THE INFLUENCE OF RESIN CHEMISTRY ON A COMPOSITE'S INHERENT BIOCHEMICAL STABILITY

By

Yoav Finer

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Graduate Department of Dentistry
The Institute of Biomaterials and Biomedical Engineering
University of Toronto

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Yoav Finer

Graduate Department of Dentistry

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Abstract

Studies have shown that inflammatory and salivary enzymes can degrade bis-phenyl glycidyl dimethacrylate (bisGMA) and triethylene glycol dimethacrylate (TEGDMA), two dental composite resin components. In other work, commercial composite resins containing a urethane modified bisGMA-TEGDMA (u-bis) based monomer showed a 10-fold reduction in the release of bisGMA derived product, bis-hydroxy-propoxyphenyl propane (bisHPPP), as compared with that found for bisGMA-TEGDMA (bis) based composites. These observations have motivated the further study of interactions between composites and enzymes.

Photopolymerized model composite resin samples based on bis or u-bis resins were incubated in pseudocholinesterase (PCE), cholesterol esterase (CE) or both, and in the presence of a specific esterase inhibitor, phenylmethylsulfonyl fluoride (PMSF), for 16 and 32 days (pH 7.0 at 37°C). Degradation rates for the monomers were assessed for each enzyme. Incubation solutions were analyzed for resin degradation products by high performance liquid chromatography (HPLC), UV spectroscopy and mass spectrometry. The composite surfaces were characterized by scanning electron microscopy (SEM) and

X-ray photoelectron spectroscopy (XPS). The vinyl group conversion was characterized by Fourier transform infra red spectroscopy (FT-IR). Hydrolase activity in human saliva was analyzed and compared to stock enzymes.

The results showed that both enzymes degrade the composite in a dose dependent manner. In the presence of CE, the u-bis system showed a 15 to 180 (depending on the specific product) fold decrease in the amount of isolated products relative to the bis system. SEM analysis confirmed the relative degradation levels. CE showed a greater ability to cleave bisGMA monomer while PCE showed more specificity towards TEGDMA. Addition of PMSF inhibited the degradation process, confirming the implication of the serine active site in the enzymes. Saliva was found to contain CE and PCE-like hydrolase activity, at levels that could degrade composite resins. The study suggests that the level of degradation products generated for a material will be dependent on the esterase make-up for an individual's saliva and the specific formulation of monomer components. Since resin formulation has a significant effect on the composite's chemical stability, manufacturers should consider testing biochemical stability as part of their routine product evaluation.

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1.0 INTRODUCTION

Interest in polymeric dental restorative materials has been stimulated by the possibility of adverse health effects from exposure to mercury in dental amalgams and the need of improved esthetic dental restorations [McHugh, 1992; Ewell, 1993; Jones, 1993]. General use of polymeric materials over the past 20 years has indicated a high benefit-to-risk ratio [McHugh, 1992]. However systemic reactions to the polymers and related degradation products have not been closely examined [Bayne, 1992].

Elution of unreacted components from dental composites is a diffusion rate-dependent process that can be enhanced by the presence of ethanol [Ferracane, 1990]. Exposure of the composite resins to chemicals such as ethanol, vegetable oil, organic acids and enzymes causes softening of the resin matrix [Wu, 1982; Mair, 1991; de Gee, 1995]. It has been recognized in the last decade that dental polymeric materials can be degraded by enzyme hydrolysis to produce toxic products such as methacrylic acid [Freund, 1990] and to undergo oxidation processes which produce formaldehyde [Øysæd, 1988].

Biodegradation studies have been used to investigate the effect of a salivary enzyme (pseudocholinesterase) [Chauncey, 1961; Yamalik, 1990; Leung, 1997] as well as the inflammatory cell-derived enzyme, cholesterol esterase [Labow, 1983] on composite dental materials [Santerre, 1999; Shajii, 1999]. Santerre et al [1999] has shown that cholesterol esterase can generate the breakdown of triethyleneglycol dimethacrylate (TEGDMA) and 2,2-bis[4-(2-hydroxy-3- methacryloxypropoxy) phenyl] propane

(bisGMA) components from composite resins while Leung et al [1997] has shown that pseudocholinesterase was also able to generate biodegradation products from commercial dental composite. It was also noted in the former study that commercial composite resins containing a urethane modified bisGMA-TEGDMA based monomer showed a 10-fold reduction in the release of bisGMA derived products, as compared with the amount found for bisGMA-TEGDMA based composites [Santerre, 1999]. However, the author was unable to determine if this observation was specifically related to the resin chemistry, filler type or their concentration.

It has been reported that the native monomers, themselves, can alter host cell DNA function at concentrations of 2.5 ppm while protein synthesis may be affected with concentrations as low as 0.25 ppm [Wataha, 1994]. Recent studies have also questioned the estrogenicity of bis-phenol A released from a bisGMA based fissure sealant [Olea 1996] and the predisposition of TEGDMA to promote bacterial growth [Hansel, 1998]. In considering the fact that enzymes can readily degrade composites and that the monomers that make up these materials can alter cell function, it is of significance to assess the potential of salivary enzymes to degrade dental composite materials and gain further insight into the parameters that influence the mechanism of degradation. As well, a measure of product concentration that could be generated as a function of material type and enzyme activity will be important in assessing the ultimate biocompatibility of the materials. The following thesis will seek to address the following hypotheses.

HYPOTHESES

1. Pseudocholinesterase (PCE) and cholesterol esterase (CE) catalyzed hydrolysis of resin components occurs via the ester bonds of the methacrylate based resin monomers, and is a dose dependent process which occurs via the same active site as natural substrates for the enzymes.

2. The chemical structure of dental composite resins affects its stability with respect to the enzyme catalyzed hydrolysis of the monomers' ester groups.

3. Different esterases have distinct specificities for defined composite resin components.

4. Saliva contains hydrolase activity levels, CE and/or PCE-like, within the defined limits of activities that affect ester containing composite resin material.

Confirmation of the above hypotheses will provide a better understanding of the enzymatic degradation of dental composite resin materials in the oral environment, their potential biocompatibility and could lead to the synthesis of more chemically stable materials.

1.2 OBJECTIVES

The following objectives have been identified to further understand the nature of the enzyme interaction with urethane and non-urethane containing materials:

1. To determine the dose response of enzyme composite resin interactions and to confirm their mode of action by using specific inhibitors to the enzymes (to test hypotheses 1, 2 and 3).

2. To determine the relative biostability of urethane modified bisGMA-TEGDMA based composite resins to bisGMA-TEGDMA based composite resins, in the presence of CE and/or PCE (to test hypotheses 2 and 3).

3. To determine the biostability of radiolabeled urethane modified bisGMA composite resin in the presence of CE or PCE (to test hypotheses 2 and 3).

4. To characterize the cured composite resin samples by SEM, XPS, Vickers Microhardness and FT-IR (to test hypotheses 1 and 2).

5. To isolate and identify degradation products using a combination of high performance liquid chromatography, UV spectroscopy, mass spectrometry and radiolabeled counting (to test hypotheses 1, 2 and 3).

6. To determine the combined effect of CE and PCE on dental composite resins (to test hypotheses 3 and 4).

7. To determine the relative biostability of TEGDMA, bisGMA and ethoxylated bisphenol A dimethacrylate (bisEMA) monomers in the presence of CE or PCE (to test hypothesis 3).

8. To determine the hydrolase activity, CE and/or PCE-like, in human saliva (to test hypothesis 4).

1.3 REVIEW OF LITERATURE

1.3.1 COMPOSITE RESIN SYSTEMS

A composite resin, by definition, consists of two or more components in which there is interatomic or molecular bonding between these components, so as to provide overall properties which are superior to the constituents alone [Dogon, 1990]. Dental composite materials consist of a resin matrix and a filler that is usually glass, quartz or a ceramic material. The molecular bonding is produced by the treatment of the filler with vinyl silane compounds to create active sites on the filler particles, enabling each particle to chemically bond to the resin matrix.

Current uses of dental composite resins are in tooth restorations, cements, orthodontic space maintainers, crown and bridge facings, inlay and onlay patterns, temporary crowns and restorations and endodontic fillings [Craig, 1993].

1.3.2 COMPOSITE RESIN SYSTEMS- HISTORY AND DEVELOPMENT

The first self-curing acrylic resins were developed in the 1940's in Germany, and this led to the introduction of acrylic filling materials in 1948, also known as poly(methyl methacrylate) or Sevriton. The structure of methyl methacrylate is given in Figure 1.1. Composite resin restorative materials were introduced to the dental world in the mid-1960's as an improved esthetic restorative material over silicate cements and the non-

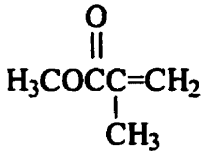


Figure 1.1 Structure of methyl methacrylate

reinforced poly(methyl methacrylate)-PMMA direct filling materials for the restoration of anterior teeth [Bowen, 1962 and 1965]. These were the first steps toward the composite restorative materials of today. The earlier acrylic dental restorative material showed poor color stability, significant polymerization contraction, lack of bonding to tooth structure (enamel and dentin) and low stiffness and hardness [Smith, 1985]. Later, it had been found that when inert fillers were incorporated into poly(methyl methacrylate), there was a reduction in the coefficient of thermal expansion and in water sorption, in proportion to the concentration of the filler [Rose, 1955a].

Composite resin restorative materials were introduced in 1962 [Bowen, 1962], however, the first materials did not yield significant improvements in the mechanical properties of the resins due to a lack of reinforcement between the inorganic filler particles and the resin matrix. Later Bowen suggested the use of silane treatments for the filler particles, in order to enhance their bonding with the resin matrix [Bowen, 1963]. The new composite resins had lower polymerization shrinkage, a lower coefficient of thermal expansion, higher compressive strength and stiffness and (depending on the filler materials used) higher radiopacity than did the non-reinforced resins [Bowen, 1992]. As compared with

the silicate cements, the composite resins exhibited lower solubility, higher tensile strength and comparable compressive strength.

In 1983, the 3M Dental Products Company introduced the first dentin-bonding agent, called Scotchbond [Feigenbaum, 1994]. It represented a major step forward. For the first time, the dentist could bond to both the etched enamel and dentin. This development was a major breakthrough in the use of composite resin materials, making it possible to restore loss tooth structure in the dentin when necessary. Restorative preparations could be extended to achieve better adhesion and esthetics. Since then, vast improvement in the bonding agent materials has been achieved, resulting in materials that can bond to virtually any substrate found in the mouth.

1.3.3. RESIN SYSTEMS

Adhesiveness was one of several intriguing characteristics of epoxy resins that led to their experimental use in conjunction with various hardening systems and fillers [Rose, 1955b; Bowen, 1956]. A compromise between 2,2-bis[4-(2,3-epoxypropoxy)-phenyl]propane or bisphenol A epoxy (Figure 1.2) and methacrylate resins was conceived in 1956 when Bowen was issued a patent for a hybrid molecule. The reaction site of the epoxy molecule was replaced by methacrylate groups, which yielded a hybrid molecule that polymerized through the latter groups [Bowen, 1985 and 1992]. This dimethacrylate monomer was synthesized by the reaction of glycidyl methacrylate and bisphenol A [Bowen, 1992] and was later synthesized by the reaction of methacrylic acid and the

diglycidyl ether of bisphenol A [Sanders, 1992]. This monomer was 2,2-bis[4,(2-hydroxy-3-methacryloxypropoxy)-phenyl]propane, more commonly known as bisGMA (Figure 1.3). The bisGMA monomer exhibited many advantages. It was suitable for use as a binder for reinforcing fillers, since it was relatively non-volatile, had relatively low polymerization shrinkage as compared to poly(methyl methacrylate), and hardened rapidly under oral conditions, when formulated with an appropriate initiator system [Smith, 1985].

A major disadvantage of bisGMA was its high viscosity, caused mainly by the hydrogen bonds between the hydroxyl groups in the alkyl chain [Sanders, 1997] and its two phenyl rings [Deb, 1998]. Hence, the use of a diluent monomer was mandatory in order to reduce the viscosity of the monomer system and to facilitate the incorporation of fillers as well as improve handling and manipulation. The most commonly used monomer included diethylene glycol dimethacrylate (Figure 1.4) and triethylene glycol dimethacrylate (TEGDMA) [Ruyter, 1981]. Alternate diluent monomers were introduced [Asmussen,

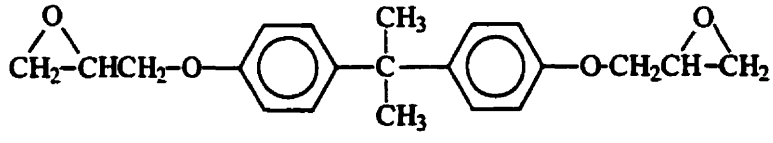


Figure 1.2 Structure of 2,2-bis[4-(2,3-epoxypropoxy)-phenyl]propane or bisphenol A epoxy [Sanders, 1992]

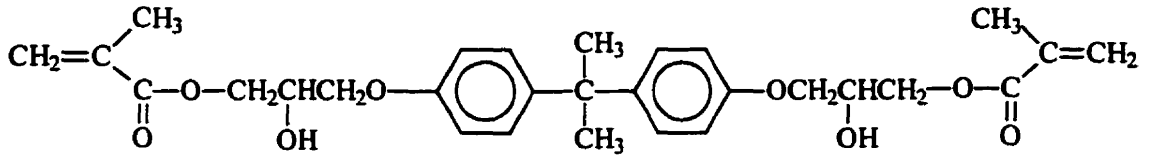


Figure 1.3 Structure of 2,2-bis[4,(2-hydroxy-3-methacryloxypropoxy)-phenyl] propane (bisGMA) monomer [Ruyter, 1981]

1998; Davy, 1998] and included 1,6-bis(methacryloxy-2-ethoxy-carbonylamino)-2,4,4-trimethyl-hexane (UEDMA) (Figure 1.5), 2,2-bis [4,(2-methacryloxyethoxy)phenyl] propane or ethoxylated bisphenol A dimethacrylate (bisEMA) (Figure 1.6) and 2,2-bis[4,(3- methacryloxyporpoxy) phenyl] propane dimethacrylate or bisphenol A propoxilated (bisPMA) (Figure 1.6). A comparison between bisGMA, UEDMA, bisEMA and bisPMA showed that the viscosities varied as follows: 1200 Pa for bisGMA, 23.1 Pa for UEDMA, 0.9 Pa for bisEMA and 1.3 Pa for bisPMA [Davy, 1998].

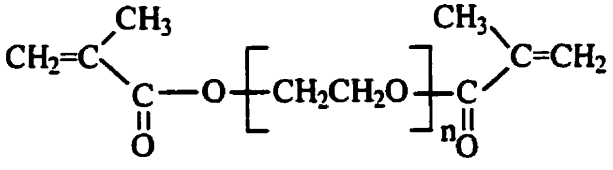


Figure 1.4 Structure of ethylene glycol dimethacrylate (n=1), diethylene glycol dimethacrylate (n=2) and triethylene glycol dimethacrylate (n=3) [Ruyter, 1981]

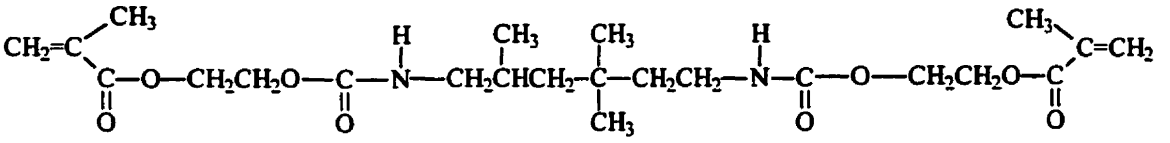


Figure 1.5 **Structure of 1,6-bis(methacryloxy-2-ethoxy-carbonylamino)-2,4,4-trimethyl-hexane or urethane ethyl dimethacrylate (UEDMA) [Ruyter, 1981]**

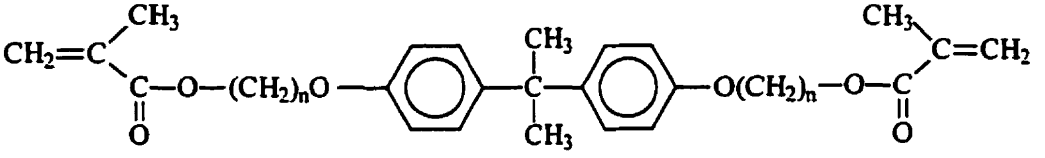


Figure 1.6 **Structure of 2,2-bis[4,(2-methacryloxyethoxy)phenyl]propane or ethoxylated bisphenol A dimethacrylate (bisEMA) (n=2) and 2,2-bis[4,(3-methacryloxypropoxy)phenyl]propane or bisphenol A propoxilated dimethacrylate (bisPMA) (n=3) [Ruyter, 1981]**

The composition of resins in different commercial dental composite systems were found to vary substantially from one product to another [Ruyter and Sjøvik, 1981; Ruyter and Øysæd, 1987a; Ruyter and Sjøvik, 1987b]. Ruyter and Øysæd showed that most of the materials contained relatively well-defined methacrylates like bisGMA, ethylene glycol dimethacrylate, TEGDMA and UEDMA [Ruyter and Øysæd, 1987a]. However, the materials such as Ful-Fill (Fu-F) (L.D. Caulk/Dentsply), Prisma-fil (PF) (L.D. Caulk/Dentsply) and Occlusin (OC) (ICI PLC) contained oligomeric components based on urethane methacrylates in addition to TEGDMA. Based on NMR spectra analysis, the

oligomeric components in Fu-F and PF were shown to be synthesized from bisGMA and hexamethylene diisocyanate, thus producing a urethane modified bisGMA oligomer (u-bis) (Figure 1.7). The number average molecular weight of this oligomer was found to be 4900 [Ruyter and Øysæd, 1987a]. In the same study, commercially available bisGMA was resolved using high performance liquid chromatography (HPLC) analysis into linear and branched components. The ratio of the linear to branched bisGMA was approximately 3 to 1, and a double-branched bisGMA was also found at a quantity of about 2 %. Ruyter showed variations in the composition of commercial polymeric crown and bridge veneering materials [Ruyter, 1987b]. Most of the materials in this latter study were found to contain methyl methacrylate, TEGDMA and UEDMA in different concentrations. In addition, the veneering material Triad K+B (TR) (DeTrey/Dentsply), was found to contain the linear polyurethane u-bis, with a number-average molecular weight of 7600 [Ruyter, 1987b]

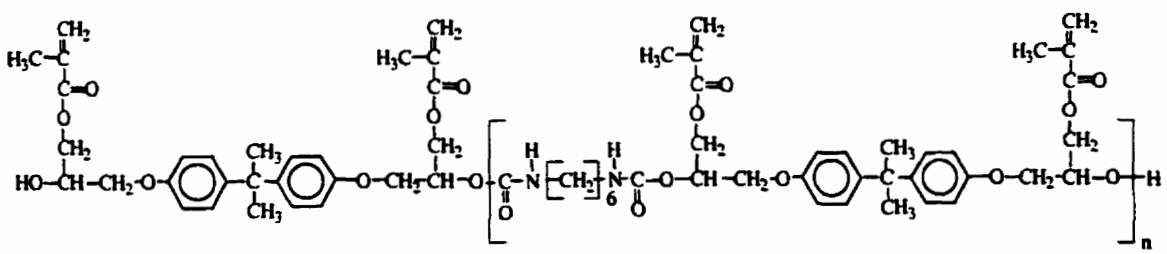


Figure 1.7 Schematic representation of the structure of the urethane modified bisGMA oligomer (u-bis) [Ruyter, 1981]

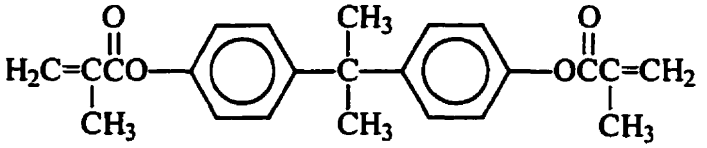


Figure 1.8 Structure of 2,2,-bis-[4-methacryloxy-phenyl]propane or bisphenol A dimethacrylate (bisMA) [Ruyter, 1981]

Ruyter and Sjøvik analyzed the composition of the resin in commercially available pit and fissure sealant and dental resin restorative materials [Ruyter, 1981]. They reported that most of the materials contained bisGMA and TEGDMA. In addition to the linear and branched bisGMA, as mentioned before [Ruyter, 1987a], the monomethacrylate derivative of bisGMA was found. The following monomers were also found in some of the materials: ethylene glycol and diethylene glycol dimethacrylates (Figure 1.4), UEDMA (Figure 1.5), 2,2,-bis-[4-methacryloxy-phenyl]propane or bisphenol A dimethacrylate (bisMA) (Figure 1.8), bisEMA and bisPMA monomers (Figure 1.6).

1.3.4 EFFECT OF RESIN CHEMISTRY ON THE PHYSICAL PROPERTIES OF COMPOSITE

Changes in the composition and the chemistry of the monomers within the resin system alter some of the physical properties of the composites. In one study [Asmussen, 1998], TEGDMA, bisGMA and UEDMA were varied. Substitution of bisGMA or TEGDMA by UEDMA resulted in an increase in the tensile and flexural strength and substitution of bisGMA with TEGDMA increased tensile, but reduced flexural strength. Similar results

were found in another study [Ferracane, 1986]. The observed increase in the tensile and flexural strength when bisGMA or TEGDMA were substituted by UEDMA was reported to have been possibly associated with the ability of the urethane linkage to form hydrogen bonds in the copolymer. This could restrict the sliding of polymer segments relative to each other. A moderate increase in the modulus of elasticity was observed when bisGMA was replaced by TEGDMA, for low content levels of the latter monomer. This was followed by a relatively steep decline in stiffness as the content of TEGDMA increased. The reduction in modulus of elasticity was explained by the natural flexibility of this molecule which results from the chain mobility of the ether groups. For the group of monomers studied, the investigation concluded that a monomer composition of 70 mol % UEDMA and 30 mol % TEGDMA (no bisGMA) would result in a resin composite of optimum strength and a relatively low modulus of elasticity, while a monomer composition of 50 mol % TEGDMA and 50 mol % bisGMA (no UEDMA) would result in a maximum modulus of elasticity.

In another study [Jones, 1996], blends of monomers involving bisGMA or bisEMA and various amounts of TEGDMA were evaluated for their modulus of elasticity. A trend showing an increase in the modulus of elasticity was found when the amount of bisGMA was increased. This was similar to the finding of others [Asmussen, 1998], although the effect was moderate relative to the finding of the latter study. The bisEMA/TEGDMA blends had a very low moduli relative to the bisGMA/TEGDMA blends, but the trend of increased moduli with the reduction of the TEGDMA was found to be the same.

Beatty et al [Beatty, 1993] investigated the effect of monomer chemistry on its properties. The resins were based on diphenyloxymethacrylate (DPMA) and TEGDMA blends, and urethane dimethacrylate (UDMA) (Figure 1.9) and TEGDMA blends in different ratios. The UDMA imparted higher compressive and tensile strength, resistance to toothbrush abrasion and higher water sorption, while the DPMA based resins showed a greater surface hardness. The higher water sorption of the urethane based resin was associated with the longer aliphatic chains between the ring structures, which increase flexibility, and the presence of the urethane (O-CO-NH) groups which procured a potent hydrogen bond former [Venz, 1991].

Another study [Chowdhury, 1996] investigated the effect of addition of an experimentally-synthesized polyfunctional urethane monomer on visible light-cured bisGMA-TEGDMA blend resin systems. The addition of the polyfunctional urethane monomer to the bisGMA-TEGDMA resin, produced a system with improved mechanical properties such as diametral and tensile strength and hardness. The improved mechanical properties with the presence of urethane monomer was associated with the increased cross-linking of the polyfunctional monomer as well as hydrogen bonding which could occur between the amide groups [Venz, 1991].

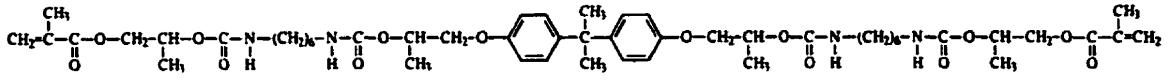


Figure 1.9 Chemical structure of urethane dimethacrylate (UDMA) [Beatty, 1993]

It was reported in another study that increasing the ratio of TEGDMA to bisGMA in a resin matrix system leads to an increase in water uptake [Kalachandra, 1987; Beatty, 1993]. That was explained by the relative hydrophilicity of TEGDMA as compared with bisGMA. In the clinical situation, this could result in the swelling of the restoration, leading to peeling stresses on the composite. Following water sorption, the deterioration of some mechanical properties, such as the modulus of elasticity, strength and hardness were associated with a water plasticizing effect [Beatty, 1993]. Other investigators [Davy, 1991; Tanaka, 1991; Sankarapandian, 1997] suggested the use of fluorinated monomers, thus increasing their hydrophobicity, in order to reduce water sorption.

1.3.5 POLYMERIZATION SYSTEMS

Methacrylate based resins polymerize via an addition type mechanism, initiated by free radicals [Cook, 1985]. In a free radical polymerization, each molecule grows by addition of a monomer to a terminal free radical reaction site [Young, 1991]. Free radicals can be generated by chemical activation or by external energy activation, such as heat or light. Chemically activated materials are typically supplied as two pastes, one of which contains a benzoyl peroxide initiator and the other a tertiary amine activator, usually N,N-dimethyl-p-toluidine. When the two pastes are mixed, the amine reacts with the benzoyl peroxide to form free radicals, and addition polymerization is initiated. Today, these materials are mainly used for restorations that are not readily cured with a light source. For over 30 years, photopolymerization of methacrylate based resins has been widely used in the dental field for composite resins restorative materials, adhesives,

denture bases and impression materials [Cook, 1985]. The following steps are involved in free radical photopolymerization reactions [Cook, 1985]:

Initiation

$$I \xrightarrow{h\nu} I^* \qquad \textbf{Equation 1.1}$$

$$I^* + A \xrightarrow{k_i} R^\bullet$$

Propagation

$$R^\bullet + M \longrightarrow RM^\bullet \qquad \textbf{Equation 1.2}$$

$$RM_n^\bullet + M \xrightarrow{k_p} RM_{n+1}$$

Termination

$$RM_n^\bullet + RM_m^\bullet \xrightarrow{k_t} RM_{n+m}R \qquad \textbf{Equation 1.3}$$

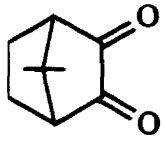
Inhibition or retardation

$$RM^\bullet + X \xrightarrow{k_x} RMX^\bullet \quad \text{Inactive} \qquad \textbf{Equation 1.4}$$

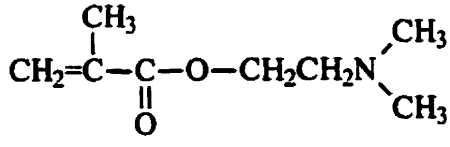
I* designates the photoinitiator, A represents a photo-reducing agent, R• represents a free radical, M represents the unsaturated site in the methacrylate molecule, and k_i, k_p, k_t and k_x are the rate constants of the respective reactions. The inhibitors (X) are present in the composite in order to prevent premature polymerization during storage [Cook, 1985]. Oxygen also inhibits radical polymerization, which can lead to the formation of an unpolymerized resin surface layer [Ruyter, 1985]. In a visible light curing system, free radicals would be formed by absorption of light having a wavelength of 468 nm [Kilian,

1979]. This photoinitiation is based on the interaction of diketones (I in Equation 1.1) such as camphorquinone (CQ) with an amine (A in Equation 1.1) such as N,N-dimethylaminoethyl methacrylate (DMAEM) (Figure 1.10). The propagation step in the reaction with dimethacrylates can lead to the formation of linear, cross-linked or cyclized polymer chains [Aso, 1959] (Figure 1.11).

A reduction in the diffusion rate of the propagating free radicals, decrease in the concentration of unreacted methacrylate molecules and the presence of pendant methacrylate species reduce the rate of polymerization [Cook, 1985]. This reduction has been attributed to the effect of steric hindrance as the molecules increase in size [Munk, 1989] and to the fact that the system's glass transition temperature increases relative to the temperature of the reacting environment [Horie, 1976; Maffezzoli, 1994]. As a consequence, when the polymerization process is terminated, not all of the monomer's active sites have reacted.



(A)



(B)

Figure 1.10 Chemical structure of (A) diketone camphorquinone (CQ) and (B) a reducing agent N,N-dimethylaminoethyl methacrylate (DMAEM) during light exposure [Craig, 1993]

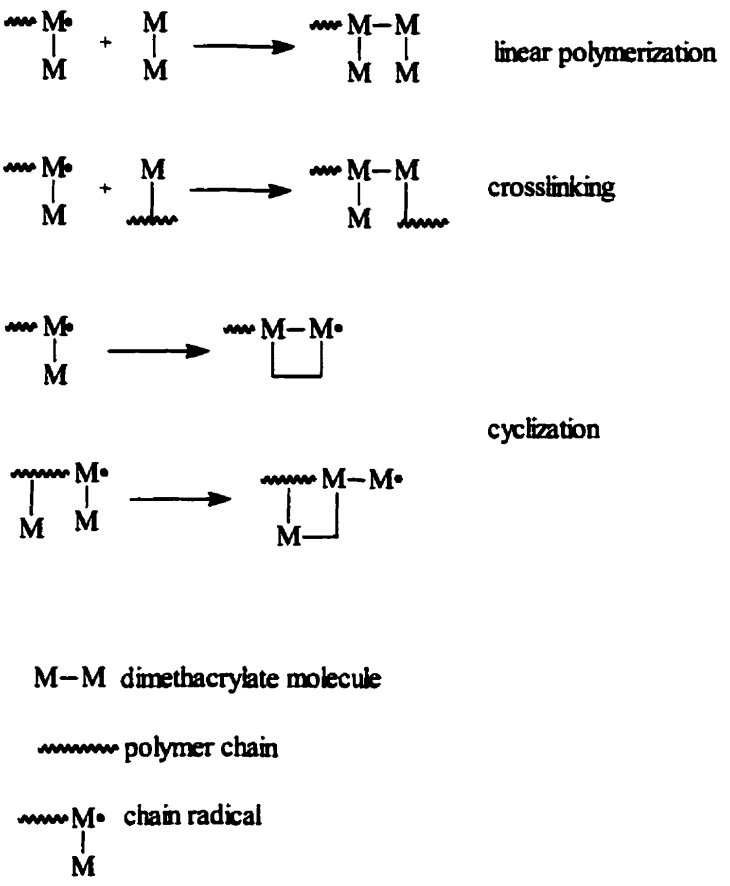


Figure 1.11 Possible reactions in the propagation step in the polymerization of a dimethacrylate [adapted from Aso, 1959]

Infra-red spectroscopy analysis of cured composites have shown that a significant percentage (15 to 60 %) of the methacrylate groups remain unreacted [Asmussen, 1982; Ruyter, 1985; Peutzfeldt, 1994; Park, 1996]. Increases in the intensity of the light source [Nomoto, 1994; Peutzfeldt, 1994], the photocuring period [Condon, 1997] and the use of pulsed laser source [Tarle, 1998] showed an increase in the degree of conversion while an increase in the distance from the materials surface [Ruyter, 1982; Tarle, 1998,] and from the light source [Ruyter, 1982; Park, 1996] caused the opposite effect.

The composition of the monomer system has also been found to effect the degree of conversion. Materials with relatively larger quantities of the rigid bisGMA monomer have shown relatively low degrees of conversion [Ruyter, 1987a; Sanders, 1997]. This lower conversion ratio has been attributed to the high viscosity of this molecule, which reduces the mobility of reactive molecules and thus lowers the probability of random encounters with free radicals [Chung, 1990]. In contrast, diluent monomers such as TEGDMA contribute to an increase in the number of cross-links in the matrix due to their lower viscosity and higher mobility [Ruyter, 1987; Asmussen, 1992]. These findings led to the development of alternate monomers such as propoxylated diphenol dimethacrylate. This latter monomer is a flexible derivative of bisGMA, which has shown an increase in the conversion ratio [Sanders, 1997]. Recent studies [Anseth, 1996], have shown that ethylene-glycol dimethacrylate monomers with varying chain lengths as comonomers, such as poly(ethylene glycol)$_{n=600}$ dimethacrylate, provide an increased level of conversion, without compromising strength.

Another factor affecting the degree of conversion is the initiator system. Previous research has demonstrated the importance of proportioning photo-active components (CQ and DMAEM) in light-cured restorative materials [Yoshida, 1993]. For unfilled resins, the degree of conversion was dependent on the CQ to DMAEM ratio, which at a value of 3:1 respectively yielded a maximum degree of conversion. In a more recent study, Ruggeberg et al found that a four fold increase in the photoinitiator level compared with the standard amount, caused an increase in the degree of conversion [Ruggeberg, 1997].

Post-cure heating of resin composites has demonstrated an increase in diametral strength [Bausch, 1981; Wendt, 1987; Convey, 1992], flexural strength [Dionysopoulos, 1989; Asmussen, 1990; Gregory, 1992] and fracture toughness [Ferracane, 1992]. These property changes are thought to arise from either an increased monomer conversion during the heating processes [Wu, 1983] or from an annealing of residual polymerization stresses generated during the initial light curing [deGee, 1990]. Post-cured treatment, generates an increase in the segmental chain vibrational amplitude, allowing near neighbor radicals and methacrylate groups to collide, thus increasing monomer conversion [Ruggeberg, 1997]. This change was also attributed to the treatments removal of volatile unreacted monomers [Bagis, 1997], thus enhancing the composite resin properties by removing the component that can act as a plasticizer, and subsequently, improving the degree of conversion.

The setting of the current cross-linked polymeric matrix systems is always accompanied by shrinkage due to the exchange of vinyl double bond for two single covalent bonds during the propagation step (Equation 1.2) and the change in the van der Waals interaction between molecules [Venhoven, 1993]. Hence, in methacrylate based resins, the number of converted vinyl groups per unit volume is an important factor in determining the polymerization contraction. Studies have shown polymerization shrinkage values ranging from 0.8 to 4.1 % volume [Tarle, 1998] and 1.67 to 5.68 % volume [Goldman, 1983] depending on the monomer composition and method of cure. The polymerization contraction may lead to the formation of a gap around the cavity

margins. When a gap does not form, there is a build up of internal stresses which may lead to adhesive or cohesive failures [Tarle, 1998].

In conclusion, an optimal degree of conversion and minimal polymerization shrinkage are generally antagonistic goals as increased monomer conversion invariably leads to elevated polymerization shrinkage values. However, both parameters are indispensable for an optimal composite resin restoration.

1.3.6 FILLER SYSTEMS

The major constituent of composite resin materials, by weight and volume, is the filler [Dogon, 1990]. The filler imparts significant physical properties to the material such as increased strength and modulus of elasticity, reduced polymerization shrinkage, coefficient of thermal expansion, heat of polymerization and water sorption (Cook, 1984; Dogon, 1990). Common commercial composite resin materials contain fillers such as quartz, colloidal silica, silica glasses with barium or lithium aluminum silicate [Söderholm, 1985]. Each filler type endows the composite resin with specific properties for defined applications.

The common classification for fillers are microfills, macrofills and hybrid [Lang, 1992]. The traditional macrofillers were prepared by grinding quartz or other inorganic material such as glass or ceramic. The particle size ranged from 0.1 to 100 μm [Roulet, 1987]. The composite materials containing these fillers suffered from particle sedimentation, poor

polishability and high wear rate [Dogon, 1990]. An improvement in the filler system was achieved by the reduction of the filler size by a factor of 10 or more, producing a filler with an average particle size ranging between 40 to 60 nm [Leinfelder, 1991]. Such fine particles are smaller than the wavelength of light and thus generate lustrous surfaces upon finishing [Smith, 1985]. This microfiller system was composed of finely dispersed glass spheres such as silica and produced by a combustion process. The preference by clinicians for materials that were polishable and which showed improved abrasion resistance, while maintaining optimal consistency led to the development of hybrid materials [Smith, 1985]. These materials combined the advantages of macrofills (high filler loading and better mechanical properties) and microfills (better esthetics and wear resistance). They had an average particle size in the range of 1 to 5 μm. Improvements in the technology of grinding and dispersion of fillers has produced highly filled materials with loading in excess of 80 % by weight or 65 % by volume.

1.3.7 FILLER-MATRIX INTERFACE

To achieve a good bond between the resin and the filler, two approaches can be taken; either to create a filler surface to which the resin can bond mechanically [Bowen, 1976a and 1976b; Ehrnford, 1983], or to provide a chemical bond [Bowen, 1963]. Mechanical bonding can be enhanced by either sintering particles or fibers together [Ehrnford, 1976], by using an appropriate shape [Jones, 1996], or by etching away a continuous phase of glass [Bowen, 1976a and 1976b]. In all cases, a porous structure will be created, into

which the resin can flow and after polymerization, the matrix will be mechanically bonded to the filler particles.

The chemical bonding approach has been a common method adopted. The chemical bonding between the matrix and the filler allows the more flexible polymer matrix to transfer stresses to the stiffer filler particles. The most common coupling agent used in dentistry is γ-methacryloxypropyltrimethoxy silane (MPS) [Söderholm, 1985] (Figure 1.12). This silane coupling agent contains a reactive organic structure with a vinyl group that subsequently reacts with the resin matrix. The methoxy groups can be hydrolyzed by water to form silanol groups, which then react at the inorganic filler surface in monomeric or oligomeric forms [Söderholm, 1985] (Figure 1.13A). Subsequent drying would complete the condensation process linking the coupling agent molecules to each other and to the filler surface by siloxane bonds (Figure 1.13B). A silane treated composite resin shows lower water solubility and is less susceptible to disintegration in water than are the untreated silica based composites [Bowen, 1963].

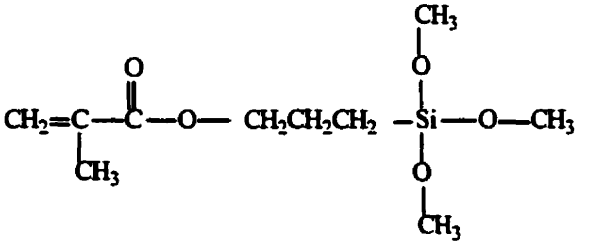


Figure 1.12 Structural formula of γ-methacryloxypropyltrimethoxy silane (MPS) [Söderholm, 1985]

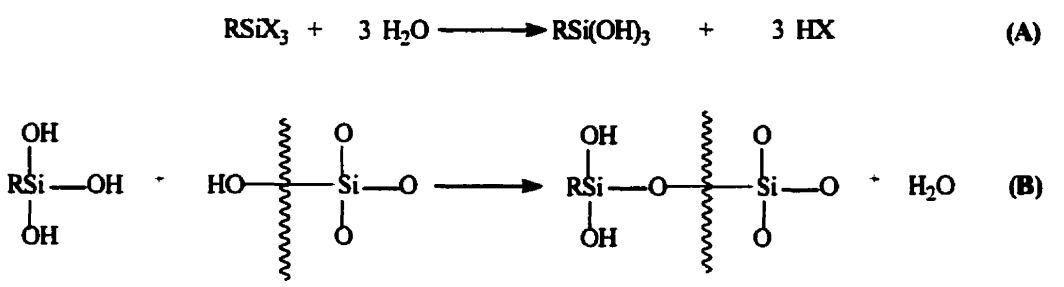


Figure 1.13 Hydrolysis of the methoxy groups of the silane to form silanol groups (A) and reaction of the silanol groups with existing groups on the filler surface through a condensation reaction (B) [Söderholm, 1985]

The optimum number of chemical bonds needed at the interface to provide lasting strength has not yet been determined. A monolayer coverage of the coupling agent usually does not yield optimum performance [Johannson, 1967]. Coverage is influenced by the surface geometry of the filler, its composition and the presence of foreign materials [Ishida, 1979]. Arkles suggested a formula to determine the amount of silane coupling agent used for filler treatment [Arkles, 1987]: $X = \frac{A}{\omega} f$, where X= amount of coupling agent in grams needed to obtain a minimum uniform coverage; f= amount of filler in grams; A= particle surface area of the filler per gram [m^2/g]; ω= wetting surface capability of the silane agent per gram [m^2/g]. Based on the latter equation, Moshen et al found that when three times the minimum calculated amount of silane (X) was used, the treatment was more effective [Moshen, 1995]. The proposed explanation was that a minimum coverage would lead to unsilanated filler or condensation of the silanes with themselves via the alkoxy groups rather than the hydroxyl groups on the surface of the filler. Another study concluded that the amount of silane needed, depended on the

number of isolated hydroxyl groups available on the filler surface [Söderholm, 1993]. Fourier transform infra-red spectroscopy revealed that MPS molecules formed two types of bonds with the hydroxyl groups on the surface of the filler; a siloxane bond (Figure 1.13B) and a hydrogen bond formed by the carbonyl groups of the MPS molecule.

1.3.8 SALIVARY AND GINGIVAL DERIVED ENZYMES

Saliva is a complex mixture of liquid and particulate matter which originates from several sources; salivary glands, oral micro-organisms, serum-like gingival crevicular fluid, polymorphonuclear leukocytes, epithelial cells and dietary constituents [Chauncey, 1961; Nakamura, 1983a]. Chauncey [1961], has classified the salivary enzymes into five major groups; the carbohydrases (such as amylase and maltase), the esterases (such as acetylcholinesterase and pseudocholinesterase), the transferring enzymes (such as catalases and oxidases), the proteolytic enzymes (such as proteinase and peptidase) and other enzymes (such as carbonic anhydrase and aldolase). It was also noted that most enzymes were partially or entirely produced by micro-organisms, which normally inhabit the oral cavity [Chauncey, 1961; Nakamura, 1983a]. Many enzymes are also present in the leukocytes, found in the saliva and large portions are contained in the pure secretions generated by the salivary glands [Chauncey, 1961]. When checked for esterase activities, *Streptococcus salivarius* was found to have moderate to intense esterase activities [Chauncey, 1961] and the level of proteolytic enzymes was associated with the existence of periodontal disease [Nakamura, 1983a]. Nakamura et al [1983a] studied the extent to

which of the sources of salivary constituents contribute to the total amount of enzymes. Oral micro-organisms were found to produce a large portion of these activities and some of the activities found in the saliva were attributed solely to micro-organisms associated with dental plaque. Esterase activity in the saliva has been found to also be derived from epithelial cells [Lindqvist, 1977].

Studies have been conducted in order to investigate the relationship between systemic conditions and enzymatic activity in the oral cavity [Güven, 1996; Lundgren, 1996; Lenander-Lumikary, 1998]. Lenander-Lumikary et al [1998] found that salivary myeloperoxidase levels increase in adult asthmatic patients compared with healthy adults. Güven et al [1996] demonstrated an elevated amount of salivary peroxidase in patients with insulin-dependent (type I) diabetes mellitus. Lundgren et al [1996] showed that patients suffering from Papillon-Lefvere syndrome (PLS) exhibit lower unstimulated and stimulated salivary flows, as well as peroxidase levels in stimulated saliva as compared with healthy patients. It should be mentioned that the aforementioned systemic conditions were also linked to a compromised periodontal health, so changes in enzymatic activities could be used as markers for periodontal disease.

Other studies have placed an emphasis on the relationship between enzymatic activity in gingival crevicular fluid and human saliva and the status of periodontal disease [Genco, 1984; Ingman, 1996]. Higher enzymatic activity levels were found in numerous investigations [Nakamura, 1983a; Yamalik 1990 and 1991; Gazi, 1996; Ingman, 1996;

Klimec, 1997]. The elevated enzyme levels have been partially attributed to the presence of a marked accumulation of bacteria associated with periodontal lesions [Slots, 1979] and dental plaque such as *Bacteroides gingivalis,* Capnocytophaga, spirochetes [Nakamura, 1983; Slots, 1983; Zambon, 1985], and *Treponema denticola* or *Porphyromonas gingivalis* [Loesch, 1990; Pederson, 1994; Gazi, 1996]. Bacteria can elaborate a number of hydrolytic and proteolytic enzymes [Fotos, 1990] along with a wide range of metabolic by-products [Schuster, 1990] on the surface of the restoration, to higher levels than those in the saliva [Munksgaard, 1990]. Cox and Eley [1987] have shown that gingival crevicular fluid contains cathepsin B and tryptase–like activities. In other studies [Cox, 1989], gingival crevicular fluid was found to possess a trypsin-like activity that was suspected to have been derived from subgingival plaque, associated also with periodontal disease.

The considerable presence of micro-organisms in periodontitis generates a cascade of host responses [Genco, 1984] which often leads to the destruction of the periodontium, mediated via enzymatic activity [Loesch, 1988; Cootauco, 1993]. The inflammatory response may also be triggered by the presence of a foreign body within the oral biological tissue, specifically in the periodontal sulcus. Such a foreign body could be a composite resin restoration, to which oral streptococci can adhere [Yamamoto, 1989; Zalkind, 1998].

