the first eight hours of incubation with HSDE (Jaffer *et al.*, 2002; Jaffer, 2005). Degradation continued at lower levels up to 180 days (Finer and Santerre, 2006; Lin *et al.*, 2004; Shokati *et al.*, 2010). Another kinetic study on biodegradation have demonstrated a higher amount of bisHPPP produced from Z250 in the initial 15 days, with a significant reduction by day nine, and a constant rate degradation that continues
beyond day 30 (Jaffer, 2005). It was hypothesized that the initial bisHPPP was largely derived from hydrolysis of the more accessible pendant groups and any free monomer. Esterases have more difficulty accessing deeper sites as the matrix is degraded, leading to a subsequent drop in bisHPPP measurement, to a baseline of 2.9 +/- 0.5 ug/cm\(^2\) each day. *In vivo*, continuous production of saliva and ingestion of fluids may continually dilute any bisHPPP present at the time of the later sample collection to below the ppb detection limits of the HPLC system.

In this study, both the Z250 and TPH resin-composite materials were subject to salivary enzyme degradation. Finer and Santerre (2004a) observed that the rate of degradation products was dependent on exposed restoration surface area, *in vitro*. Therefore, the results in this study were normalized to amount of degradation product, bisHPPP, per square millimeter of restoration in each patient.

**Effects of Resin Formulations on Biodegradation**

BisHPPP was detected at levels comparable to the degradation of Z250 in previous *in vivo* studies (Jaffer, 2005; Chiu, 2007). Samples collected immediately after the restorative procedure, were collected within 10 minutes of being checked and approved by a clinic instructor. In the Z250 samples, 0.59 \(\mu g/mm^2\)\(\pm\) 0.16 of bisHPPP/mm\(^2\) was detected at this time, which is similar to those levels detected in previous pilot studies (\(p= 0.443\)). The TPH samples produced 0.68 \(\mu g/mm^2\)\(\pm\) 0.16 of bisHPPP, which was not significantly different from the Z250 (\(p=0.767\)). This is in marked contrast to previous *in vitro* studies, where significantly more bisHPPP was detected from the Z250 samples as
compared with TPH. Jaffer, et al (2002) found a 10 fold higher level of bisHPPP from the \textit{in vitro} breakdown of Z250 than TPH. The authors attributed this difference in degradation to increased resistance of the urethane-modified bisGMA in TPH to hydrolysis by CE or HSDE (Finer and Santerre, 2004b).

The differences in results mentioned above may be partially a reflection of the fundamental differences between the controlled \textit{in vitro} conditions and the variable clinical setting. In the \textit{in vitro} study, the photo-polymerization protocol was very regimented and included a period of vacuum curing. It is possible that the two materials have differing sensitivities to light under normal clinical conditions, and the enhanced polymerization procedure benefited the TPH more than the Z250, resulting in less degradation product. As well, the polymerization kinetics of the materials may be different, which in part may compensate for the inherent biostability differences between the two commercial materials.

There was also a marked difference in the duration of incubation and timing of sample collection between the \textit{in vitro} and \textit{in vivo} studies. \textit{In vivo} collection occurred for 2 minutes less than one hour after initial polymerization, while \textit{in vitro} collection occurred much longer after initial polymerization. Additionally, \textit{in vitro} samples were soaked for 24 hours to remove leachable components prior to incubation. Although bisGMA demonstrates limited leachability, it is possible that some product leached from either one or both of the composites \textit{in vivo}.
HSDE demonstrated a stable CE-like activity, and once samples were collected it may have continued to degrade any residual monomer in solution. All samples were frozen within 15 minutes of collection (range 2-15 minutes). Some dental students completed the finishing and polishing procedure more quickly than others, and while samples were collected within 10 minutes of approval by an instructor, the overall time from initial polymerization freezing of the collected sample may have ranged from 10-45 minutes. *In vitro* and *in vivo* biodegradation kinetics in the periods immediately following initial polymerization are not well known, and the *in vivo* kinetics may be significantly different from *in vitro*. Perhaps the early kinetics of Z250 and TPH degradation are more similar and it is not until later stages that differences in susceptibility to degradation appear.