Pseudocholinesterase is one of the numerous enzymes present in the saliva. PCE was shown in a previous study to significantly reduce the surface micro-hardness of two commercial dental restorative composite resins compared to the control [Leung, 1997]. Cholinesterases (ChE) constitute a group of esterases that hydrolyze choline esters at a higher rate than they do other esters [Ryhänen, 1983a]. Various types of ChE can be differentiated by the use of either specific substrates or selective inhibitors [Ryhänen, 1983a]. In humans, two main types of ChE exist: acetylcholinesterase, which is physiologically important and highly specific to acetylcholine; and pseudocholinesterase (PCE), which hydrolyzes a few other choline esters as well [Ryhänen, 1983a]. ChE are generally accepted as being synthesized in the liver, and although the exact function of PCE is not known, it can serve as a useful indicator of organophosphate poisoning and liver disorders [Foldes,1966; Brown, 1981].

The PCE activity in several body fluids has been analyzed by Ryhänen et al [1983b]. The level of the enzyme activity was approximately the same in sweat, lacrimal fluid, urine, parotid fluid and whole saliva, but it was only 1/1500 of that of plasma [Ryhänen, 1983b]. Ryhänen et al [1983a], studied the existence and origin of ChE in human oral fluids. PCE activity was measured by using butyrylthiocholine iodide as a substrate. Males had approximately twice as much salivary PCE activity as did females [4.8 ± 2.4 S.D. units/L and 2.2 ± 1.5 S.D. units/L respectively]. Salivary PCE activity showed diurnal variation; activities were about three times greater at four a.m. than at four p.m.. Parotid PCE activity correlated with that of the whole saliva, while the enzyme activity in gingival crevicular fluid (GCF) was much higher at 120 ± 48 S.D. units/L. Induced

gingivitis did not show a significant elevation in the PCE activity in the saliva but showed an increase in the enzyme GCF activity levels as compared with that of the healthy patients. Furthermore, no PCE activity was found in sonicated samples of plaque, indicating that the enzyme activity originated primarily from saliva. These results were in contrast to other studies [Chauncey, 1961; Yamalik, 1990], which reported that bacteria and food particles might be responsible for part of the ChE activity in oral fluids. Yamalik et al [1990], investigated the PCE activity levels in the GCF, saliva and serum from patients with juvenile periodontitis (JP) and rapidly progressive periodontitis (RPP). The PCE activity levels in the GCF samples for the JP, RPP and the control groups were 181 ± 48, 588 ± 135 and 88.5 ● 29.1 units/L respectively, while the salivary PCE activity levels were respectively 9.1 ± 1.7, 21.8 ± 4.5 and 12.7 ± 0.8 units/L. The authors also found that GCF contained a higher PCE activity than saliva but lower than serum. The RPP group has a significantly higher enzyme activity in both GCF and saliva than that of the JP and the control, although no significant difference could be found in their serum levels. The authors suggested that the increase in the PCE activity seen in the RPP patients might be caused by either the direct production of esterase by bacteria or the induction of esterases during periodontal destruction. In a latter study, Yamalik et al [1991] observed the effect of different stages of periodontal therapy on salivary PCE activity in patients with periodontitis. The mean PCE activity in whole saliva was 17.95 units/L in the pre-treatment phase, 4.65 units/L following scaling and oral hygiene instructions and 1.93 units/L following surgical procedure. It was observed that the mean salivary PCE activity in patients with periodontitis showed a tendency to decrease in both

phases of treatment, and PCE levels at the end of periodontal therapy were similar to those of healthy subjects.

Mononuclear phagocytic cells, i.e. macrophages and monocytes, are presented in normal and inflamed gingiva both in human and animal periodontal diseases [Payne, 1975; Tenovuo, 1990; Lappin, 1999]. The origin of such cells is with blood monocytes [Ebert, 1939], which are chemotactically attracted into inflammatory sites by numerous substances and become activated in tissue to form macrophages. In inflamed gingiva, macrophages show a striking positive activity, both for non-specific esterase and acid phosphatase activities [Seymour, 1978]. Most of the esterase related activity in mature macrophages is cholesterol esterase (CE) [Li, 1997]. Cholesterol esterase generation increases in macrophages under a variety of conditions, both during the nonspecific immune response in acute inflammation [Lindhorst, 1997] and following exposure to a biomaterial. CE was shown in previous work to significantly reduce the surface micro-hardness of three commercial dental restorative materials [Santerre, 1999] and caused the release of elevated levels of degradation products from both commercial and experimental [Shajii, 1999] dental restorative composite resins compared to the control samples incubated with buffer solutions at pH 7.0. Although CE has not been isolated from human saliva, CE-like activity has been found during the course of this thesis [Pershad, 1999]. Its presence in macrophages, located within the gingiva may be of great importance, especially when composite resin restorations have gingival or subgingival margins.

1.3.9 BIODEGRADATION OF DENTAL COMPOSITE RESINS

Biodegradation is defined as the gradual breakdown of a material, mediated by specific biological activity [Williams, 1987]. Degradation of composites in the oral cavity has been associated with several sources; enzymatic hydrolysis [Munksgaard, 1990], thermal cycling [Mair, 1989], mineralization [Asmussen, 1984], pH changes [Lefebvre, 1995] oxidation [Ruyter, 1988] and salivary types [Kao, 1989; Mair, 1991]. In the mouth, this is a complex process, including disintegration and dissolution in saliva and other types of chemical or physical degradation, wear and erosion caused by food, chewing, drugs, and salivary and bacterial activities. No *in vitro* test is capable of reproducing this complex and synergistic process [Øilo, 1992].

Studies have investigated the degradation or material loss in dental composites via three principle modes [Ferracane, 1994]; wear [Condon, 1997; Jaarda, 1997; Söderholm, 1998], elution processes [Hamid, 1997; Nathanson, 1997; Geurtsen, 1998a and 1988b; Lee, 1998; Spahl, 1998; Vaubert, 1999], and chemical degradation [Øysæd, 1988; Freund, 1990; Larsen 1991 and 1992].

Mechanical wear is a principal factor contributing to degradation and accounts for most of the material loss [Winkler, 1991]. It has also been associated with a reduction in the composites' diametral tensile strength and hardness values [Truong, 1988]. Anusavice et

al [1990] have criticized the use of these measures of degradation since the wear process is complex in nature and includes more than just mechanical loading but also chemical reactions and fluid sorption. For instance, Chadwick et al [1990], showed an increase in the wear rate of commercial composite resin material following incubation in citrate buffer as compared with distilled water. Specimens that had been stored in lactate buffer (pH 4) also exhibited signs of increased degradation as measured by surface hardness changes. It was suggested that possible explanation for these changes was related to the hydrolysis of the ester groups within the resin matrix. This reaction is acid-catalyzed and hence is pH-dependent. In addition to the effects upon the resin matrix, degradation of the particulate fillers may also be involved. All composites leach silicon as a result of stress corrosion attacks upon the glass fillers [Söderholm, 1983]. The leakage of filler constituents produces cracks at the resin-filler interface [Roulet, 1984, Söderholm, 1984], which may weaken the material. In another investigation, Wu et al [1982] demonstrated an increase in the initial wear rate and decrease in the Knoop Hardness values once the composites had been immersed for two weeks in chemicals known to soften the cross-linked matrix material. These included cyclohexanone and various ethanol-water mixtures, and were compared with water. An increase in composite resin wear rates [Kula, 1997] as well as surface degradation [Papagiannoulis, 1997], has been reported also following the application of topical fluoride gels. In conclusion, intra-oral conditions can be expected to be more complex than those achieved by employing 37°C in distilled water, as is frequently done in accelerated laboratory wear measurements use [Wu, 1982].

Elution of unused/unreacted components from dental composites is a diffusion rate-dependent process. Diffusion is dependent upon polymer type, surface treatment of the filler particles and the type of solvent [Øilo, 1992; Feracanne, 1994]. Tests in water have shown that approximately 50 % of the leachable species are eluted within three hours, while when soaked with an ethanol/water mixture, 75 % of the leachable molecules were eluted during the same time [Ferracane, 1990]. Elution of nearly all-leachable components was completed within 24 hours in both solvents. Numerous studies have focused on the leaching of unreacted monomers from dental resins [Gerzina, 1994; Hamid, 1997; Geurtsen, 1998a and 1998b; Vaubert, 1999]. Gerzina et al [1994] reported the release of TEGDMA and bisGMA monomers by means of HPLC. Another study [Tanaka, 1991], have utilized gas-liquid chromatography and mass spectrometry (GC-MS) to identify and quantify unreacted TEGDMA and bisGMA that eluted in water. Recent studies detected other species in addition to TEGDMA and bisGMA [Vaubert, 1999]. These included such comonomers as hydroxyethyl methacrylate (HEMA) [Geurtsen, 1998a], bisPMA, bisEMA, UDMA, MMA and more [Geurtsen, 1998b], bisMA [Nathanson, 1997], as well as additives and initiators such as camphorquinone (CQ) [Geurtsen, 1998b], tryphenyl stibine and ultra-violet stabilizers [Lee, 1998]. Other studies have detected the elution of methacrylic acid, MMA and benzoic acid from acrylic denture base materials, in distilled water [Koda, 1988], artificial saliva at different pH's [Koda, 1990] and whole human saliva [Baker, 1988]. Shintani et al [1993] reported on the presence of the additive N,N-dimethyl p-toludine leaching from acrylic denture base materials.

The leaching of components from dental composites has a potential impact on the structural stability of the material. A 75 vol. % ethanol/water solution was shown to soften a bisGMA based resin [Wu, 1982]. As well, decreases in diametral tensile strength have been reported for composite exposed to various solutions [Lee, 1994]. Lee et al [1998] demonstrated that a decrease in the diametral tensile strength of bisGMA-UDMA and bisEMA-TEGDMA based commercial composite resin materials after 30 days immersion in ethanol (35- 50 %), proprionic acid (25-30 %) and acetic acid (40–60 %) accompanied the leaching of components from the resin. For most of the elution studies, the materials were incubated for relatively short periods, up to a maximum of two weeks. There is now concern that many of the components that are leached out during laboratory tests would not necessarily be found clinically, because of chemical changes occurring with time during intra-oral surface interactions with salivary components, among which are included enzymes.

The hydrolytic action of enzymes on the surface of composite resins has been the subject of various investigations [Munksgaard, 1990; Bean, 1994; Santerre, 1999]. Micro-hardness has been used as a method for assessing the effect of enzymatic hydrolysis on the materials *in vitro* [Munksgaard, 1990; Larsen, 1992; Tsang, 1994; Santerre, 1999] and *in vivo* [van Groeningen, 1986]. Larsen et al [1991] reported a decrease in the hardness of the surface of bisGMA-TEGDMA based polymers after incubation with porcine liver esterase for 48 hours. Since the softening effect was significant only with the use of a light test load, the author attributed this to a superficial action of the esterase on the samples, which caused the release of methacrylic acid. This lead to a plasticizing effect

since the release of methacrylic acid increased the hydrophilicity of the surface, which was suspected to have enhanced water uptake by the polymer matrix and yielded a greater softening of the material. Since the softening effect is associated with the loosening of matrix polymer structure, an increased propensity to surface staining may also be associated with the softening of composites [Asmussen, 1984]. Material discoloration is a clear marker of chemical change, which has been consistently observed around the margins of restorations that exhibited microleakage [Prati, 1990].

Hydrolytic degradation [Munksgaard, 1990; Larsen, 1991; Santerre, 1999] can predispose the material to mechanical wear during mastication [Munksgaard, 1990; Larsen, 1991]. When a softened surface layer is removed, new exposed material can be subjected to enzymatic attack and a repeated wear process. Munksgaard et al [1990] observed a substantially greater mean loss of dry weight when bisGMA-TEGDMA based samples were incubated with esterase than those incubated with buffer only. In another study, simulating the proximal wear processes in commercial composites, an increase in the wear was observed for Z-100 incubated with porcine liver esterase, suggesting that the enzymatic attack could start at the surface of the composites [deGee, 1995]. For commercial materials with smoother surfaces, Heliomolar and Silux-Plus, the effect was not significant, possibly because of the lower friction with these materials.

The degradation products of the resin-enzyme interactions can be specifically identified using chromatography techniques combined with mass spectrometry [Tanaka, 1991;

Wang, 1997]. Bean et al [1994] used high performance thin layer chromatography (HPTLC) to analyze degradation products following incubation with non-specific porcine liver esterase. Methacrylic acid and alcoholic components were proposed as degradation products of the commercial composites, and resulted from an enzymatic attack of unreacted methacrylate groups. The author suggested that these unreacted groups could be derived from unreacted monomers or they may come from methacrylic acid terminals of oligomers. When the rate of enzyme catalyzed hydrolysis for different monomers were measured, TEGDMA showed a higher rate of degradation, as compared with bisGMA. Other studies have confirmed this [Larsen, 1991]. The effect of non-specific esterase on the degradation of model bisGMA-TEGDMA based polymerized resins has been investigated [Munksgaard, 1990; Larsen, 1991 and 1992] and methacrylic acid was also detected by HPLC. In the latter study, the polymers were crushed and extracted with ethanol in order to remove unreacted monomer from the matrix prior to the degradation. Hence, the results led the authors to conclude that the source of methacrylic acid was related to partially reacted monomers (or oligomers) that were bonded in the matrix only by one methacrylate group. The study also demonstrated that an increase in the amount of TEGDMA in the resin caused an increase in the methacrylic acid production following incubation of cured composite mixtures with the enzyme.

Biodegradation studies have investigated the effect of the inflammatory cell-derived enzyme, cholesterol esterase, on composite dental materials [Santerre, 1999; Shajii, 1999] and on the degradation of various polymers [Santerre, 1993 and 1994; Labow, 1994 and 1995]. It was also shown that a salivary enzyme, pseudocholinesterase, was able to

generate biodegradation products from commercial dental composite [Leung, 1997]. Santerre et al [Santerre, 1999] had studied the effect of cholesterol esterase on the degradation of three commercial composite resins differing in their monomer chemistry and content, as well as filler type and content. The data showed the nature of that degradation products varied in content across the materials. A urethane-modified bisGMA system (TPH, DENTSPLY/L.D. Caulk, Milford, DE) was found to show a 10 fold lower release of bisGMA and TEGDMA derived products relative to that of two bisGMA-TEGDMA based systems (Silux-Plus and Z-100, 3M Corp., Minneapolis, MN). Furthermore, the latter two materials, each distinct in their filler type and content, showed a different product release profile. Shajii et al [1999] investigated the effect of filler content, in microfilled composites, on the liberation of biodegradation products following incubation with cholesterol esterase. It was found that after 8 days of incubation with the enzyme, higher amounts of bisGMA and TEGDMA derived products were isolated in the lower filler model material, while the opposite effect was observed between 8 and 16 days. They concluded that the biodegradation product release profiles were dependent on the filler/resin ratio.

Oxidation processes have been reported on by Øysæd et al [1988]. Using HPLC combined with UV spectroscopy, they demonstrated the release of formaldehyde from commercial composite resins immersed in water for up to 115 days. The authors suggested two different mechanisms resulting in formaldehyde formation. 1) The primary oxidation of unreacted methacrylate groups, in the presence of air, was seen as a possibility in both unreacted monomers or pendant vinyl groups (originating from

partially reacted monomers within the matrix). 2) the decomposition of an alternating copolymer of methacrylate with oxygen could also occur, presumably formed during the initial stage of polymerization. There was some concern that the detected level of formaldehyde (up to 0.5 $\mu g/cm^2$) could possibly lead to allergic reactions.

1.3.10 BIOCOMPATIBILITY OF COMPOSITE RESINS

Modern dental composite resin materials contain a great variety of different monomers and additives. Because of the complex chemical composition, the incomplete conversion of monomers and biodegradation processes, it is found that numerous substances, such as comonomers, additives, polymerization products and degradation products may be released from the restoration into the adjacent tissues and the oral cavity [Söderholm, 1983 and 1984; Øysæd 1988; Ferracane, 1990 and 1994; Santerre, 1999; Shajii 1999]. The interaction of these components with cells at the molecular level could cause tissue reactions such as inflammation [Hansasuta, 1993], necrosis, immunogenesis and carcinogenesis [Wataha, 1994]. In many studies, it was found that composite resin fillings may cause pulp inflammation. Stanley [1992] reported that the degree of pulpal alterations increases in deeper cavities. Furthermore, it has been determined *in vivo* that several composite components such as dibenzoyl-peroxide or 2-hydroxy-4-methxy-benzophenone may cause pulp inflammations if protective cavity liners are not used [Stanley, 1979]. The risk that leachable components from a composite resin could affect tissues adjacent to composite restoration (pulp or periodontium) is correlated to several

factors such as the liberated amounts of products, the dentinal contact area, the diffusion of products through the dentine as well as their accumulation in the pulp [Wataha, 1994].

Genotoxic, mutagenic or estrogenic effects due to extracts from composite resins, light curing glass-ionomer cements, dentin bonding agents and various composite resin ingredients have been investigated in several *in vitro* studies [Heil, 1996; Olea, 1996; Schweikl, 1998]. The cellular toxicity of aqueous extracts from four dental composite resins after diffusion through dentine was investigated by Hanks et al [1988] using Balb/c 3T3 cells. During the first 24 hours, cytotoxic responses were observed. Nakamura et al [1983b], determined the long-term biocompatibility of consecutive extracts from two composite resins over a period of 20 weeks by means of HeLa cells. They found severe cytotoxic reactions with the 2-week extracts. Polymerized composite specimens were tested by Geurtsen [1986, 1987] for up to 47 months in primary human gingival fibroblasts and permanent L 5178y cells. The set resin material revealed a moderate cytotoxicity, which did not decrease during the entire period of the investigation. HPLC analyses of the extracts indicated that TEGDMA was the component eluted from the composites. By extraction of the unreacted components with several organic solvents, the cytotoxicity of polymerized composite specimens in Balb/c 3T3 cells was reduced to 10 % [Rathbun, 1991]. Based on these findings, it was suggested that an increase in the degree of conversion should be applied in order to improve the biocompatibility of composites. This work was further supported by Caughman et al [1990].

The reversibility of cytotoxicity of 11 isolated composite ingredients (comonomers, precursors, initiators and coinitiators) was determined by Hanks et al [1991] using permanent Balb/c 3T3 cells. The toxicity values could be rated into three different concentration ranges, with ethoxylated bisphenol A dimethacrylate being the most toxic substance. A very high toxicity was determined for bisGMA, UDMA and EGDMA. Lehmann et al [1993] reported that bisGMA was highly toxic in permanent and primary human oral fibroblast cell cultures types. In addition, it was found that the primary cells were more sensitive to the toxic effects than the permanent lines. The toxicity of urethane dimethacrylate was investigated with a cell line and primary human foreskin fibroblasts, using flow cytometry [Nassiri, 1994]. At a concentration of 50 μM, which was lethal, cell growth was inhibited significantly. However, cellular alterations caused by sublethal concentrations were reversible after withdrawal of the monomer from the culture medium. Geurtsen et al [1998c] evaluated the cytotoxicity of 35 dental composite resin components in permanent 3T3 and three human primary fibroblast cultures. Within the groups of comonomers, initiators, and coinitiators, severe (e.g. bisGMA, UDMA) or moderate (HEMA, CQ) cytotoxic effects could be observed. Morphological changes of periodontal ligament cells in the presence of commercial composites were observed [Adams, 1994, 1995]. Vacuolation, cell disruption and cell loss were evident for most materials, while each material exhibited a distinctive pattern of cell injury.

As mentioned before, the polymerization contraction may lead to a gap formation around the cavity margins, resulting in micro-leakage and its possible sequelae; passage of fluids, bacteria molecules, ions or air between the restorative material and the cavity walls.

These events usually result in pulpal irritation, thermal sensitivity and recurrent caries [Asmussen, 1975; Condon, 1998; Kinomoto, 1999]. Mutans streptococci and lactobailli were specifically identified in plaque found at the margins of composite fillings and during cavity preparations [Svanberg, 1990; Kidd, 1993]. In addition, it has been documented that comonomers such as EGDMA and TEGDMA may promote the proliferation of important cariogenic microorganisms, *Lactobacillus acidofilus* and *Streptococcus sobrinus* [Hansel, 1998]. These observations corroborate the hypothesis that bacteria proliferating within the gap between the composite resin restoration and the cavity walls might be a main causative factor for alteration of the pulp [Brännstrom, 1981; Cox, 1992].

Exposure to estrogen-mimicking compounds (xenoestrogens) has been judged to be a potential problem for humans as well as for wildlife [Raloff, 1994; Safe, 1997]. As early as 1936, Dodds and Lawson [1936] reported the estrogenicity of some diphenyl compounds containing two hydroxyls in the para position. One such derivative, bearing two methyl groups and known as bisphenol A, has been used by some manufacturers for the synthesis of bisGMA [Bowen, 1965] and bisphenol A dimethacrylate or bisMA [Ruyter, 1981]. The bisGMA molecule itself may not be an estrogen agonist if the bisphenol A groups present in the bisGMA molecule are sterically hindered and if the monomer is pure and does not contain any impurities. However, this assumpion may not be correct [Glenn, 1979], due to impurities in the material. Olea et al [1996] reported that significant quantities of bisphenol A leached from dental resins and sealants into the saliva of treated patients. They tested the estrogenicity of this compound and its

dimethacrylate ester (bisMA) *in vitro* using the estrogen-responsive MCF-7 breast cancer cell line as an indicator system, and found that both compounds effected an increase in cell proliferation. BisGMA, by itself, was unable to stimulate proliferation. The stimulation effect on the proliferation of cell lines were corroborated by Schafer et al [1999]. It is important to note *in vitro* experiments in two subsequent studies to the Olea work failed to confirm the presence of bisphenol A in eluates of dental resins [Hamid, 1997; Nathanson, 1997], but the elution of bisMA from two fissure sealant brands was reported [Nathanson, 1997]. However, it should be noted that the materials in these *in vitro* studies, were incubated either in ethanol (95 %) [Nathanson, 1997] or water [Hamid, 1997], which do not exhibit strong hydrolase activities onto bisphenol A derived dimethacrylates. Such activities, which are present in human saliva [Nakamura, 1983a] may cleave bisMA molecules to produce bisphenol A [Fung, 1999].

In summary, the estrogenicity of substances present in dental composite substances is a relevant issue since xenoestrogens are fat soluble and can gain access to other cells via the oral or gastrointestinal routes [Nagel, 1997; Ben-Jonathan, 1998]. It is not known whether bisphenol A and other xenoestrogens accumulate in the human body or how they are metabolized. Also controversial are: 1) the level of exposure of the population to these compounds; 2) exactly what levels are sufficient to cause harmful effects *in vivo* [vom Saal, 1998]; and 3) whether xenoestrogens have additive effects. However, current studies indicate that both bisphenol A and bisphenol A dimethacrylate have the potential to be estrogenic at micromolar concentrations *in vitro*.

2.0 MATERIALS AND METHODS

2.1 MATERIAL SYNTHESIS

All of the composite resin materials used in this study were synthesized at the Biomedical Polymer Science Laboratory located within the Faculty of dentistry at the University of Toronto. The materials used for the synthesis of the composite resins are listed in Table 2.1, along with their abbreviations, manufacturer and manufacturer catalog number.

2.1.1 RESIN PREPARATIONS

BisGMA and TEGDMA were supplied in kind by Esschem (Linwood, PA) and the urethane-modified bisGMA-TEGDMA-bisEMA (u-bis) resin was supplied in kind by L.D. Caulk/ DENTSPLY (Milford, DE). All monomers were stored at 4°C until use. A bisGMA-TEGDMA (bis) model resin was formulated to contain 55 % w/w bisGMA and 45 % w/w TEGDMA. The weight fraction for this latter model was based on current resin compositions for commercial restorative composite resins [3M- Z-100 (MSDS), 3M-Silux Plus (MSDS), [Ferracane, 1992]. In order to facilitate the handling of the high viscosity bisGMA monomer, the monomer was heated to 37°C for one hour prior to the addition of TEGDMA. The bisGMA and TEGDMA monomers were mixed at room temperature, in a glass vessel for 20 minutes at 500 rpm, using a shear dispersion rotor blade (Type P4, Bowers, Stratford, ON).

Table 2.1 Chemicals used in the synthesis of model composite resins

Materials	Abbreviation	Manufacturer catalog number	Manufacturer
2,2-bis[4-(2-hydroxy-3-methacryloyloxypropoxy) phenyl] propane	BisGMA	X 9500000	Esschem (Essington, PA)
Triethyleneglycol dimethacrylate	TEGDMA	X 9437446	Esschem (Essington, PA)
BisGMA/diisocyanate resin (urethane modified bisGMA)	u-bisGMA	999452	DENTSPLY/ Caulk (Milford, DE)
Ethoxylated bis-phenol-A dimethacrylate	BisEMA	X 970000	Esschem (Essington, PA)
Camphoroquinone	CQ	1073-78-1	Aldrich (Milwaukee, WI)
2(dimethylamino)ethyl-methacrylate	DMAEM	2867-47-2	Aldrich (Milwaukee, WI)
1,6 Diisocyanatohexane	HDI	D 12,470-2	Aldrich (Milwaukee, WI)
Hexamethylene Diisocyanate (1,6 ^{14}C) (8.80 mci/mmol)	^{14}C-HDI	CUS-030C	Du-Pont New England Nuclear (Boston, MA)
Silanated, milled SF BABG glass	Barium glass filler	999451	DENTSPLY/ Caulk (Milford, DE)

Prior to the addition of the initiator and reducing agent, both resin systems (i.e. bis and u-bis) were warmed to room temperature for one hour. To each resin system, 0.3 % w/w (relative to the resin component) of the initiator Camphorquinone-CQ (12489-3, Aldrich, Milwaukee, WI) was added, followed by the addition of 0.1 % w/w 2-(Dimethylamino) Ethyl Methacrylate-DMAEM (23490-7, Aldrich, Milwaukee, WI) as the reducing agent. The weight ratio of DMAEM to CQ was established at 1:3 in order to have a maximum degree of conversion [Yoshida, 1993]. The mixtures were blended for an additional 20 minutes until a homogeneous mixture was obtained, while being covered with aluminum foil to prevent light polymerization.

2.1.2 COMPOSITE MATERIAL PREPARATION

Four composite resin materials were prepared. For each resin type (bis or u-bis) two composite resin materials with different filler contents were prepared: 10 % w/w and 60 % w/w. To each of the resin-initiator-reducing agent mixtures, the appropriate amount of silanated barium glass filler (1 micron average diameter), supplied in kind by L.D. Caulk /DENYSPLY, Milford, DE, was added. This was achieved by gradually adding the filler into the resin over a period of 30 minutes using a shear dispersion rotor blade (Type P4, Bowers, Stratford, ON) at 500 rpm. Table 2.2 contains the amounts of each component used in the synthesis of the different composite resin materials. The freshly made composite resin mixtures were degassed in a vacuum oven (-760 mmHg gauge pressure, 30°C) overnight. After degassing, the composite resins were transferred to glass containers and stored refrigerated (at 4°C) until required.

2.1.3 PREPARATION OF CURED COMPOSITE MATERIAL

Prior to the preparation of the cured composite resin samples, the composite resin mixtures were warmed at room temperature for one hour. All tools and molds were disinfected with 70 % ethanol solution. Standardized samples of the composite resin were made using a Teflon^{tm} mold (4mm diameter, 4mm long) and Mylar^{tm} covered glass clamped from both sides (Figure 2.1). The mold was specifically designed to allow

Table 2.2 Composition of model bisGMA-TEGDMA (bis) and urethane modified bisGMA-TEGDMA-bisEMA (u-bis) based composite resins (all amounts are in grams and related to a total weight of 100 grams)

Component	bis 10	Bis 60	u-bis 10	u-bis 60
BisGMA	49.30	21.90	-	-
TEGDMA	40.34	17.93	-	-
u-bisGMA-TEGDMA-bisEMA	-	-	89.64	39.84
Camphorquinone	0.27	0.12	0.27	0.12
Dimethylaminoethyl methacrylate	0.09	0.04	0.09	0.04
Barium glass filler	10.00	60.00	10.00	60.00

for an optimal curing depth of 2 mm [Ruyter, 1982; Caughman, 1995]. The composite resin inserts were initially photocured using a light oven with self calibration curing process (AC UNILUX, Kulzer, Germany) that was set to program number 1. Following this cure, the mylar[tm] strips were removed and the samples were pushed out of the mold using a clean plastic instrument. Additional photocuring, using program #10 on the curing unit was then carried out. The samples were post cured in a vacuum oven (-760 mmHg gauge pressure, 60°C) for 48 hours to promote the removal of volatile residual monomers and to increase the degree of monomer conversion [Ferracane, 1992; Bagis, 1997].

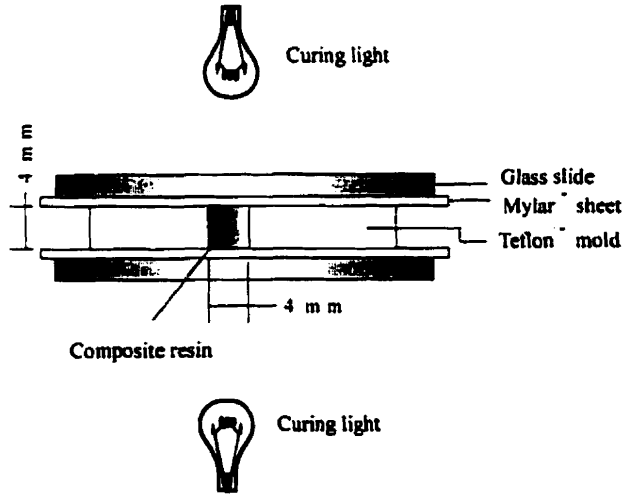


Figure 2.1 Cross-sectional view of the sample preparation apparatus

2.1.4 SYNTHESIS OF RADIOLABELED U-BISGMA-TEGDMA-BISEMA (^{14}C-u-bis)

The commercial u-bis resin, supplied in kind by L.D. Caulk, contained u-bisGMA, bisEMA and TEGDMA in a weight ratio of approximately 45:45:10 respectively, as analyzed by gel permeation chromatography (GPC) and high performance liquid chromatography (HPLC). In order to provide a sensitive measure of degradation products associated with the urethane bisGMA monomer itself, the analog of the commercial u-bis material was synthesized at the Biomedical Polymer Science Laboratory. A final radiolabel concentration of approximately 5 x 10^5 counts per minute (CPM) per 100 mg of composite resin material was targeted. This value was based on previous experiments, which yielded detectable levels of degradation with polyurethane materials [Santerre, 1994]. To the bisGMA monomer, bisEMA (supplied in kind by Esschem, Essington, PA) and TEGDMA monomers were added as diluents. The weight ratio for the monomers was 40:45:15 respectively. The monomers were mixed at room temperature, in a glass vessel for 20 minutes using a shear dispersion rotor blade (Type P4, Bowers, Stratford, ON) at 500 rpm. The mixture was then degassed in a vacuum oven (-760 mmHg gauge pressure, 55°C) for 24 hours. 30.5 grams of the monomer mixture was then blended and heated to 60-70°C in a glass vessel, using a magnetic stirrer on a heated mixing plate, placed in a glove box and purged with dry nitrogen gas.

To the monomer mixture, 0.25 mCi of ^{14}C-1,6 hexamethylene diisocyanate (^{14}C HDI, CUS-030C, Du-Pont, sealed ampoule in dry N_2) and of 2.6 grams non-labeled distilled

HDI (D12,470-2, Aldrich, Milwaukee, WI) were gradually added and mixed together over a period of 60 minutes. Prior to use, the non-labeled HDI was distilled in order to remove trace impurities and separate residual dimerized diisocyanate from the monomer. The stoichiometry of the bisGMA to HDI was 1:0.65 respectively. After an additional 60 minutes, the reaction was quenched by adding methanol (20% stoichiometry of the isocyanate groups). The mixture was then degassed in a vacuum oven (~760mmHg, 55°C) for 24 hours. A schematic diagram of the reaction pathway for the ^{14}C-u-bis is presented in Figure 2.2. The synthesized resin mixture was analyzed for molecular weight by gel permeation chromatography (GPC) and for chemical bond analysis by Fourier transform infra red (FT-IR) spectroscopy, as described in sections 2.2.1 and 2.2.2 respectively.

To the ^{14}C-u-bis resin, CQ, DMAEM, and barium glass filler (60 % w/w of the total composite resin mass) were added and cured samples were prepared as described in sections 2.1.1, 2.1.2 and 2.1.3. The radiolabel concentration of the ^{14}C-u-bis composite resin was 1.120 x 10^6 CPM per 100mg. After addition of the filler, the radiolabel concentration was approximately 4.5 x 10^5 CPM per 100 mg composite resin, a value reasonably close to the targeted value. Radioactivity was measured after dissolving resin samples in 0.5 mL of ethanol, contained within scintillation vials, and adding 4 mLs of scintillation cocktail (Ready Safe P/N 141349, Beckman, Fullerton, CA). The vials were then counted for radiolabeled activity (LS 6500 Multi-Purpose Scintillation Counter, Beckman, Fullerton, CA).

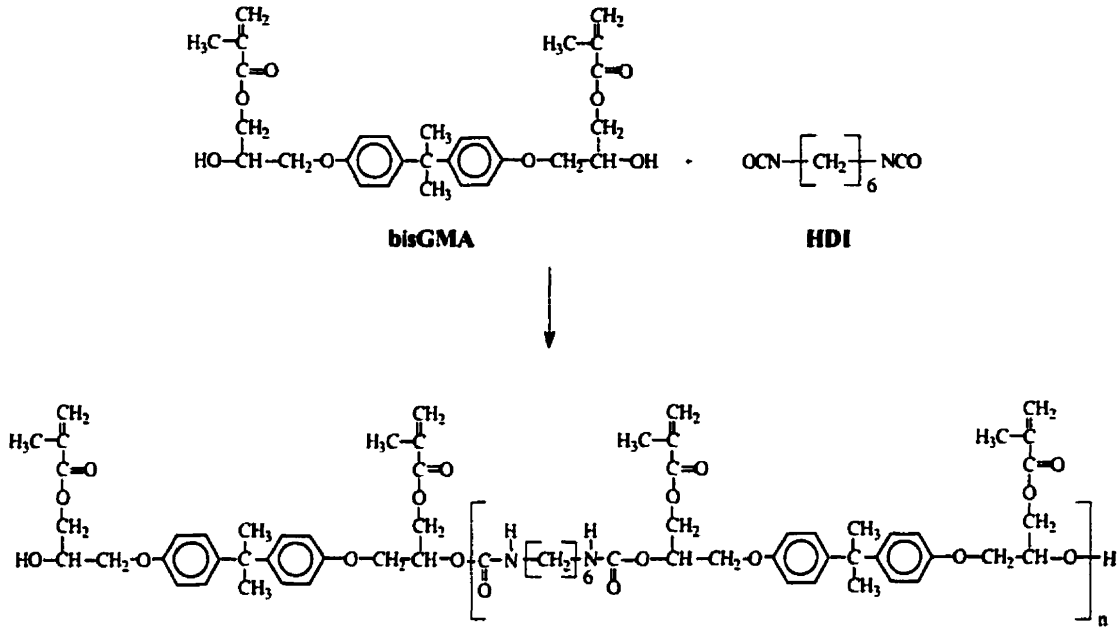


Figure 2.2 Reaction schematic for the ^{14}C-u-bis resin synthesis

2.2 ANALYSIS OF THE RESINS

2.2.1 GEL PERMEATION CHROMATOGRAPHY

Gel permeation chromatography (GPC) is a method of molecular weight determination that utilized the principle of size exclusion chromatography to separate molecules according to their relative size. The sample is carried through packed columns by a suitable solvent. The porous packing in the columns permits relatively low molecular weight species to diffuse into the particles, thus slowing their passage through the columns. In this manner, the different molecular weight species are separated and the different retention times can be correlated to their respective molecular weight. The

mobile phase used for GPC analysis was HPLC grade *N,N*-dimethylformamide (DMF) (27,054-7, Aldrich, Milwaukee, WI). The mobile phase was filtered using a 0.45 μm Teflon® filter (Chromatographic Specialties Inc., Brockville, Ontario). Waters™ Styragel™ HR1 and HR2 HPLC columns (WAT044235 and WAT044238, Waters, Milford, MA) with styrenedivinyl-benzene copolymer packing were heated to 80°C and flushed overnight with the mobile phase at a rate of 0.1 mL/minute to establish a stable baseline. Resin samples were dissolved in mobile phase at a concentration of 0.2 % w/v (2 mg/mL) and filtered using a 0.45 μm syringe Teflon® filter (Chromatographic Specialties Inc., Brockville, Ontario). A Waters™ 510 HPLC pump was used to deliver the solutions through the columns and to a Waters™ 410 Differential Refractometer detector. TEGDMA, bisGMA, bisEMA (supplied in kind by Esschem, Linwood, PA), and the commercial u-bisGMA-TEGDMA-bisEMA (supplied in kind by L.D.Caulk, Milford, DE) monomers were used as references. The synthesized radiolabeled resin was then analyzed by comparing it to the references, using a Baseline 810 Chromatography Workstation Version 3.30 software (Dynamic Solutions, a division of Millipore Corporation, Bedford, MA).

2.2.2 FOURIER TRANSFORM INFRA RED SPECTROSCOPY (FT-IR)

Most compounds having covalent bonds are found to absorb various wavelengths of electromagnetic radiation in the infrared band of the spectrum. Specifically, the vibrational region of the infra red band has been used for chemical group analysis. This region is defined at wavelengths between 2.5 μm (4000 cm^{-1}) to 15 μm (700 cm^{-1}).

Fourier Transform Infra red Spectroscopy (FT-IR) is a procedure by which the data is collected and converted from an interference signal to a spectrum. FT-IR analysis was carried out in the Ontario Laser and Lightwave Research Centre, University of Toronto. The IR spectra were recorded by means of a Bomem Hartmann & Braun system in the transmission mode. The IR transmission was measured between 4000 cm^{-1} to 700 cm^{-1}. In order to improve the signal to noise ratio, 30 scans were averaged to obtain one representative scan for each sample. The deconvolution of the data and their analysis were carried out using Bomem Grams / 386^{TM} software version 2.04 (Galactical Industrial Corporation). The spectra were obtained via transmission through thin films (~30 micron) of uncured composite resin [Ferracane, 1992] and placed on a NaCl disk (13X2mm, Spectra-Tech, Shelton, CT) for analysis. Each of the different monomer systems, bisGMA-TEGDMA, the commercial u-bisGMA-TEGDMA-bisEMA and the ^{14}C-u-bisGMA-TEGDMA-bisEMA were analyzed in the same manner.

2.3 SURFACE ANALYSIS OF THE COMPOSITE RESIN SAMPLES

Since enzyme/material interactions are prominent on the surface of biomaterials, and because surface properties cannot always be predicted from bulk observations, it becomes important to evaluate those characteristics unique to the surface. In this study, X-ray photoelectron spectroscopy, scanning electron microscopy and Fourier transform infra red spectroscopy were used as surface analysis techniques.

2.3.1 X-RAY PHOTOELECTRON SPECTROSCOPY

X-ray photoelectron spectroscopy (XPS), also known as Electron Spectroscopy for Chemical Analysis (ESCA), is a non destructive technique that provides an analysis for all elements, except hydrogen and helium, within the top 1-25 nm of any solid surface, which is vacuum stable [Andrade, 1985]. XPS provides identification of elements present at concentrations greater than 0.1 % and chemical shifts for bonding studies.

The theoretical foundation of XPS lies in Einstein's photoelectric effect

Equation 2.1 $E_b \cong h_v - E_k$

where E_b is the electron binding energy, E_k is the electron kinetic energy and hv is the photon energy. Hence, given that the photon energy is greater than the binding energy of the electron in the atom, the electron can then be ejected from the atom with a kinetic energy approximately equal to the difference between the photon energy and the binding energy of the electron. Hence, different elements can be identified by the characteristic energy required to release a specific electron from a particular element.

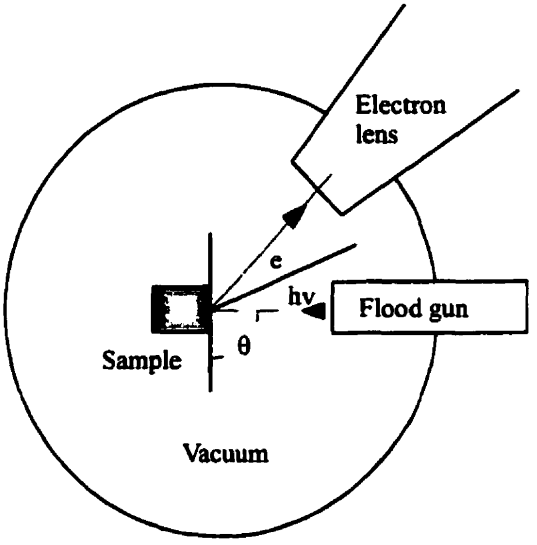


Figure 2.3 Schematic diagram of an X-ray photoelectron spectroscopy apparatus [adapted from Beamson and Briggs, 1992]

The principle of XPS is schematically illustrated in Figure 2.3. XPS is a very useful technique in characterizing polymeric biomaterial surfaces due to its strong surface sensitivity and high information content [Ratner, 1983], and has been shown to complement other methods of material characterization used in surface studies, such as transmission FT-IR and scanning electron microscopy (SEM).

The samples were mounted on a holder with the surface to be analyzed facing upward, and placed in the high vacuum analysis chamber. The output was a spectrum of photoelectron intensity versus binding energy, which allowed for elemental analysis to be carried out. Quantitation was achieved by measuring the area under each peak and

applying the correct sensitivity factor for the element. The latter is a measure of the likelihood of an electron being emitted. The depth of analysis varies depending on the sample type and the angle, theta, at which the sample is placed. The angle could be changed by tilting the sample from 15 to 90°.

From each composite resin group, three pre-incubation composite resin samples were rinsed with 1,1,3 trichlorotrifluoroethane (TCTFE) for 30 minutes, covered with aluminum foil and then dried in a vacuum oven overnight (-760 mmHg, 30°C) to remove possible traces of silicone contamination, which have a common presence in laboratories. The samples were studied at 90° theta angles, representing a 10nm depth into the samples. The samples were analyzed by Dr. Rana Sodhi at the Centre for Biomaterials, University of Toronto, using a Leybold Max 200 X-ray photoelectron spectrometer. For the methacrylate based composite resins an Al Kα source was employed. Low-resolution scans were performed for carbon, oxygen, silicon and barium. High-resolution scans were performed between –278 eV and –292 eV, which targets the C1s bonding energies. Data was processed by the Ph.D. candidate using ESCA Tools interfaced with MatLAB® for Windows, Version 4.2b.

2.3.2 SCANNING ELECTRON MICROSCOPY

Scanning electron microscopy (SEM) provides high resolution, three dimensional images with a significant depth of field. By applying a high voltage electron beam (>5 KVolt) to the surface of the sample and spatially reconstructing the intensity of the secondary

electron emissions produced by the irradiation, SEM images can be generated. The intensity of the secondary electron emission is a function of the geometry of the sample's surface.

SEM was used in this study to investigate the effect of the biodegradation experiments on the surface morphology of the different composite resin groups. Composite resin samples were analyzed prior to and following incubation with the different media. The samples were mounted on the holder using double-sided adhesive carbon tape. Prior to analysis, the samples were coated with 4 nm of platinum deposited using an SC515 SEM coating system (Polaron equipment LTD). The composite resin samples were analyzed by Mr. Robert Chernecky at the Faculty of Dentistry, University of Toronto, using a Hitachi S 2500 scanning electron microscope (Hitachi LTD, Mito City, Japan), viewed at X1000, X5000, X10000 and X20000 magnification ratio at 10 KVolt operating voltage.

2.3.3 MICRO-HARDNESS ANALYSIS OF THE COMPOSITE RESIN SAMPLES

Six composite resin samples from each of the experimental groups were selected for Vickers micro-hardness analysis. The samples were washed with double distilled water to remove impurities and residual proteins from the surface. Then they were dried with sterile gauze pads. A Miniload hardness tester (Leitz Wetzlar, Germany) was employed to perform Vickers indentations. The lamp was switched on for 15 minutes before using the instrument. A microscopic image of the surface was focused on, using the x 20 magnification objective with the coarse adjustment. A smooth surface was selected for

indentation. The testing diamond was moved into the indenting position by a rotating turret, and the load and testing diamond were released. The load was 200 grams. Following 30 seconds, the indenting unit was raised and the sample was initially focused on with the 20× objective, and subsequently re-focused on with the 40× objective. At this magnification, the length of the indentation diagonal was measured according to the Vickers formula, which assumes that both diagonals are equal in length.

Equation 2.2

$$Hv = \frac{1854 \times M}{d^2}$$

Where H_V= Vickers Hardness [Kg/mm^2]

M= Mass of the load [grams]

d= length of the indentation diagonal [μm]

2.3.4 DEGREE OF VINYL GROUP CONVERSION AT THE SURFACE

The degree of vinyl group conversion within the different cured composite resin sample groups was measured using FT-IR analysis in the transmission mode as described in Section 2.2.2. In order to measure the degree of vinyl group conversion after curing the composite resin samples, the absorbance ratio of the band intensity (i.e. peak area) for the carbon double-bond (C=C methacrylate saturation) stretch at 1636 cm^{-1} and the aromatic ring in the bisGMA at 1608 cm^{-1} was calculated by use of a baseline technique. The baseline spectra were obtained via transmission through thin films (~30 microns thick) of uncured composite resin [Ferracane, 1992], placed on a NaCl disk (13X2mm, Spectra-

Tech, Shelton, CT) for analysis. The spectra of the cured composite resins were obtained by shaving a small sliver (approximately 500 μm long by 30 μm thick) with a scalpel from the samples' surface. In order to obtain the degree of conversion, the results were compared to the baseline. The following equation was used to determine the degree of conversion (D.C.):

Equation 2.3 D.C. [%]=[1-(cured sample bands ratio/ baseline band ratio)] x 100%

Six slivers from each of the different composite resin group were analyzed in this manner, so that comparative values of degree of conversion could be provided for each group.

2.4 ENZYME STUDIES

2.4.1 ENZYME PREPARATION

In-vitro biodegradation studies focused on the use of biological activities that have been documented to be associated with human saliva and/or produced by leukocytes or oral bacteria when these cells are in an active state [Chauncey, 1961; Nakamura, 1983a]. Cholesterol esterase (CE) is of specific interest to this study and is a member of the serine enzyme family produced by monocytes and their derived macrophages, which are found, at sites of inflammation [Tenovuo, 1990]. CE was shown in previous work to significantly reduce the surface micro-hardness of three commercial dental restorative materials

[Tsang, 1994; Santerre, 1999] and caused the release of elevated levels of degradation products from both commercial and experimental dental restorative composite resins compared to the control samples incubated with buffer solutions at pH 7.0 [Santerre, 1999; Shajii, 1999]. An alternate enzyme used in this study and that has been found in saliva was represented by pseudocholinesterase (PCE) [Chauncey, 1961; Ryhänen, 1983a and 1983b; Yamalik, 1990 and 1991]. PCE was shown in previous work in our laboratory to generate biodegradation products and significantly reduce the surface micro-hardness of two commercial dental restorative composite resins compared to the control samples incubated with buffer solutions at pH 7.0. [Leung, 1997].

CE (Item No. 70-1081-01, Lot No. 9750, Genzyme, Cambridge, MA) and PCE (C-5386, Sigma, St. Louis, MO) were prepared by dissolving the enzymes at the required concentrations in phosphate buffered saline (D-PBS, 21600-010, Gibco, Grand Island, N.Y.). All solutions were sterile filtered using a 0.22 μm filter (Millex®-GP, 0.22mm Filter unit, Cat. No. SLGPR25LS, Millipore, Bedford, MA). The prepared CE and PCE solutions for replenishing were stored at -80°C.

2.4.2 ENZYME ACTIVITY ASSAYS

2.4.2.1 CE ACTIVITY ASSAY

The units of CE per mL were initially calculated based on the specification obtained from the supplier (> 10 units per mg powder; a unit is defined as the amount of enzyme that will catalyze the formation of 1.0 μmol of cholesterol per minute at 37°C under specific assay method conditions). The actual activity for this study was determined using p-nitrophenylacetate as a substrate (Labow, 1983). The reaction involved is as follows:

$$\textbf{p-nitrophenyl acetate (p-NPA)} + H_2O \xrightarrow{\text{Cholesterole esterase}} \textbf{p-nitrophenol (yellow)} + \textbf{acetatic acid}$$

This substrate was prepared by dissolving 22 mg para-nitrophenylacetate (*p*-NPA) (N-8130, Sigma, St. Louis, MO) in 1 mL methanol, which was then diluted with 100 mLs of 0.1 M sodium acetate, pH 5.0 in order to achieve a final concentration of 1 mM. Enzyme activity was determined by incubating 50 μL of the enzyme solution with a solution containing 1.0 mL of phosphate buffer, pH 7.0 and 0.5 mL of the prepared substrate solution in a 1.5 mL optical cuvette. Spectrophotometric measurements were taken at room temperature (25°C) and obtained every 30 seconds for 5 minutes, starting immediately after the enzyme addition and while the reaction rate was linear. The spectrophotometer unit was an Ultrospec® II, (LKB Biochrom, Cambridge, England). For this volume of CE, one unit of activity was defined as a change of absorbance of 0.01 O.D. per minute at 410 nm using *p*-NPA as a substrate at pH 7.0, 25°C.

2.4.2.2 PCE ACTIVITY ASSAY

The units of PCE activity per mL were initially calculated based on the specification obtained from the supplier (3.4 units/mg solid; a unit is defined as the hydrolysis of 1.0 μmol of butyrylcholine to choline and butyrate per min at pH 8.0, 37°C). The actual activity for this study was determined by using butyrylthiocholine (Figure 2.5, page 68) as a substrate [Ellman, 1961] (cholinesterase (BTC) activity kit, Sigma, Procedure No. 421). With this kit, the reactions involved were as follows:

$$\textbf{Butyrylthiocholine} + H_2O \xrightarrow{\text{Cholinesterase}} \textbf{Butyrate} + \textbf{Thiocholine}$$

$$\textbf{Thiocholine} + \textbf{5,5'-Dithiobis-2-nitrobenzoic Acid} \longrightarrow \textbf{5-Thio-2-Nitrobenzoic-Acid}$$

Cholinesterase hydrolyzes butyrylthiocholine (BTC) to yield thiocholine, which reacts with 5,5'-Dithiobis-2-nitrobenzoic acid (DTNB) (Figure 2.6, page 68) to form the yellow 5-thio-2-nitrobenzoate with an absorbance maximum at 405nm. The rate of change in the absorbance at 405 nm is directly proportional to cholinesterase activity. The reconstituted BTC kit contains approximately the following concentrations of active ingredients: Butyrylthiocholine iodide 5 mM, DTNB 0.25 mM and Buffer pH 7.2 ± 0.1. Spectrophotometric measurements were obtained on an Ultrospec® II, (LKB Biochrom, Cambridge, England) spectrophotometer at room temperature (25°C). Measurements were recorded every 30 seconds for 5 minutes, starting immediately after the enzyme addition, while the reaction rate was linear. For PCE, a unit of enzyme activity was

defined as 1 mmol butyrate released per 1 mL enzyme solution per minute. The calculation of the PCE activity was computed as follows:

Equation 2.4

$$\textbf{PCE [unit/mL]} = \frac{\textbf{O.D. x T.V.x 1000}}{\textbf{E.C. x L.P. x S.V.}}$$

Where:

O.D. = Change in the absorbance per minute at 405 nm.

T.V. = Total volume [mL].

E.C. = Molar absorptivity of 5,5'-Dithiobis-2-nitrobenzoic acid at 405 nm [13,600 M^{-1} cm^{-1}].

L.P. = Light path [1 cm].

S.V. = Sample volume [mL].

1000 = Correction factor.

2.4.3 ENZYME STABILITY ASSAYS

Prior to the incubation of samples with the enzymes, stability studies were conducted to assess the required replenishing schedule, the effect of the composite resin samples on the activity and the mutual influence of the enzymes on each other. Since multiple esterase activities are present within saliva it would be of value to determine if synergistic effects could be observed between the two enzymes investigated in this study. In both CE and PCE stability experiments, the ratio between the composite's resin sample surface area

and the volume of the incubation solution was kept identical to that of the biodegradation experiment (section 2.5.2).

2.4.3.1 CE STABILITY ASSAY

The CE-like activity found in initial solutions of CE (1 unit/mL), PCE (1 unit/mL) and CE + PCE (1 unit/mL+1 unit/mL) incubated (pH 7.0, 37°C) with and without cured bis and u-bis composite resin samples for 96 hours was measured. Measurements were taken at the following incubation times: 0, 2.0, 4.0, 6.0, 8.0, 10.0, 24.0, 48 and 96 hours. Collected aliquots from each of the incubation solutions were analyzed using *p*-NPA, as described for CE in section 2.4.2.1.

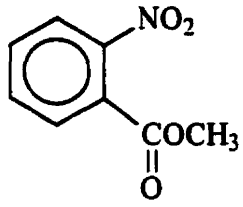
2.4.3.2 PCE STABILITY ASSAY

The stability of PCE preparations was studied using solutions of, PCE (1 unit/mL), CE (1 unit/mL) and PCE + CE (1 unit/mL + 1 unit/mL), incubated (pH 7.0, 37°C) with and without cured bis or u-bis composite resin samples for 32 days. Based on the CE stability experiment and preliminary data, the CE containing groups were replenished for CE activity daily (24 hours) in order to maintain the above specified activity. The activity of the PCE at 37°C was determined at the following times: 0, 1.0, 2.0, 5.0, 10.0, 16.0 and 32 days. The collected aliquots from the incubation solutions were analyzed using butyrylthiocholine, as described for PCE in section 2.4.2.2.

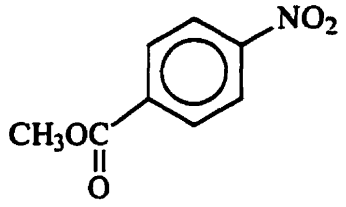
2.4.4 ENZYME SUBSTRATE SPECIFICITIES

Commonly used substrates for assaying esterases include *o*- and *p*-nitrophenylbutyrate [Saboori, 1990] and *o*- and *p*- nitrophenylactate [Labow, 1983]. The structures of the nitrophenyl esters are given in Figure 2.4. The *o*- and *p*-isomers of these substrates were used to assess the different substrate specificities of CE and PCE. The rationale behind using these isomers was to compare the activity profiles for CE and PCE when the location and the length of the side chain was changed. As well, the substrates could be used to identify the possible presence of CE and PCE-like activities in human saliva. The addition of the butyrilthiocholine (BTC) substrate permited the specific measure of PCE activity.

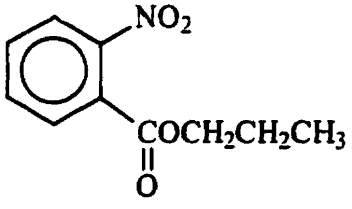
The nitrophenyl-isomers, *o*-nitrophenylacetate (*o*-NPA)(N-9001 Sigma, St. Louis, MO), *p*-nitrophenylacetate (*p*-NPA)(N-8130, Sigma, St. Louis, MO), *o*-nitrophenylbutyrate (*o*-NPB)(N-9751, Sigma, St. Louis, MO) and *p*-nitrophenylbutyrate (*p*-NPB)(N-9876, Sigma, St. Louis, MO), were prepared by dissolving 22 mg of the acetate isomers and 25.4 mg of the butyrate isomers in 1mL methanol which was then diluted with 100 mLs of 0.1 M sodium acetate, pH 5.0 in order to yield a final concentration of 1 mM. CE and PCE activities were determined by incubating the enzymes in a solution containing 1.0 mL of 0.05 M phosphate buffer, pH 7.0 and 0.5 mL of the prepared nitrophenyl ester substrate solution.



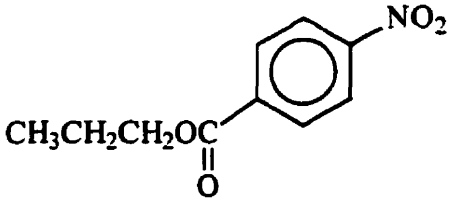
ortho nitrophenyl acetate



para nitrophenyl acetate



ortho nitrophenyl butyrate



para nitrophenyl butyrate

Figure 2.4 Structure of para-nitrophenyl acetate, ortho-nitrophenyl acetate, para-nitrophenyl butyrate and ortho-nytrophenyl butyrate

The amount of protein (solid) required for the assay of CE was 7.49 μg, when using the acetate derivatives and 0.749 μg when using the butyrate derivatives. Since the latter derivatives are more sensitive to CE, less enzyme was required in order to keep the O.D. changes per minute within the range of 0.01-0.015, where the conversion rate of the substrate is in the linear range. The amount of protein for PCE was 57.72 μg when using the nitrophenyl esters. Spectrophotometric measurements at 410 nm were taken, as described in section 2.4.2. For the nitrophenyl esters, a unit of enzyme activity was expressed as a nmol nitrophenol released per μg protein per minute. Calculation of the enzyme activities were done using Equation 2.5.

Equation 2.5

$$\textbf{Enzyme Activity [nmol/µg/min.]} = \frac{\textbf{O.D. x } 10^6}{\textbf{E.C. x L.P. x S.M.}}$$

Where:

O.D. = Change in the absorbance per minute at 410 nm.

E.C. = Molar absorptivity of nitrophenol at pH 7.0 at 410 nm [16,300 M^{-1} cm^{-1}].

L.P. = Light path [1 cm].

S.M. = Sample mass [µg].

10^6 = Correction factor.

The BTC substrate was prepared as described in section 2.4.2. The amount of protein from the commercial CE preparation was 14.98 µg and the amount of the commercial PCE was 11.55 µg for the BTC substrate. The structures of butyrylthiocholine (BTC) and 5,5'-Dithiobis-2-nitrobenzoic acid (DTNB) are presented respectively in Figures 2.5 and 2.6. Spectrophotometric measurements at 405 nm were taken, as described in section 2.4.2. For BTC, a unit of enzyme activity was expressed as mmol butyrate released per µg protein per minute. Calculation of the enzyme activities were carried out using Equation 2.6.

Equation 2.6

$$\textbf{Enzyme Activity [mmol/µg/min.]} = \frac{\textbf{O.D. x 1000}}{\textbf{E.C. x L.P. x S.M.}}$$

Where:

O.D. = Change in the absorbance per minute at 405 nm.

E.C. = Molar absorptivity of 5,5'-Dithiobis-2-nitrobenzoic acid at 405 nm [13,600 M^{-1} cm^{-1}].

L.P. = Light path [1 cm].

S.M. = Sample mass [μg].

1000 = Correction factor.

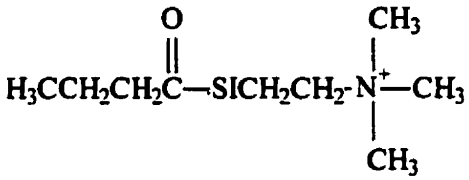


Figure 2.5 Structure of butyrylthiocholine iodide (BTC).

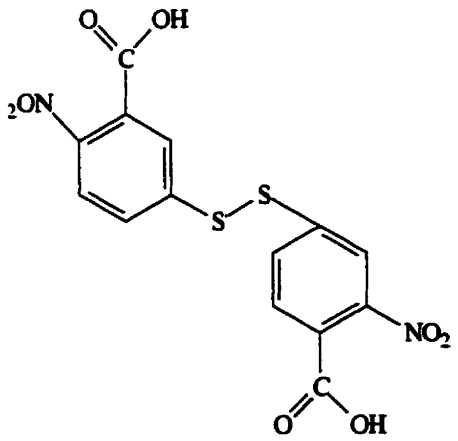


Figure 2.6 Structure of 5,5'-Dithiobis-2-nitrobenzoic acid (DTNB)

2.4.5 HYDROLASE ACTIVITY IN HUMAN SALIVA

Since enzymes are present in human saliva [Nakamura, 1983a, Chauncey, 1961], it is of great interest to determine its enzymatic activity profile and compare it to the activity profiles of the stock CE and PCE. Unstimulated human saliva was collected into 50 mLs centrifuge tubes and processed based on the methods described by Munksgaard [1990]. Micro-organisms and debris were removed by centrifugation, using a clinical model centrifuge (Centrifuge international equipment Co., Needham, MA) at 2400 R.P.M for 10 minutes and the supernatant was filtered (Millex®-GP, 0.22mm Filter unit, Cat. No. SLGPR25LS, Millipore, Bedford, MA). Aliquots of the filtered saliva were tested for hydrolase activity, and compared with the stock CE and PCE enzymes using five substrates, *p*-NPA, *o*-NPA, *p*-NPB, *o*-NPB and BTC, as described in sections 2.4.2.1, 2.4.2.2 and 2.4.4. For the nitrophenyl esters, saliva hydrolase activity was determined by incubating 50 μL of filtered saliva with a solution containing 1.0 mL of 0.05 M phosphate buffer, pH 7.0 and 0.5 mL of the prepared substrate solution. Spectrophotometric measurements at 410 nm were obtained using an Ultrospec® II (LKB Biochrom, Cambridge, England) spectrophotometer at room temperature (25°C). Data was recorded every 30 seconds for 5 minutes, starting immediately after the saliva/enzyme addition while the reaction rate was linear. One unit of activity per one mL of saliva was defined as a change of absorbance of 0.01 O.D. per minute.

For the BTC substrate, saliva hydrolase activity was determined by incubating 10μL of filtered saliva in the cholinesterase (BTC) activity kit (Sigma, Procedure No. 421), as described in section 2.4.2.2. Saliva activity was calculated using Equation 2.4.

2.4.6 ENZYME INHIBITION ASSAYS

The mechanism of the enzymatic attack on the model composite resin samples was investigated using a specific esterase inhibitor, phenylmethylsulfonyl fluoride (PMSF). The structure of PMSF is given in Figure 2.7. Prior to the biodegradation experiments, the activity of the enzymes with and without PMSF (P-7626, Sigma, St. Louis, MO), was measured. The inhibition of CE and PCE was determined by adding the PMSF, dissolved in ethanol, to the enzyme solutions prior to their activity measurement [Labow, 1994]. Activity measurements were described above for CE (section 2.4.2.1) and PCE (section 2.4.2.2). The enzymes were also assayed with the same volume of ethanol alone in order to assess if the latter influenced the enzyme's activity. PMSF dissolved in ethanol was also assayed alone. The final concentration range of PMSF was adjusted to 1-2 mM in the CE solution (1 unit/mL) and 0.5-1 mM for PCE (1 unit/mL) and provided measurable inhibition values.

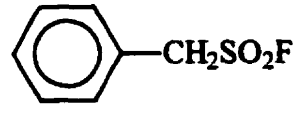


Figure 2.7 Structure of phenylmethylsulfonyl fluoride (PMSF)

2.5 BIODEGRADATION STUDIES

2.5.1 COMPOSITE RESIN SAMPLE CONDITIONING

Prior to the biodegradation experiments, all the composite resin samples were incubated in D-PBS (phosphate saline buffer made up from distilled water) solution (pH 7.0, 37°C) for 48 hours. The latter step was carried out in an attempt to remove a significant fraction of unreacted monomers available through leaching processes from the samples [Santerre, 1999, Shajii, 1999]. The samples were then removed, washed with ddH_2O and dried with sterile gauze.

2.5.2 INCUBATION

Each treated group (i.e. bis10, bis60, u-bis10 or u-bis60) contained three composite resin samples, placed in a 2 mLs sterile vial. The total surface area of the samples for each of these groups was 2.26 cm^2. To each vial (group), 1 mL D-PBS and 10μL of the selected enzyme or buffer solutions were added. Based on the enzyme stability experiments (section 2.4.3), the enzyme groups were replenished daily (every 24 hours) with 10μL of replenishing solutions, with the appropriate enzyme concentrations to maintain the activity at the specified level. 10μl D-PBS was added daily to the buffer treated groups in order to maintain an equal volume with the enzyme groups. All solutions were sterile filtered using 0.22 μm filter (Millex®-GP, 0.22mm Filter unit, Cat. No. SLGPR25LS, Millipore, Bedford, MA). The experiments were run in triplicates (N=3).

In addition, the following control groups were used:

A. Buffer control group (D-PBS, glass tubes).

B. CE control group (1 unit/mL, glass tubes).

C. PCE control group (1 unit/mL, glass tubes).

Each control group contained glass tubes with the same total surface area as the composite resin samples, incubated with the specified incubation media.

2.5.2.1 DOSE DEPENDENT EFFECT OF CE AND PCE ON THE BIODEGRADATION OF THE COMPOSITE RESIN SAMPLES

In order to assess the dose dependence of CE and PCE biodegradation of the composite resin samples, composite resin samples containing 60 % w/w filler content and using either the bis or u-bis resin formulation were prepared as described in sections 2.1.1, 2.1.2 and 2.1.3. For each composite resin material, the experiment was divided into the following groups:

A. Buffer treated groups.

B. CE enzyme treated groups (0.01, 0.05, 0.1 and 1 unit/mL).

C. PCE enzyme treated groups (0.01, 0.05, 0.1 and 1 unit/mL).

The samples were incubated for 16 days as described in section 2.5.2. After 16 days, incubation solutions were collected for HPLC analysis and the samples were kept for further SEM and micro-hardness analysis.

2.5.2.2 THE EFFECT OF INCUBATION PERIODS AND FILLER CONTENT ON THE BIODEGRADATION OF THE COMPOSITE RESIN SAMPLES

In order to determine the effect of incubation period and filler content on the biodegradation of the composite resin samples, composite resin samples containing 10 % w/w or 60 % w/w content filler and using either bis or u-bis resins were prepared as described in sections 2.1.1, 2.1.2 and 2.1.3. For each composite resin group, the experiment was divided into the following groups:

A. Buffer treated groups.

B. CE enzyme treated groups (1 unit/mL).

C. PCE enzyme treated groups (1 unit/mL).

The samples were incubated for a total of 32 days as described in section 2.5.2. After 16 days, incubation solutions were collected for HPLC analysis while the samples were re-incubated in their corresponding solutions for the subsequent 16 day period. After 32 days the incubation solutions were collected for HPLC analysis and the samples were kept for further SEM analysis.

2.5.2.3 MUTUAL INFLUENCE BETWEEN CE AND PCE ON THEIR BIODEGRADATION OF THE COMPOSITE RESIN SAMPLES

It is well documented that saliva contains different enzymatic activities [Chauncey, 1961] and these activities may originate from different sources including oral microorganisms, salivary glands and gingival crevicular fluid [Nakamura, 1983a]. Since these activities co-exist in saliva, it is of a great interest to determine if there could be a mutual influence

between different esterases. Composite resin samples containing 60 % w/w content filler and using either bis or u-bis resins were prepared as described in sections 2.1.1, 2.1.2 and 2.1.3. For each composite resin material, the experiment was divided into the following groups:

A. Buffer treated groups.

B. CE enzyme treated groups (0.01 unit/mL for the bis composite and 0.1 unit/mL for the u-bis composite).

C. PCE enzyme treated groups (0.01 unit/mL for the bis composite and 0.1 unit/mL for the u-bis composite).

D. CE + PCE enzyme treated groups (0.01 unit/mL CE + 0.01 unit/mL PCE for the bis material, 0.1 unit/mL CE + 0.1 unit/mL PCE for the u-bis material).

The samples were incubated for a total of 16 days as described in section 2.5.2. After 8 days, incubation solutions were collected for HPLC analysis while the samples were re-incubated in their corresponding solutions for the subsequent 8 day period. After 16 days the incubation solutions were collected for HPLC analysis The samples were kept for further SEM analysis.

2.5.2.4 EFFECT ON THE BIODEGRADATION OF THE COMPOSITE RESIN SAMPLES WHEN CE AND PCE WERE INHIBITED

The mechanism of enzymatic attack on the model composite resin samples was investigated using a specific esterase inhibitor, phenyl methyl sulfonyl fluoride (PMSF). Prior to the biodegradation experiments, the activity of the enzymes with and without

PMSF was measured as described in section 2.4.6. Composite resin samples containing 60 % w/w filler content and using either bis or u-bis resins were prepared as described in sections 2.1.1, 2.1.2 and 2.1.3. The inhibition of the CE or PCE catalyzed biodegradation of the composite was carried out by adding PMSF dissolved in ethanol, or ethanol alone (as a control), to the replenishing solutions, prior to their addition to the incubation solutions of the composite resin samples as described below. For each composite resin material, the experiment was divided into the following groups:

A. Buffer treated groups

B. Buffer + PMSF treated groups (0.1 mM PMSF)

C. CE treated groups (0.1 unit/mL)

D. CE + ethanol treated groups (0.1 unit/mL +10µl ethanol)

E. CE + PMSF treated groups (0.1 unit/mL CE + 0.1 mM PMSF)

F. PCE treated groups (0.1 unit/mL)

G. PCE + ethanol treated groups (0.1 unit/mL + 10µl ethanol)

H. PCE + PMSF treated groups (0.1 unit/mL + 0.05mM PMSF)

The samples were incubated for 16 days as described in section 2.5.2. After 16 days, incubation solutions were collected for HPLC analysis. The samples were kept for further SEM analysis.

2.5.2.5 BIODEGRADATION OF ^{14}C-u-bis COMPOSITE BY CE OR PCE

Radioactivity monitoring was used to assess the quantity of radiolabeled HDI and HDI-bisGMA derivatives released from the ^{14}C-u-bis composite resin samples. Composite

resin samples containing 60 % w/w filler content and using ^{14}C-u-bis resin were prepared (section 2.1.4). The experiment was divided into the following groups:

A. Buffer control groups.

B. CE treated groups (1 unit/mL)

C. PCE treated groups (1 unit/mL)

The samples were incubated for a total of 32 days as described in section 2.5.2. After 16 days, 0.5 mL from each of the incubation solutions were collected into scintillation vials, 4.5 mLs of scintillation cocktail (Ready Safe P/N 141349, Beckman, Fullerton, CA) were added and the vials were read for radiolabeled activity (LS 6500 Multi-Purpose Scintillation Counter, Beckman, Fullerton, CA). For HPLC analysis, 0.4 mL from each of the incubation solutions were filtered with a Millipore centrifuge filter device (Ultrafree®–MC UFC3LGC00, Millipore, Bedford, MA) that removes molecules with a molecular weight greater than 5,000 in a centrifuge (H-25FI, Silencer) at 2400 R.P.M., and kept refrigerated. The samples were re-incubated in their corresponding solutions for a subsequent 16 day period. After 32 days, the incubation solutions were collected for HPLC analysis and radioactivity monitoring as described above.

2.5.3 ENZYMATIC HYDROLYSIS OF MONOMERS

Since the cured composite resin samples contained unreacted, partially and fully reacted monomers, it was important to determine the enzymes' ability to catalyze the hydrolysis of the monomers that were used in the preparation of the resins. Hence, in conjunction with the enzyme substrate specificity experiments (section 2.4.4), a more comprehensive

activity profile could be assembled for each of the enzymes. The CE and PCE catalyzed hydrolysis of bisGMA, bisEMA and TEGDMA were determined as follows:

CE or PCE (1 unit/mL) were added to 2.0 mL 10^{-4} M bisGMA, 10^{-4} M bisEMA or 5 x 10^{-5} M TEGDMA dissolved in D-PBS, pH 7.0 at 37°C. Aliquots (200μL), in which the reaction was stopped by the addition of 133μL of methanol were taken after various time periods and used for detection of products by HPLC. The methanol was used to deactivate the enzymes [Munksgaard, 1990]. Since methacrylic acid is an end product of the biodegradation of all the monomers, its production rate was measured in order to assess the hydrolysis rate of the different monomers with the different enzymes.

2.5.4 PRODUCT COLLECTION

The collected incubation solutions were filtered with a Millipore centrifuge filter device (Ultrafree®-CL, UFC4LCC00 5000 NMWL, Millipore, Bedford, MA) that removes molecules with a molecular weight greater than 5,000. Samples were centrifuged using an international clinical model centrifuge (Centrifuge international equipment Co., Needham, MA) at 2400 R.P.M. and kept refrigerated at 4°C until required for analysis.

2.6 ISOLATION AND IDENTIFICATION OF BIODEGRADATION PRODUCTS

The techniques that were in use to separate and identify the biodegradation products of the composite resin samples were high performance liquid chromatography (HPLC) coupled with ultraviolet (UV) spectroscopy and mass spectrometry (MS).

2.6.1 PRODUCT ISOLATION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC was used to separate and isolate the biodegradation products of the composite resin samples. The separation process is based on interactions between a stationary phase (column), a mobile phase (solvent) and the solute molecules (sample). A Waters™ HPLC system (Waters, Mississauga, Ontario) was employed in the separation of biodegradation products. A schematic of the HPLC apparatus is presented in Figure 2.8. Filtered and pressurized mobile phase is passed from a pump (model 600 pump, Waters™, Mississauga, Ontario) to an injector (model U6K, Waters™, Mississauga, Ontario) and into a column (μBondpack™ C_{18} 4.6 X 250, Waters™, Mississauga, Ontario; or Phenomenex Luna 5μ C18(2) 4.6 X 250, Phenomenex, Torrance, CA), where the solvent equilibrates with the solid column packing and exits to a flow cell detector (model 996 Photo Diode Array (PDA) detector, Waters™, Mississauga, Ontario). The final sample is then transferred out to the waste or fraction collection module. Pressure,

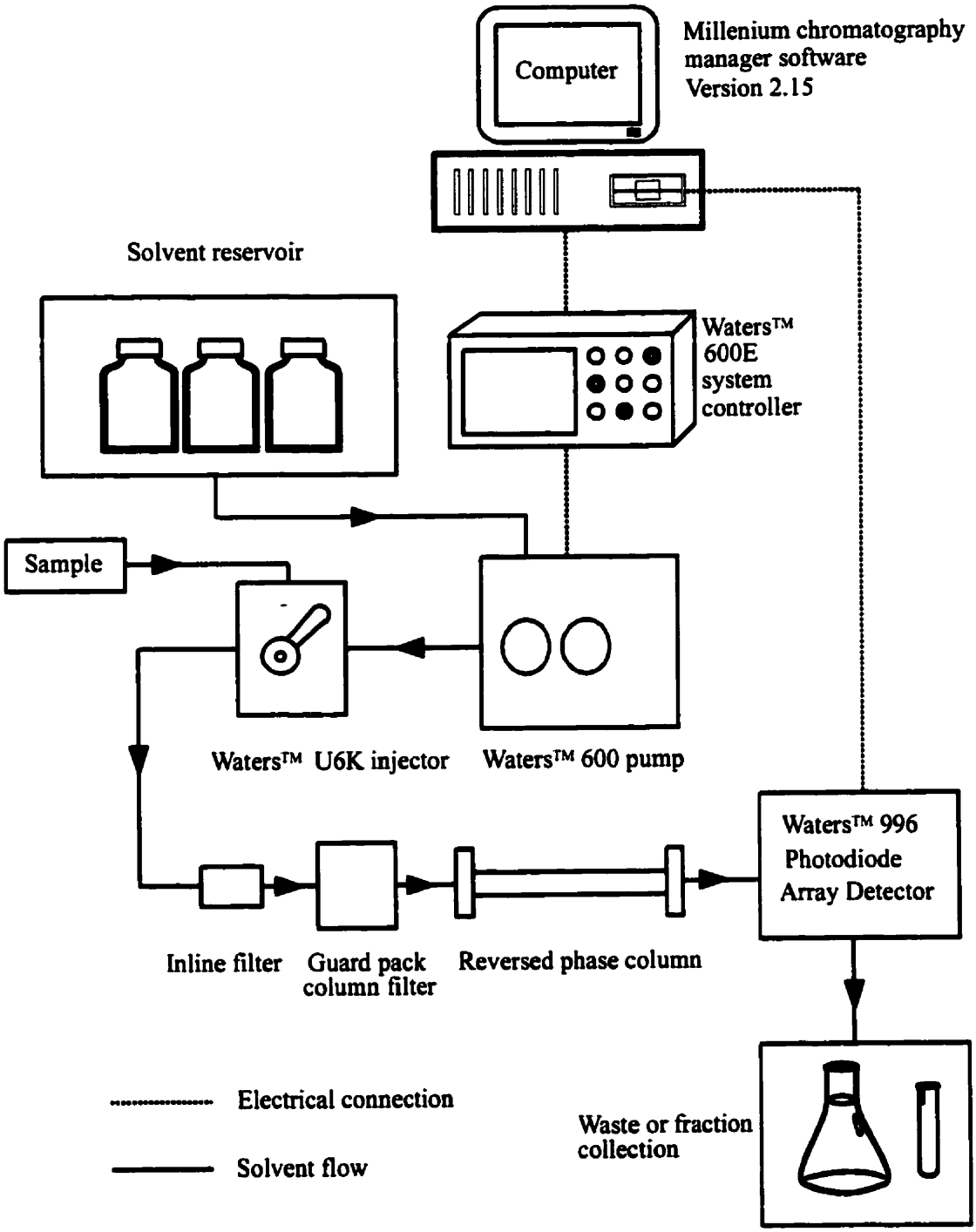


Figure 2.8 Schematic of the HPLC apparatus

flow rate, and composition of the solvent mixture are programmed through the software (Millenium chromatography manager software Version 2.15) and controlled by a controller (model 600E system controller, Waters™, Mississauga, Ontario). Prior to use, the mobile phase was filtered through a 0.45 μm Teflon® filter (Chromatographic Specialties Inc., Brockville, Ontario) to remove particulate contamination and then degassed by sonication for 20 minutes prior to its use. During the HPLC cycles, the mobile phase was further degassed by helium sparging. Each sample was dissolved into the mobile phase and was delivered to the system through the injector. A Waters™ inline column filter (SS, 0.22μm), and a guard pack column filter (μbondapak, C18) served to remove residual micro-particles and proteins from the sample. Separated compounds passed through the detector flow cell which translated concentration changes to signal voltage. Data were recorded into the computer hard drive using software (Millenium chromatography manager software Version 2.15) as a chromatogram and was represented in terms of an absorbance signal (μVolt) given as a function of retention time. Quantitation was achieved by measuring the area under each peak (μVolt x second) and then these values were converted to amounts per composite resin sample area using calibration curves generated from appropriate standards (see Appendices 8.1-8.5).

Product isolation in this study was based on a reverse phase chromatographic process. In reverse phase chromatography, the polarity of the stationary phase is lower relative to that of the solvent. In these systems, the mobile phases consist of relatively polar solvent mixtures such as methanol/water or acetonitrile/water. Different solutes were distributed between the non-polar stationary phase and the polar mobile phase according to their

polarities. In this way, the distribution of the solutes between the phases could be changed by changing the difference in the polarity between the stationary phase (column packing) and the mobile phase. The following parameters can affect the polarity of the mobile phase: composition, concentration, type, pH and temperature.

Table 2.3 represents the optimized mobile phase method applied for the separation of the biodegradation products in this study. The buffer consisted of a 2 mM ammonium acetate 99.999% (37,233-1, Aldrich, Milwaukee, WI) solution adjusted to pH

Table 2.3 HPLC gradient method for separation of composite resin sample biodegradation products

Time [Minutes]	Flow Rate [mL/minute]	Methanol [%]	Buffer [%]	Water [%]
0	1.0	40	60	0
8	1.0	60	40	0
16	1.0	100	0	0
30	1.0	100	0	0
30.1	1.0	0	0	100
35	1.0	0	0	100
35.1	1.0	40	60	0
60	1.0	40	60	0

3.0 with hydrochloric acid 6.00 N (VW3204-1, VWR, West Chester, PA) or acetic acid HPLC grade (double distilled, PPB/Teflon® 38,012, Aldrich, Milwaukee, WI). Each chromatogram ran for a cycle of 30 minutes. Following the sample separation cycle, the system was flushed with water for 5 minutes. After that, re-equilibration was performed with the initial mobile phase mixture until the next sample injection.

2.6.2 ULTRAVIOLET (UV) SPECTROSCOPY

Most organic molecules and functional groups are transparent in the UV electromagnetic region. This region spans wavelengths between 190 nm to 400 nm. In some cases, useful information can be obtained from this region of the spectrum. An empirical expression, also known as the Beer-Lamber Law, is written below. (This law is obeyed only for a single species at a time).

Equation 2.7 $\log(I_o/I) = \varepsilon c l$

Where:

I_o = Intensity of light incident upon the sample cell

I = Intensity of light leaving the sample cell

c = Molar concentration of the solute

l = Length of the sample cell [cm]

ε = molar absorptivity for a certain a wavelength [M^{-1} cm^{-1}]

The HPLC system used in this study was equipped with a photodiode array detector (model 996 Photo Diode Array (PDA) detector, Waters™, Mississauga, Ontario). In this detector, a polychromatic UV spectrum passes through the sample cell. The light leaving the sample cell falls upon an array of photodiodes, each measuring a narrow band of wavelengths in the spectrum. The data acquisition is parallel so all the spectrum points are measured simultaneously. In this manner, the spectra of each peak can be obtained and compared to a known standard, which aids in product identification. Since the PDA detector provides a full spectrum for a given product, it is an effective aid for selecting a wavelength in which product peaks have their maximum absorbance. For this study, the peaks were reported at a wavelength of 215 nm.

2.6.3. PRODUCT IDENTIFICATION BY MASS SPECTROMETRY

Mass spectrometry (MS) is a technique used for molecular mass determination. In this system, the samples are evaporated under vacuum in an ionization chamber. During this process, energy is transferred to the molecules in the vapor state , resulting in the production of fragments of positive ions [Pavia, 1979]. These ions are accelerated through an electric field and separated by quadrupoles. A quadrupole is an arrangement of four charged rods surrounding the path of the ions , thus directing their path by means of an electrostatic field. At this point, a molecular mass spectrum is produced and scanned by the mass analyzer, which generates peaks in which the relative intensities are proportional to the abundance of the ions. The spectrum is then plotted as relative intensities versus mass to charge (m/z) ratio. Additional products associated with the

same mass ion, can be observed if salts are present in the mobile phase or in the sample, thus producing [molecular species] with Na^+, K^+ and NH^+_4 ions. A typical mass spectrometry apparatus is presented in Figure 2.9.

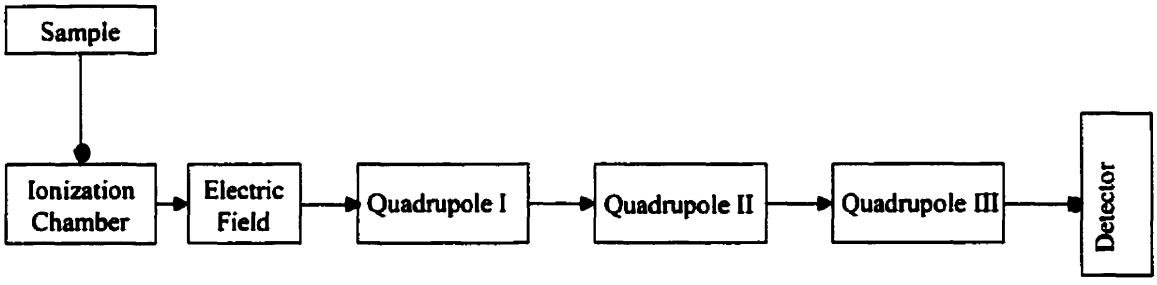


Figure 2.9 Block diagram of a triple quadrupole mass spectrometer

In order to further investigate the structure of peaks related to the parent spectrum, a tandem mass spectrometry (MS/MS) method was employed. The ions are passed into the first quadrupole, where the specific molecular species are separated and passed to the second quadrupole. Undesired molecular species are eliminated by forced collision with the quadrupole rods. In the second quadrupole, the parent ions are bombarded with argon gas, which causes ion fragmentation, which can be controlled by the pressure of the collision gas. Fragmented ions are called daughter ions. The third quadrupole separates the resulting daughter ions and directs them to the detector.

Peak samples isolated using the HPLC system, were collected and analyzed by MS. Depending on the product concentration, injection volumes were varied between 1 to 20 μLs. The mobile phase consisted of HPLC grade methanol at a flow rate of 0.02

mL/minute using an HPLC pump (LKB, Broma, Sweeden). A 5.0 KVolt potential was applied at the tip of the spray needle to ionize the samples and an 80 Volt potential was applied at the diaphragm of the spectrometer. Ions with an m/z ratio between 100 to 2000 atomic mass unit (a.m.u.) typically have a standard deviation of ± 0.5 a.m.u..

In order to increase the sensitivity of the detection with low concentration species, the MS system was used to detect and analyze products. The mass spectrometry analysis of biodegradation products was carried out by Ms. Doreen Wen at the laboratory located in the Carbohydrate Research Centre, University of Toronto, using an API-III biomolecular mass analyzer (Perkin-Elmer Sciex Instruments, Concord, Ontario). TUNE 2.5 and MACSPEC 3.3 software (Perkin-Elmer Sciex Instruments, Concord, Ontario) were used to collect and analyze the data. All m/z assignments were done by the Ph.D. candiadate.

2.7 RADIOACTIVITY MONITORING OF HPLC FRACTIONS

In an attempt to isolate and identify the different radiolabeled degradation products of the ^{14}C-u-bis composite resin samples, HPLC fractions of the incubation solutions were collected every minute and counted for radiolabel contents in addition to the whole solution radioactivity monitoring. The fractions (1 mL) were collected into scintillation vials, 4.5 mLs of scintillation cocktail (Ready Safe P/N 141349, Beckman, Fullerton, CA) were added and the vials were read for radiolabeled activity (LS 6500 Multi-Purpose Scintillation Counter, Beckman, Fullerton, CA).

2.8 STATISTICAL ANALYSIS

The amount of product that was released by the model materials was expressed as the amount per composite resin surface area for the HPLC analysis and as counts per minute (CPM) per composite resin surface area for the radiolabeled material analysis. A Sceffe multiple comparison after one way analysis of variance was applied for each group, with the dependent variable being amount per composite resin surface area or CPM per composite resin surface area. The independent variables were: the composite resin types and/or incubation media and/or incubation period. The amount per composite resin surface area or CPM per composite resin surface area was expressed as a mean ± standard error. The confidence interval was set at 95 %. The number of samples in each group was at least three. When more than the three samples were used, a special note was made.

Micro-hardness values were expressed as Vickers scale readings [Kg/mm^2]. A Sceffe multiple comparison after one way analysis of variance was applied for each group. The dependent variable was the Vickers hardness reading. The independent variables were the composite resin type and/or the incubation media as follows: pre-incubation, 32 days buffer incubated, 32 days CE incubated and 32 days PCE incubated. The number of samples in each group was 6. Micro-hardness results were expressed as the mean ± standard error. The confidence interval was set at 95 %.

Enzyme activities were expressed in unit/mL (by the definition of each enzyme activity, see section 2.4.2) or as a percentage of a selected reference activity. A Sceffe multiple comparison after one way analysis of variance was applied for each group. The dependent variable was the enzymes' activity. The number of samples in each group was six. Enzyme activities were expressed as mean ± standard error. The confidence interval was set at 95 %.

3.0 RESULTS

3.1 RESIN CHARACTERIZATION

In this study, three resin formulations, namely bisGMA-TEGDMA (bis), commercial urethane modified bisGMA-TEGDMA-bisEMA (u-bis) and ^{14}C-urethane modified-bisGMA-TEGDMA-bisEMA (^{14}C-u-bis), with varying filler components were synthesized as described in sections 2.1.1, 2.1.2, 2.1.4 and in Tables 2.1 and 2.2. The composite resin materials were characterized using Fourier transform infra-red spectroscopy (FT-IR) and gel permeation chromatography (GPC) to determine their molecular weight distribution and chemical bond structure. The results of these characterization studies are described in the following sections.

3.1.1 FOURIER TRANSFORM INFRA RED SPECTROSCOPY

Fourier transform infra red spectroscopy (FT-IR) of bis, u-bis and ^{14}C-u-bis was carried out in transmission mode. Figure 3.1 shows the plots of absorbance versus wavenumbers for bis, u-bis and ^{14}C-u-bis, analyzed using infra-red spectroscopy. Peak assignment was based on the existing literature [Rueggeberg 1990; McCarthy J., 1997; Xu J. 1997]. The main differences were noted in the 3320-3450 cm^{-1} and 3300-3600 cm^{-1} wavenumber regions, which are attributed to N-H and O-H groups respectively. The O-H bond, which originated from the bisGMA molecule within the bis resin, is replaced in the commercial

u-bis and ^{14}C-u-bis materials with a urethane bonded (N-H) HDI-bisGMA. Some of the other principal peaks in Figure 3.1, which were related to the methacrylate monomers of the composites are attributed to the stretching of the carbonyl group located at 1722 cm^{-1} (associated with the esters in the methacrylate units and the urethane for the u-bis and ^{14}C-u-bis), the aliphatic C=C stretching at 1638 cm^{-1}, the aromatic C=C stretching at either 1608 or 1580 cm^{-1} and the C-H stretching of the methyl group at 2890 and 2930 cm^{-1}. In both systems, the aliphatic C-O stretch mode, usually observed between 1050 to 1300 cm^{-1} region is dominated by a peak at 1176 cm^{-1}. Note the similarities for the N-H groups for the commercial u-bis and the ^{14}C-u-bis, confirming that the model u-bis is representative of the commercial material.