Some *in vitro* specimens were highly polished in a controlled laboratory setting, while the *in vivo* samples were much less regimented. Polishing removes the matrix rich surface and presents less matrix available for degradation (Finer and Santerre, 2004). In the Finer and Santerre study, unpolished bisGMA specimens exhibited rougher surface than that of the urethane modified samples. Since the biodegradation of bisGMA is surface dependent, this can explain to some extent the lower release of bisHPPP from the urethane modified composites *in vitro*. In the current *in vivo* study, all restorations were polished prior to rinse sample collection, potentially resulting in more similar restoration surfaces. In addition particulate dust, created during the *in vivo* finishing and polishing procedure, may alter the overall surface area and increase the amount of available bisGMA to enzyme interaction. It was hypothesized that the use of a rubber dam could influence the amount of detectable degradation product by preventing excess particulate
from entering the oral cavity. There was no difference in the use of rubber dam between groups, and no correlation with use of rubber dam and amount of degradation product produced.

In enzyme degradation of resin composites there are three main steps: 1) enzyme transport through the solution to the surface of the resin, 2) reaction of enzyme with the resin, and 3) release of degradation product into solution (Finer and Santerre, 2006). In the in vitro system there is limited physical flow of enzyme and biodegradation product to and from the surface of the resin. The in vivo environment on the other hand involves many more forces related to the flow of saliva and enzymes, which may all contribute to differences in the amount of degradation occurring in vivo vs. in vitro, and also to the amount of degradation product detected at any set time point.

Another possible contributing factor in vivo is the effect of adhesive on the degradation product detected. The adhesive used in the current study contained bisGMA. A previous study demonstrated the susceptibility of the adhesive subject to enzymatic degradation, with 1.8 times more bisHPPP released from adhesive than from resin composite (Z250) (Shokati et al, 2010). The surface area of adhesive available for degradation should be very small and it is unlikely to contribute a significant amount of bisHPPP to the overall amount measured. However, there were a wide variety of operators, the vast majority of whom were dental students who may have differed in the amount of adhesive initially placed and accuracy with which the adhesive was confined to the cavity preparation or excess removed. Any remaining adhesive would likely be poorly converted and available
for immediate hydrolysis, thus affecting the post-restoration samples. By seven days, the adhesive would be completely consumed and no longer contribute to any bisHPPP collected at this time.

The results of the current study suggest that there is no detectable difference in the \textit{in vivo} biostability of the two composite and therefore we were unable to reject the null hypothesis, that “the \textit{in vivo} biostability of resin composites, as measured by the levels of biodegradation products, is not affected by their formulations.

\textbf{Potential Estrogenicity of Degradation Products}

BPA has received a lot of recent media coverage for its reported estrogenic activity, and has been reported as a degradation product from resin sealants (Olea \textit{et al}, 1996; Arenhold-Binslev \textit{et al}, 1999, Fung \textit{et al}). However, \textsc{Concise}\textsuperscript{TM} (3M, ESPE) is the only commercial resin composite containing BPA as a listed ingredient. BPA is not a degradation product of bisGMA or bisGMA-based composites. Production of BPA from bisGMA would require the cleavage of the stable ether bond near the phenol rings. This does not happen under physiological or physiologically relevant conditions. BPA can be produced from materials containing bisphenol-A dimethacrylate (bisDMA), in a manner similar to the production of bisHPPP from bisGMA, i.e. via hydrolysis of the ester bonds at either side of the molecule. However, bisDMA is not a component of either Z250 or TPH.
The degradation of monomers in Z250 and TPH will thus not produce BPA, and if any BPA were present it would be as a contaminant since BPA is used in the initial formulation of bisGMA. As BPA exposure is of concern to many patients today, all post restoration rinse samples were checked for the presence of BPA. BPA was not detected in any patients at any time periods from either resin composite, at a detection limit of ppb.