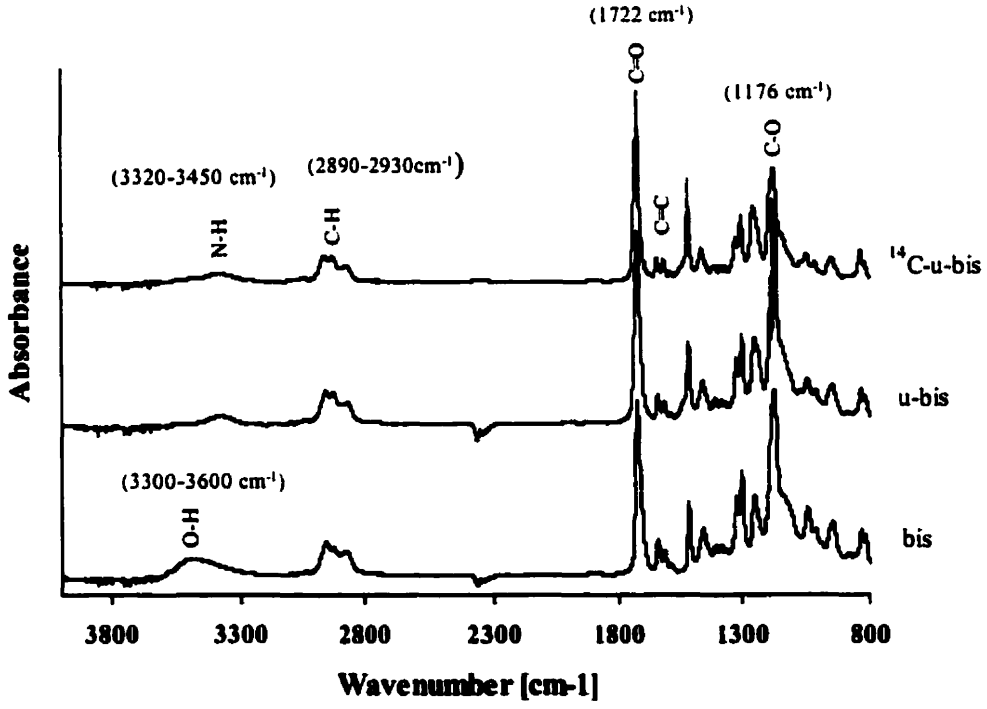


Figure 3.1 **Fourier transform infra red spectra for bisGMA-TEGDMA (bis), commercial urethane modified bisGMA-TEGDMA-bisEMA (u-bis) and ^{14}C-urethane modified bisGMA-TEGDMA-bisEMA (^{14}C-u-bis) resins**

3.1.2 GEL PERMEATION CHROMATOGRAPHY

Gel permeation chromatography (GPC) analysis of the model ^{14}C-u-bis and the commercial u-bis were compared to TEGDMA, bisGMA and bisEMA monomers used as references and representative monomers of the principal resin components (i.e. bisEMA, TEGDMA and bisGMA) in the u-bis system (Figure 3.2). The data are presented as changes in refraction index (proportional to concentration) versus retention time.

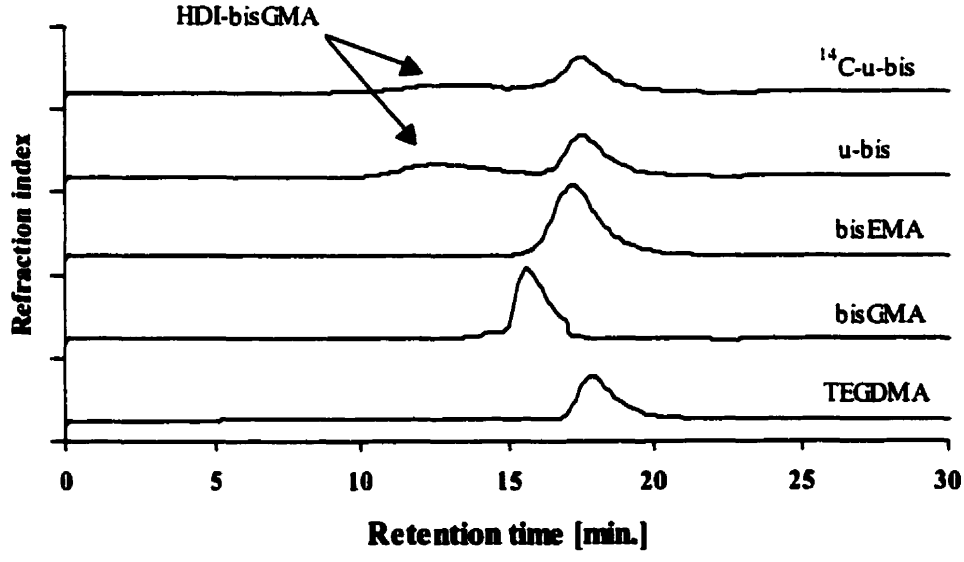


Figure 3.2 **Gel permeation chromatography analysis of TEGDMA, bisGMA, bisEMA, commercial urethane modified bisGMA-TEGDMA-bisEMA (u-bis) and a model radiolabeled urethane modified bisGMA-TEGDMA-bisEMA (^{14}C-u-bis)**

It is noted that the ^{14}C-u-bis and u-bis are composed mainly of a mixture containing bisEMA monomer, a smaller amount of TEGDMA monomer and an oligomer, synthesized from a urethane linked HDI and bisGMA molecules. Based on the chromatogram data it is also observed that there is very little bisGMA remaining in the monomer mixture following the reaction with HDI. The urethane linked HDI-bisGMA oligomer is composed of an average of 7-9 linked bisGMA molecules, similar to the range found in other studies [Ruyter 1981, 1987a and 1987b]. The model ^{14}C-u-bis is very similar, in terms of monomer and oligomer distribution, to the commercial u-bis. This character should permit the ^{14}C-u-bis system to reasonably reproduce the character of u-bis system in the biodegradation studies.

3.2 SURFACE ANALYSIS OF THE COMPOSITE RESIN SAMPLES

The cured composite resin samples were analyzed by XPS to determine surface elemental composition, by SEM to determine surface morphology and by FT-IR to determine the degree of vinyl group conversion at the surface as described in section 2.3.1, 2.3.2 and 2.3.4 respectively.

3.2.1 SURFACE ELEMENTAL ANALYSIS OF THE COMPOSITE RESIN SAMPLES

Elemental surface analysis of three specimens from each of the composite resin groups (i.e. bis10, bis60, u-bis10 and u-bis60) was performed prior to the incubation of the

samples in their corresponding solution, using X-ray photoelectron spectroscopy (XPS). Table 3.1 shows the surface elemental composition [%, mean ± standard error] of the composite resin samples at a 90° take off angle which corresponds to an approximate analysis depth of the top 10 nm.

It is observed that changing the resin type as well as increasing the amount of filler from 10 to 60 w/w % does not alter the surface elemental composition of the samples. Although the amount of Si 2p shows a slight increasing trend with the higher filler contents samples, this increase is small in absolute values. Furthermore, taking into account that the resolution limit of the instrument is 1 %, and the amounts shown are within this limit, this leads to the conclusion that most of the samples' surface is pure resin. A theoretical calculation of carbon and oxygen content (based on the original monomer mixtures) in the pure resin yields values of approximately 76 % and 24 % respectively. These values are very similar to the values reported in Table 3.1.

Table 3.1 Quantitative elemental analysis of composite resin samples

Composite Resin	Elements [%]			
	C 1s	O 1s	Si 2p	Ba 3d5
bis 10	74.03±0.16	25.48±0.12	0.32±0.06	0.17±0.02
bis 60	75.31±0.53	23.90±0.49	0.68±0.12	0.11±0.03
u-bis 10	77.67±0.83	21.93±0.85	0.32±0.07	0.08±0.02
u-bis 60	75.10±0.56	23.30±0.33	1.48±0.21	0.13±0.04

A more detailed surface analysis was obtained from the high-resolution spectra of the C 1s peak (Table 3.2). The C 1s binding energies at 285.0, 286.5, 289 and 289.6 electron Volts (eVolts), correspond respectively to the C-C, C-O, C=O and OCONH (urethane) bonds. An example of the curve fitting is shown in Figure 3.3. For both materials, a higher content of C-C is evident, followed by C-O and C=O. The urethane bond at 289.6 eVolts is evident only for u-bis materials. The presence of C=O is assigned primarily to the ester bond of the resin and identifies that ester groups are present near and at the surface of both materials. Since these groups are particularly sensitive to hydrolytic reactions, it is anticipated that they will be available to undergo cleavage in the biodegradation studies. A higher content of this bond is presented in the bis groups, for example 11.54 ± 0.68 % for bis10 compared with 5.65 ± 0.17 % for u-bis10 material.

Table 3.2 C 1s high-resolution analysis of composite resin samples

Composite resin	Chemical Bonds [%]			
	C-C	C-O	C=O	OCONH
Bis10	58.77±0.69	29.69±0.14	11.54±0.68	0.00
Bis60	65.47±2.01	24.68±2.85	9.85±1.10	0.00
u-bis10	64.04±2.59	28.49±2.04	5.65±0.17	1.82±0.87
u-bis60	64.43±1.10	25.74±0.81	6.76±0.22	3.07±0.53

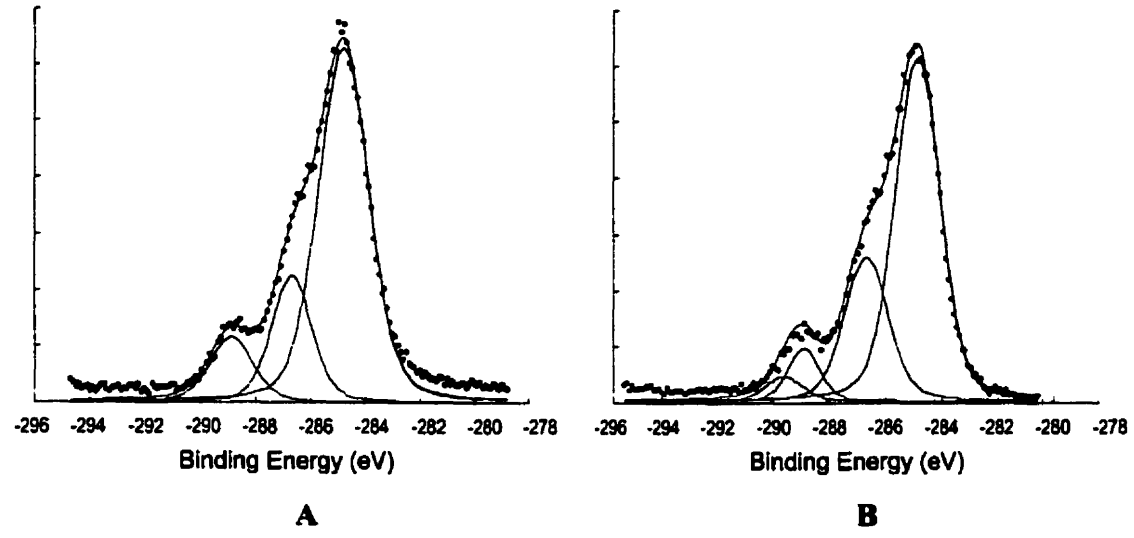
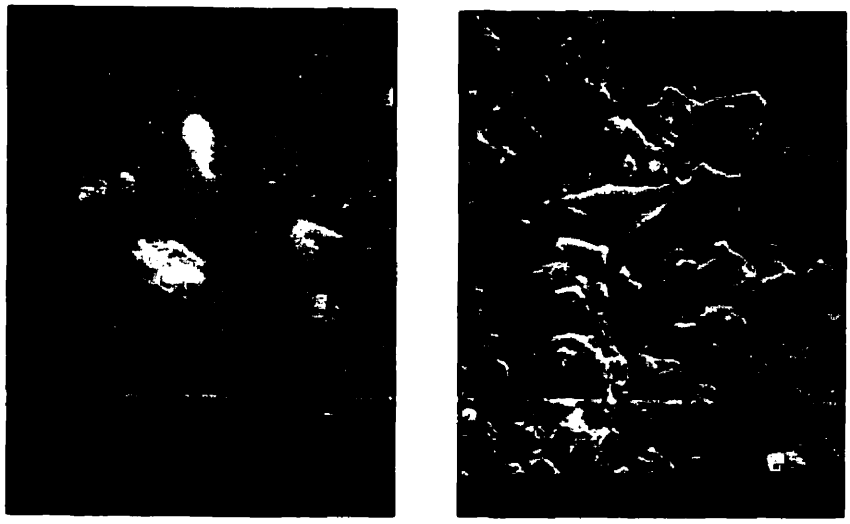


Figure 3.3 XPS high resolution curve fitting for 285.0, 286.5, 289 and 289.6 eVolts, corresponding respectively to C-C, C-O, C=O and OCONH (urethane) bonds in bis60 (A) and u-bis60 (B) composite samples

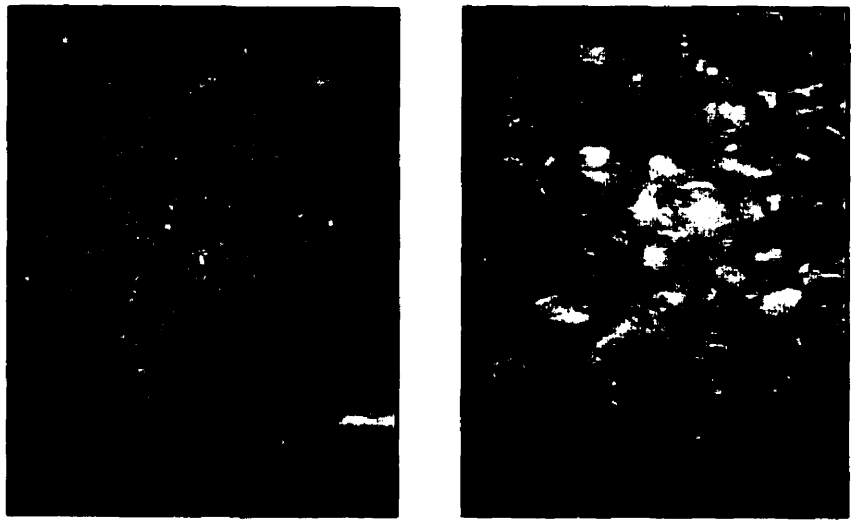
3.2.2 SURFACE MORPHOLOGY OF THE COMPOSITE SAMPLES

The surface morphology of the composite resin samples was studied using scanning electron microscopy (SEM), as described in section 2.3.2. Photomicrographs of the bis10, bis60, u-bis10 and u-bis60 prior to incubation in the aqueous solutions are presented in Figures 3.4 and 3.5. The photomicrographs support the XPS analysis, which suggested that the surfaces for all the samples are composed mainly of resin (Figures 3.4 and 3.5). The surfaces of u-bis10 and u-bis60 samples are relatively smoother than those of their non-urethane analogs, bis10 and bis60 formulations. Increasing the filler content from 10 % to 60 % produced a rougher surface morphology and this is particularly relevant for the bis samples (compare Figures 3.4A-B to Figures 3.5A-B respectively).



A B

Figure 3.4 **Scanning electron micrograph of bis10 (A) and bis60 (B) prior to incubation ($1x10^4$ original magnification)**



A B

Figure 3.5 **Scanning electron micrograph of u-bis10 (A) and u-bis60 (B) prior to incubation ($1x10^4$ original magnification)**

3.2.3 DEGREE OF VINYL GROUP CONVERSION AT THE SURFACE

The degree of vinyl group conversion for the cured composite resin samples was measured using FT-IR spectroscopy in transmission mode as described in Section 2.3.4. FT-IR analysis of 6 specimens from each type of composite resin was performed prior to the incubation of the samples. The composite resins were analyzed before and after the photocuring, in order to calculate the degree of conversion. An example of the curve fitting for the 1608 cm^{-1} and 1636 cm^{-1} wavenumber peaks, corresponding respectively to the aromatic C=C and the vinyl group C=C bonds is presented in Figure 3.6. The use of the aromatic C=C bond to normalize the vinyl group has been reported by others [Asmussen 1982; Ferracane J.L. 1992; Nomoto, 1994; Peutzfeldt, 1994].

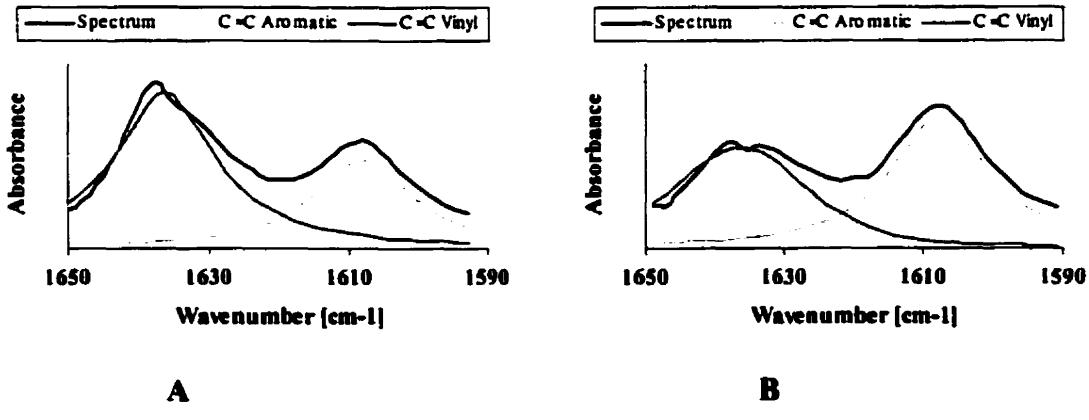


Figure 3.6 Curve fitting for the 1608 cm^{-1} and 1636 cm^{-1} wavenumber peaks, corresponding respectively to the C=C aromatic and C=C vinyl bonds, prior (A) and following (B) photocuring of bis10 sample

The 1636cm^{-1} to 1608cm^{-1} peak area ratio prior to the photocuring (Figure 3.6A) is the ratio when 100 % of vinyl groups are unreacted, while the same peak area ratio following the photocuring process (Figure 3.6B) represents the remaining unreacted vinyl groups. Using Equation 2.3 (section 2.3.4), the degree of vinyl group conversion was calculated for each type of composite resin (i.e. bis10, bis60, u-bis10 and u-bis60) prior to incubation of the samples (Figure 3.7).

It is observed that the degree of conversion is about 70% (Figure 3.7), with no significant difference found between the composites with different filler contents or resin type.

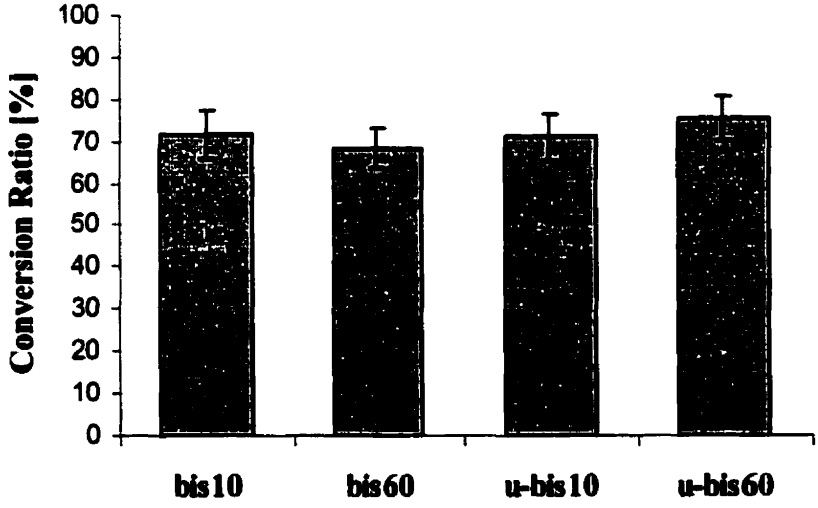


Figure 3.7 Degree of vinyl group conversion for the bis10, bis60, u-bis10 and u-bis60 composite resin materials

3.3 ENZYME STUDIES

3.3.1 ENZYME STABILITY ASSAYS

Prior to the incubation of samples with the enzymes, stability studies were conducted in order to assess the required replenishing schedule, the effect of the composite resin samples on the activity and the mutual influence of the enzymes on each other. Since multiple esterase activities are presented within human saliva, it would be of value to determine if synergistic effects could be observed in the presence of two or more enzymes. These data were acquired with the assistance of Mr. Fayaaz Jaffer, an undergraduate summer student, under my supervision.

3.3.1.1 CE STABILITY ASSAY

The activity of CE with and without the presence of PCE, with and without the addition of bis60 or u-bis60 composite resin samples, following incubation in D-PBS (37°C at pH 7.0) was measured at different time points using *p*-NPA as a substrate, as described in sections 2.5.2.1 and 2.5.3.1. The data are presented in Figures 3.8 and 3.9 as a percentage of the initial activity.

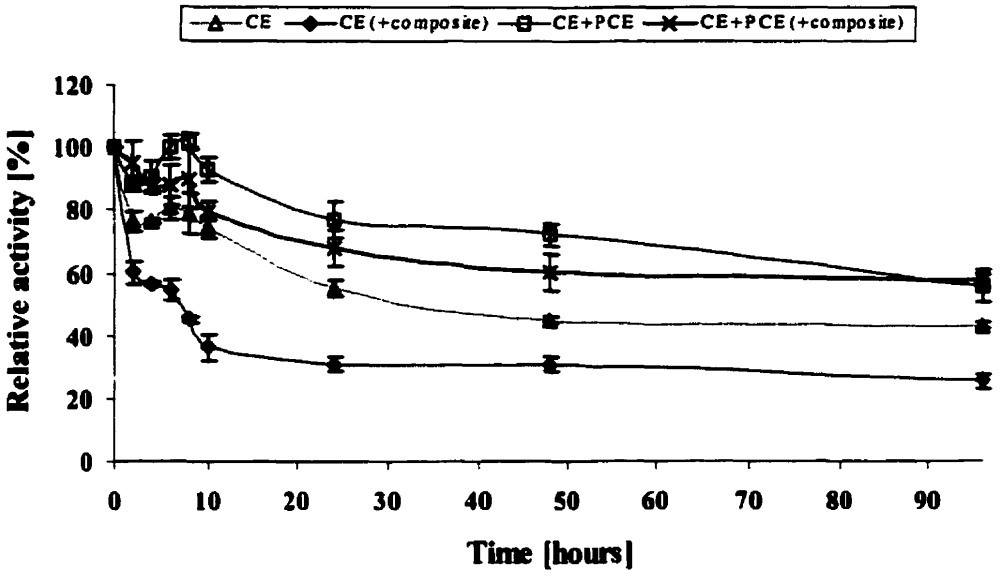


Figure 3.8 Relative activity of CE incubated in D-PBS (37°C at pH 7.0) and with the addition of bis60 composite and/or PCE

It is observed that at the start of the measurements, the activity of CE undergoes a rapid decrease (Figure 3.8). After 24 hours the relative activity of CE incubated alone was approximately 55 ± 2.6 % of the initial activity ($p<0.001$) and the half life was 32 hours.

The addition of bis60 composite samples into the incubation media, caused a greater loss of enzyme activity over the same time period, i.e. 31 ± 2.34 % of the initial activity ($p<0.001$), while the half life showed a decrease to 8 hours. Between the 6 to 24 hour time periods, the relative activity of the enzyme incubated with the composite, showed a reduction compared with the relative activity of the enzyme alone ($p<0.05$). Beyond this time point, the activity changes appeared to be similar for both the CE alone and CE

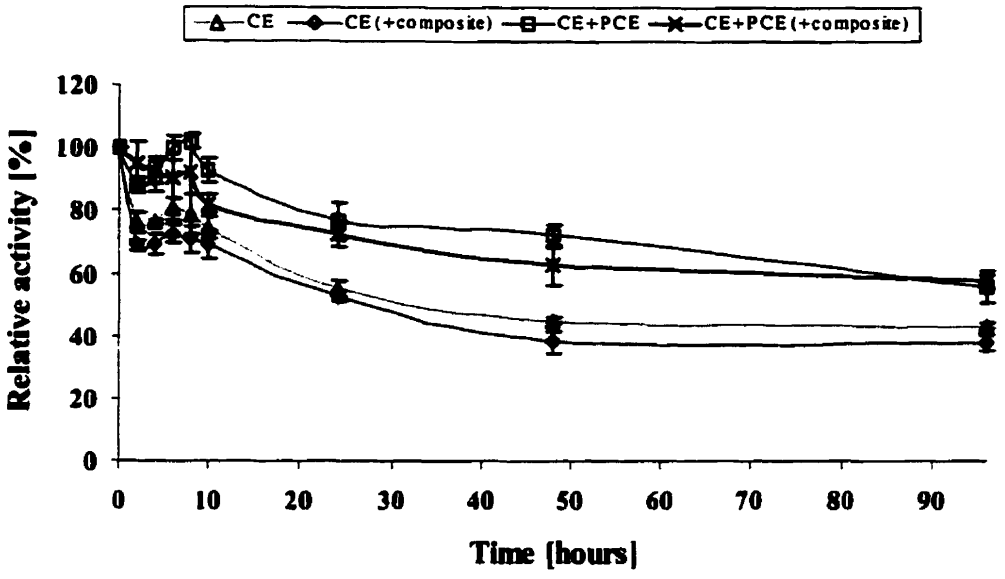


Figure 3.9 Relative activity of CE incubated in D-PBS (37°C at pH 7.0) and with the addition of u-bis60 composite and/or PCE

incubated with bis composite material. The addition of PCE to the incubation solution eliminates the effect of the composite on the CE activity and increased the half-life of the *p*-NPA type activity to over 96 hours (Figure 3.8). The u-bis60 composite shows a small effect on the stability of CE between 2 and 4 hours, but it was not statistically significant (Figure 3.9), however, the influence of PCE on the *p*-NPA type activity was still observed.

3.3.1.2 PCE STABILITY ASSAY

The activity of PCE with and without the presence of CE, with and without the addition of bis60 or u-bis60 composite resin samples, following incubation in D-PBS (37°C at pH 7.0) was measured at different time points using BTC as a substrate. The methods were described in sections 2.5.2.2 and 2.5.3.2. The data are presented in Figure 3.10 and 3.11 as a percentage of the initial activity.

It is observed that PCE incubated alone did not show a decrease in the relative activity during the 32 days incubation period (Figure 3.10) but showed an increase in the relative activity between days 1 and 16 ($p<0.05$) (Figure 3.10). However, PCE incubated with bis60 composite samples showed a reduction in the relative activity as compared with the PCE that was incubated alone. This effect was significant ($p<0.05$) at 32 days. Daily replenishment of CE eliminates the effect of the composite on the PCE activity and eliminated the increase in the relative activity between days 1 and 16. The addition of u-bis60 composite samples did not show any effect on PCE activity (Figure 3.11). For all groups, the half-life was greater than 32 days.

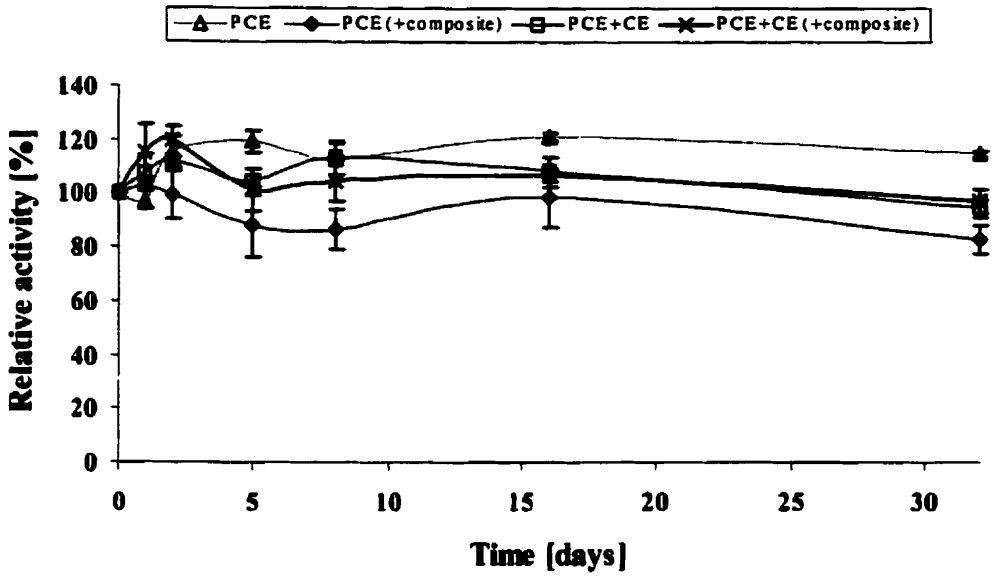


Figure 3.10 Relative activity of PCE incubated in D-PBS (37°C at pH 7.0) and with the addition of bis60 composite and/or CE

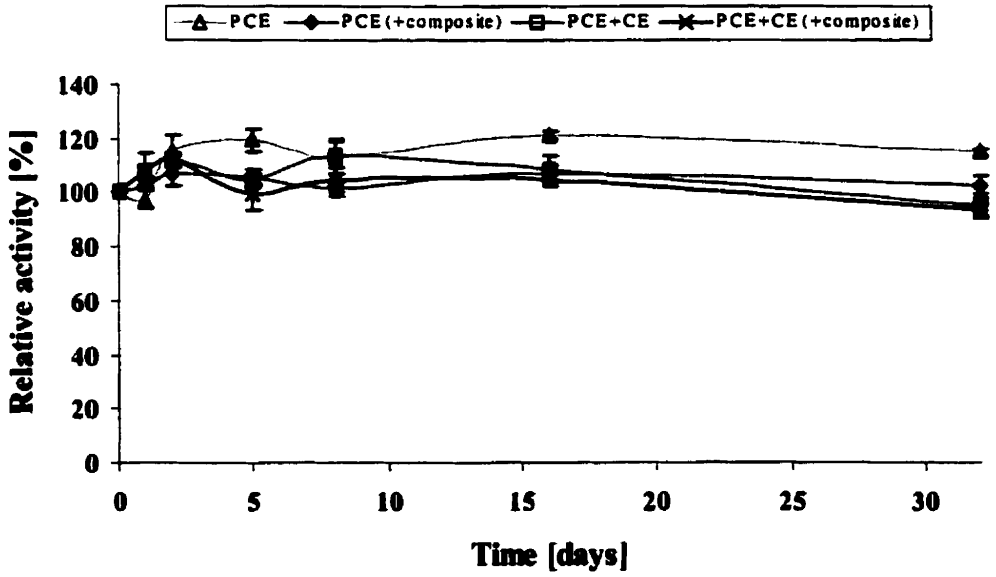
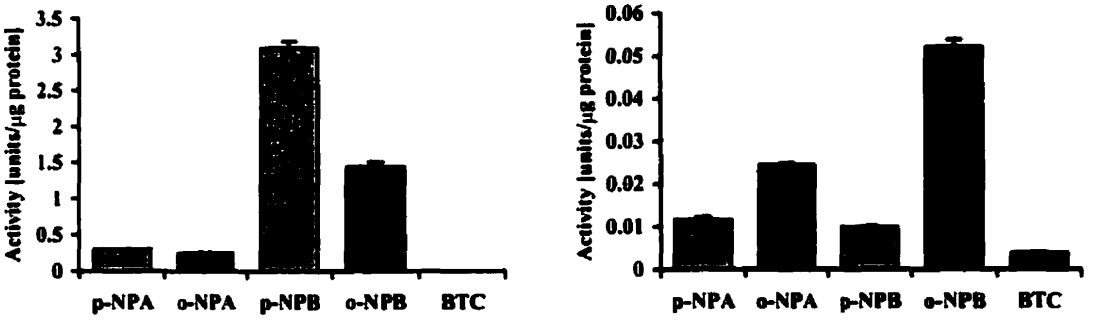


Figure 3.11 Relative activity of PCE incubated in D-PBS (37°C at pH 7.0) and with the addition of u-bis60 composite and/or CE

3.3.2 ENZYME SUBSTRATE SPECIFICITIES

The activity of CE or PCE was measured using *p*-NPA, *o*-NPA, *p*-NPB, *o*-NPB and BTC as described in section 2.5.4. By using these substrates, an activity profile for each enzyme could be assembled and compared with activities measured from human saliva. The activity profiles of CE and PCE are presented in Figure 3.12. and the data are recorded as units of enzyme activity per μg of protein. The activities were calculated using equations 2.5 and 2.6 (section 2.5.4) respectively for nitrophenyl and BTC substrates.



A B

Figure 3.12 Activity profiles for CE (A) and PCE (B) using para-nitrophenylacetate (*p*-NPA), ortho-nitrophenylacetate (*o*-NPA), para-nitrophenylbutyrate (*p*-NPB) and ortho-nitrophenylbutyrate (*o*-NPB). One unit of activity was defined as the production of one nmol of nitrophenol from the nitrophenyl substrates and one mmol production of butyrate from the BTC substrate.

CE was 10 to 314 fold more active than PCE with respect to the nitrophenyl substrates (Figure 3.12A and B). The difference in CE activity between the shorter chain acetate substrates and their analogous longer chain butyrate was greater than the difference for PCE ($p<0.05$). The difference in PCE activity between the para position side chain substrates and the ortho position side chain substrate was greater than the difference observed for the same substrates with CE ($p<0.05$).

When a factorial analysis for the length and position of the side chain was performed, only the length of the side chain was a significant variable for CE activity ($p<0.001$), while for PCE, only the location was a significant variable for the enzyme activity ($p<0.01$). CE showed no activity with the BTC substrate.

3.3.3. HYDROLASE ACTIVITY IN HUMAN SALIVA

Since enzymes are present in human saliva [Chauncey, 1961; Nakamura 1983a], it is of a great interest to determine its esterase activity profile and compare it to the activity profiles of the stock CE and PCE. The enzymatic activity profile of human saliva was determined using *p*-NPA, *o*-NPA, *p*-NPB, *o*-NPB and BTC as described in section 2.4.5. These data were acquired with the assistance of Mr. Neeraj Pershad, an undergraduate summer student, under my supervision.

Hydrolase activities in human saliva, collected from seven different subjects, were measured using nitrophenyl ester substrates and compared with the stock CE data (Figure 3.13). For the nitrophenyl substrates, one unit of activity was defined as a change of 0.01 O.D. per minute, under the assay conditions as described in section 2.4.2.1.

It is observed that all of the subjects exhibit a hydrolase activity when analyzed with the different nitrophenyl substrates (Figure 3.13). The average activity level measured using *p*-NPA as a substrate is 0.19 ± 0.02 unit/ml with a range between 0.09 to 0.26 unit/ml. This range is within the activity level range (0.01-1 unit/ml) used in the enzyme dose response biodegradation studies for the composites (section 2.5.2.1). The highest

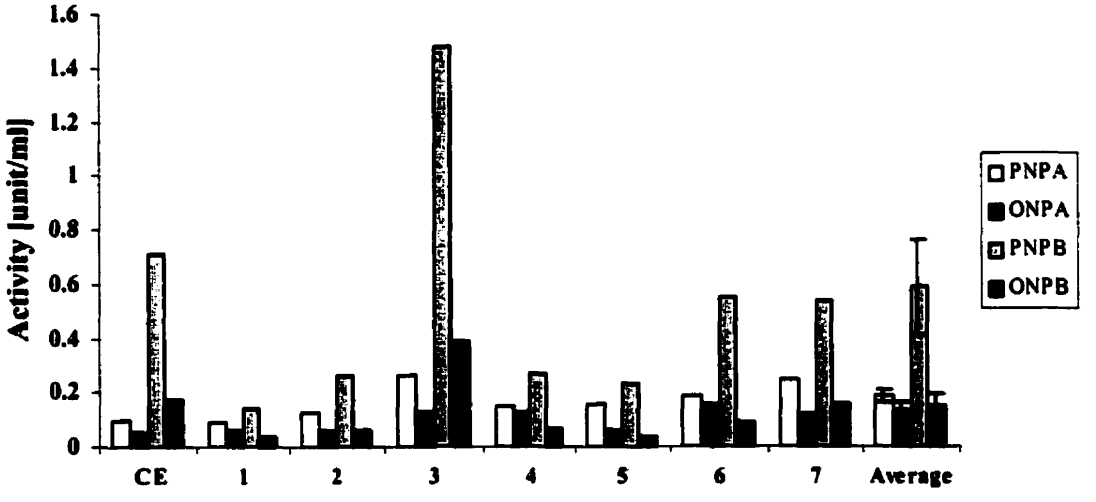


Figure 3.13 Hydrolase-like activity measured in human saliva, collected from different subjects and measured using para-nitrophenylacetate (*p*-NPA), ortho-nitrophenylacetate (*o*-NPA), para-nitrophenylbutyrate (*p*-NPB) and ortho-nitrophenylbutyrate (*o*-NPB), (pH 7.0 at 25°C). One unit of activity was defined as a change of 0.01 O.D. per minute under the specified test conditions (section 2.4.2.1)

measured activity for all subjects is observed using *p*-NPB, with an average of 0.59 ± 0.18 unit/ml and a range between 0.14 unit/ml to 1.48 units/ml. The sensitivity of the *p*-NPB substrate over that of the other substrates is similar to that observed for the CE stock sample (Figure 3.13) and reported by others for CE [Labow 1994].

Hydrolase activities in human saliva, collected from seven different subjects and measured using BTC as a substrate were also compared to the stock PCE and are presented in Figure 3.14. For BTC, the activities were calculated using equation 2.4 (section 2.4.2.2).

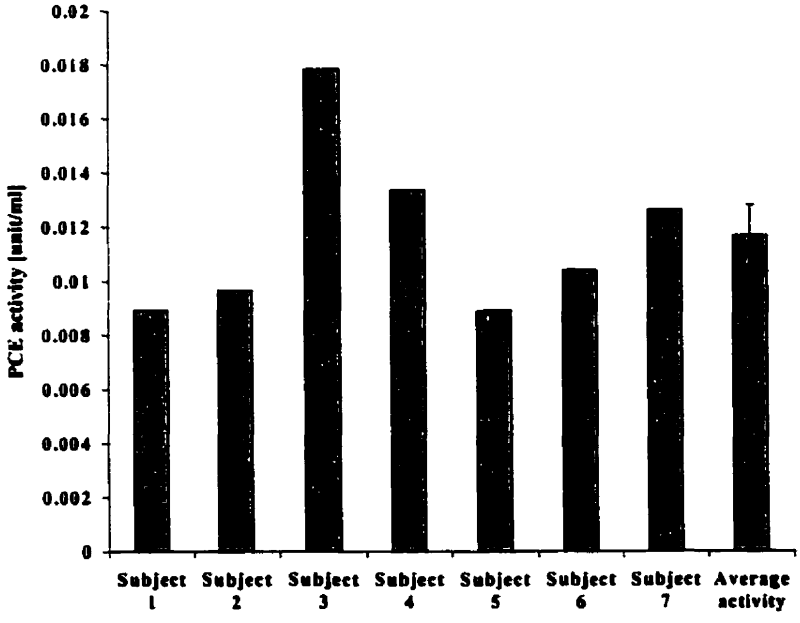


Figure 3.14 Hydrolase activity measured in human saliva collected from different subjects measured using butyrylthiocholine (BTC) as a substrate (pH 7.2 at 25°C). One unit of activity was defined as a production of one mmol butyrate per minute (see section 2.4.2.2)

It is observed that all the subjects show a hydrolase PCE-like activity when analyzed with butyrylthiocholine (BTC) (Figure 3.14). The average activity level measured using BTC as a substrate is 0.011 ± 0.001 unit/ml with a range between 0.004 to 0.018 unit/ml. This range is slightly above the lower limit of PCE used in the enzyme dose response biodegradation studies for the composites (section 2.5.2.1).

3.3.4 ENZYME INHIBITION ASSAYS

The active site of the enzyme involved in the enzymatic attack onto the model composite resin samples was investigated using a specific esterase inhibitor, phenylmethylsulfonyl fluoride (PMSF). Prior to the biodegradation experiments, the activity of the enzymes with and without PMSF was measured as described in section 2.4.6. The data are shown in Figure 3.15 as the relative activity of the corresponding enzyme incubated alone. The activities were measured using *p*-NPA as a substrate for CE (section 2.4.2.1) and BTC as a substrate for PCE (section 2.4.2.2).

The addition of ethanol (the inhibitor's carrier solvent) to the assay solution had no effect on the activity of the enzyme, incubated alone (Figure 3.15). The addition of 0.5 or 1 mM PMSF (final concentration) to the PCE solution (1 unit/mL) showed a decrease in the relative activity of the enzyme to 58 ● 4.7 % and 30 ± 2.9 % respectively ($p<0.05$). The addition of 1 or 2 mM PMSF to the CE solution (1 unit/mL) showed a decrease in the activity to 63 ± 0.5 % and 50 ● 2.7 % respectively ($p<0.05$). According to these data, the

PMSF concentrations used in the biodegradation studies (see section 3.4.6) were adjusted for the different enzymes.

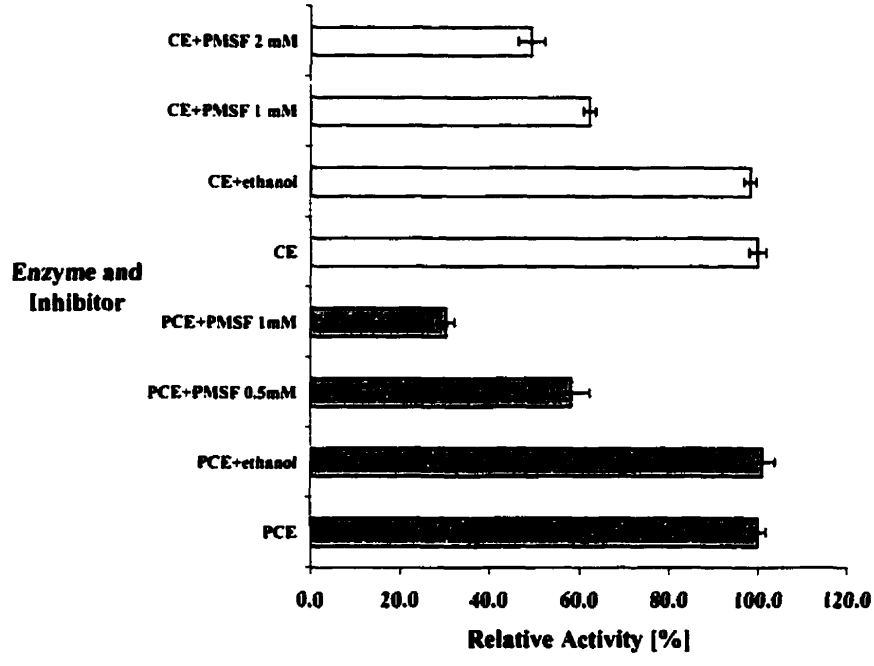


Figure 3.15 The inhibition effect of PMSF, at different concentrations, on the activities of CE and PCE (pH 7.0 at 25°C). The activities were measured using para-nitrophenylacetate and butyrylthiocholine as substrates for CE and PCE respectively

3.4 BIODEGRADATION STUDIES

3.4.1. SURFACE MORPHOLOGY OF THE COMPOSITE SAMPLES

The surface morphology of the composite resin samples following incubation with PBS, CE (1 unit/ml) or PCE (1 unit/ml) for 32 days was assessed using scanning electron microscopy (SEM) as described in section 2.3.2. The data are shown in Figures 3.16 to 3.19.

Figures 3.16A and 3.17A demonstrate bis10 and bis60 samples respectively following incubation in D-PBS. The surfaces for these samples are rougher than those of u-bis10 and u-bis60 that incubated in D-PBS (Figures 3.18A and 3.19A). The effect of the enzymatic attack on the materials is illustrated by Figures 3.16B-C, 3.17B-C, 3.18B-C and 3.19 B-C. The surfaces of the bis samples appear to be much more porous and exhibit exfoliated filler particles when compared with the D-PBS incubated samples (compare Figures 3.16B-C to 3.16A, and 3.17B-C to 3.17A). The effect of CE or PCE on the surface morphology of the u-bis samples show less change as compared with the bis materials (compare Figures 3.18B-C and 3.19B-C with 3.16B-C and 3.17B-C).

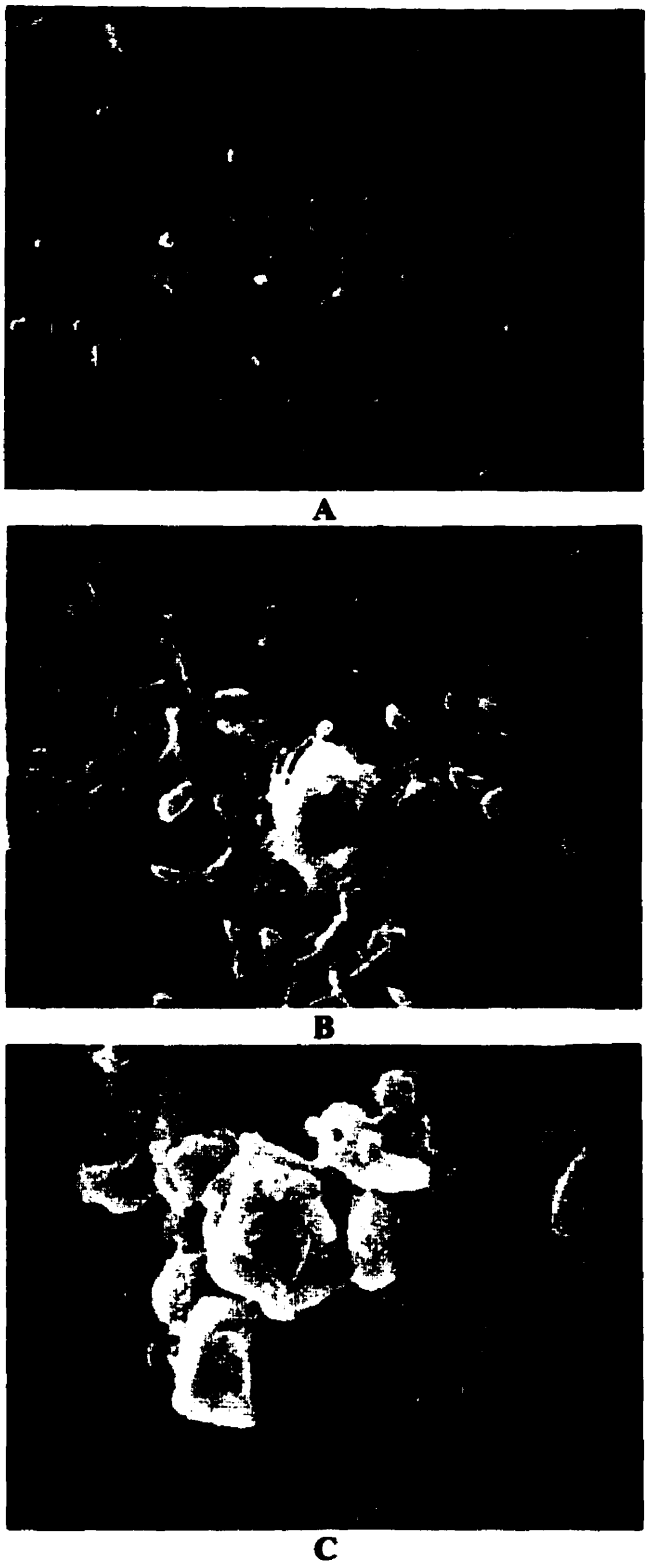


Figure 3.16 **Scanning electron micrographs of bis10 following 32 days incubation in (A) D-PBS, (B) CE and (C) PCE (1×10^4 original magnification)**

A

B

C

Figure 3.17 **Scanning electron micrographs of bis60 following 32 days incubation in (A) D-PBS, (B) CE and (C) PCE (1×10^4 original magnification)**

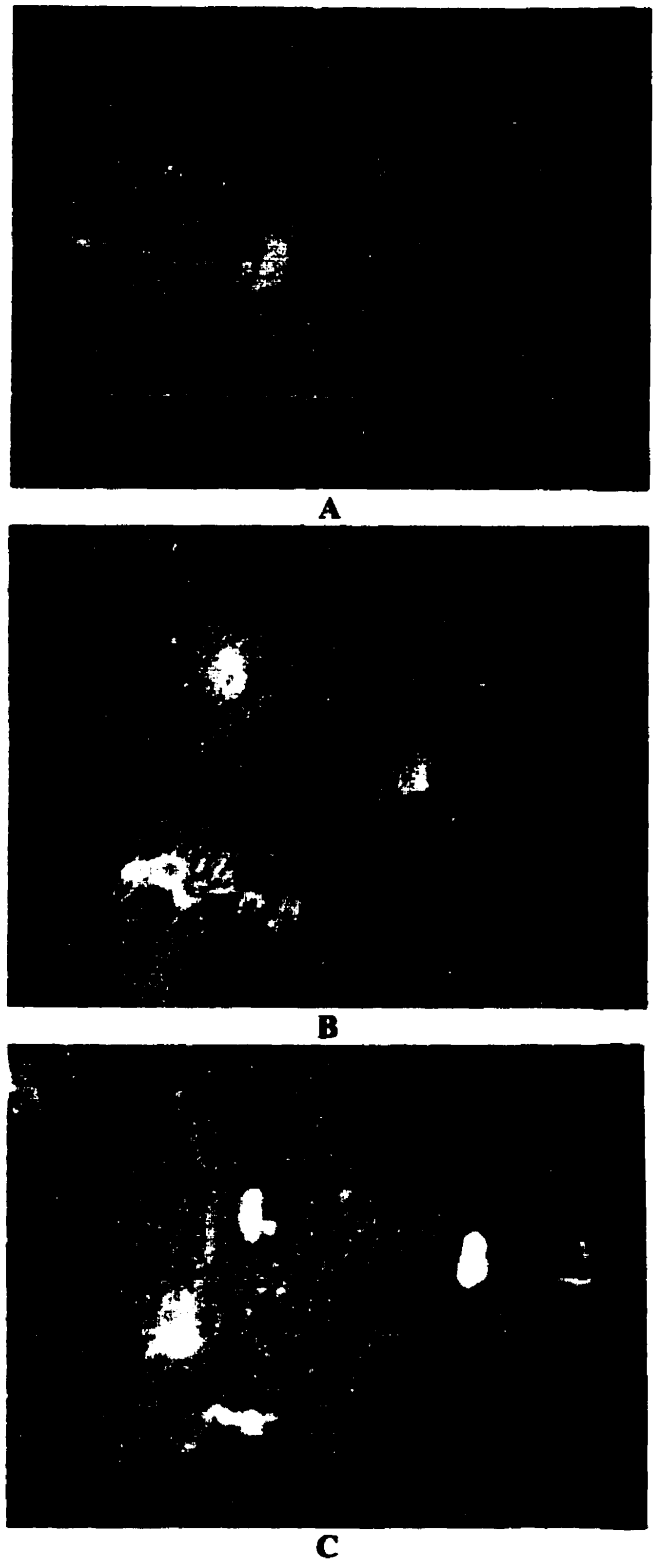


Figure 3.18 Scanning electron micrographs of u-bis10 following 32 days incubation in (A) D-PBS, (B) CE and (C) PCE (1×10^4 original magnification)

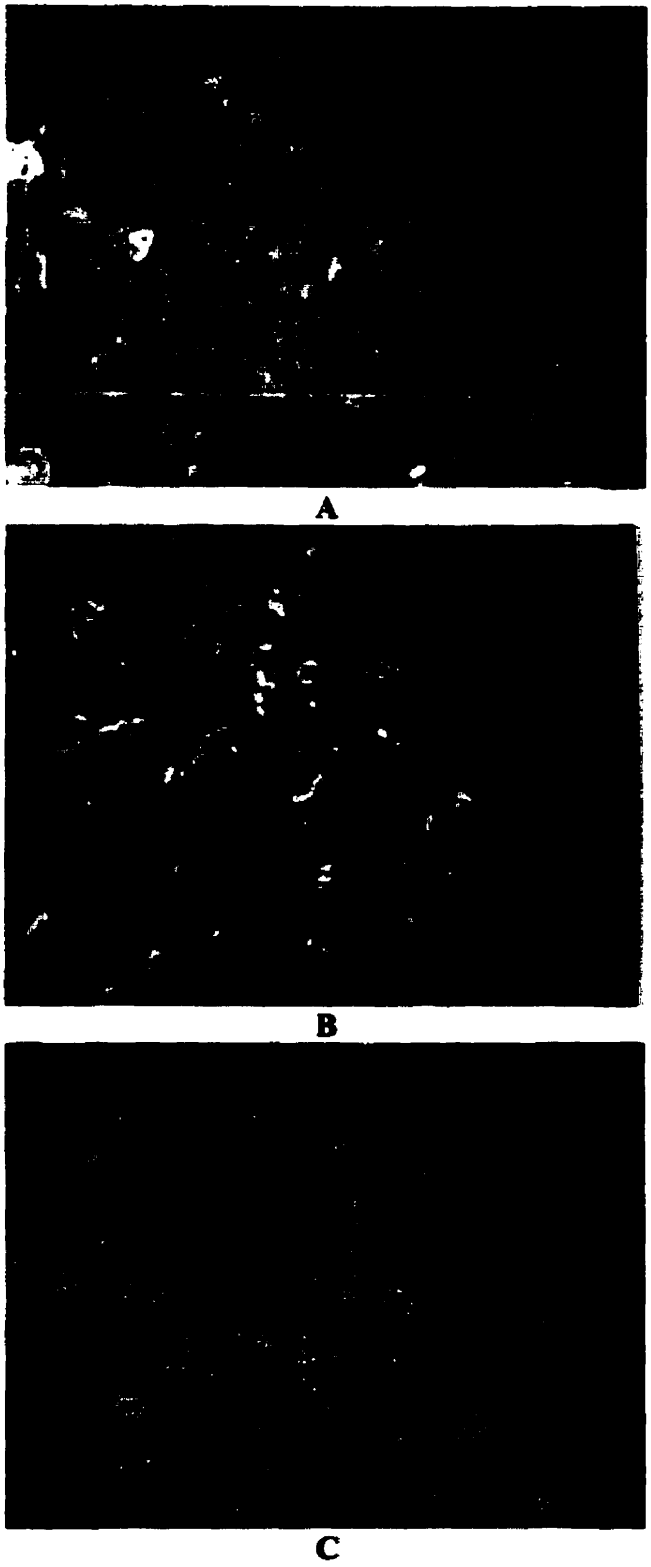


Figure 3.19 Scanning electron micrographs of u-bis60 following 32 days incubation in (A) D-PBS, (B) CE and (C) PCE (1×10^4 original magnification)

3.4.2. SURFACE MICRO-HARDNESS ANALYSIS OF THE COMPOSITE RESIN SAMPLES

Mean Vickers surface micro-hardness analysis for six samples from each composite resin sample group, i.e. bis10, bis60, u-bis10 and u-bis60, was performed as described in section 2.3.3. The data are reported in Table 3.3 as mean ± standard error [Kg/mm^2]. The load was 200 grams. The baseline data correspond to composite resin samples for which micro-hardness values were measured following their preparation and post cure treatment in a vacuum oven as described in section 2.1.3. Micro-hardness values of samples following 32 days incubation in PBS, CE (0.1 unit/ml) or PCE (0.1 unit/ml) are labeled accordingly.

Table 3.3 Mean Vickers surface micro-hardness [Kg/mm^2] results for composite resin samples before and following incubation in enzymes and control media for 32 days (pH 7.0, 37°C)

	bis10	bis60	u-bis10	u-bis60
Baseline	11.00±0.37	42.26±1.73	16.10±0.25	32.84±0.53
PBS	12.37±0.20	43.61±1.15	16.54±0.28	30.97±1.56
CE	12.13±1.17	36.93±0.16	15.07±0.80	29.31±0.31
PCE	12.61±0.36	35.68±0.72	15.81±0.41	27.21±1.23

Following incubation in PBS, CE or PCE, no significant difference was found between the different incubation media for bis10 and u-bis10 samples. In previous studies, composite resin materials that exhibited less than a threshold of 40 Kg/mm^2 of Vickers hardness, showed a lack of sensitivity in this type of measurement [Shajii, 1997]. For bis60 samples, following incubation in CE or PCE for 32 days, a significant decrease in micro-hardness values ($p<0.05$) is observed when comparing these to the baseline or the D-PBS groups. While the u-bis60 group shows a decrease in hardness following incubation with either CE or PCE, the differences were not statistically significant ($p>0.05$). Since the micro-hardness of both bis60 and u-bis60 groups are close to the threshold value that was mentioned above, the reliability of this test as a procedure for quantitative analysis of the degradation process for these materials is questionable as has been previously mentioned in other work [Santerre, 1999].

3.4.3 ISOLATION AND IDENTIFICATION OF DEGRADATION PRODUCTS

Pseudocholinesterase (PCE) and cholesterol esterase (CE) catalyze the hydrolysis of the composite resin samples in aqueous solution, leading to the release of degradation products. Micro-hardness analysis demonstrated a limited ability to quantify the degradation process, since statistically significant differences were only observed in one of the composite resin samples (bis60). Even for this composite group, the changes in micro-hardness did not necessarily represent the extent of the biodegradation process since they could be partially attributed to the swelling of the polymer matrix following water sorption. Thus, micro-hardness analysis is not considered an accurate measure of

the biodegradation process [Santerre, 1999]. Alternatively, HPLC in combination with UV spectroscopy and mass spectrometry are considered to be valuable tools for the evaluation of the biodegradation process. By using these techniques, isolation, identification and quantification of the degradation products can be effectively carried out.

Figure 3.20 contains HPLC chromatograms of the incubation solutions for bis60 and u-bis60 composite samples, following incubation in D-PBS or CE for 16 days, 37°C at pH=7.0. The selected chromatograms represent typical chromatograms obtained from the different materials studied. Since the u-bis60 composite incubated with D-PBS did not show any unique products, when compared with the bis60 composite under the same conditions, the latter's chromatogram also represents a typical chromatogram for the u-bis60 group incubated in D-PBS. Similarly, the PCE incubated composites did not show any additional products when compared with the CE incubated groups, hence the latter's chromatograms also represent the corresponding product for PCE incubated groups. Chromatograms for the incubated enzymes (no composite), methacrylic acid and TEGDMA standard are also shown in Figure 3.20. The UV absorbance of the chromatograms are recorded at a wavelength of 215 nm although during data collection, the full UV spectrum of each product was recorded. The top chromatogram (in Figure 3.20) represents a methacrylic acid standard with the main component (i.e. pure methacrylic acid) showing a retention time near 8 minutes. The second chromatogram from the top represents a standard TEGDMA monomer with its principal component

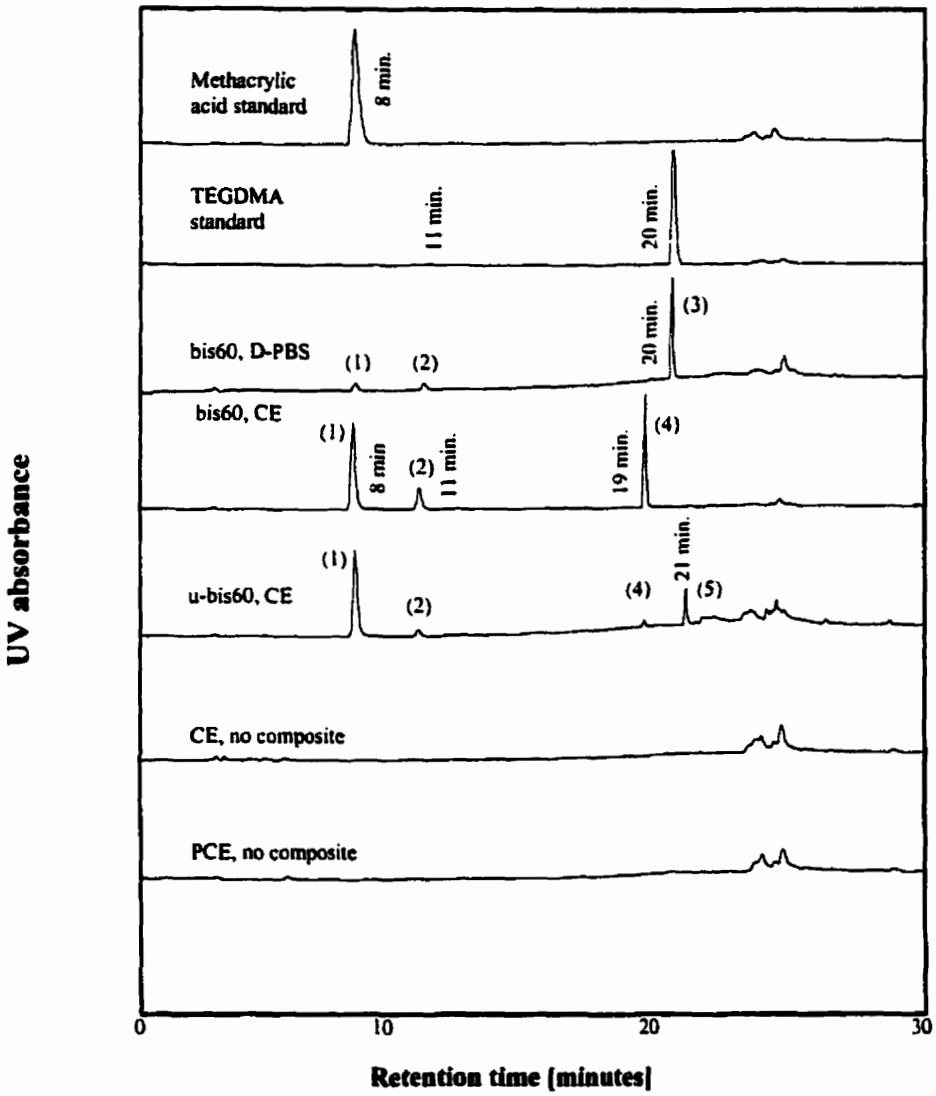


Figure 3.20 **HPLC chromatograms (Y axis not to scale) of methacrylic acid and a standard TEGDMA monomer, compared with incubation solutions for bis60 following incubation in D-PBS or CE, and u-bis60 following incubation in D-PBS (16 days, 37°C at pH=7.0). Also included are CE and PCE incubation solutions (no composites).**

(i.e. pure TEGDMA) showing a retention time near 20 minutes while a small peak near 11 minutes is also associated with this standard.

There are five main peaks specifically associated with derivative component of the composite resin samples. None of the peaks have similar retention times to the compounds associated with the pure enzyme only solutions (CE or PCE, no composite). Furthermore, the latter two chromatograms show very similar peaks, thus associating them with the buffer and not necessarily with the enzymes themselves. The peaks with retention times of 8 and 11 minutes are presented in both D-PBS and CE incubated composite samples. The peak having a retention time of 19 minutes is specifically associated with the enzyme incubated composite samples, while the peak at 20 minutes is detected only in the D-PBS incubated groups. The chromatogram for the u-bis60 incubated with CE shows an additional peak at 21 minutes. This peak appeared only in the CE incubated u-bis composite samples and was not isolated in the bis samples or the PCE incubated u-bis samples (PCE with composite data are not shown in Figure 3.20).

The UV spectra of the methacrylic acid standard isolated at 8 minutes and degradation product (1), isolated at a retention time 8 minutes (Figure 3.20), are presented in Figure 3.21. The 8 minute peak showed an identical UV absorbance spectrum to the methacrylic acid standard, indicating that this product was likely methacrylic acid. The identity of the isolated degradation product with the retention time of 8 minutes was confirmed using mass spectrometry, as described in section 2.6.2 (Figure 3.22). When comparing this to

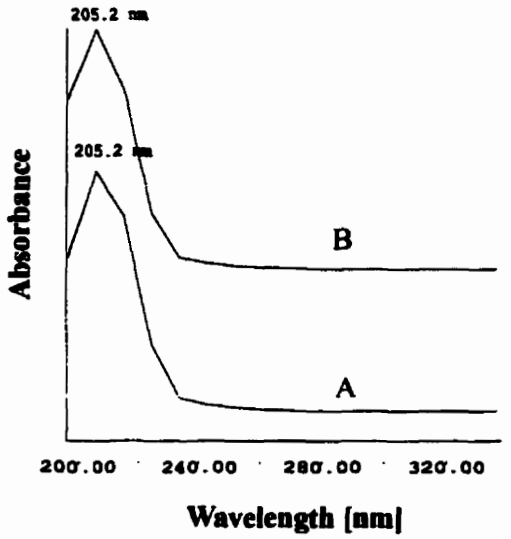


Figure 3.21 **UV spectra of the isolated products related to (A) methacrylic acid standard and (B) product with 8 minutes retention time (Figure 3.20)**

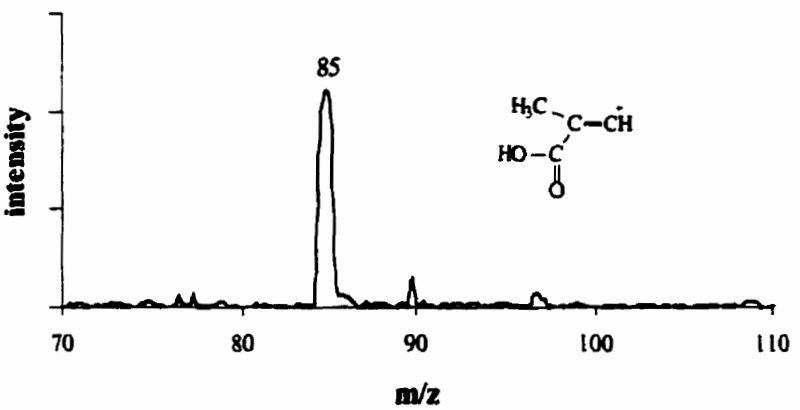


Figure 3.22 **Mass spectrum (MS) of the product isolated at a retention time of 8 minutes (Figure 3.20) in the enzyme incubated composite samples**

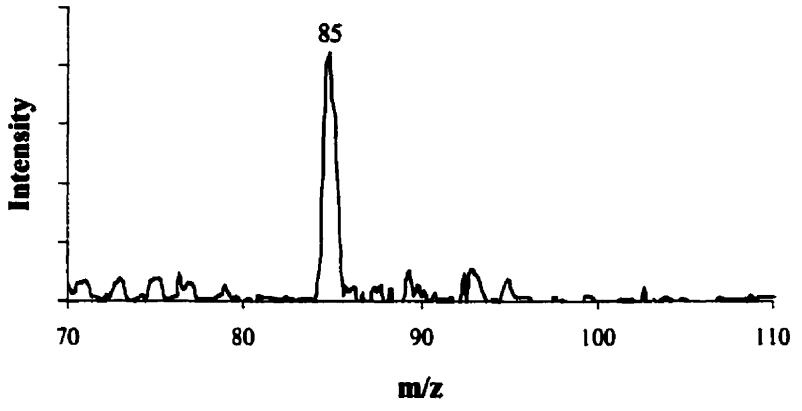


Figure 3.23 Mass spectrum (MS) of the standard methacrylic acid isolated at a retention time of 8 minutes (Figure 3.20)

the mass spectrum of the standard methacrylic acid, presented in Figure 3.23, it can be seen that both contain the same dominant mass to charge (m/z) peak of 85.

The UV spectra for the standard TEGDMA monomer, isolated at a retention time of 20 minutes and the degradation products isolated at 11 minutes and 20 minute from the composite samples are presented in Figure 3.24. Both the 11 and 20 minutes peaks from Figure 3.20 show UV absorbance spectra very similar to the standard TEGDMA monomer, indicating that these products were suspected of being related to the latter.

The mass spectrum for the 11 minute product is shown in Figure 3.25. Based on the fragmentation pattern and the mass to charge ratio (m/z) of 219, the proposed chemical structure of the product is referred to as triethyleneglycol methacrylate (TEGMA). The

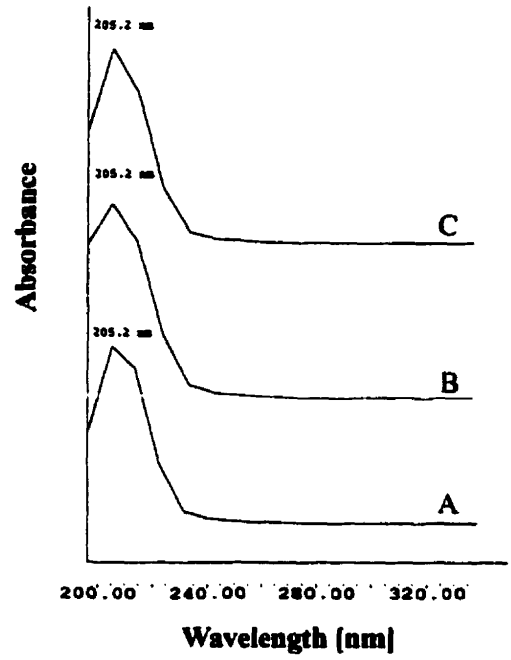


Figure 3.24 **UV spectra of the isolated products related to (A) standard TEGDMA monomer, (B) 11 minute product (Figure 3.20) and (C) 20 minute product (Figure 3.20)**

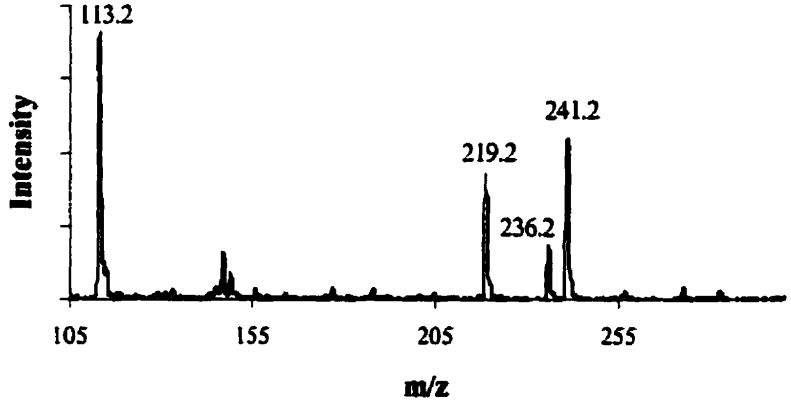


Figure 3.25 **Mass spectrum (MS) of the product isolated at a retention time of 11 minutes (Figure 3.20) in the enzyme incubated composite samples**

chemical structures of the principal fragmented ions related to the product with the retention time of 11 minutes (i.e. TEGMA) are given in Table 3.4. The three ionic states in Table 3.4 correspond to H^+, Na^+ and NH_4^+ ions originating from the D-PBS and the ammonium acetate buffer used as the mobile phase for the HPLC runs (see Table 3.4).

Table 3.4 The chemical structure of the principal fragmented ions related to the product with a retention time of 11 minutes (i.e. TEGMA)

m/z	Ion chemical structure
113	$CH_2=C(CH_3)CO_2CH_2CH_2$
219	$[CH_2=C(CH_3)CO_2(CH_2CH_2O)_3H]H^+$
236	$[CH_2=C(CH_3)CO_2(CH_2CH_2O)_3H]NH_4^+$
241	$[CH_2=C(CH_3)CO_2(CH_2CH_2O)_3H]Na^+$

The peak at 20 minutes (Figure 3.20) was confirmed by mass spectrometry to be TEGDMA, and had a mass to charge ratio (m/z) of 287 for the parent peak (Figure 3.26). This mass spectrum was compared with the spectrum of the standard TEGDMA monomer in Figure 3.27 and confirmed as being this monomer. The corresponding fragmented ions (i.e. H^+, NH_4^+, Na^+ and K^+) are presented in Table 3.5.

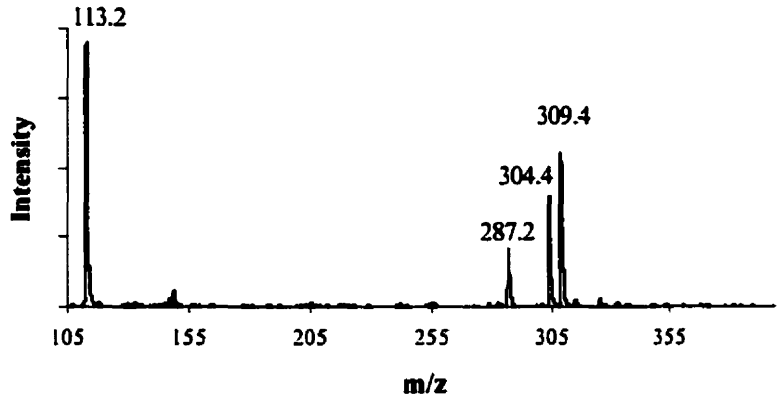


Figure 3.26 **Mass spectrum (MS) of the product isolated at a retention time of 20 minutes (Figure 3.20) in the D-PBS incubated composite samples**

Table 3.5 **Chemical structure of the TEGDMA monomer isolate at a retention time of 20 minutes for the bis and u-bis composite samples**

m/z	Ion chemical structure
113	$CH_2=C(CH_3)CO_2CH_2CH_2$
287	$[CH_2=C(CH_3)CO_2(CH_2CH_2O)_3OC(CH_3)C=CH_2]H^+$
304	$[CH_2=C(CH_3)CO_2(CH_2CH_2O)_3OC(CH_3)C=CH_2]NH_4^+$
309	$[CH_2=C(CH_3)CO_2(CH_2CH_2O)_3OC(CH_3)C=CH_2]Na^+$
325	$[CH_2=C(CH_3)CO_2(CH_2CH_2O)_3OC(CH_3)C=CH_2]K^+$

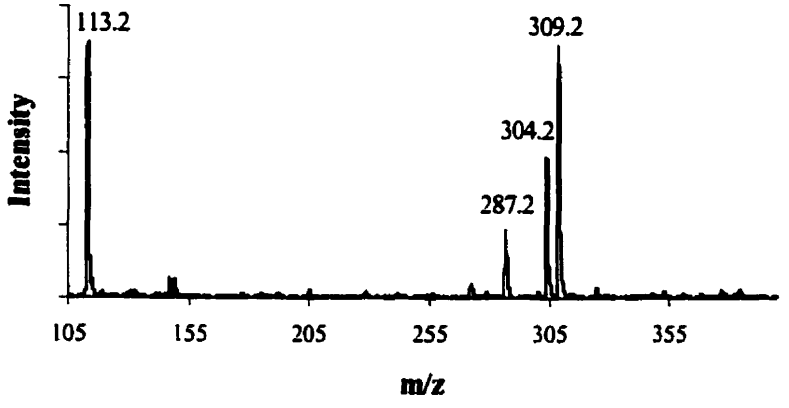


Figure 3.27 Mass spectrum (MS) of the standard TEGDMA monomer isolated at a retention time of 20 minutes (Figure 3.20)

The UV spectrum of the degradation product at 19 minutes, isolated from the bis and u-bis composite samples is presented in Figure 3.28 B. The product peak at 19 minutes differs from the 8, 11 and 20 minute peaks (Figures 3.21 and 3.24) in that it exhibits UV absorbance values near 230 and 280 nm in addition to the 205 nm value. These high absorbance values are usually associated with conjugated chemical structure (Peters, 1974). One of the main components in the bis and u-bis composites which contains conjugated double bonds is bisGMA. Spectrum A in Figure 3.28 corresponds to a bisGMA standard. The 19 minute peak (Figure 3.20) shows a UV absorbance spectrum (Figure 3.28 B) very similar to the standard bisGMA monomer, indicating that this product may be related to the latter.

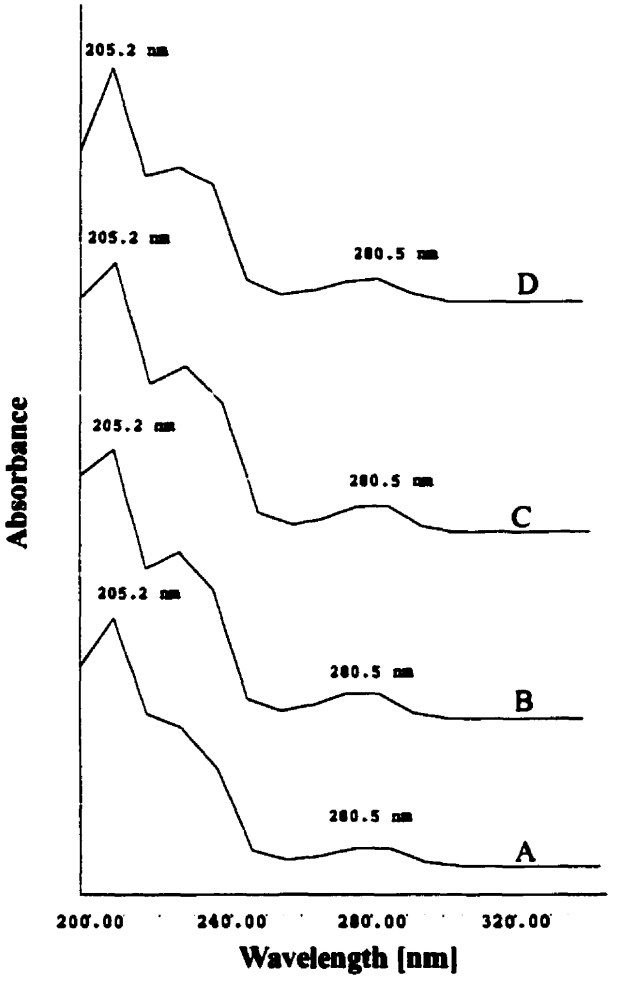


Figure 3.28 UV spectra of the isolated products related to (A) a standard bisGMA monomer, (B) 19 minute peak (Figure 3.20), (C) 21 minute peak (Figure 3.20) and (D) a standard bisEMA monomer product

In a similar manner, the peak at 21 minutes, isolated for the u-bis composite samples incubated with CE (Figure 3.28 C) shows a similar spectrum to the 19 minute peak, indicating that it may also be similar in nature to the latter. The spectrum of bisEMA, a major component of the u-bis resin is shown in Figure 3.28 D. The 21 minute peak shows a UV absorbance spectrum very similar to the standard bisEMA monomer. In order to further differentiate the four compounds, the use of a technique such as mass spectrometry becomes critical.

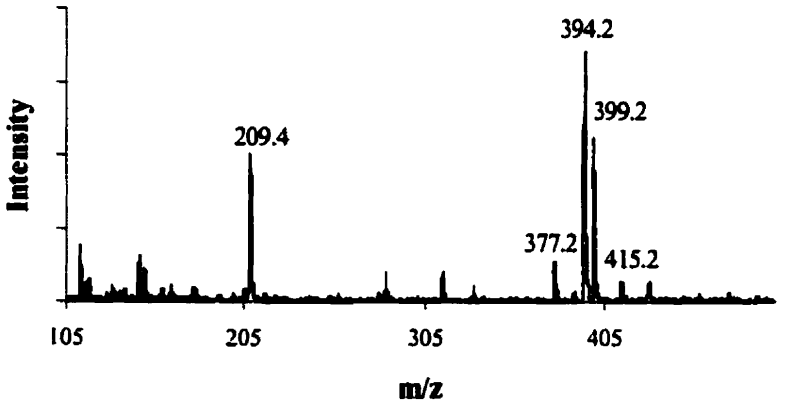


Figure 3.29 **Mass spectrum (MS) of the bisGMA derived product, bisHPPP, isolated at a retention time of 19 minutes (Figure 3.20) from bis and u-bis composite samples**

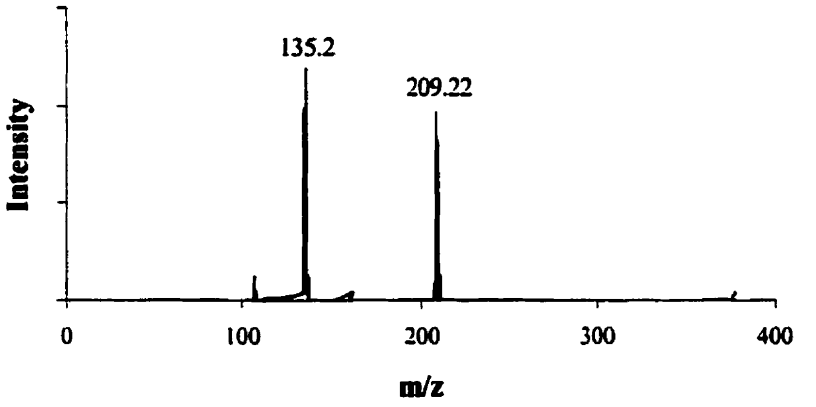
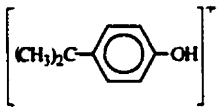
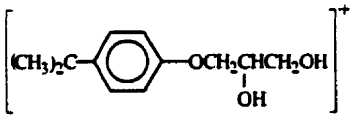
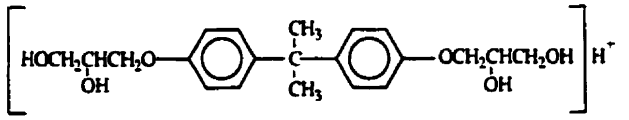
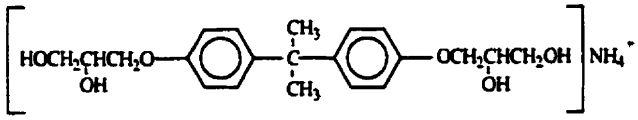
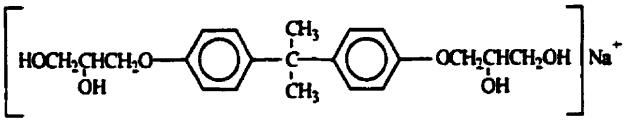
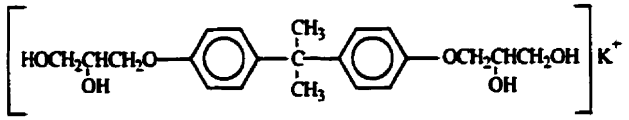


Figure 3.30 **MS/MS spectra of the 394.2 isolated peak associated with the product having a retention time of 19 minutes (Figure 3.20), for the bis and u-bis composite samples**

The MS and MS/MS mass spectra for the 19 minute peak, hypothesized to be related to bisGMA, are given in Figures 3.29 and 3.30 respectively. Based on the fragmentation

pattern and a mass to charge ratio of 377 (Figure 3.29), a molecule with the structure of 2,2-bis[4(2,3-hydroxy-propoxy)phenyl]propane (bisHPPP) is proposed for this product. The chemical structure of bisHPPP and its fragmented ions from Figures 3.29 and 3.30 are presented in Table 3.6.

Table 3.6 Chemical structure of the bisGMA derived product, bisHPPP, isolated at a retention time of 19 minutes for bis and u-bis composite samples

m/z	Ion chemical structure
135	
209	
377	
394	
399	
415	

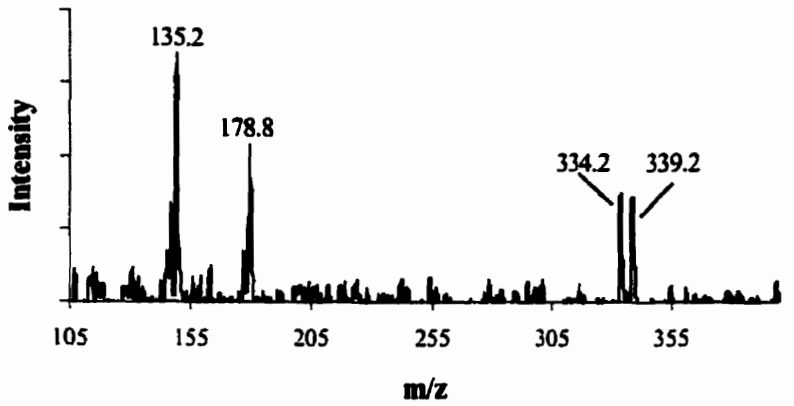


Figure 3.31 **Mass spectrum (MS) of the bisEMA derived product, E-bisPA, isolated at a retention time of 21 minutes (Figure 3.20) from u-bis composite samples incuabted in CE**

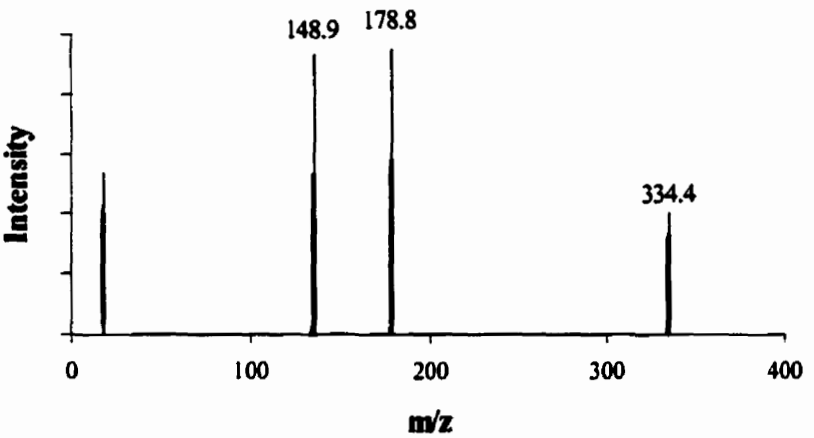
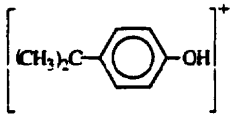
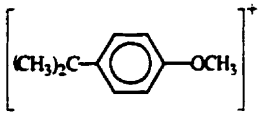
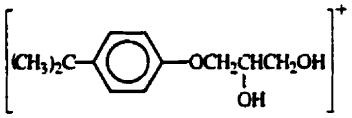
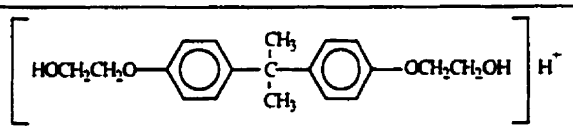
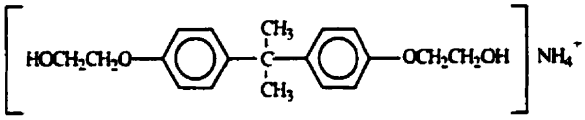
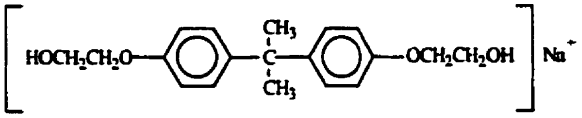
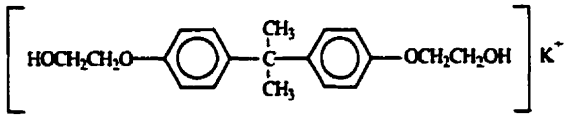


Figure 3.32 **MS/MS spectra of the 334.4 isolated peak associated with the product having a retention time of 21 minutes (Figure 3.20), for the u-bis composite samples incubated with CE**

The mass spectrum for the 21 minutes peak is given in Figures 3.31 and 3.32. Based on the fragmentation pattern and a mass to charge ratio of 317, a molecule with the structure

Table 3.7 **Chemical structure of the bisEMA derived product, E-bisPA, isolated at a retention time of 21 minutes for u-bis composite samples incubated with CE**

m/z	Ion chemical structure
135	
149	
179	
317	
334	
339	
355	

of 2,2-bis[4(2-hydroxy- ethoxy) phenyl]propane or ethoxylated bis phenol A (E-bisPA) is proposed for this product. The chemical structure of E-bisPA and its fragmented ions from Figures 3.31 and 3.32 are presented in Table 3.7.

3.4.4 ENZYMATIC HYDROLYSIS OF THE MONOMERS

Since the cured composite resin samples contained unreacted as well as partially and fully reacted monomers, it was important to determine the enzymes' ability to catalyze the hydrolysis of the monomers that were used in the preparation of the resins. The CE and PCE catalyzed hydrolysis of TEGDMA, bisGMA and bisEMA monomers was carried out as described in section 2.5.3. HPLC data for the incubation solutions are presented in Figure 3.33, as the amount of methacrylic acid production per hour per mL for each of the three monomers.

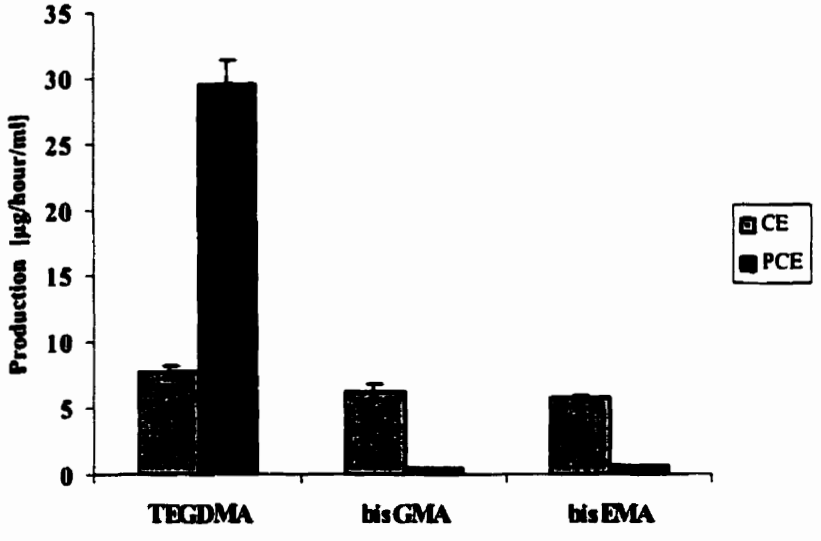


Figure 3.33 Methacrylic acid production rate (pH 7.0 at 37°C), for CE (1 unit/mL) and PCE (1 unit/mL) catalyzed hydrolysis of TEGDMA, bisGMA and bisEMA monomers

It is observed that PCE catalyzed hydrolysis rates for TEGDMA, bisGMA and bisEMA were respectively 29.55 ± 1.78, 0.49 ± 0.01 and 0.57 ± 0.03 μg/hour/mL. While the latter varied for the different monomers, the CE hydrolysis rates were relatively similar for the three compounds, having values of 7.7 ± 0.5, 6.3 ± 0.65 and 5.83 ± 0.16 μg/hour/mL for TEGDMA, bisGMA and bisEMA respectively. The PCE catalyzed hydrolysis rate for TEGDMA was 3.8 fold higher than that of the CE, while CE catalyzed hydrolysis of bisGMA and bisEMA were respectively 12.79 and 10.23 fold higher than that for the PCE.

3.4.5 DOSE DEPENDANT EFFECT OF CE AND PCE ON THE BIODEGRADATION OF THE COMPOSITE RESIN SAMPLES

The effect of D-PBS or different concentrations of CE and PCE on the biodegradation of bis60 and u-bis60 composite samples incubated for 16 days (37°C at pH=7.0) was carried out as described in section 2.5.2.1. HPLC data for the incubation solutions are presented in Figures 3.34 to 3.37. The extent of biodegradation is represented by the amount of biodegradation products relative to the composite samples' surface area and as a function of the enzyme's concentration.