**Summary**

This study demonstrated the enzymatic biodegradation of two resin composite restorative materials _in vivo_ by the detection of biodegradation product in the oral cavity at µg or ppm levels. Although _in vitro_ differences have been reported, there were no significant differences in the amount of biodegradation products from the two resin composite materials _in vivo_. If a difference in biodegradation between different formulations of resin composites exists, it may have been masked by the presence of confounding factors such as operator variation, adhesive and sample collection methods variation. The two composite materials that were used are based on the most commonly used formulations for resin matrices in dentistry and it can be postulated that enzymatic biodegradation of other commonly used resin composite restorative materials will occur _in vivo_. The duration and degree of biodegradation observed in this _in vivo_ study, however, was very limited. _In vivo_, the effect of biodegradation on the bulk properties of resin-composite restorations has not been completely elucidated. Other physical processes, such as mechanical wear as a result of mastication, may play a greater role in the
breakdown of resin-composite bulk. The effect of biodegradation may have a more critical effect at localized susceptible regions of the restoration than throughout the bulk of the composite material. There is evidence that suggests that biodegradation may play a more significant role at the interfacial margin or in regions of bacterial accumulation. The creation of defects at these critical regions could readily propagate and reduce significantly the clinical service of these restorations. The effects of the measured *in vivo* biodegradation product, bisHPPP, on the host, as well as on the bacteria, are not well investigated. BPA, associated with estrogenic activity, is not a degradation product of TPH or Z250 and was not detected in the oral rinse solution from patients with recently placed resin composite restorations.

**CONCLUSIONS**

1. The resin composite biodegradation product, bisHPPP, was measurable in rinse samples obtained from the oral cavity immediately after resin composite placement. BisHPPP was not detected in baseline or seven-day post-restoration rinse samples.

2. There was a wide range of bisHPPP levels measured from the post-restoration rinse sample of patients (0.07-57.6 µg/mm² of restoration surface area).

3. Baseline HSDE activity ranged from 5.6-67.3 units/mL, well within the ability of the enzyme to degrade the restorations. The level of bisHPPP detected did not correlate to the patient’s salivary enzyme activity level.
4. Both Filtek Z250 and Spectrum TPH resin composite materials were subject to salivary enzyme degradation. In contrast to previous *in vitro* studies, the type of resin composite did not have a significant effect on the amount of measurable bisHPPP. This reflects the fundamental differences between the controlled *in vitro* conditions and the heterogenous *in vivo* clinical conditions and confirms the need for more *in vivo* studies.

5. Evidence of biodegradation was determined, *in vivo*, at levels that have previously been associated *in vitro* with surface deterioration of resin composite, interfacial restoration deterioration and restoration marginal leakage. The effects of the measured *in vivo* biodegradation products on the host, as well as on oral bacteria, are unknown.

6. BPA was not detected in any patient at any time point. BPA is not a degradation product of bisGMA and use of bisGMA based resin composites is not a source of systemic exposure to BPA.

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RECOMMENDATION AND FUTURE STUDY DIRECTIONS

1. In the current study enzyme activity levels were measured only at baseline. Since natural circadian variation occurs in salivary flow with possible effects on esterase activity, future studies should include measurement at each collection time point. This would provide a more accurate representation of esterase activity in human saliva, and its relationship with the biodegradation process.

2. Studies have shown that leachable monomers and degradation products accumulate in plaque. The plaque collection protocol used in the current study did not result in detection of bisHPPP both immediately after restoration placement, as well as at 7 days post-restoration time points. Since accumulation of degradation products within the plaque may require additional time, future studies may include plaque sampling at later time points post-restoration.