In the bis60 system, both enzymes degraded the samples in a dose dependant manner ($p<0.01$) (Figures 3.34 and 3.35). Increasing the amount of CE (Figure 3.34) or PCE (Figure 3.35) showed an increase in the amount of released methacrylic acid and a bisGMA derived product, 2,2- Bis [4(2,3-hydroxy-propxy)phenyl]propane (bisHPPP),

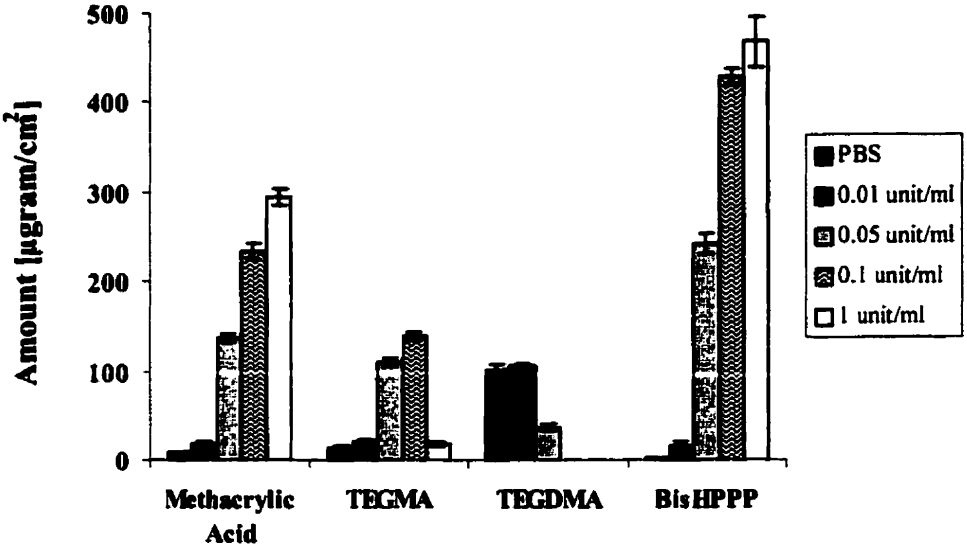


Figure 3.34 The effect of D-PBS and different concentrations of CE on the biodegradation of bis60 composite samples incubated for 16 days, pH 7.0 at 37°C

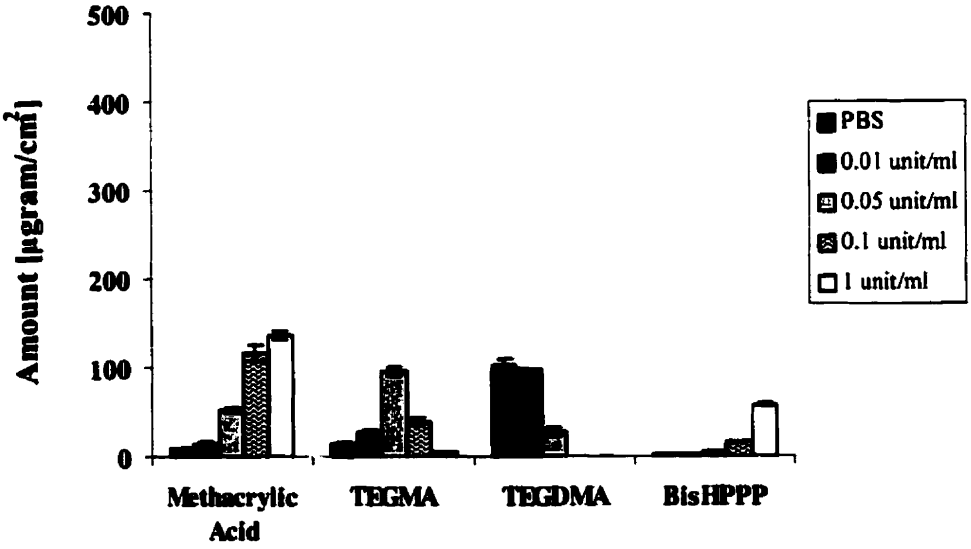


Figure 3.35 The effect of D-PBS and different concentrations of PCE on the biodegradation of bis60 composite samples incubated for 16 days, pH 7.0 at 37°C

but showed a decrease in the amount of TEGDMA monomer compared with D-PBS ($p<0.001$). Increasing the amount of CE or PCE showed an increase in the amount of triethylene glycol methacrylate (TEGMA), a product originating from unreacted or partially reacted TEGDMA, with a maximum at 0.1 unit/mL for CE ($p<0.01$) (Figure 3.34) and 0.05 unit/mL for PCE ($p<0.001$) (Figure 3.35). At 1 unit/mL, following 16 days incubation the amount of bisHPPP released by CE (470 ± 28 $\mu g/cm^2$) is 8 fold higher as compared with that released by PCE (57 ± 1.2 $\mu g/cm^2$) ($p<0.001$), while the amount of methacrylic acid released by CE (295 ± 9 $\mu g/cm^2$) is only double ($p<0.001$) the amount released by PCE (136 ± 3 $\mu g/cm^2$).

In the u-bis60 system, both enzymes also degraded the composites in a dose dependant manner ($p<0.01$) (Figures 3.36 and 3.37). Increasing the amount of CE (Figure 3.36) showed an increase in the release of methacrylic acid and a bisEMA derived product, ethoxylated bisphenol-A (E-bisPA) ($p<0.01$). Although higher amounts of bisHPPP were isolated after incubation with CE, the increase in bisHPPP was not statistically significant at all concentrations of CE, due to the high variability of the results relative to the small amount of isolated bisHPPP. Incubating the u-bis60 samples with 0.05, 0.1 and 1 unit/mL PCE (Figure 3.37) showed an increase in the amount of isolated methacrylic acid ($P<0.001$), while only 1 unit/mL enzyme showed a significant increase in the amount of bisHPPP ($p<0.001$). Increasing the amount of CE or PCE showed an increase in the amount of TEGMA, with a maximum at 0.05 and 0.1 unit/mL for CE ($p<0.001$) (Figure 3.36) and 0.05 unit/mL for PCE ($p<0.05$) (Figure 3.37), but showed a decrease in the amount of TEGDMA as compared with D-PBS ($p<0.01$). No E-bisPA was detected for the PCE treated samples.

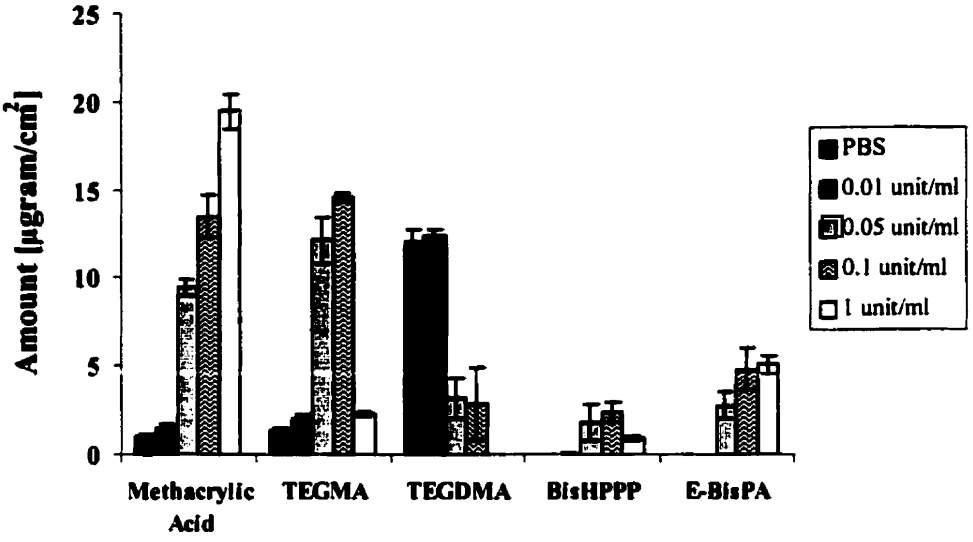


Figure 3.36 **The effect of D-PBS and different concentrations of CE on the biodegradation of u-bis60 composite samples incubated for 16 days, pH 7.0 at 37°C**

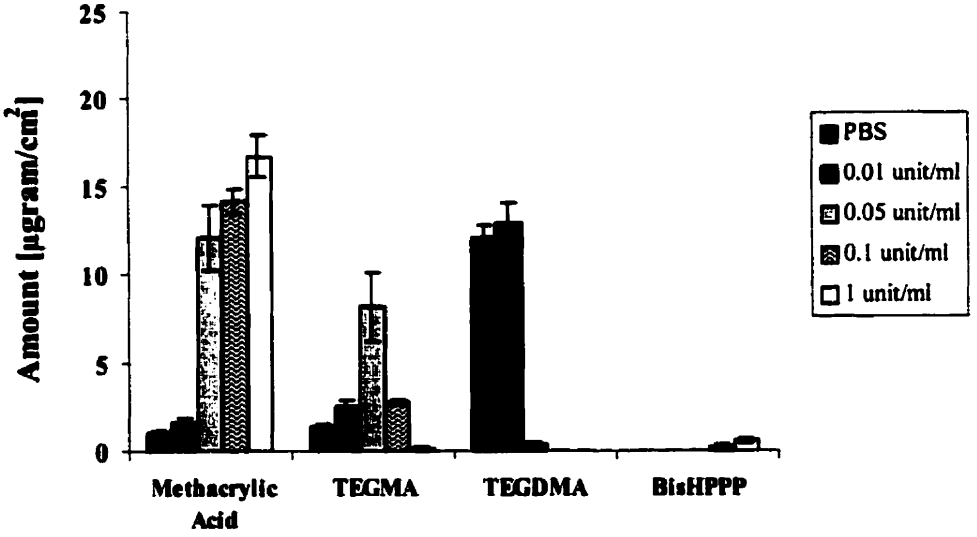


Figure 3.37 **The effect of D-PBS and different concentrations of PCE on the biodegradation of u-bis60 composite samples incubated for 16 days, pH 7.0 at 37°C**

Following 16 days of incubation in D-PBS, it is observed that the amount of released methacrylic acid, TEGMA, and TEGDMA is between 8 to 10 fold higher for the bis60 system as compared with the amount found in the u-bis60 system ($p<0.01$) (Figures 3.34-3.37). For instance, following 16 days incubation in D-PBS, the amount of released methacrylic acid and TEGMA, relative to the surface area of the composites were respectively 8 ± 0.36 $\mu g/cm^2$ and 14 ± 0.56 $\mu g/cm^2$ for the bis60 system, while in the u-bis60 system the amount was 1 ± 0.1 $\mu g/cm^2$ and 1.4 ± 0.04 $\mu g/cm^2$. Following 16 days incubation in D-PBS, bisHPPP was only isolated in the bis60 system.

Incubation in CE or PCE showed an increase between 15 to 180 fold in the amount of isolated products for the bis60 system as compared with the u-bis60 ($p<0.001$). For instance, following 16 days incubation in 0.1 unit/mL CE, the released methacrylic acid per cm^2 of the composite is respectively 233 ± 9 $\mu g/cm^2$ and 14 ± 1 $\mu g/cm^2$ for bis60 and u-bis60 respectively. The difference between bis60 and u-bis60 systems for the bisHPPP production is even more pronounced, showing values of 430 ± 10 $\mu g/cm^2$ and 2.4 ± 0.6 $\mu g/cm^2$ for the respective materials.

3.4.6 EFFECT ON THE BIODEGRADATION OF THE COMPOSITE RESIN SAMPLES WHEN CE AND PCE WERE INHIBITED

The mechanism of the enzymatic attack on the model composite resin samples was investigated using a specific esterase inhibitor, phenylmethylsulfonyl fluoride (PMSF). Prior to the biodegradation experiments, the activity of the enzymes with and without

PMSF was measured. The results were shown in section 3.3.4. The effect of the inhibitors on the biodegradation of bis60 and u-bis 60 composite samples when CE and PCE were inhibited by PMSF was carried out as described in section 2.5.2.4. Based on the enzymes' linear range of activities, with respect to the production of methacrylic acid from the composite (section 3.4.5), CE and PCE concentrations of 0.1 unit/mL were chosen for incubation. The results for CE are shown in Figures 3.38 and 3.39 as a relative amount of product isolated following 16 days incubation with D-PBS, D-PBS+PMSF, CE+ethanol and CE+PMSF. All groups were normalized using the data for the CE+ethanol group.

It is observed that the addition of PMSF (0.1 mM) to the D-PBS solutions had no effect on the biodegradation of the samples. However, the addition of 0.1 mM PMSF to the CE incubated bis60 system, showed a reduction in the relative amount of methacrylic acid and bisHPPP to 47 ± 3.6 % and 48 ± 5.4 % ($p<0.05$) respectively (Figure3.38).

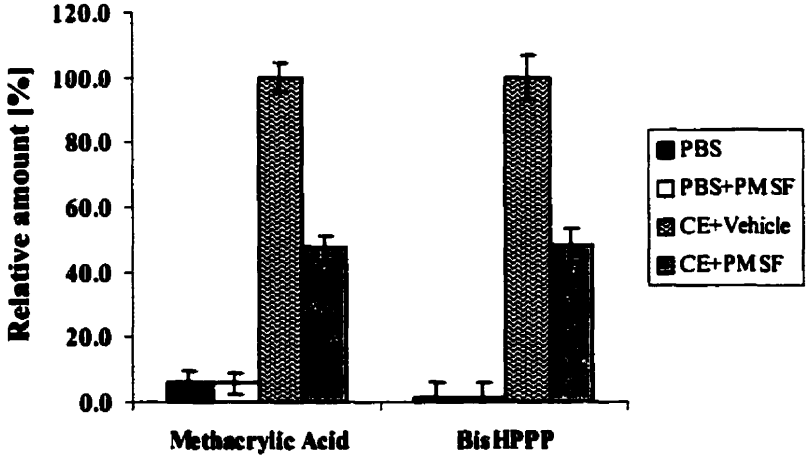


Figure 3.38 Inhibition of CE catalyzed biodegradation by PMSF, in the bis60 system following 16 days incubation (pH 7.0 at 37°C)

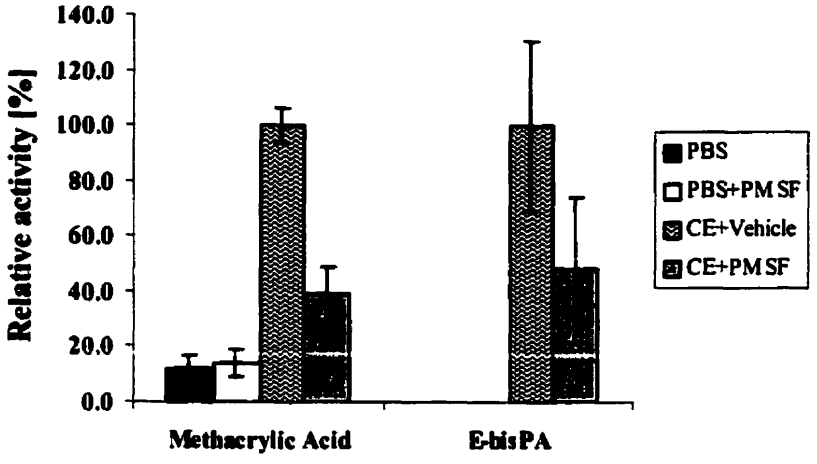


Figure 3.39 Inhibition of CE catalyzed biodegradation by PMSF, in the u-bis60 system following 16 days incubation (pH 7.0 at 37°C)

In the u-bis60 system, the addition of 0.1 mM PMSF to the CE incubated samples showed a reduction in the relative amount of methacrylic acid to 39 ± 9.7 % ($p<0.05$) of the value obtained for the CE+ethanol group (Figure 3.39). Although the amount of released E-bisPA is decreased to 48 ± 26 % respectively , it is not statistically significant.

PMSF inhibition of the PCE biodegradation effect for the composite resin samples following 16 days incubation is presented in Figure 3.40. Again, the results are presented as the relative amount of product isolated following incubation with D-PBS, D-PBS+PMSF, PCE+ethanol and PCE+PMSF with respect to the amount for the PCE+ethanol group.

The addition of 0.5 mM PMSF to the PCE incubated group showed a reduction in the amount of isolated methacrylic acid to 70 ± 3.7 % in the u-bis60 system. In the bis60

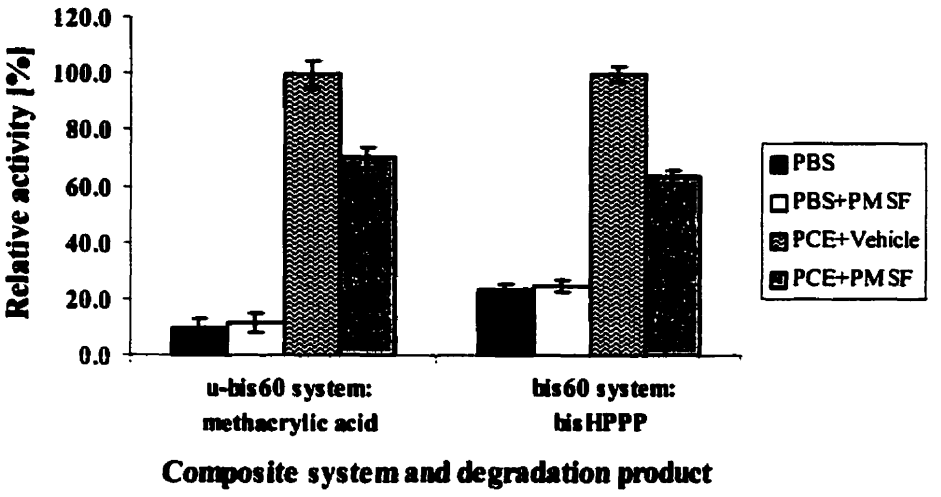


Figure 3.40 Inhibition of PCE catalyzed biodegradation by PMSF, in the u-bis60 and bis60 systems following 16 days incubation (pH 7.0 at 37°C).

system, the same condition showed a reduction in the amount of bisHPPP production to 64 ± 2.3 % relative to the amount isolated for the PCE+ethanol group.

3.4.7 THE EFFECT OF INCUBATION PERIODS AND FILLER CONTENT ON THE BIODEGRADATION OF THE COMPOSITES RESIN SAMPLES

The effect of incubation period and filler content on the biodegradation of composite resin samples containing 10 % w/w (bis10) or 60 % w/w (bis60) content filler and using either bis or u-bis resins was studied as described in section 2.5.2.2. The composite resin samples were incubated for 16 and 32 days in D-PBS, CE (1 unit/mL) or PCE (1 unit/mL). The data are shown in Figures 3.41-3.47. The extent of the biodegradation for

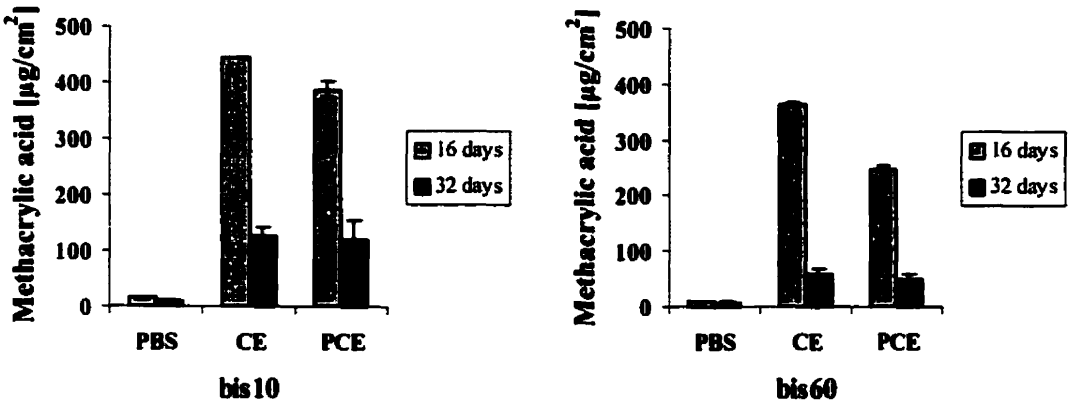


Figure 3.41 The effect of D-PBS, CE (1 unit/mL) and PCE (1 unit/mL) on methacrylic acid production in bis10 and bis60 composite samples incubated for 16 and 32 days (pH 7.0 at 37°C). All data are incremental.

each composite is represented by the amount of degradation product relative to the composite's surface area.

In the bis system, incubating both filler content groups with CE or PCE for 16 and 32 days showed an increase in the amount of released methacrylic acid and bisHPPP ($p<0.05$) relative to the D-PBS incubated samples, but showed a decrease in the amount of TEGDMA ($p<0.001$) (Figures 3.41, 3.43 and 3.44).

In both the bis10 and bis60 samples incubated in CE for 16 days, the amount of released bisHPPP (respectively 395 ± 42 and 437 ± 12 µg/cm^2) was 6-8 fold higher as compared with that released by PCE (respectively 47 ± 0.7 and 70 ± 3 µg/cm^2) ($p<0.01$) (Figure 3.44). In contrast, the amount of methacrylic acid released by CE in the bis10 samples

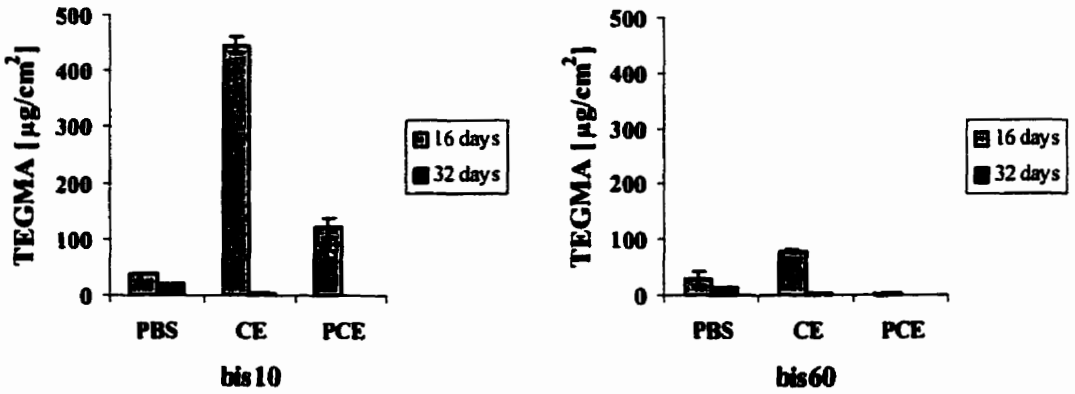


Figure 3.42 **The effect of D-PBS, CE (1 unit/mL) and PCE (1 unit/mL) on TEGMA production in bis10 and bis60 composite samples incubated for 16 and 32 days (pH 7.0 at 37°C). All data are incremental.**

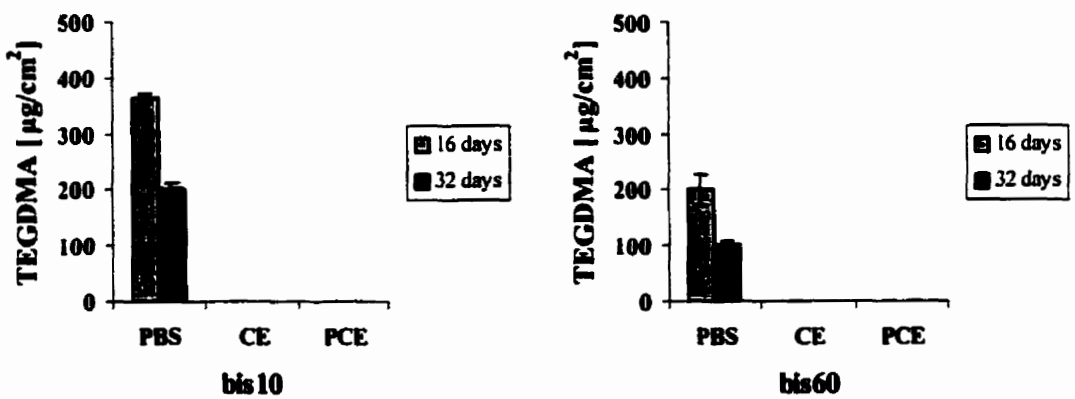


Figure 3.43 **The effect of D-PBS, CE (1 unit/mL) and PCE (1 unit/mL) on TEGDMA production in bis10 and bis60 composite samples incubated for 16 and 32 days (pH 7.0 at 37°C). All data are incremental.**

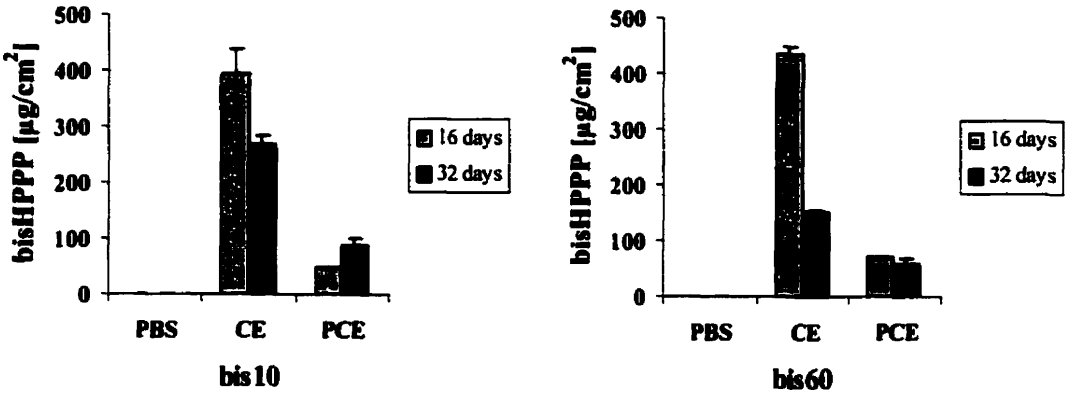


Figure 3.44 The effect of D-PBS, CE (1 unit/mL) and PCE (1 unit/mL) on bisHPPP production in bis10 and bis60 composite samples incubated for 16 and 32 days (pH 7.0 at 37°C). All data are incremental.

showed no difference with that of the PCE incubated samples. For the bis60 samples, the amount of methacrylic acid production was only approximately 50% higher (362 ± 4 µg/cm^2) than with PCE (248 ● 13 µg/cm^2) ($p<0.01$) (Figure 3.41). As well, after 32 days, the amount of bisHPPP in both bis samples was still 3 fold higher in CE incubated samples as compared with that for PCE ($p<0.01$), however there was no difference in the amount of methacrylic acid released by both enzymes.

Following 32 days of incubation for both bis10 and bis60 in CE or PCE, a decrease in the amount of methacrylic acid as compared with that for 16 days incubation ($p<0.001$) was shown (Figure 3.41). Following the incubation of both bis groups for 32 days, only CE treated groups showed a decrease in the amount of released bisHPPP as compared with 16 days incubation groups ($p<0.01$) (Figure 3.44) and the reduction for the bis60 composite was greater than that of the bis10 samples ($p<0.05$).

Following incubation in D-PBS for 16 and 32 days, the bis10 group showed a greater amount of TEGDMA compared with bis60 for both incubation periods ($p<0.01$) (Figure 3.43). Both groups showed a decrease in the amount of TEGDMA ($p<0.01$) following 32 days incubation as compared with the 16 days incubation period (Figure 3.43).

Incubation of bis samples in CE for 16 days shows a higher amount of isolated TEGMA as compared with that for PCE or D-PBS ($p<0.01$), while after 32 days, there is no difference in the amount for both enzymes (Figure 3.42).

In the u-bis system (Figures 3.45, 3.46), all enzyme groups show an increase in the amount of released methacrylic acid when compared with D-PBS ($p<0.01$). As with the bis system, a decrease in the amount of TEGDMA is observed in the presence of the enzymes ($p<0.001$). Following incubation for 16 days with CE, only u-bis60 groups showed a higher amount of methacrylic acid when compared with PCE ($p<0.01$). At 32 days, u-bis biodegradation data showed an increase in the amount of methacrylic acid with CE as compared with PCE, for both u-bis10 and u-bis60 groups ($p<0.01$) (Figure 3.45, 3.46). The u-bis10 group, which was incubated in D-PBS for 16 days, showed a greater amount of TEGDMA release in comparison to u-bis60 ($p<0.01$), while both the u-bis materials showed a decrease in the amount of TEGDMA ($p<0.01$) between 16 and 32 days of incubation (Figures 3.45, 3.46).

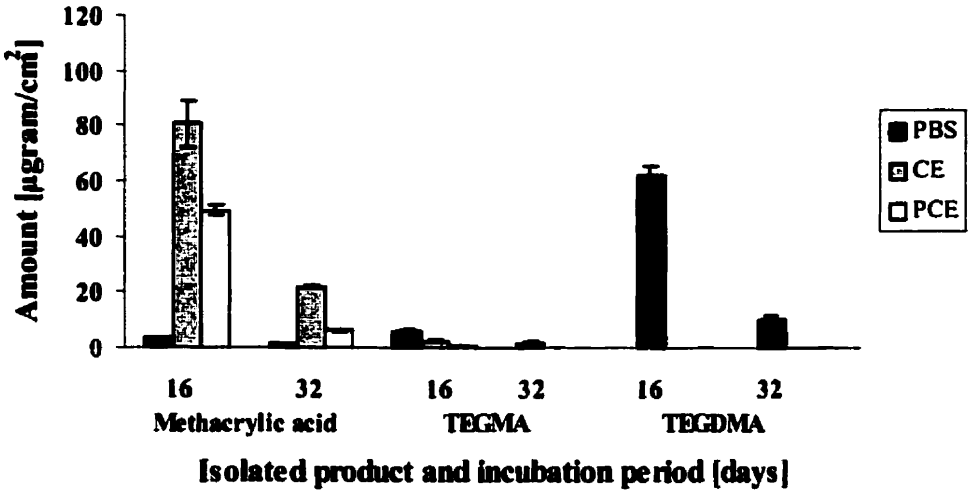


Figure 3.45 **The biodegradation effect of D-PBS, CE (1 unit/mL) and PCE (1 unit/mL) on u-bis60 composite samples incubated for 16 and 32 days (pH 7.0 at 37°C). All data are incremental.**

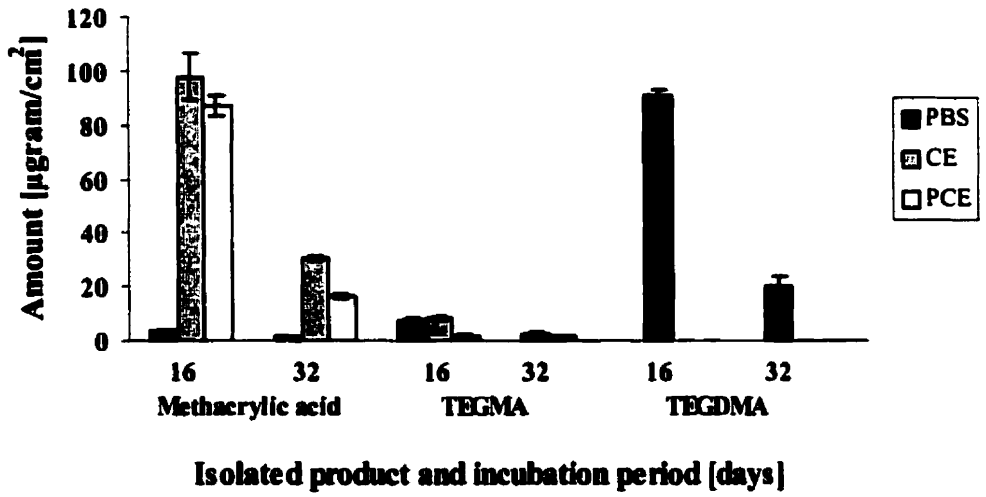


Figure 3.46 **The biodegradation effect of D-PBS, CE (1 unit/mL) and PCE (1 unit/mL) on u-bis10 composite samples incubated for 16 and 32 days (pH 7.0 at 37°C). All data are incremental.**

The incubation of the u-bis systems in CE showed a higher amount of released bisHPPP compared with D-PBS at both incubation periods ($p<0.01$), while PCE only showed an elevated bisHPPP release following 16 days of incubation ($p<0.01$) (Figures 3.47, 3.48).

Following 16 and 32 days of incubation, both u-bis systems show a higher amount of released bisHPPP with CE as compared with that for PCE ($p<0.01$) (Figures 3.47, 3.48). When comparing 32 versus 16 days of incubation with CE or PCE, a decrease in the amount of methacrylic acid (Figures 3.45 and 3.46) and bisHPPP (Figures 3.47, 3.48) ($p<0.01$) is noted at the longer time. When comparing the amount of released bisHPPP, no significant difference was found between both filler content groups.

The presence of ethoxylated bisphenol-A (E-bisPA) was only found in the u-bis systems and was only released in the presence of CE (Figure 3.47 and 3,48). There was no statistical difference between the amount of E-bisPA found for the first 16 day incubation period versus the subsequent 16 day incubation period. In addition, no significant difference in its release was found between the two filler content groups.

Following 16 and 32 days of incubation in D-PBS, it is observed that the amount of released methacrylic acid, TEGMA and TEGDMA is between 3 to 10 fold higher for the bis system (Figure 3.41, 3.42 and 3.43) in comparison with the amount found in the u-bis system (Figures3.45 and 3.46) ($p<0.01$). For instance, following 32 days incubation, the amount of released methacrylic acid and TEGDMA relative to the surface area of the composites were respectively 8.7 ● 0.3 $\mu g/cm^2$ and 202 ± 8 $\mu g/cm^2$ for the bis10

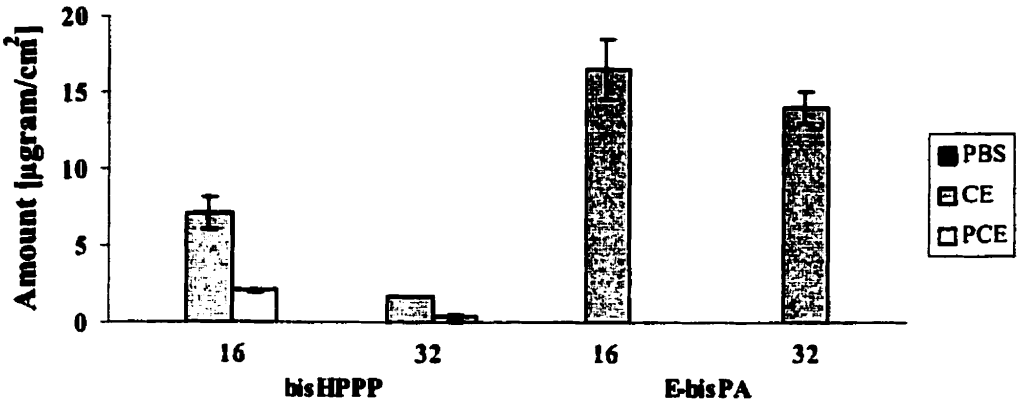


Figure 3.47 Incremental release of bisHPPP and E-bisPA from the u-bis60 system after incubation in D-PBS, CE or PCE for 16 and 32 days (pH 7.0 at 37°C).

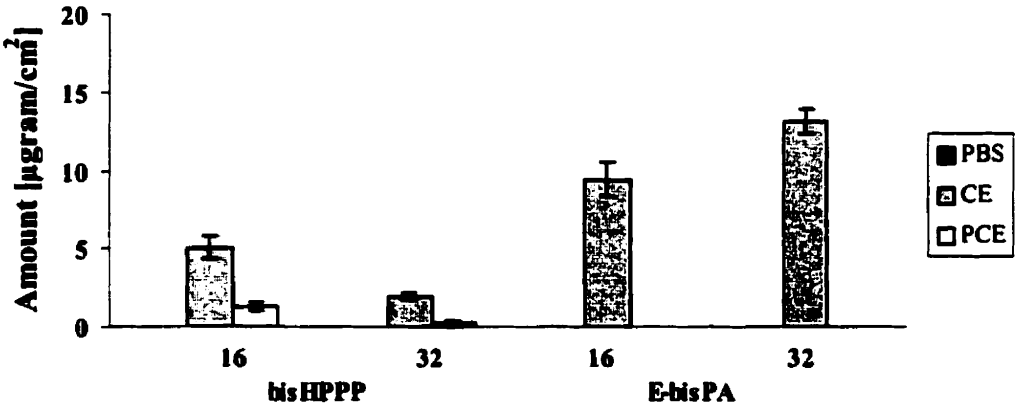


Figure 3.48 Incremental release of bisHPPP and E-bisPA from the u-bis10 system after incubation in D-PBS, CE or PCE for 16 and 32 days (pH 7.0 at 37°C).

composite (Figures 3.41 and 3.43), while in the u-bis10 composite the amounts were 1.1 ± 0.18 μg/cm^2 and 20 ± 3.5 μg/cm^2 (Figure 3.46).

For the enzyme incubated samples, the bis systems showed (Figures 3.41 to 3.44) an increase between 2.6 to 133 fold in the amount of isolated products relative to the u-bis system (Figures 3.45 to 3.48) ($p<0.01$). For instance, following 32 days incubation in CE, the amounts of released methacrylic acid and bisHPPP relative to the surface area of the composites were respectively 127 ● 16 μg/cm^2 and 266 ± 3.7 μg/cm^2 for the bis10 composite (Figures 3.41 and 3.44), while in the u-bis10 composite the amounts were 30 ± 0.61 μg/cm^2 and 1.99 ± 0.16 μg/cm^2 (Figures 3.46 and 3.480. It should be noted that the u-bis system generated a product (E-bisPA, 14 ● 0.78 μg/cm^2 at 16 days for u-bis10) which was not found in the bis system, however the levels were still comparatively lower than the bisHPPP levels of the bis system.

3.4.8 BIODEGRADATION OF ^{14}C-U-BIS COMPOSITE BY CE OR PCE

Radioactivity monitoring was used to assess the quantity of radiolabeled HDI and HDI-bisGMA derivatives released from the ^{14}C-u-bis composite resin samples containing 60 % w/w filler content. The material was incubated with D-PBS, CE (1 unit/mL) or PCE (1unit/mL) as described in section 2.5.2.5. The data are shown in Figure 3.49. The extent of biodegradation is represented by the number of counts per minutes [CPM] relative to the surface area of the composite samples.

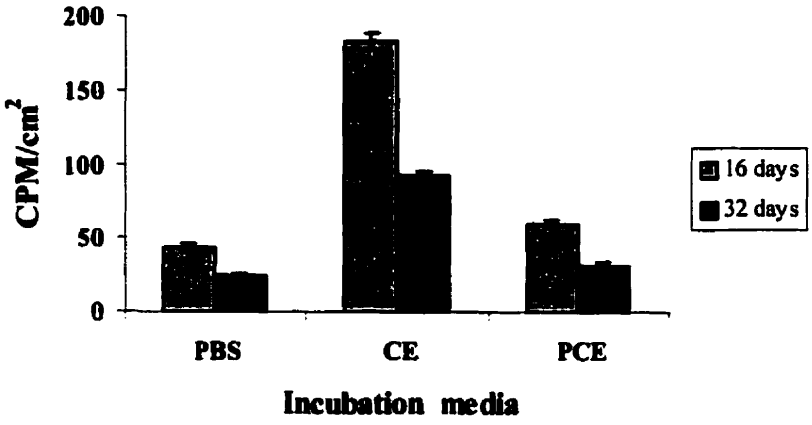


Figure 3.49 The effect of CE or PCE on the biodegradation of radiolabeled ^{14}C-u-bis composite (60 % w/w filler) following incubation for 16 and 32 days (pH 7.0 at 37°C). All data are incremental.

It is observed following the incubation of ^{14}C-u-bis with CE or PCE, for either incubation period, that an increase in the amount of radiolabel release is detected relative to the measurement for the D-PBS solution ($p<0.05$). As well, the amount of radiolabel following incubation in CE for 16 and 32 days is higher than that of PCE ($p<0.001$).

For all these incubation solutions, D-PBS, CE or PCE, the incremental amounts of radiolabel release between 16 and 32 days was less than for the first 16 day incubation period ($p<0.05$).

HPLC analysis of the ^{14}C-u-bis composite samples showed a similar pattern of degradation products when compared with the non-radiolabeled u-bis composite system (results not shown). In attempt to isolate and identify the different radiolabeled

degradation products, HPLC fractions were collected every minute and counted for radiolabel content. Since the radioactive products were soluble, it was assumed that they would be relatively low molecular weight oligomers as well as possibly some 1,6 hexane diamine (HDA). If present, HDA would be a hydrolysis product of the original HDI molecule. Attempts to find a specific radioactive product failed due to the low total radiolabel count that came out of the column and was counted in the HPLC fractions. All the count values were in the order of the background count, i.e. between 20-30 CPM.

3.4.9 MUTUAL INFLUENCE BETWEEN CE AND PCE ON THE BIODEGRADATION OF COMPOSITE RESIN SAMPLES

Since multiple esterase activities are present within human saliva [Chauncey, 1961; Nakamura, 1983a] it would be of value to determine if synergistic effects could be observed between the two enzymes, CE and PCE. Prior to the incubation of samples with the enzymes, stability studies were conducted to assess the mutual influence of the enzymes on each other. These data were shown in section 3.3.1.

Composite resin samples containing 60%w/w content filler and either bis or u-bis resins were incubated in D-PBS, CE, PCE or CE+PCE, as described in section 2.5.2.3. Based on the enzymes' linear range of activities, with respect to the production of methacrylic acid from the composite (section 3.4.5), CE and PCE concentrations of 0.01 unit/mL (bis composite) or 0.1 unit/mL (u-bis composite) were chosen for incubation. The results of the HPLC analysis for the solutions are shown in Figures 3.50-3.53. The extent of the

biodegradation for each composite is represented by the amount of degradation product relative to the composite's surface area and as a function of the incubation media.

For the bis60 material, incubated in CE for 8 days (Figure 3.50A), an increase in the amount of the bisGMA derived product, bisHPPP, is observed relative to the D-PBS group ($p<0.05$). An even greater amount of bisHPPP was found in the CE+PCE incubated group ($p<0.05$) when compared to CE alone. The amount of methacrylic acid, a product derived from unreacted or partially reacted monomers, is only showing a significant increase after incubation in CE+PCE solution ($p<0.05$).

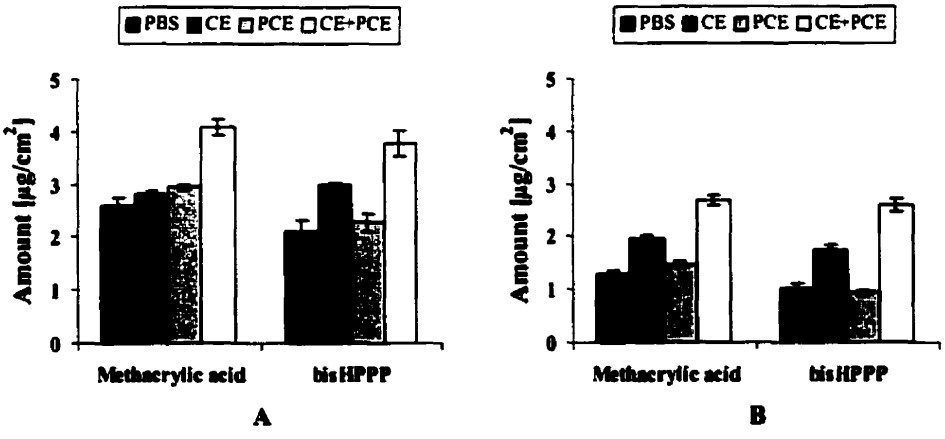


Figure 3.50 HPLC analysis of bis60 composite following (A) 8 days and (B) 16 days of incubation in PBS, CE (0.01 unit/mL), PCE (0.01 unit/mL) or CE+PCE (0.01 unit/mL+0.01 unit/mL) (pH 7.0 at 37°C). All data are incremental.

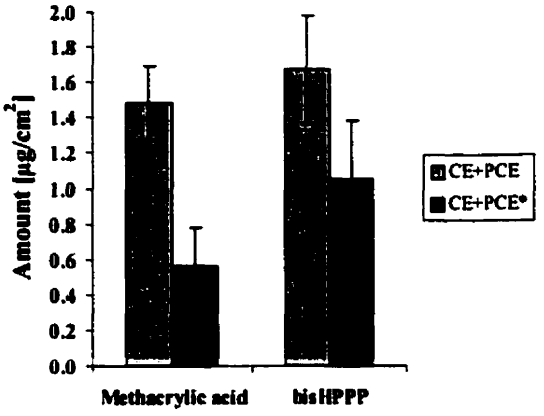


Figure 3.51 Bis60 system-Comparison of the combined CE+PCE (0.01 unit/mL+0.01 unit/mL) effect with the arithmetic sum (CE+PCE*) of the individual effects of CE (0.01 unit/mL) and PCE (0.01 unit/mL) following 8 days incubation (pH 7.0 at 37°C)

A further analysis of the bis60 data comprising a comparison between the combined net effect (PBS release subtracted) for CE+PCE and the arithmetic sum of the individual net effects of CE or PCE (CE+PCE*) is shown in Figure 3.51. Incubation with CE+PCE caused a greater increase in the amount of methacrylic acid produced as compared with that of the CE+PCE* group ($p<0.05$). Although the amount of bisHPPP isolated from the CE+PCE solution is greater than the amount from CE+PCE*, it is not statistically significant ($p>0.05$).

The HPLC analysis data for the solutions collected following a subsequent 8 days of incubation in the different solutions are presented in Figure 3.50B. An increase in the

amount of methacrylic acid and bisHPPP is observed for the CE incubated group ($p<0.05$) when compared with the D-PBS group. Again, an even greater increase in the amount of these products is observed for the CE+PCE group ($p<0.05$).