3. *In vitro* studies suggested that there was a higher degree of degradation during initial time periods compared with later time periods in resin composite incubated with CE or HSDEs. The highest amount of bisHPPP was produced in the first 24 hours, with ~ 85% of bisGMA hydrolyzed in the first eight hours of incubation with HSDE (Jaffer *et al*, 2002; Jaffer, 2005). In this study, a difference in degradation products between Z250 and TPH was not detected immediately post-restoration. Future studies collecting rinse samples multiple times over the first 24 hours may provide more information about the early degradation kinetics *in vivo*.
4. The adhesive used in the current study contained bisGMA, which is capable of producing 1.8 times more bisHPPP than from resin composite (Z250) (Shokati et al, 2010). Future studies exploring the *in vivo* biodegradation of adhesive may provide some insight into the extent of the effect of adhesive on overall degradation *in vivo*. As well, the utilization of non-bisGMA containing adhesives will provide better elucidation on the processes involved in the degradation of adhesives and resin composites.
APPENDICES
Appendix 1 - CONSENT FOR TREATMENT

Biodegradation of dental composite resins: Marginal breakdown and clinical investigations: Consent Form

Introduction

The researchers of this study include Dr. P. Santerre [Associate Dean {Research}], Dr. L. Tam (Associate Professor, Restorative Dentistry), Dr Y. Finer (Assistant Professor, Restorative Dentistry) and a graduate student (Dr. M. MacAulay, licensed to practice dentistry). They are affiliated with and can be contacted at the Faculty of Dentistry, University of Toronto, 124 Edward Street, Toronto, ON, M5G 1G6.

I understand that part of my treatment plan at the Faculty of Dentistry includes a resin composite (tooth-coloured) restoration on a non-biting surface of my tooth. For this reason, I have been asked to participate in a study, which will assess the long-term stability of this type of restoration after it is placed.

The purpose of this informed consent document is to provide information about the proposed study so that I may better make a decision as to whether I wish to participate. This consent form gives detailed information about the study and the possible risks and benefits. This proposed study will not change my treatment plan at the Faculty of Dentistry. My regular student dentist will complete the treatment in a standard manner regardless of whether or not I agree to participate in the study.

Purpose of the Study:

The purpose of the study is to determine whether resin composites undergo breakdown (degradation) and release breakdown products into the saliva, gingival fluid and/or plaque that is in the mouth, in very small amounts, after a resin composite restoration is placed. The levels and types of breakdown products will be measured and compared with the level of breakdown enzymes in my mouth. The information acquired from such studies could help investigators determine the level of resin composite breakdown that occurs in people’s mouths and whether different people have different propensities to break down different resin composite restorations.

Description of the Treatment and Study Method:

If I choose to participate in the study, the restoration and data collection procedures will consist of two clinic appointments and two saliva rinse collections as described below. The appointments will take place in the Faculty of Dentistry.

Tooth Restoration

1st appointment. My regular student dentist will perform the treatment of the tooth in a standard manner. If I choose to participate in the study, one of two widely used brands of resin composite, Z250 (3M Dental Products) or Spectrum TPH (Dentsply), will be selected as the restorative resin composite material. If I choose not to participate in the study, the resin composite material will likely be Spectrum TPH (Dentsply), the standard resin composite material used in the Faculty of Dentistry Clinics.

Data Collection

The following procedures will be done for research purposes only and are not required as part of your usual dental treatment.

You will be asked to rinse before the restorative treatment at the first appointment. The instructions for rinsing will be collected as follows:

- 1. Rest quietly for 3 minutes
• 2. Rinse mouth with 5 mLs (1 teaspoon) of 0.9% saline rinse solution (may contain 20% ethyl alcohol as is commonly used in commercial mouthwashes) for up to 2 minutes, then expectorate (spit) into a labeled tube.

• 3. Repeat Step 2 three more times.

In some cases, we will also collect a sample of your gingival crevicular fluid (the fluid that naturally seeps out of the gums). 1.0 µL of gingival crevicular fluid can be collected by contacting a filter paper strip to your gumline around the tooth to be restored. Plaque samples (if present) may also be taken from around the tooth to be restored using a standard hand instrument that is used for professional teeth cleanings. These two additional specimen collections will be carried out at the same appointment as the rinse collection.

A photograph of the prepared and restored tooth will be taken. The photograph will compose the teeth only and will not include any of the face. The photographs will be coded to ensure patient confidentiality and blind assessment. Your oral hygiene (excellent, good, fair, poor) and periodontal health as determined by your student dentist during the periodontal examination will also be recorded. The data collected will be used for research purposes only.

2nd appointment: I will return to the Faculty clinic for a second saliva rinse and if applicable, gingival crevicular fluid and plaque collection (as per protocol given above) at 7 days following completion of the restoration. I will be called for a reminder and requested (if possible) not to have any food or drink within 1 hour of the appointment. A monetary incentive ($30/return visit) will be provided for the patients after their 2nd visit for their participation.

Possible risks:
There are no apparent possible risks to participating in this study. Both resin composite restorative materials are widely used in practice with no known differences in clinical outcomes between the two. There are no reasonably foreseeable risks or emotional distress associated with the method of collecting saliva, gingival crevicular fluid or plaque. There is a potential for tissue irritation while rinsing. I will need to spend extra time (10-15 minutes) in the dental chair for the saliva rinse collection and clinical photographs.

Possible Benefits:
I will not benefit from participating in this study. Information from this study may help other dental patients in the future by helping in the selection of resin composite products or developing new resin composite materials with less susceptibility to breakdown.

Other dental treatment:
I may proceed with the remainder of my dental treatment plan, which I would arrange with my student dentist. If it is determined that I need to have another resin composite restoration to be done in my mouth during the 1 week study period, I may proceed with the needed treatment. However, my participation in the study may no longer be necessary. I should immediately inform the investigator if another resin composite restoration has been placed in my mouth during the study period so that the investigator may assess the impact that this change may have on the study.

Voluntary Participation:
If I choose to take part in this study, I do so of my own free will. I may refuse to take part now or can stop participating in this study at any time during the study period. If I do not
I wish to participate in any aspect of this study, my student dentist will continue to treat me in the usual manner.

**Compensation/ Expenses:**
I will not be compensated for the resin composite restoration, which is part of my prescribed treatment plan, or for the saliva, gingival crevicular fluid and plaque collections, and clinical photographs. Travel costs will be the patient’s responsibility. I will be compensated for the saliva rinse, gingival crevicular fluid and plaque collections to be done and for the extra time commitment related to the additional appointment by a monetary stipend ($30) to be given to me after the 1 week dental appointment.

**Confidentiality and Access to Medical and Dental Records:**
Information from my medical and dental records will be maintained at the Faculty of Dentistry. At the conclusion of the research (2006), the saliva rinse, gingival crevicular fluid and plaque samples, if any, will be discarded. The coded photographs and data obtained from the specimens will be kept on file by one of the investigators until publication of the research findings are complete. Only the investigators will have access to the code. I will be informed in a timely manner if information becomes available that may be relevant to my willingness to continue participation in the study. I will not be informed of the results of the research. The researchers intend to publish the results of this research and if they do, my identity will remain confidential. Upon request to the investigators, I will have access to the list of scientific publications generated from this study following such publication.

**Financial Support for the Study**
This study is funded by a Canadian Institutes of Health Research Grant MOP 68947

**Further Questions:**
I have been given a copy of this consent form. After reading this, if I have questions, I should ask my student dentist, the investigator, or Dr. D. Mock, Dean, Faculty of Dentistry, at 979-4910 x4383
Dr. D. Haas, Associate Dean of Clinical Sciences, at 979-4922 X4577
who is not associated with this study, with whom I can discuss my rights as a research subject.

**TO BE SIGNED PRIOR TO REGISTRATION**
I have read and understand this consent form. My signature in this section of the consent form means that I agree to register into the study. I will keep a copy of this consent form.