A further analysis on the net effect of CE+PCE as compared with the arithmetic sum of the individual net effects for CE and PCE (CE+PCE*) is presented in Figure 3.52 for the 16 days experiment. The increase in the amount of bisHPPP during the second 8 day incubation period of the 16 day experiment was highly significant ($p<0.01$). The effect of CE+PCE as compared with that for the CE+PCE* on the amount of methacrylic acid produced, remained greater in the CE+PCE group ($p<0.05$) for the second 8 day incremental period.

Figure 3.53 shows the analysis of the net effect of CE+PCE as compared with the arithmetic sum of the individual net effects for CE and PCE (CE+PCE*) in the u-bis60 composite system incubated for 8 and 16 days. It is observed that following 8 days of incubation, the amount of bisHPPP isolated in the CE+PCE group is higher than that of the CE+PCE* group ($p<0.05$). Following 16 days incubation, the amount of E-bisPA is higher for the CE+PCE group ($p<0.05$). Again, it is noted that this latter product is only isolated in the u-bis system.

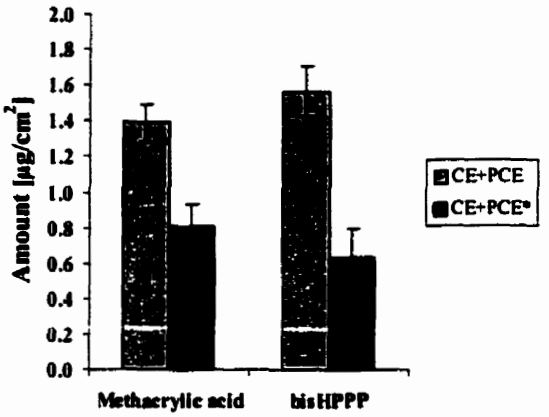


Figure 3.52 **Bis60 system-Comparison of the combined CE+PCE (0.01 unit/mL+0.01 unit/mL) effect with the arithmetic sum (CE+PCE*) of the individual effects of CE (0.01 unit/mL) and PCE (0.01 unit/mL) following 16 days incubation. All data are incremental.**

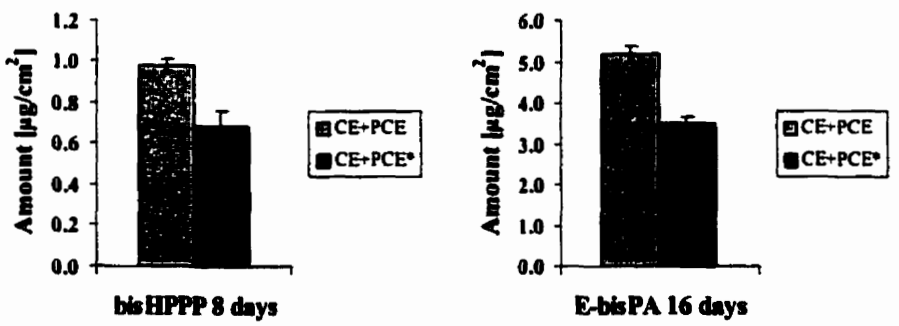


Figure 3.53 **u-bis60 system-Comparison of the combined CE+PCE (0.01 unit/mL+0.01 unit/mL) effect with the arithmetic sum (CE+PCE*) of the individual effects of CE (0.01 unit/mL) and PCE (0.01 unit/mL) following 8 and 16 days incubation (pH 7.0 at 37°C). All data are incremental.**

4.0 DISCUSSION

The growing application of composite resin materials in the dental field as an alternative to amalgam and cast restorations requires a greater understanding of the interactions between the composite materials, gingival tissue and saliva. Of specific interest are the interactions between the materials and salivary or cellular enzymes. This is particularly an important factor due to the higher failure rate of composite resin restorations *in vivo* versus *in vitro* studies, when compared with amalgam [Bayne, 1989; Letzel, 1989].

4.1 DEGREE OF VINYL GROUP CONVERSION

Numerous studies have reported that the presence of residual monomers as well as unreacted pendant methacrylate groups on the monomers is inherent in the nature of free radical polymerization reactions, such as those systems investigated in the current study [Asmussen, 1982; Ruyter, 1985; Peutzfeldt, 1994; Park, 1996]. The diffusion rates of the propagating free radicals, the unreacted methacrylate molecules and the pendant methacrylate species are drastically reduced as the polymerization proceeds [Cook, 1985]. This occurs as a consequence of the lengthening of the polymer chains, which limits mobility and also causes steric hindrance [Munk, 1989]. This is further reflected by an increase in the glass transition temperature of the resin over that of the curing temperature [Horie, 1976; Maffezzoli, 1994]. As a consequence, when the polymerization process is terminated, not all of the monomer's active sites have reacted.

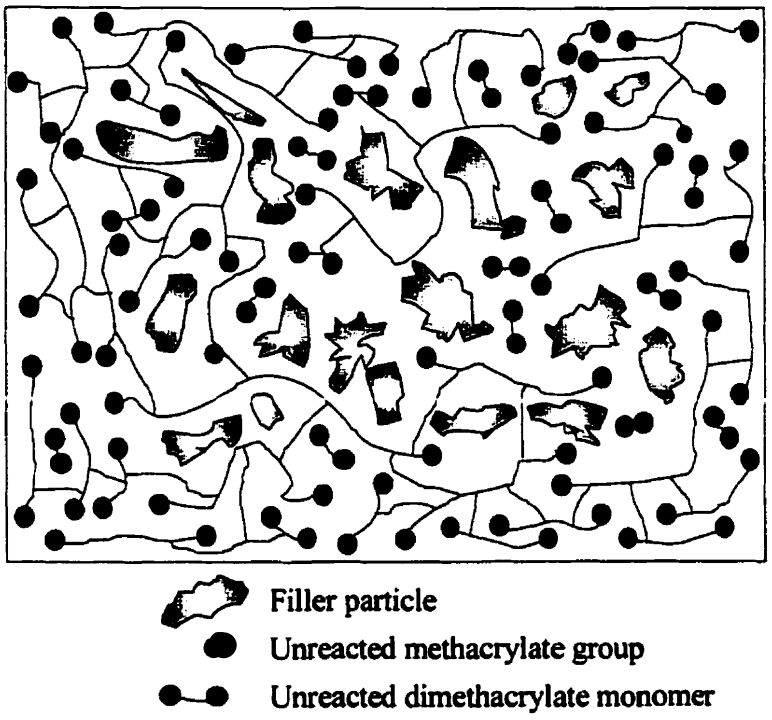


Figure 4.1 Schematic representation of a cured composite resin sample

In the current study, the degree of vinyl group conversion was found to be near 70 % (Figure 3.7) for all model composite resins. Hence, no significant difference was observed between the different resin systems or between different composite resin with varying filler content. This study corroborates other investigations that have reported a lack of correlation between filler content and the degree of vinyl group conversion [Chung, 1990]. The values are in the higher range of conversion ratios, i.e. 40 to 85 %, reported by others using similar infra-red spectroscopy technique [Asmussen, 1982; Ruyter, 1985; Peutzfeldt, 1994; Park, 1996].

Figure 4.1 depicts a schematic representation of the composite resin materials, based on the above results. As indicated in the schematic diagram, it is difficult to assess whether

the unreacted vinyl groups are associated with the unreacted pure monomers that are trapped in the set composite resin matrix or with the pendant monomers, reacted with the resin matrix via one methacrylate group. Several studies have quantified the amount of unreacted monomer trapped in dental composites. Inoue and Hayashi [1982] found that the amount of the unreacted bisGMA was in the range of 0.4 to 1.2 % of the initial weight of the composite, or 2 to 6 % of the resin only. Similar results have been found by Ferracane [1990]. Tanaka et al [1991] reported residual values of TEGDMA and bisGMA, relative to their original amounts, to be between 12.8 to 23.5 % and 1.3 to 4.1 % respectively, depending on the curing period. It should be noted that the above analyses were done based on the amount of residual monomer eluted into organic solvents immediately following the curing process and for relatively short periods (up to 7 days). When the composite samples were not immersed immediately following the curing process, the residual monomers ranged between 0.25 to 0.85 % of the composite mass or 1.6 to 5.5 % of the resin [Pearson, 1989]. Because the polymerization reaction continues for hours and days after light-curing, it is possible that the lower levels of unreacted monomers found in the latter study were due to further consumption of these molecules during the post-curing process [Ferracane, 1994]. Other studies have estimated that approximately 90 % of the unreacted vinyl groups are found on pendant molecules bound to the resin matrix via one methacrylate group [Ferracane, 1994]. Hence, based on the FT-IR data for the materials in this study, it could be hypothesized that most of the unreacted methacrylate groups (~30 % of the initial vinyl groups) are associated with pendant monomers and could be available for cleavage and production of methacrylic acid following the hydrolysis of ester bonds in the monomers.

4.2 FORMATION OF BIODEGRADATION PRODUCTS

Previous studies have reported that unhindered ester bonds are very susceptible to hydrolytic degradation [Coury, 1996]. Based on this information, it was highly probable that the enzyme catalyzed hydrolysis of resin components, by CE and PCE, would occur at the ester bonds of the methacrylate based resin monomers. The work in the thesis further hypothesized that the hydrolysis of these sites would be enzyme dose dependent and occurs via the same active sites as natural substrates for the enzymes. The nature of degradation products isolated from the model composite resin materials was assessed using HPLC, UV spectroscopy and MS spectrometry and the data are discussed below.

HPLC analysis showed that all the materials investigated in this study generated similar products with the exception of E-bisPA, which is a bisEMA derived product that was not incorporated into the bis system (Figure 3.20). It should be noted that the u-bis materials consistently showed a lower amount of released product (degradation products and residual monomers), by an order of magnitude, in comparison with the bis materials (compare Figures 3.36, 3.37, 3.45, 3.46, 3.47 and 3.48 to 3.36, 3.37, 3.41, 3.42, 3.43 and 3.44). Although both composite resin material systems contained identical silanated inorganic filler, initiator and reducing agent, no products associated with the latter three agents were detected in the incubation solution.

4.2.1 DEGRADATION PATHWAYS OF TEGDMA

Figure 3.24B depicts the UV spectrum of a product derived from TEGDMA, which was isolated for the incubation solution of all composite resin materials. This product had a mass to charge ratio of 219 (Figure 3.25) and was derived from TEGDMA monomer hydrolyzed at one of its ester bonds. The product is referred to as triethylene glycol methacrylate or TEGMA and was previously shown to be isolated from microfilled composite materials [Shajii, 1999]. While TEGMA can be derived from the unreacted monomer TEGDMA, hydrolyzed at one of the ester bonds (Figure 4.2A), it can also be formed following cleavage of pendant TEGDMA molecules, which have only reacted with the resin matrix via one vinyl group (Figure 4.2B) [Freund, 1990; Larsen, 1992].

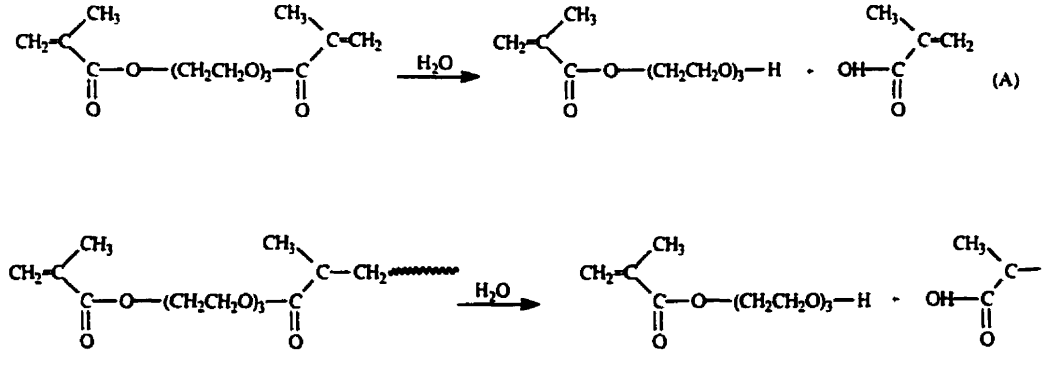


Figure 4.2 Degradation pathways of TEGDMA (A) hydrolysis of unreacted TEGDMA monomer and (B) hydrolysis of pendant TEGDMA from the matrix

The TEGMA molecule can be further hydrolyzed to an alcohol, triethylene glycol, and methacrylic acid (Figure 4.3) [Munksgaard, 1990; Larsen, 1992].

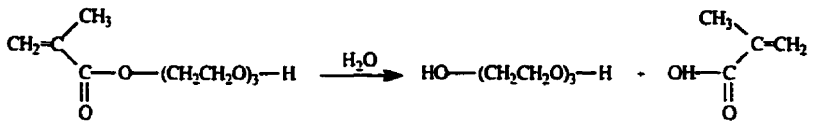


Figure 4.3 Degradation of triethylene glycol methacrylate (TEGMA) [Munksgaard, 1990; Larsen, 1992]

In the current HPLC study, triethylene glycol was not detected using the PDA detector, due to its lack of absorbance at the working wavelength range (i.e. 190-500 nm) for the instrument. However, using the mass spectrometer as an additional detector on the HPLC system, triethylene glycol was detected (see appendix 8.6), but not quantified due to constraints in the experimental apparatus. In this thesis, the hydrolysis of TEGMA was indicated by its gradual elimination with increasing concentrations of enzyme. This occurred in parallel with an increase in the amount of methacrylic acid when the composite resin samples were incubated in the presence of high concentrations of CE or PCE (Figures 3.34-3.37).

Increasing the amount of CE or PCE produced a decrease in the amount of isolated TEGDMA and an increase in the amount of isolated TEGMA with peak levels achieved at 0.1 unit/mL for CE and 0.05 unit/ml for PCE (Figures 3.34-3.37). In theory, each molecule of hydrolyzed TEGDMA can produce one molecule of TEGMA. Figure 4.4 depicts a comparison of the theoretical calculation for TEGMA, i.e. the potential amount available from the consumed TEGDMA, with the actual isolated TEGMA. The consumed TEGDMA was calculated relative to changes from the level of TEGDMA released in D-PBS incubated samples. For bis60 incubated with 0.05 and 0.1 unit/mL CE

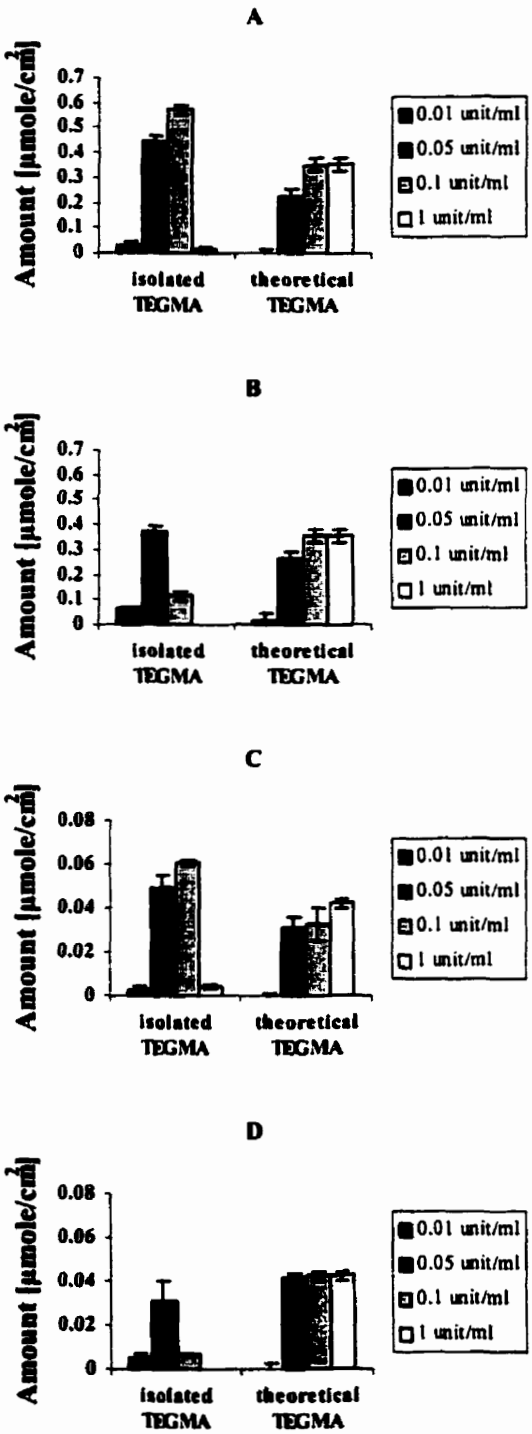


Figure 4.4 **Comparison of the isolated TEGMA with its theoretical production, calculated from the amount of consumed TEGDMA for bis60 incubated with different concentrations of (A) CE or (B) PCE, and u-bis60 incubated with different concentrations of (C) CE or (D) PCE (16 days incubation, 37°C at pH 7.0)**

or 0.05 unit/mL PCE (respectively Figure 4.4A and B) and u-bis60 incubated with 0.05 and 0.1 unit/mL CE and 0.01 unit/mL PCE (respectively Figure 4.4C and D), the actual isolated TEGMA is higher than that predicted by the cleavage of TEGDMA. This indicates that a certain number of the isolated TEGMA molecules must have originated from pendant TEGMA molecules within the resin matrix. This is an important observation because it supports the claim that the enzyme is actually degrading components of the matrix resin and not solely leached monomer.

It should be noted that while TEGMA itself is produced by both CE and PCE, it is concurrently hydrolyzed by these enzymes, as indicated by its gradual elimination when the composites are incubated with high levels (1 unit/mL) of CE or PCE. Hence, the actual isolated TEGMA only represents the balance between its production and elimination. This indicates that the level of TEGMA production would actually be higher than the isolated TEGMA, thus indicating an even greater degradation of the pendant TEGMA from the resin matrix. This makes sense, in view of the fact that an estimated 90 % of unreacted methacrylate groups are reported to be associated with partially reacted monomers [Ferracane, 1994].

The levels of TEGDMA monomer isolated in D-PBS for the different composite resin groups are different from one model material to the other, and vary for different incubation periods (Figures 3.43, 3.45 and 3.46). Figures 4.5A and B provide a better

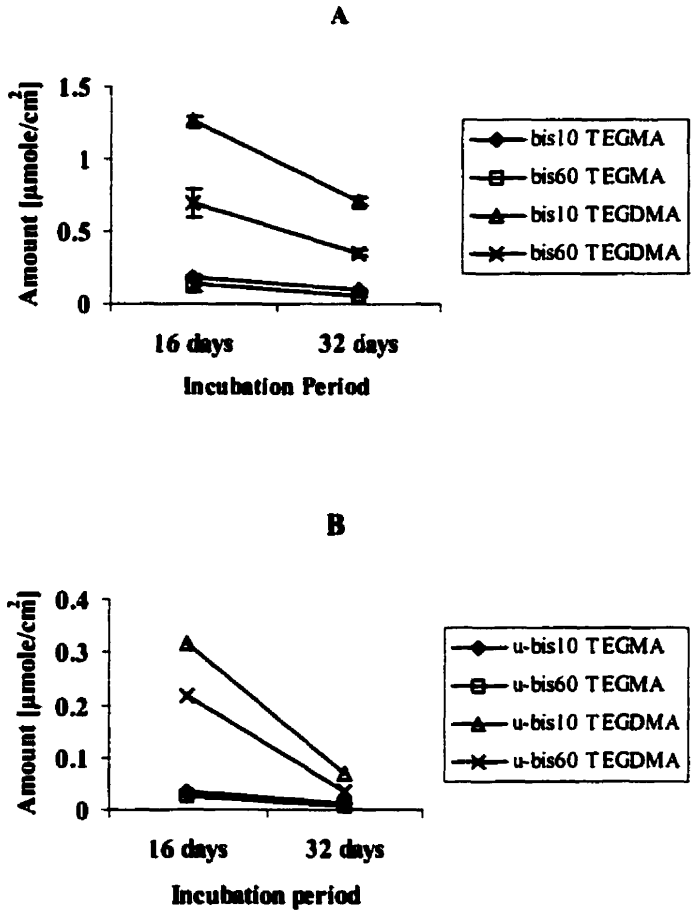


Figure 4.5 **Incremental amount of TEGMA and TEGDMA isolated for bis and u-bis composite samples incubated in D-PBS for 16 and 32 days (37°C at pH=7.0)**

illustration of the amounts of TEGDMA and TEGMA isolated for the different systems following incubation with D-PBS. The amount of TEGDMA isolated for the 60 % filler content groups is significantly lower ($p<0.05$) than that found for the 10 % filler content groups in both systems at both time points. For both resin systems at both time points, the amount of TEGDMA was correlated ($r \leq -0.953$) with the amount of filler ($p<0.05$). It is seen that there is a very strong association ($r \geq 0.988$) between the release of TEGMA

and the amount of available TEGDMA for both systems at both incubation periods ($p < 0.01$). This suggests that the TEGMA degradation product in the D-PBS incubated groups was derived in large part from the release of unreacted TEGDMA monomer.

In summary, while the literature estimates that approximately 90 % of unreacted methacrylate groups are associated with pendant molecules bound to the resin matrix [Ferracane, 1994], the above data suggest that the additional contribution of the unreacted TEGDMA monomer cannot be overlooked.

4.2.2 DEGRADATION PATHWAYS OF bisGMA

Unlike TEGMA, only negligible amounts of the bisGMA-derived product, bisHPPP, were isolated from the buffer solution with the bis system and no bisHPPP was detected in the buffer treated u-bis system. These data compare well with buffer control samples analyzed in previous studies [Santerre, 1999; Shajii, 1999]. For all composite materials used in this study, increasing the amount of CE or PCE, showed an increase in the amount of isolated bisHPPP as compared with the amount found for the D-PBS incubated samples (Figures 3.34-3.37, 3.44, 3.47, 3.48). The low levels of bisHPPP isolated in the D-PBS incubated composite samples suggests that the aqueous solution alone has a limited capability to hydrolyze the monomeric segment and pendant bisGMA molecules from the resin matrix. This is supported by other studies [Munksgaard, 1990; Bean, 1994] that found a lower hydrolysis rate for bisGMA than for TEGDMA in the presence or absence of esterase.

BisHPPP can be derived from the unreacted bisGMA monomer, hydrolyzed at both its ester bonds (Figure 4.6); by hydrolysis of pendant bisGMA molecules, which have only reacted with the resin matrix via one vinyl group (Figure 4.7); or from the hydrolysis of the ester bonds within the bisGMA repeat segments of the matrix (Figure 4.8).

As indicated in the review of the literature, Tanaka et al [1991] reported values for residual unreacted trapped bisGMA remaining in the composite resin following photocuring. These values were between 1.3 and 4.1% of the original amount of bisGMA, depending on the curing period. Other studies [Ferracane, 1994]

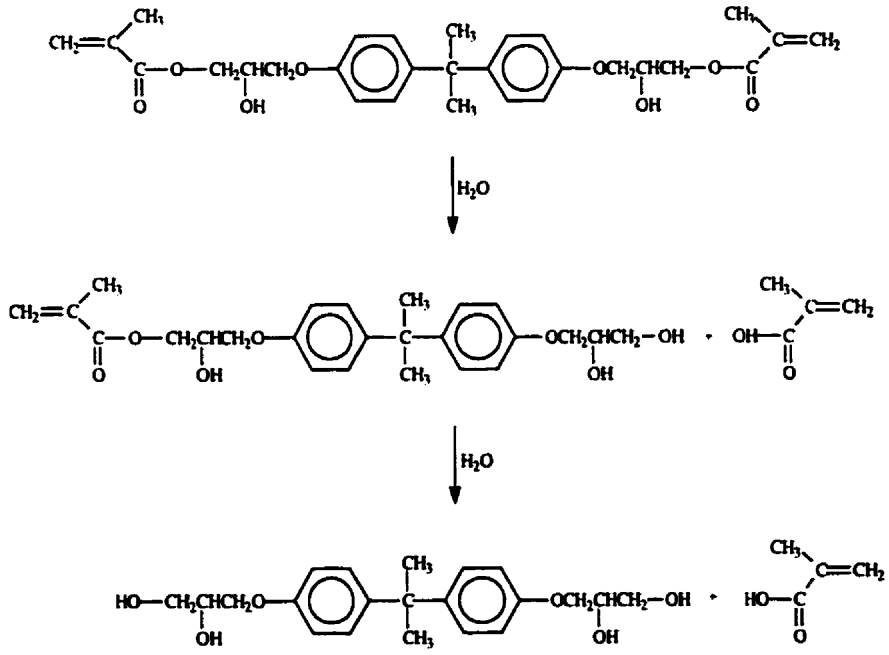


Figure 4.6 Degradation pathways of bisGMA: hydrolysis of unreacted bisGMA monomer

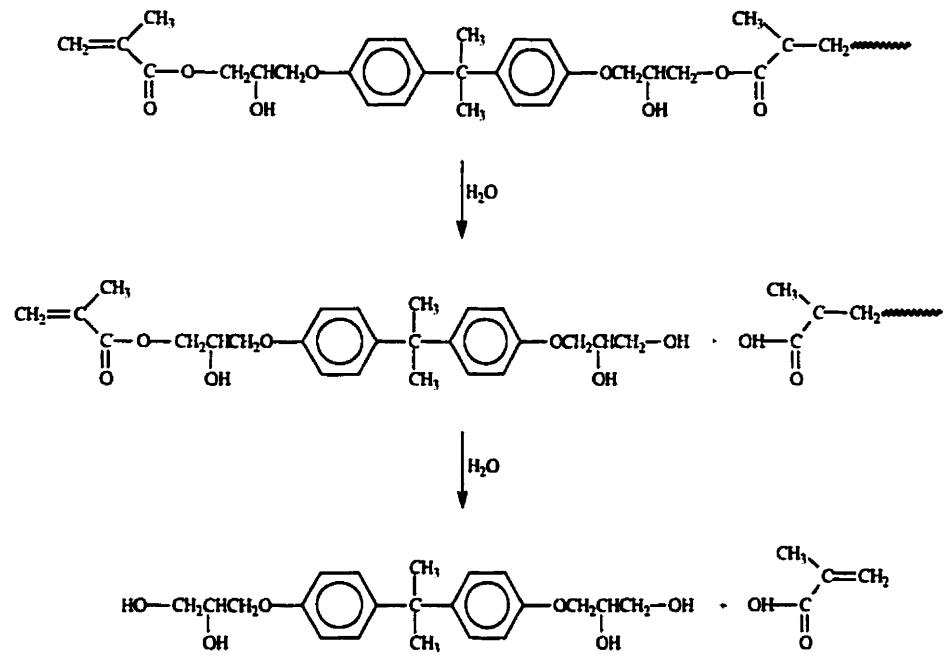


Figure 4.7 **Degradation pathways of bisGMA: hydrolysis of pendant bisGMA from the matrix**

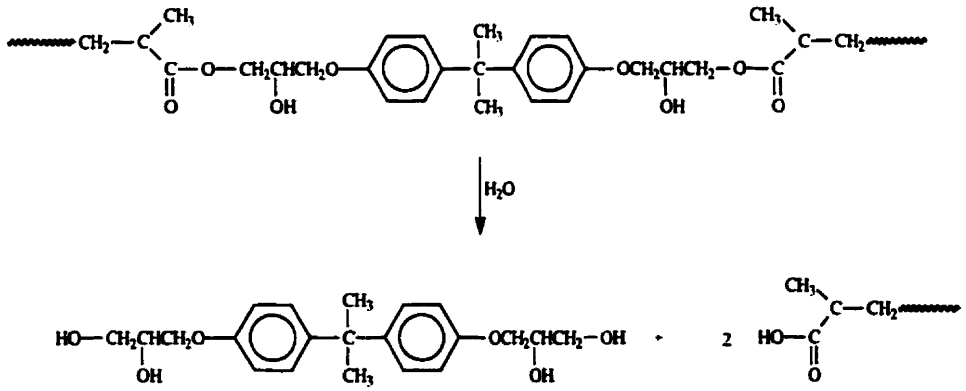


Figure 4.8 **Degradation pathways of bisGMA: hydrolysis of monomeric bisGMA segment within the polymer chain**

have estimated that approximately 10 % of the unreacted vinyl groups are associated with trapped monomers within the resin matrix. Using the latter values, it can be assumed that approximately 3 % (10 % of the 30 % conversion ratio) of the bisGMA was trapped as a monomer within the resin matrix. This amount could lead to the production of 673 and 432 μg bisHPPP respectively for bis10 and bis60 materials. When comparing these values to the isolated amount of bisHPPP in the incubation solutions of bis10 and bis60 (Figure 3.44), it was shown that following 32 days of incubation with CE, the total accumulations of bisHPPP are 661 and 586 μg respectively for bis10 and bis60. When considering the amount isolated for the bis60 material following 16 days (i.e. 432 μg), there is more released bisHPPP than what could be accounted for by the expected bisHPPP production from bisGMA monomer. Hence, again the data support the thought that cured resin matrix components are undergoing degradation.

Furthermore, the high molecular weight and the rigid nature of the phenol rings, as well as the high hydrogen bonding capacity of the bisGMA molecule, limit the diffusion of this monomer within the resin matrix [Ferracane, 1994]. Therefore, it can be assumed that most of the unreacted trapped bisGMA monomer was not available for hydrolysis by CE and hence most of the bisHPPP product probably originated from the hydrolysis of the ester bonds within the bisGMA repeat segments (Figure 4.8) or from the pendant bisGMA molecules (Figure 4.7) within the resin matrix. This is also supported by the SEM micrographs (Figures 3.16 and 3.17), which show a significant degradation of the resin matrix following 32 days of incubation with CE.

With PCE, the amount of isolated bisHPPP following 32 days treatment were lower (133 and 128 μg for respectively bis10 and bis60) than the expected total values of bisHPPP (Figure 3.44). In addition, the accumulation rate of bisHPPP showed no significant difference between the two incubation periods, suggesting that the production of bisHPPP by PCE may be primarily related to unreacted trapped bisGMA monomer that diffused through the resin matrix. However, the SEM analysis shows that the matrix of the bis samples incubated in PCE for 32 days was degraded to a considerable level. Accordingly, it can be hypothesized that PCE produced bisHPPP from both unreacted bisGMA monomer diffusing through the resin matrix and pendant bisGMA monomer, reacted with the resin polymer via one methacrylate group. Since PCE showed a lower ability to hydrolyze bisGMA monomer as compared with CE (Figure 3.33), some of these sources would not appear to have been depleted as rapidly and were equally available throughout the 32 day incubation period.

In the current study, the mono-vinyl derivative of bisGMA (Figures 4.6 and 4.7), produced from bisGMA, following hydrolysis of only one ester bond, was not detected in the HPLC system using the UV detector. However, using the mass spectrometer as an additional detector for the HPLC system, this product was isolated and identified in the incubation solutions of bis composite samples following incubation in D-PBS (see appendix 8.7). Since the latter system was not equipped for quantification, the amount of this product was unknown. The low amounts of this product in the incubation solutions can be explained by the molecule's low solubility in aqueous solutions [Kababoura, 1996; Li, 1996] and its low diffusion through the composite polymer matrix [Ferracane,

1994]. The presence of this product in the D-PBS could be attributed to the mild hydrolysis conditions of the buffer and to impurities in the bisGMA monomer used in the synthesis of the bis samples, as identified by HPLC (Appendix 8.8). This product was only detected in the absence of enzymes.

4.2.3 DEGRADATION PATHWAYS OF bisEMA

Figure 3.28C depicts a UV spectrum for a product derived from bisEMA, which was only isolated for the u-bis system. The identification of this product, having a mass to charge ratio of 317 (Figure 3.31), was provided by the fragmented ions defined in Figure 3.32 and Table 3.7. This product has never been previously isolated in composite resin biodegradation studies. E-bisPA can be derived from the fully unreacted monomer bisEMA, hydrolyzed at both its ester bonds (Figure 4.9); following hydrolysis of pendant bisEMA molecules within the polymeric resin matrix (Figure 4.10) or from the hydrolysis of both ester bonds within the bisEMA repeat segments of the matrix (Figure 4.11).

E-bisPA shows some similarity to bisHPPP, in that it was not found in the D-PBS incubation solutions (Figures 3.36, 3.37, 3.47 and 3.48). However, while bisHPPP was detected in the PCE and CE incubated u-bis samples, E-bisPA was only found in the CE incubated u-bis groups. The absence of this product in both D-PBS and PCE indicates that the latter have a very poor ability to hydrolyze the bisEMA repeat segments and monomers from the polymeric resin matrix.

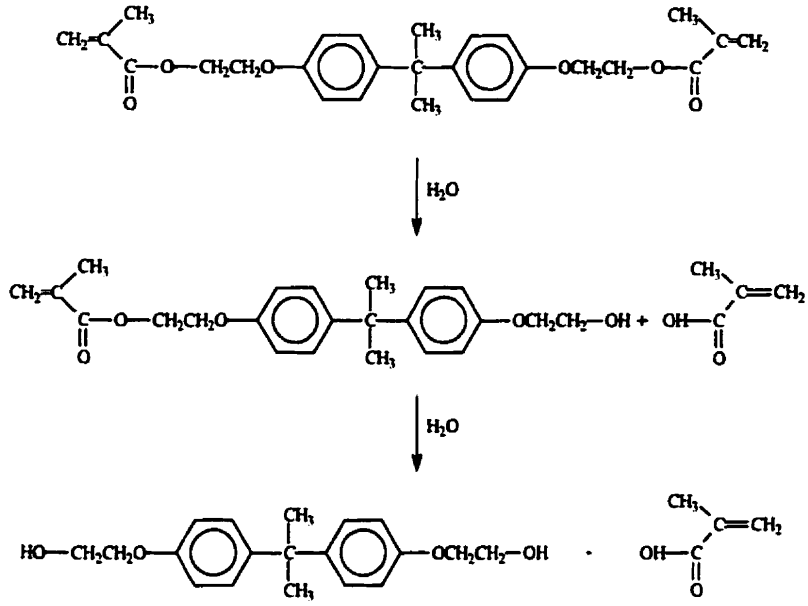


Figure 4.9 **Degradation pathways of bisEMA: hydrolysis of unreacted bisEMA monomer**

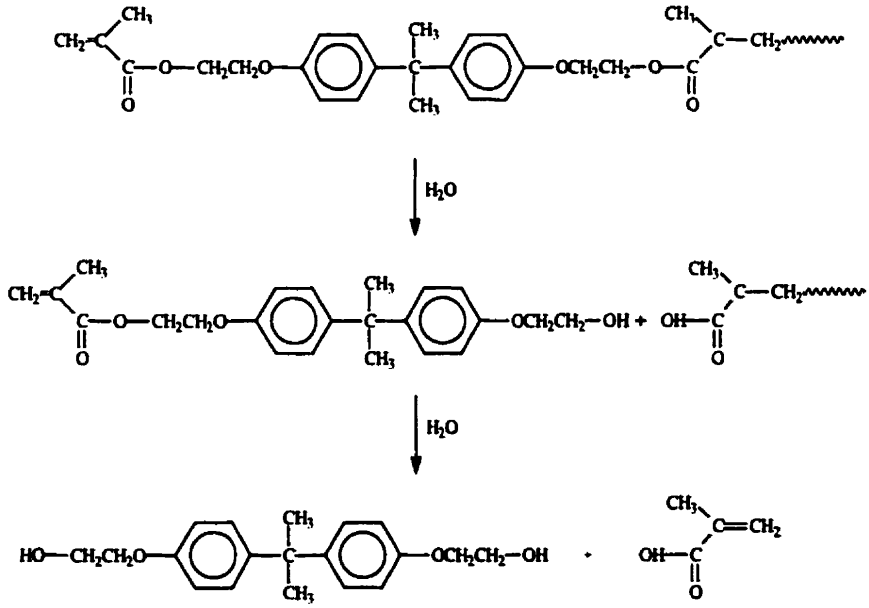


Figure 4.10 **Degradation pathways of bisEMA: hydrolysis of pendant bisEMA from the matrix**

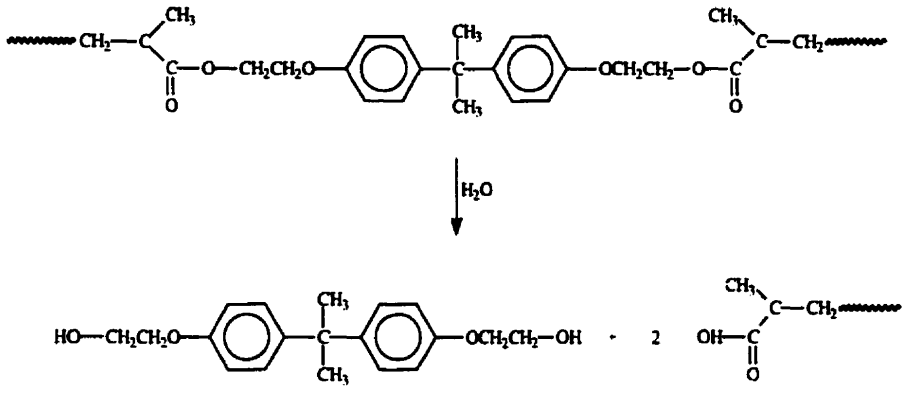


Figure 4.11 Degradation pathways of bisEMA: hydrolysis of monomeric bisEMA segment within the polymer chain

In order to determine the possible sources from which the E-bisPA degradation product was derived, a similar calculation to that of the bisHPPP was carried out. Assuming that about 3 % of the original amount of bisEMA was trapped in the resin polymer, the expected values of E-bisPA originating from trapped bisEMA would be 471 and 286 μg respectively for u-bis10 and u-bis60 materials. However, the actual accumulations of E-bisPA for 32 days of incubation were much lower, having values of 22.64 and 30.63 μg respectively for u-bis10 and u-bis60 (Figures 3.47 and 3.48). Although CE showed a significant ability to hydrolyze bisEMA monomers (Figure 3.33), the low overall amount of the latter composite degradation product, E-bisPA, as well as its constant accumulation rates for both incubation periods, suggests that the reaction between CE and the bisEMA pendant molecules, as well as its repeat segments from the resin matrix may be quite low in comparison to degradation of unreacted monomer, that may have a greater ability to diffuse to the surface. This is further supported by the SEM micrographs, which show

that CE had only a minor effect on the degradation of the u-bis system (Figures 3.18 and 3.19).

It should be noted that although CE showed a similar ability to hydrolyze both bisEMA and bisGMA monomers (Figure 3.33), and the amount of bisGMA and bisEMA molecules incorporated into the u-bis system are roughly the same (see sections 2.1.1, 2.1.3 and 2.1.4), the amounts of the bisEMA product, E-bisPA, were higher than the amounts of the bisGMA degradation product, bisHPPP (Figures 3.47 and 3.48). It is thought that the urethane coupling of the bisGMA molecules has contributed to this difference by increasing the cross-linkage within the monomer system. The effect of the urethane coupling will be further discussed in section 4.5.

In a similar manner to the bisGMA system, the mono-vinyl derivative of bisEMA (Figures 4.9 and 4.10), was not detected in the incubation solutions. Since the HPLC method was capable of detecting this product, as evident in the ability to detect bisEMA monomer in standard solutions, it can be assumed that this product was not present in the incubation solutions of the u-bis system in any measurable amount. Due to the similarity of this product to the mono-vinyl derivative of bisGMA, its absence in the incubation solutions of the u-bis system can be explained in part by its low solubility in aqueous solutions [Kababoura, 1996; Li, 1996] and its low diffusion through the polymer matrix [Feracane, 1994]. While the mono-vinyl derivative of bisGMA was present in the D-PBS incubation of the bis system, the u-bis system which shows a lower overall product

formation, yielded no mono-vinyl derivatives for either bisGMA or bisEMA. This again reflects the relatively lower degree of resin monomer degradation.

4.2.4 METHACRYLIC ACID PRODUCTION

Methacrylic acid, with a mass to charge ratio of 85 (Figure 3.23), is a common end product of all methacrylate based monomers hydrolyzed at the ester bond. As mentioned in section 4.1, a significant number of unreacted vinyl groups (~30 %) (Figures 3.7 and 4.1) are available to be cleaved and form methacrylic acid. For both of the resin systems under study with varying filler content, increasing the amount of the enzymes showed an increase in the amount of methacrylic acid (Figures 3.34, 3.35, 3.36 and 3.37). These data are comparable to the results reported in other studies [Koda, 1990; Munksgaard, 1990].

While methacrylic acid can be derived from the fully unreacted pure monomers such as TEGDMA (Figure 4.2 A), bisGMA (Figure 4.6) or bisEMA (Figure 4.9) and their intermediate degradation products such as TEGMA (Figure 4.3) or the mono-vinyl derivatives of bisGMA and bisEMA (respectively Figures 4.6 and 4.9), it can also be derived from the cleavage of their pendant monomers which have only reacted with the resin matrix via one vinyl group [Freund, 1990; Santerre, 1999] (Figures 4.2, 4.7 and 4.10).

To further investigate the sources of methacrylic acid, a theoretical calculation of methacrylic acid production (based on the assumption that all bisHPPP, E-bisPA (in the

u-bis system only) and TEGMA products were derived from leached unreacted bisGMA, bisEMA (u-bis system only) and TEGDMA) was compared with the actual amount of isolated methacrylic acid in the incubation solutions. Following the hydrolysis of the ester bonds, dimethacrylate molecules, such as TEGDMA, bisGMA or bisEMA are capable of producing two molecules of methacrylic acid, while pendant molecules are capable of producing only one molecule and repeat segments of the polymer resin matrix will not produce any methacrylic acid (Figures 4.6-4.11).