_________________________________________ Date
Signature of Patient

_________________________________________ Date
Signature of Investigator

_________________________________________ Date
Signature of Witness
Appendix 2 – SPECTROPHOTOMETRY

The CE-like activity of the filtered saliva samples was measured using spectrophotometer (DU800, Beckman Coulter, Inc. Fullerton, CA USA) with a CE activity protocol with para-nitrophenol butyrate (pNPB) as the enzyme substrate for measurement of baseline human salivary derived esterase activity (HSDEA) (Lin, 2005). One unit of esterase activity was defined as capable of generating 1 nmol of p-nitrophenol per minute, as measured at 410 nm, pH 7.0 and 25°C.

Chemical Structure of the substrate, para-nitrophenyl butyrate

\[
\text{CH}_3\text{C}_2\text{H}_2\text{C}_2\text{O} \quad \text{NO}_2
\]

Chemical reaction of CE with para-nitrophenyl butyrate (Saboori and Newcombe 1990)

\[
\text{p-nitrophenyl butyrate} + \text{H}_2\text{O} \xrightarrow{\text{CE catalysis}} \text{p-nitrophenol (yellow)} + \text{butyrate}
\]

The Tungsten source of the spectrophotometer (DU800, Beckman Coulter) was turned on 20 min before starting the measurements of the CE activity. The wavelength was then set to 401 nm. The CE activity was measured by adding the reagents below to a 1.5 mL cuvette:

- 50 µL of the supernatant which was prepared from saliva rinse sample
- 950 µL of the 50 mM Sodium phosphate buffer
- 500 µL of the 0.4 mM p-NPB

Parafilm was used to seal the cuvette, and the cuvette was inverted to mix the saliva with p-NPB. Each saliva rinse sample was measured in triplicate. OD values of the solutions were measured every thirty seconds for five minutes.

CE activity was calculated with the following equation:

\[
\text{CE Activity (units/mL)} = \frac{\text{E.C.} \times \text{L.P.} \times \text{S.V.}}{\text{O.D.} \times \text{T.V.}}
\]
Where:
O.D.  = change in absorbance per minute at 401 nm
T.V.  = total volume (L)
E.C.  = molar absorptivity of pNPB at 401 nm (16000 M⁻¹ cm⁻¹)
L.P.  = Light path (1 cm)
S.V.  = Sample volume (mL)
Appendix 3
HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) SYSTEM
HPLC Principles
The mobile phase flows from the solvent reservoir to the pump (model 600E multisolvent delivery system, Waters™, Mississauga, ON), and then mixes with the sample, which is delivered to the system through the injector (model U6K, Waters™, Mississauga, ON). Residual contaminants and proteins are removed from the sample when the sample passes through a Waters™ inline column filter (SS, 0.22 µm) and a guard pack column filter (µbondapak, C18). The sample then flowed through a Phenomenex Luna 5µm C\textsubscript{18}(2) 4.6 X 250 (Phenomenex, Torrance, CA) used to separate and isolate the products. Separated compounds then pass into the detector (model 996 Photo Diode Array (PDA) detector, Waters™, Mississauga, ON) flow cell, which translates concentration changes to signal voltages. This information is passed transferred to the computer and processed using the Millennium32 chromatography manager software, Version 3.05. For each injection, a chromatogram was constructed where the absorbance signal (µvolt) was plotted as a function of time (minutes). Product concentrations were calculated from the area under representative peaks and were converted to concentrations using calibration curves.

For this study, separation of the resin composite biodegradation products used a thirty-minute gradient method developed by Finer and Santerre (2003, 2004).

HPLC Gradient method for separation of biodegradation products

<table>
<thead>
<tr>
<th>Time [Minutes]</th>
<th>Flow Rate [mL/minute]</th>
<th>Methanol [%]</th>
<th>Buffer [%]</th>
<th>Water [%]</th>
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<tbody>
<tr>
<td>0</td>
<td>1.0</td>
<td>40</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>0.10</td>
<td>1.0</td>
<td>40</td>
<td>60</td>
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</tr>
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</tr>
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</tr>
<tr>
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<td>1.0</td>
<td>40</td>
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<tr>
<td>60.00</td>
<td>1.0</td>
<td>40</td>
<td>60</td>
<td>0</td>
</tr>
</tbody>
</table>
Appendix 4
PRODUCT IDENTIFICATION BY MASS SPECTROMETRY (MS)
Mass spectrometry was used to confirm the identity of products eluted at specific time points from the HPLC. The mass spectrometer separates gas phase ions according to their m/z (mass to charge ratio) value. It creates charged particles (ions) from molecules and analyzes those ions to provide information about the molecular weight and chemical structure of the compound. Mass spectrometers consist of three distinct regions: 1) Ionizer 2) Ion Analyzer 3) Detector

Schematic of the Mass Spectrometer

Mass spectrum (MS) of the bisGMA derived product, bisHPPP, isolated at a retention time of 17 minutes from Z250 and TPH composite samples
Chemical structure of the bisGMA derived product, bisHPPP, isolated at a retention time of 17 minutes for Z250 and TPH composite samples

<table>
<thead>
<tr>
<th>m/z</th>
<th>Ion chemical structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>135</td>
<td>( \text{CH}_3\text{C-} \text{OH} )</td>
</tr>
<tr>
<td>209</td>
<td>( \text{CH}_3\text{C-} \text{OCH}_2\text{CHCH}_2\text{OH} )</td>
</tr>
<tr>
<td>377</td>
<td>( \text{HOCH}_2\text{CHCH}_2\text{OH} ) ( \text{CH}_3\text{C-} \text{OCH}_2\text{CHCH}_2\text{OH} ) ( \text{CH}_3\text{C-} \text{OCH}_2\text{CHCH}_2\text{OH} ) \text{H}^+</td>
</tr>
<tr>
<td>394</td>
<td>( \text{HOCH}_2\text{CHCH}_2\text{OH} ) ( \text{CH}_3\text{C-} \text{OCH}_2\text{CHCH}_2\text{OH} ) ( \text{CH}_3\text{C-} \text{OCH}_2\text{CHCH}_2\text{OH} ) \text{NH}_4^+</td>
</tr>
<tr>
<td>399</td>
<td>( \text{HOCH}_2\text{CHCH}_2\text{OH} ) ( \text{CH}_3\text{C-} \text{OCH}_2\text{CHCH}_2\text{OH} ) ( \text{CH}_3\text{C-} \text{OCH}_2\text{CHCH}_2\text{OH} ) \text{Na}^+</td>
</tr>
<tr>
<td>415</td>
<td>( \text{HOCH}_2\text{CHCH}_2\text{OH} ) ( \text{CH}_3\text{C-} \text{OCH}_2\text{CHCH}_2\text{OH} ) ( \text{CH}_3\text{C-} \text{OCH}_2\text{CHCH}_2\text{OH} ) \text{K}^+</td>
</tr>
</tbody>
</table>
Appendix 5 - STANDARD CURVE FOR bisHPPP

\[ y = 4 \times 10^{-11}x \]

\[ R^2 = 0.997 \]
Appendix 6 – HPLC chromatograms for bisHPPP standard (A) and representative product (B)
Appendix 7 – HPLC chromatogram for bisphenol-A standard
Appendix 8 - Spectrum of bisHPPP standard and representative product
Appendix 9 – Spectrum of bisphenol-A standard
Appendix 10 – Resin Composite Contents

<table>
<thead>
<tr>
<th>Resin Type</th>
<th>Z250</th>
<th>TPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomers</td>
<td>BisGMA/UDMA/BisEMA</td>
<td>Urethane Modified BisGMA/TEGDMA/BisEMA</td>
</tr>
<tr>
<td>Filler Type (particle size)</td>
<td>Zirconia/silica (0.01-3.5μm, mean = 0.6μm)</td>
<td>Bariumaluminiumborosilicate (0.04-5μm, mean &lt; 1.5μm)</td>
</tr>
<tr>
<td>Filler % volume (%weight)</td>
<td>60 % (82%)</td>
<td>57% (77%)</td>
</tr>
</tbody>
</table>