Figures 4.12 and 4.13 depict a comparison between the theoretical calculation and the actual isolated amount of methacrylic acid for bis and u-bis composite resin systems with different filler content following 16 or 32 days of incubation in CE or PCE (37°C at pH=7.0). The fact that there is a relatively consistent imbalance of theoretical and measured methacrylic acid indicates that this product cannot be derived solely from leached components but must include degradation of the repeat segments in the cured matrix. For the bis system following 16 days incubation in either CE or PCE, it is noted that the actual amount of isolated methacrylic acid is higher than the theoretical values ($p<0.01$). The higher amounts of isolated methacrylic acid as compared with the theoretical values suggest that in addition to degrading leached monomers, for the first 16 days of incubation, both enzymes are cleaving a significant amount of methacrylate molecules from pendant moieties which have only reacted with the resin polymeric matrix via one methacrylate group [Munksgaard, 1990]. Consequently, during this phase, cleavage of the repeat segments (i.e. triethylene glycol or bisHPPP) from the

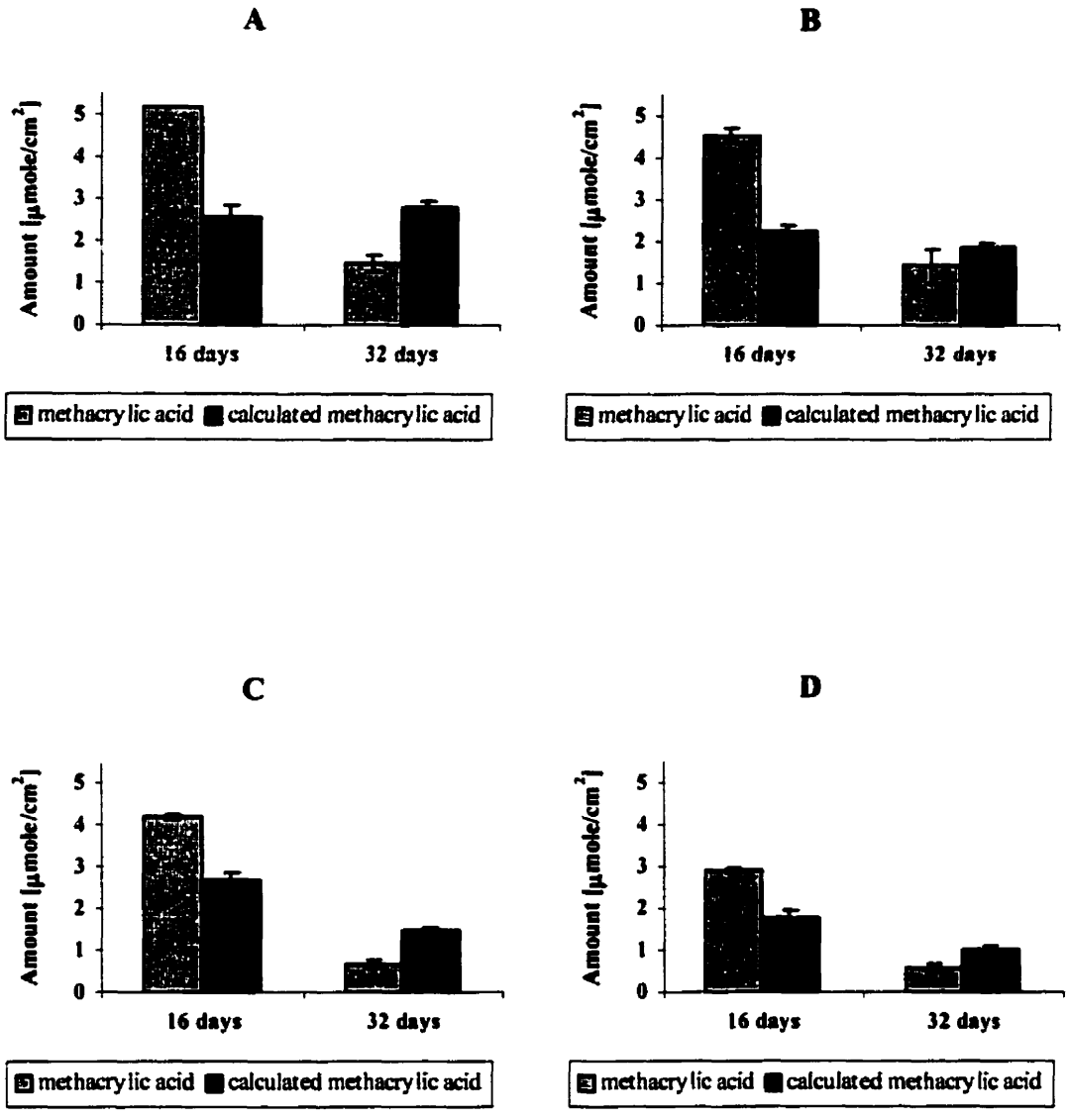


Figure 4.12 **Comparison of isolated methacrylic acid with the theoretical production by CE or PCE (1 unit/mL) following incubation for 16 and 32 days (37°C at pH=7.0); A) bis10 incubated with CE, B) bis10 incubated with PCE, C) bis60 incubated with CE and D) bis60 incubated with PCE**

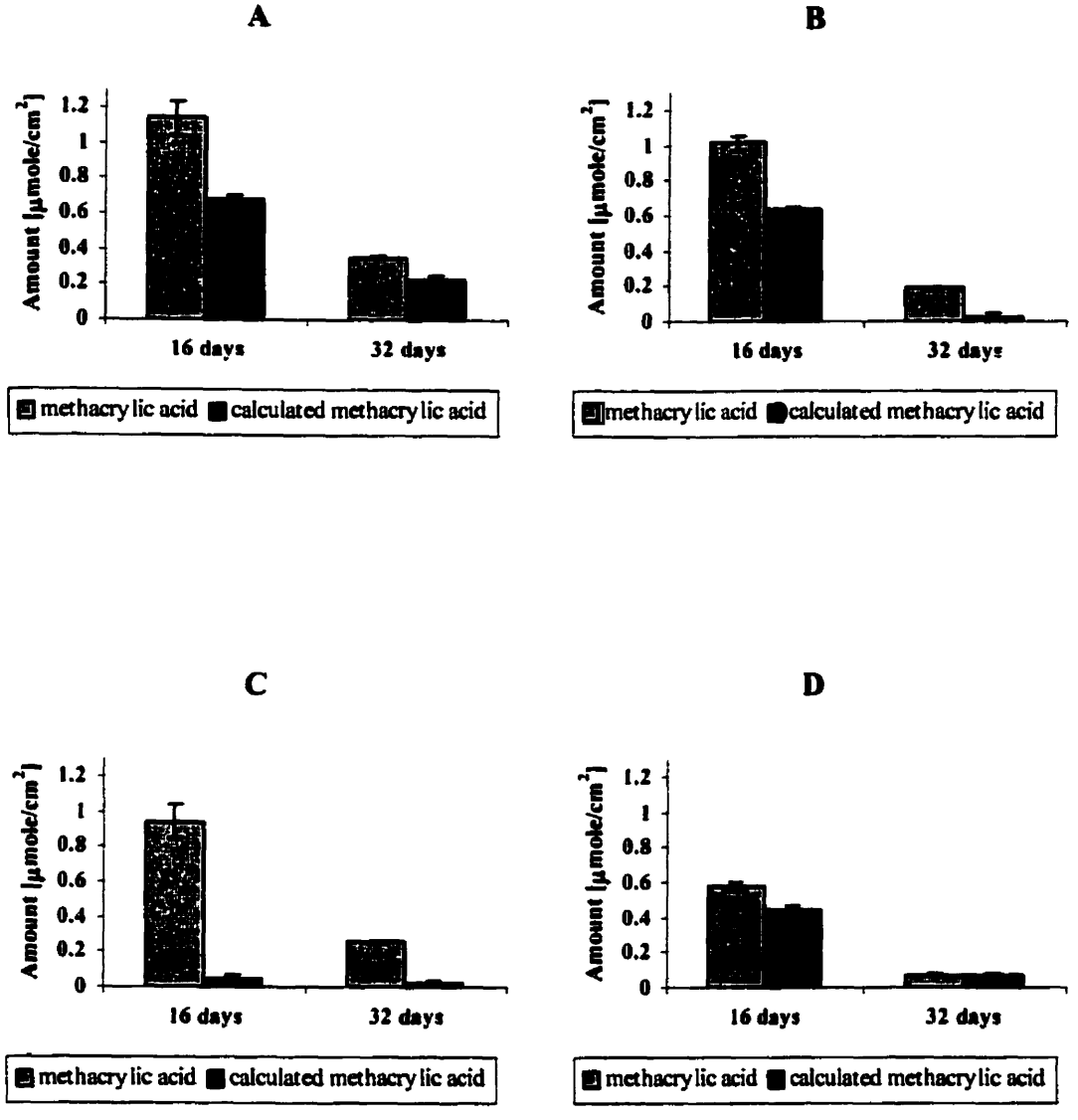


Figure 4.13 **Comparison of isolated methacrylic acid with the theoretical production by CE or PCE (1 unit/mL) following incubation for 16 and 32 days (37°C at pH=7.0); A) u-bis10 incubated with CE, B) u-bis10 incubated with PCE, C) u-bis60 incubated with CE and D) u-bis60 incubated with PCE**

matrix is relatively lower. However, following incubation for 32 days the results are quite different. The theoretical values of methacrylic acid exceed or equal those of the isolated methacrylic acid, suggesting that in the second 16 days of incubation, more of the monomeric segments within the polymeric resin matrix are being cleaved. Hence, the contribution of methacrylic acid from the second hydrolysis reaction of the moieties is minimal. The fact that the matrix is actually degraded by the enzymes is further supported by the SEM analyses, which show that the resin matrix of the bis system is degraded and filler particles are exposed following incubation in aqueous solutions for 32 days (Figures 3.16 and 3.17).

In the u-bis system, the first 16 days of incubation show a similar trend to that observed with the bis system, suggesting that the enzyme is degrading methacrylate groups from the pendant molecules within the resin matrix (Figure 4.13). However, unlike the results for the bis system, the trend of higher levels for isolated methacrylic acid as compared with the theoretical values is still observed following the additional 16 days of incubation. This indicates that for the u-bis system, the enzymes are mainly degrading methacrylate groups from the resin matrix as opposed to releasing partially reacted monomers. This also further suggests that the repeat units and the pendant molecules within the polymeric matrix of the u-bis system are less susceptible to the enzyme catalyzed hydrolysis by CE and PCE. The SEM analyses support this by showing that for the u-bis system, the effect of CE and PCE on the degradation is minimal (Figures 3.18 and 3.19).

4.2.5 LEACHING OF UNREACTED MONOMERS

As mentioned in section 4.1, the presence of residual monomer as well as unreacted methacrylate groups is inherent in the nature of the polymerization [Asmussen, 1982; Ruyter, 1985; Peutzfeldt, 1994; Park, 1996]. Although most of the unreacted methacrylate groups are reported to be associated with pendant molecules that have reacted with the resin matrix via one methacrylate group, a significant amount of unreacted pure monomers become trapped in the resin matrix (Figure 4.1). In the remainder of this section, the term unreacted monomers refers to those monomers that did not polymerize at either of the methacrylate ends.

For all composite resin materials incubated in D-PBS, a decrease in the isolated amount of isolated TEGDMA was observed as the composite samples were incubated for longer periods (Figures 3.43, 3.45, 3.46 and 4.5). Figure 4.14 provides an illustration of the variation in rates of TEGDMA accumulation, in the D-PBS incubated bis60 composite, relative to the surface area for different time periods. The rate of TEGDMA accumulation for the bis60 composite incubated in D-PBS starts at 86.5 $\mu g/(cm^2 \times day)$ for the first 2 days (48 hours of pre-incubation), while the rates for the subsequent 16 days and the last 16 days are respectively 12.5 ± 1.7 and 6.3 ± 0.3 $\mu g/(cm^2 \times day)$ (Figure 4.14). The significant release of this monomer into the incubation solution is associated with its relatively high solubility in aqueous solution and its relative ease of mobility to diffuse through the resin matrix and into the D-PBS solution [Ferracane, 1990; Tanaka, 1991;

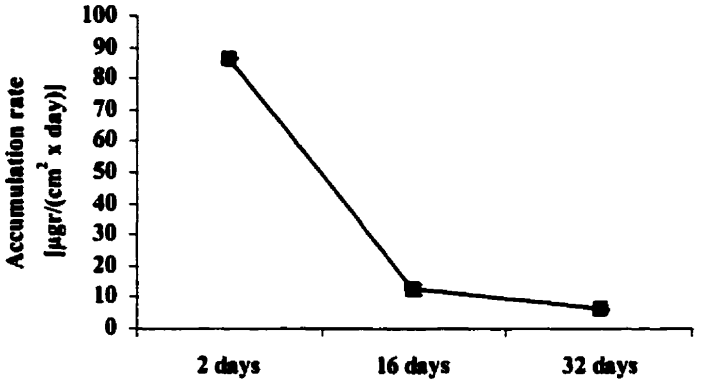


Figure 4.14 Accumulation rates of TEGDMA following 2, 16 and 32 days incubation of bis60 composite samples in D-PBS (37°C at pH=7.0). All data are incremental.

Ferracane, 1994]. This process of diffusion will proceed as long as the TEGDMA concentration gradient between the matrix and the incubation solution is present [Treybal, 1987]. As TEGDMA at the surface is rapidly removed from the resin (as shown initially in Figure 4.14 by the elevated rate of release at day 2), the amount of this monomer available for immediate release from the surface is diminished. Its replenishment at the surface is highly dependent on diffusion rates within the cured matrix, thus the gradient between the matrix and buffer becomes smaller and consequently, the accumulation rate will decrease as well.

The decrease in the accumulation rate may also be attributed to post-curing following the initial photocuring [Ferracane, 1994]. This process, which continues for hours and days, consumes some of the unreacted monomers trapped in the resin matrix, thus reducing the amount of available monomer and reducing diffusion since by virtue of the polymer's

crosslinked network being extended, the accumulation rate is ultimately affected. Hence, the mechanism of transport appears to be highly dependent on diffusion within the matrix and not on a saturated boundary layer at the liquid/solid interface.

The polymer network is composed of cross-linked molecules within which the unreacted monomers reside (Figure 4.1). As the buffer penetrates the matrix and swells the polymer matrix, monomers diffuse out. Since the complete saturation of the composite with the buffer requires weeks and months [Pearson, 1979], it is not clear whether the accumulation rate of TEGDMA in the D-PBS solution will continue to decrease following incubation for more than 32 days or would it begin to increase with an enhancement of diffusion rate, resulting from the degradation or release of repeat segments from the matrix. Following 32 days of incubation, the level of isolated TEGDMA in the bis system was between 100 to 200 $\mu g/ cm^2$, depending on the filler content, which is well above the detection threshold of the HPLC/UV detector (ngs). Hence, it could still be expected that the process could be continuously monitored beyond 32 days. Therefore, further experiments should be conducted in order to answer the above question regarding the kinetics of TEGDMA release over extended period of time.

Studies based on weight loss techniques have estimated that approximately 50 % of the leachable species are eluted within three hours in water, [Ferracane, 1990]. In the same studies, elution of nearly all-leachable components was completed within 24 hours. However, the HPLC analysis in the current thesis shows that the release of TEGDMA persists throughout the 32 days. Here, some consideration must be given to this apparent

conflict. It should be emphasized that weight loss measurements do not specify the nature of leaching components that contribute to the weight loss. These can include, in addition to the resin components, other ingredients of the composite resin, such as filler and residual water [Söderholm, 1996 and 1998]. In addition, water sorption into the composite samples may interfere with the measurements [Söderholm, 1998]. In comparison, the HPLC analysis is a more sensitive method, which can be used to detect specific products that are directly associated with resin [Santerre, 1999]. Hence, the data reported in this thesis are believed to be a better representation of the levels of monomer release than what would be anticipated based on weight loss alone.

For the bis60 composite, the sum of accumulated TEGDMA during the 2 days of pre-incubation and the 32 days of the experiment was about 3.9 % of the total amount of this monomer in the sample and makes up about 12.45 % of the sample's unreacted vinyl groups. These data are within the range of values recorded in other studies. Ferracane [1994] reported values between 5 to 10 % of the unreacted monomer, when the composite was immersed in the incubation solution immediately following photo-curing. Another study [Pearson, 1989] has reported values of released TEGDMA between 1.6 to 5.5 % of the resin, when the composite was subject to a post-curing effect process before immersion in the incubation solution. This is comparable to the cumulative isolated TEGDMA values following the 32 day of incubation period (i.e. not including the pre-incubation release values). The values for the latter were 2.5 % for bis60 and bis10. It should also be noted that the values for the current study, following 32 days of incubation, do not represent the total amount of free TEGDMA, since significant levels

of TEGDMA are still found in the final sample at the 32 day incubation period. There is every indication that the release of this monomer is likely to continue for a longer period.

Since the degree of vinyl group conversion for all composite resin groups was found to be similar (Figure 3.7), it can be assumed that the composites with lower filler content would release a higher amount of TEGDMA. The latter composites contain more resin and as a consequence, more unreacted monomer within their polymeric matrix. Therefore, it is not surprising that the values of released TEGDMA in the incubation solutions for the different composite resins, following incubation in D-PBS are correlated ($r \leq -0.953$) (see section 4.2.1) with the amount of filler in the composite resin matrix (Figures 3.43, 3.45, 3.46 and 4.5). For example, the amount of TEGDMA isolated with the lower filler (higher resin) content composite groups in both systems are significantly higher than that found for the higher filler (lower resin) groups ($p<0.05$) (Figures 3.43, 3.45, 3.46 and 4.5). In addition, the u-bis system, which contains a smaller amount of TEGDMA in the resin, shows a proportional reduction in the release of the latter monomer into the incubation solution as compared with the bis system (Figures 3.43, 3.45, 3.46 and 4.5).

It must also be considered that the accumulation of TEGDMA in the incubation solutions is equal to its release from the composite resin matrix minus its degradation to TEGMA, triethylene glycol and methacrylic acid. Therefore, the level of released TEGDMA in the D-PBS incubation solutions at any time point, as presented in Figures 4.6 and 4.14, do not solely represent the total amount released from the resin matrix, since fractions of this

monomer are readily hydrolyzed in the solution. Hence, the amounts in the incubation solution are lower than the levels of TEGDMA monomer actually released.

Although the HPLC method used in this study was capable of detecting all of the monomers used in the study, no residual bisGMA, bisEMA or urethane-modified bisGMA monomers were isolated in either the D-PBS or enzyme incubated samples (Figure 3.20). This was similar to previous findings for commercial composites [Santerre, 1999] and model composites [Shajii, 1999]. The absence of these monomers in the D-PBS incubation solutions is probably related to their limited solubility in water [Kababoura, 1996; Li, 1996], their high molecular weight, and the rigid nature of the phenol rings as well as the high hydrogen bonding capacity of bisGMA molecule which both limit the diffusion of the monomers within the composite resin matrix [Ferracane, 1994]. The low solubility in the aqueous solution and the low diffusion rate of bisGMA and bisEMA are overcome in their relevant degradation products, respectively bisHPPP and E-bisPA, by the presence of additional hydroxyl groups at both ends of the molecules as well as their lower molecular weight. This latter feature enhances their polar character and thus solubility in water.

4.3 MECHANISM OF THE ENZYMATIC ACTION

In this study, the mechanism of the enzymatic attack on the composite's resin polymeric matrix was investigated using two esterases, CE (EC 3.1.1.13) and PCE (EC 3.1.1.8). Both enzymes act on substrates via an acyl-enzyme intermediate, which is typical of serine esterases [Lombardo, 1981; Quin, 1987 and 1992; Sutton, 1990; Taylor, 1990]. Both enzymes catalyze the hydrolysis of nitrophenylesters (*p*-NPA, *o*-NPA, *p*-NPB and *o*-NPB), which are water-soluble substrates (Figures 2.4 and 3.12) [Sutton, 1986]. PCE is also capable of catalyzing the hydrolysis of butyrylthiocholine and butyrylthiocholine iodide (BTC) (Figures 2.5 and 3.12) [Soreq, 1992]. Figure 4.15 outlines the catalytic mechanism for CE and PCE. In the scheme, E-OH represents the active serine site whose γ-OH group attacks the carbonyl carbon of the scissile bond of the substrate via a nucleophilic reaction. The mechanism involves tetrahedral intermediates in both acylation and deacylation stages [Sutton, 1986; Quin, 1987; Quin, 1992]. Finally the acylated enzyme complex is hydrolyzed, freeing the enzyme and liberating acetic, butyric or other acids, depending on the nature of the substrate. Phenylmethylsulfonyl fluoride (PMSF) is a serine esterase inhibitor, which alkylates the hydroxyl of the active serine site in the esterases [Labow, 1994].

In order to investigate if CE and PCE catalyze the hydrolysis of the composite resin matrix via the same active site (i.e. serine) as other substrates for the enzymes (such as nitrophenylesters and BTC respectively for CE and PCE), the effect of PMSF on the CE and PCE biodegradation activity was assessed. These studies were compared with the

activity of the enzymes with and without PMSF, as measured using *p*-NPA and BTC substrates for CE and PCE respectively.

The inhibition, by PMSF, on the generation of biodegradation products from the composite's polymeric matrix (Figures 3.38, 3.39 and 3.40) was comparable to the inhibition of the hydrolysis with other substrates (*p*-NPA and BTC for respectively CE and PCE) (Figure 3.15), providing evidence that hydrolysis of the resin matrix occurred at the same active site as the more usual substrates of the enzymes.

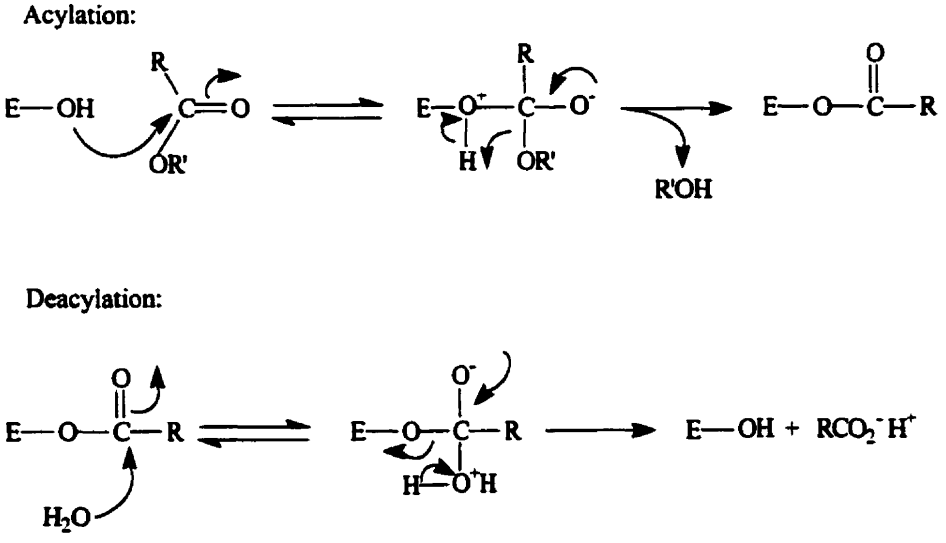


Figure 4.15 Catalytic mechanism for CE and PCE [adapted from Sutton, 1986; Soreq, 1992].

Hence, the first hypothesis of this study- "that the enzyme catalyzed hydrolysis of the resin components by CE and PCE occurs at the ester bonds of the methacrylate based resin monomers, is dose dependent and occurs via the same active enzyme site expressed onto other substrates of the enzymes"- was shown to be true. As a result, the release of hydrolysis degradation products, such as methacrylic acid, TEGMA, triethylene glycol, E-bisPA and bisHPPP, as well as unreacted monomers (TEGDMA), becomes of biological relevance. The literature has reported on the presence of enzymatic activity in human saliva [Chauncey, 1961; Nakamura, 1983a; Zambon, 1985; Ryhänen, 1983a and 1983b; Yamalik, 1990], which has been associated with the degradation of composite resin materials [Freund, 1990; Munksgaard, 1990; Larsen, 1991 and 1992]. The current thesis has demonstrated that enzyme levels, between 0.09 to 0.26 unit/mL and 0.004 to 0.018 unit/mL for CE and PCE-like activities respectively in human saliva (Figures 3.13 and 3.14), are within the range capable of degrading the composite's polymers (Figures 3.34-3.37). Since the degradation process showed a dependence with the enzymes' activity levels, the difference in the salivary hydrolase activity for different subjects, will affect the release of degradation products from composite restorations placed in different recipients.

The above findings are particularly important since recent studies have demonstrated that bisphenol-A derivatives, isolated in the saliva of fissure sealer recipients [Olea, 1996], have shown xenoestrogenic effects *in vitro* [Olea, 1996; Schafer, 1999] while comonomers such as EGDMA and TEGDMA may promote the proliferation of important

cariogenic microorganism, *Lactobacillus acidofilus* and *Streptococcus sobrinus* [Hansel, 1998]. This latter observation corroborates the hypothesis that bacteria proliferating within the gap between the composite resin restoration and the cavity walls might be a principal causative factor for alteration of the pulp as well as the cause of recurrent caries [Brännstrom, 1981; Cox, 1992]. Other studies have shown that bisEMA, bisGMA and TEGDMA caused a depression in DNA synthesis with concentrations of approximately 1 μmol/L, 10 μmol/L and 100 μmol/L for the respective monomers [Hanks, 1991]. The latter concentrations are lower than the concentrations established in the current studies, however, the concentrations to be found *in vivo* have yet to be determined. The possibility of elevated local concentrations as well as diluted salivary concentration have to be considered.

4.4 INFLUENCE OF FILLER CONTENT ON THE BIODEGRADATION OF COMPOSITES

As mentioned before in section 4.2.1, a significant correlation ($r \leq -0.953$) was found between the amount of released unreacted monomer (TEGDMA) and the amount of the filler (Figures 3.43 and 4.5). While this correlation was also observed for some of the biodegradation products in the enzyme catalyzed hydrolysis (Figure 3.41 and 4.16), it was not observed for others (Figures 3.44 and 4.17). It is seen in Figure 4.16 that the methacrylic acid content is correlated ($r = 0.9380$, $p < 0.001$) at different times with the differences in resin content. On the other hand, bisHPPP showed no correlation ($r = 0.3361$, $p = 0.312$) with the resin content of the composite (Figure 4.17).

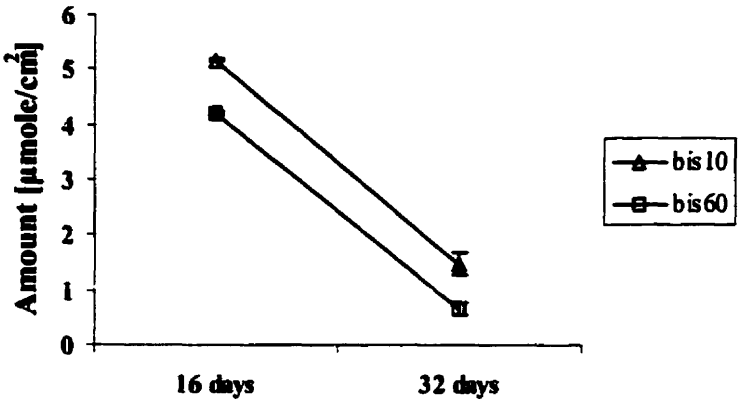


Figure 4.16 **Comparison of methacrylic acid isolated in the incubation solutions of bis10 and bis60 following 16 and 32 days incubation with CE (37°C at pH=7.0). All data are incremental.**

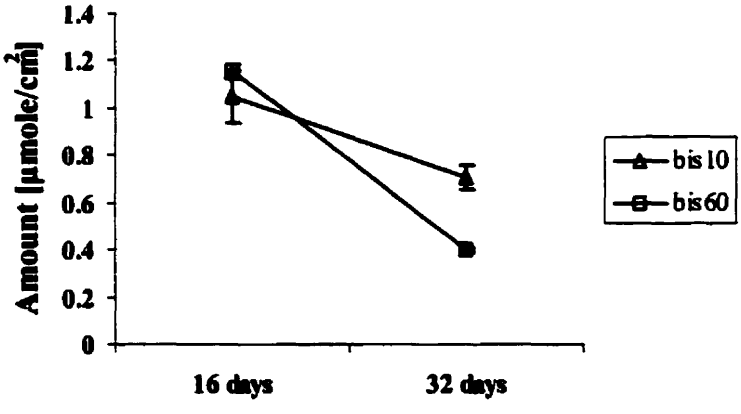


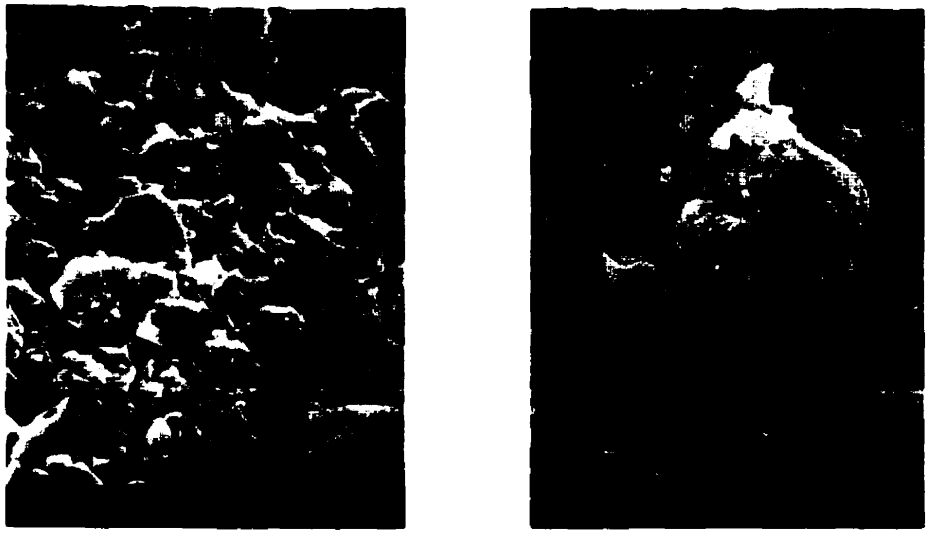
Figure 4.17 **Comparison of bisHPPP isolated in the incubation solutions of bis10 and bis60 following 16 and 32 days incubation with CE (37°C at pH=7.0). All data are incremental.**

Since no bisGMA and only negligible amounts of its mono-vinyl derivative were isolated in the incubation solutions, the bisHPPP isolated in the incubation solutions is suspected to have primarily originated from the degradation of unreacted bisGMA monomer located within the matrix, pendant bisGMA molecules or repeat segments of the polymerized bisGMA. The release of bisHPPP from any of the latter would require polymer chain degradation. Thus, the amount of isolated bisHPPP in the incubation solution is a good indicator of resin matrix degradation. On the other hand, methacrylic acid is a common end product for all methacrylate-based monomers that did not react on either side of the monomer. Its release is therefore also dependent on the amounts of TEGDMA and TEGMA available in the incubation solution, which was shown to be correlated with the monomer content of the composite (Figure 4.5). Since the presence of leached TEGDMA monomer is relatively high even in the absence of clear matrix degradation, the rate of methacrylic acid production is not as good an indicator of resin matrix degradation as is bisHPPP.

XPS analysis (Table 3.1) of the surface on cured bis composites, prior to the incubation in the aqueous solution, suggests that the surface is composed mainly of resin and no correlation between the filler content and the surface concentration of the corresponding silicon element was found. The dominant elements found by XPS were carbon and oxygen, corresponding to the methacrylate-based resin system. SEM studies (Figures 3.5 A and B) confirm the XPS analysis and indicate that the immediate surface of all samples consist mainly resin. Hence, one could expect a similar level of degradation for both high and low filler materials. In fact, the amount of isolated bisHPPP following the first 16

days of incubation in CE, for the high filler content group is equal to the amount isolated for the lower filler content group (Figure 4.17). However, this is not the case at 32 days. This observation requires some further discussion and elaborates on the relationship of bisHPPP release with matrix degradation.

The enzyme associated biodegradation mechanism of the resin matrix can be divided into three major steps: 1. Transport of the enzyme through the incubation solution to the surface of the resin. 2. Chemical reaction of the enzyme with the resin and 3. Release of degradation products into the incubation solution. The CE that was used in this study has been reported to have molecular weight values between the range of 45,000 [Labow, 1983] to about 173,000 Kd [Sonnenborn, 1982]. Due to its large size, which prevents its absorption into solid substrates, the enzyme is thought to exert predominantly an attack at the surface of the materials. The SEM studies (Figures 3.16 and 3.17) show that filler particles are completely exposed on the surface, for both filler content groups following 32 days of incubation in CE solutions. However, incubation in CE for 16 days shows less particle exposure (compare Figure 4.18 A with B). This suggests that for a significant part of the initial 16 days, the enzymatic degradation occurred primarily within the top surface layer of the composite resin samples, which was composed mainly of resin. In this case, for the initial 16 days of incubation, the two composites with different filler content should expose a similar surface area of resin and hence, both materials incubated in CE should exhibit similar amounts of bisHPPP, since this latter product is representative of matrix degradation.



A B

Figure 4.18 Scanning electron micrographs of bis60 composite following (A) 16 days and (B) 32 days incubation CE (1×10^4 original magnification)

Between 16 to 32 days of incubation with CE, a significant decrease is observed in the generation of bisHPPP for both filler content groups, however the decrease for the higher filler content group is significantly ($p<0.05$) greater than the decrease found for the lower filler content group (Figure 4.17). It is believed that once the upper surface layer has been eliminated, then the process becomes dependent on the presence of the filler content. Therefore, it is anticipated that the highly filled material, which contains less exposed resin and thus less effective surface area available for degradation, will show fewer degradation products associated with bisHPPP.

The results for the enzymatic attack of CE on bis10 and bis60, following incubation for 32 days (Figures 3.16 and 3.17), would indicate that the degradation process in both materials is not limited to the external surface of the composites and suggest that with

time, the enzyme would penetrate further into the matrix while creating pores in the material. In such a case, the first step of the degradation process, i.e. the transport of enzyme to the surface of the resin, is taking place within the pores created into the material. Again, the higher filler content composite should show more resistance to the enzymatic degradation, since in a given volume, the amount of filler is higher in bis60, and thus interfere with the enzymatic attack.

The trend depicted from these results contradicts the observations found in another study [Shajii, 1999], which demonstrated that higher filler content composites show a greater release of bisHPPP following similar incubation periods. However, it must be noted that the composite resin system in the latter study was quite different from that of the current thesis. The Shajii study used smooth (colloidal silica) surfaced, unsilanated sub-micron filler particles, which could lead to easy release of the filler particles following the initial degradation, and exposure of fresh resin and concomitantly an increase in surface area which could lead to rapid product formation as degradation proceeds. In contrast, the average particle size used in the current study was 50 times larger (1 micron average particle diameter) than that of the Shajii study. Furthermore, the particles in this study contained relatively hydrophobic silanizing agents on their irregular surfaces. Both the shape and the hydrophobic nature of the particles would lead to reduced loss of the filler in a polar media.

For all bis composite materials, the accumulation rates of bisHPPP are decreased during the last 16 days of incubation with CE (Figures 3.44 and 4.17). This is primarily thought

to be a temporal phenomenon, dependent on the transport of the enzyme, degradation products and filler [Shajii, 1999]. This phenomenon would be affected by the ability of the enzyme and the degradation products to adsorb onto the polymeric material. *In vivo*, the materials are subject to physical forces, which may promote transport of the enzyme and degradation products away from the materials thus increasing the degradation process. These physical forces are also capable of removing filler particles from the attacked composite and exposing fresh resin to the enzymatic attack. In the latter scenario, product formation for the higher filler content material would be more elevated as compared with the amount found when no forces are applied. In this latter case, the results may more closely reflect those found by Shajii et al [1999].

In the u-bis system, the SEM studies show that both CE and PCE only had a minor effect on the degradation of samples, following the 32 days of incubation (Figures 3.18 and 3.19). The surfaces of the composite samples are still composed mainly of resin and no filler particles were exposed. In this case, it would be anticipated that both u-bis10 and u-bis60 should expose a similar surface area of resin. Hence both materials incubated with CE or PCE should show similar amounts of bisHPPP for each enzyme in both incubation periods, since the latter product is a good indicator of polymeric resin degradation. Therefore, it is not surprising to see that the levels of isolated bisHPPP for both u-bis10 and u-bis60, following incubation with CE or PCE are similar to each other, at both time points (Figures 3.47 and 3.48). In conclusion, the enhanced stability of the polymeric resin in the u-bis system, as also suggested in section 4.2.4, has delayed the impact of filler content on the degradation profile of the composite within the 32 day experiment.

In a similar manner to the bis system incubated with CE, the accumulation rates of bisHPPP for the u-bis samples are decreasing during the last 16 days of incubation with both CE and PCE. This can be explained by the fact that the u-bis system showed greater stability as compared with the bis system, hence limiting product diffusion with the gradual depletion of unreacted bisGMA monomer and partially reacted pendant molecules from the polymeric resin surface.

4.5 INHERENT BIOSTABILITY OF DIFFERENT RESIN SYSTEMS

A recent study [Santerre, 1999] has shown that CE can generate the breakdown of TEGDMA and bisGMA components from commercial dental composite resins. In the latter study, HPLC analysis had shown that the levels of released bisGMA and TEGDMA-associated biodegradation products were almost one order of magnitude greater for Silux-Plus and Z-100 (3M) as compared with that found for TPH (DENTSPLY/L.D. Caulk), a composite with a resin formulation similar to that used in the u-bis system of the current study. The three commercial materials differed in their composition with respect to monomer types, monomer ratios, monomer content, monomer chemistry and filler type. While any one or combination of the above parameters could have been responsible for the observed differences, it was hypothesized that the most significant factor was related to differences is the monomer composition and their ratios. Silux-Plus and Z-100 are both based on bisGMA-TEGDMA monomers, while TPH differs in its resin by the presence of urethane linked bisGMA oligomer and

the substitution of a significant portion of the TEGDMA monomer for bisEMA. Therefore it was anticipated that the chemical structure of a dental composite resin would dictate the stability of the polymeric matrix with respect to enzyme catalyzed hydrolysis via the ester groups. Since the current thesis used composites that differed only in their resin chemistry, it was possible to test the above hypothesis.

The u-bis composite resin material in both filler content groups, exhibited a lower amount of released products (degradation products and residual monomer) as compared to the bis system after incubation in D-PBS, CE or PCE. The differences ranged between 2.6 to 180 fold, depending on the specific product (Figures 3.34-3.37 and 3.41-3.48). Since no difference was found in the number of unreacted vinyl groups between the bis and u-bis composite resin groups (Figure 3.7), the difference in the amounts of released products could only arise from their differences in chemical structure. While the amount of bisGMA molecules in both bis and u-bis composites are roughly the same (see sections 2.1.1, 2.1.3 and 2.1.4), the amounts of the bisGMA derived product, bisHPPP, is 100 to 180 fold higher in the bis composites compared with the amounts found in the u-bis groups (compare Figures 3.34, 3.35, and 3.44 against Figures 3.36, 3.37, 3.47 and 3.48). It is thought that the urethane coupling of the bisGMA molecule has contributed significantly to this enhanced stability. This is further explained below.

Braden [1986] compared the water absorption of unfilled bisGMA based resin and a series of vinyl urethane resins. BisGMA resin was shown to undergo greater sorption as compared with the urethane resins. Watts [1986] showed that a commercial composite

resin, Ful-Fil (L.D. Caulk), which contained the same urethane modified bisGMA as found in TPH and the u-bis system, exhibited a greater water stability and lower water uptake. This could be explained by the decreased flexibility of the urethane modified bisGMA oligomer as well as the elimination of the hydroxyl groups from the bisGMA monomer by the urethane links (Figure 2.2), thus increasing the hydrophobicity of the monomer. As discussed earlier, the penetration of water into the resin matrix will lead to swelling of the polymer and ultimately an increase in the diffusion of monomers from within the polymeric materials. Hence, the reduction in water uptake that could potentially be achieved by the presence of the urethane coupling agent may be partially responsible for the improved performance of the u-bis system.

Santerre and Labow (1997) reported that the presence of urethane structures within polymeric materials could reduce the susceptibility of polar groups to hydrolysis within the vicinity of the urethane. The urethanes were shown to contribute to the formation of hydrogen bonded structures which provided a cohesive system that minimized the enzyme's access to the cleavage sites. In the u-bis system, the urethane groups may possibly be generating hydrogen-bonded bridges with the hydrolyzable ester groups, thus inhibiting or delaying the enzymatic reaction. Hence, this imparts u-bis composites with increased chemical stability. XPS and SEM data (Table 3.2 and Figures 3.16-3.19) render some support for this hypothesis. The presence of urethane groups at the surface of the u-bis system was confirmed by the XPS analysis (Table 3.2) and the FT-IR analysis (Figure 3.1). XPS data show that the ester bonds (C=O) of the resin were present at the surface of both bis and u-bis materials and hence were potentially available for hydrolysis. While a

higher content of these bonds were present at the surface of the bis composite (between 9.85 ± 1.10 % to 11.54 ± 0.68 %) as compared with the amount for the u-bis system (between 5.65 ● 0.17 % to 6.76 ± 0.22 %), the ratio of these two values was only about 2 fold. This ratio is significantly less than the 100-180 fold difference in bisHPPP products isolated for bis versus u-bis systems. Hence, the available surface esters must be configured in a manner which does not render them as readily hydrolyzed. SEM data (Figures 3.16-3.19) demonstrates the stability of the u-bis configuration, showing minimal changes to their surface following 32 days of incubation with either CE or PCE, while the bis system shows extensive degradation of the resin matrix around the filler.

Another factor contributing to the stability of the u-bis system is the fact that most of the bisGMA is incorporated as an oligomer containing 7-9 repeated bisGMA units coupled with hexane diisocyanate (see GPC data, Figure 3.2, section 3.1.2) [Ruyter and Øysæd, 1987a]. No products associated with the urethane-linked bisGMA molecules were isolated in the degradation solution of the u-bis system. This could be attributed to the increased stability of the resin and/or the low solubility of such oligomeric products in aqueous solutions. Their low solubility can be explained by their high molecular weight, 4900-7600 (GPC data, Figure 3.2, section 3.1.2) [Ruyter 1987a and 1987b], which prevents their diffusion through the resin matrix and into the incubation solution. Hydrolysis of the ester bond of the monomeric segment within the resin matrix would not necessarily allow the release of such products, since the molecule would be still connected to the resin polymeric matrix via the urethane linked HDI molecules. This urethane bond is relatively more stable to hydrolysis [St. Pierre, 1986]. The latter

consideration is supported by the results of the ^{14}C-u-bis experiment. A higher amount of radiolabel release was found in the incubation solution following treatment with CE and PCE (Figure 3.46) as compared with D-PBS incubation. However, the data suggests that the amount of the water-soluble radiolabeled products are relatively small. In an attempt to isolate and identify the different radiolabeled degradation products, HPLC fractions were collected every minute and counted for radiolabel content. Since the radioactive products were water soluble, it was assumed that they would be relatively low molecular weight oligomers as well as possibly some 1,6 hexane diamine (HDA). If present, HDA would be a hydrolysis product of the original HDI molecule. Attempts to find a specific radioactive product failed due to the low total radiolabel count that came out of the column and was counted in the HPLC fractions. All the count values were in the order of the background count, i.e. between 20-30 CPM.

In addition to the incorporation of urethane-linked bisGMA molecules in the resin matrix, the u-bis system contains less TEGDMA as compared with the amount in the bis system (10 to 20 % for the u-bis resin as compared with 45 % weight fraction for the bis resin). The literature has reported that increasing the ratio of TEGDMA to bisGMA in a resin matrix systems leads to an increase in their water uptake [Kalachandra, 1987; Beatty, 1993], while TEGDMA monomer was shown to have a higher degree of enzymatic hydrolysis as compared with other monomers, such as bisGMA [Munksgaard, 1990]. Furthermore, an elevation in the amount of methacrylic acid production was shown when the amount of TEGDMA in the bisGMA-TEGDMA based composite resin samples was increased. As discussed earlier, the penetration of water into the resin matrix will lead to

swelling of the polymer and ultimately an increase in the diffusion of monomers from within the polymeric materials. The above results may well reflect the relative hydrophilicity of TEGDMA [Deb, 1998]. Hence, the substitution of TEGDMA with bisEMA (which is less water-soluble) into the u-bis system likely has contributed to the overall chemical stability of the resin. While it was noted that the u-bis system did generate an additional product, ethoxylated bis-phenol A, which was a bisEMA derived product that was not found in the bis system, the levels of this product were still comparatively lower than the bisHPPP levels of the bis system (Figures 3.36, 3,37, 3.47 and 3.48).

In summary, the u-bis system shows an increase in its chemical stability over that of the more traditional bis system. Since the model composite resins differ only in their monomers, this change in chemical stability has been associated with the resin chemistry. Therefore, the second hypothesis of this thesis- "that the chemical structure of a dental composite resin affects its stability with respect to the enzyme catalyzed hydrolysis of the monomers' ester group"- is shown to be true.

4.6 PCE VERSUS CE SPECIFICITY

Although both CE and PCE show similarity in their active sites, as discussed in section 4.3, their reactivity toward natural and synthetic substrates are quite different. CE catalyzes the interfacial hydrolysis of long chain fatty acid esters of cholesterol, while PCE catalyzes the turnover of low molecular weight choline esters, such as

butyrylcholine [Sutton, 1991]. The composite resin matrix contains different comonomers that differ in their chemical structure and may show similarities or dissimilarities with respect to their interaction with the esterases. Therefore, it can be hypothesized that the different esterases have distinct specificities for defined composite resin components.

The differences in the specificities between CE and PCE are reflected in the composite biodegradation experiments. Following 16 days of incubation with bis60 or bis10 composite samples, the amount of bisHPPP released by CE (1 unit/mL) was 7-9 fold higher than that released with PCE (1 unit/mL). The elevated ratio for bisHPPP implies that CE must have some degree of specificity over PCE for the cleavage of bisGMA components and hence degradation of the composite itself, since it was indicated earlier (section 4.4) that the release of bisHPPP was reasonably well correlated with the extent of matrix degradation. Interestingly, for the same conditions, the bis60 group exhibited only a two fold increase in the amount of methacrylic acid released by CE as compared with that released by PCE, while in the bis10 groups there was no difference for levels of methacrylic acid (Figures 3.34, 3.35, 3.41 and 3.44).

As mentioned before (section 4.2.4), methacrylic acid is a common end product of all methacrylate based monomers hydrolyzed at the ester bond. Among the possible sources for methacrylic acid production are unreacted TEGDMA, its intermediate degradation product TEGMA, as well as its pendant moieties within the resin polymeric matrix. It is to be noted that for each of the enzymes, an identical amount of unreacted TEGDMA was

available for cleavage in each of the filler content groups. All of this TEGDMA was eliminated for both enzyme systems at 1 unit/mL (Figures 3.34, 3.35 and 3.42). In the bis60 composite at 0.1 unit/ml, PCE showed a greater elimination of TEGMA compared with CE (compare Figures 3.34 and 3.35). This suggests a more rapid generation of methacrylic acid from TEGDMA, TEGMA and the matrix, for PCE versus CE. This may explain in part why the methacrylic acid ratio between CE and PCE was not as high as the product ratio for bisHPPP in the bis60 or the bis10 groups. Also, the increased amount of TEGDMA in the bis10 composite as compared with the bis60 material may explain the total elimination of the difference in the methacrylic acid production between CE and PCE. In the latter experiment it is thought that PCE has more of the available TEGDMA to further compensate for its poor ability to hydrolyze methacrylic acid from the bisGMA components.

Data for 32 days of incubation with the bis samples and CE showed a decrease in the amount of isolated bisHPPP as compared with the 16 days period, while the amounts for PCE showed no changes (Figure 3.44). This possibly suggests that CE rapidly consumed the readily available unreacted bisGMA monomer and pendant bisGMA molecules, and caused a depletion of the latter molecules in the resin/enzyme interface zone. After the initial degradation, the hydrolysis rate is limited by the diffusion rate of bisGMA molecules within the composite resin matrix, which is relatively slow [Ferracane, 1994]. On the other hand, the slower hydrolysis rate of bisGMA by PCE relative to CE (Figure 3.33) does not generate a depletion of available bisGMA and therefore does not establish

a diffusion rate limited system for the release of bisHPPP. Hence there is no change in the amount of bisHPPP between 16 and 32 days (Figure 3.44).

The above discussion is supported by the measurements of monomer hydrolysis rates (Figure 3.33). PCE shows a higher specificity with respect to the to cleavage of the relatively water-soluble substrate, TEGDMA, at 29.55 ± 1.78 μg/hour/ml of methacrylic acid production. In contrast, the respective hydrolysis rates of bisGMA and bisEMA are much lower, 0.49 ± 0.01 and 0.57 ● 0.03 μg/hour/ml. Contrary to this behavior, the CE hydrolysis rates are relatively similar, with respective values of 7.7 ± 0.5, 6.3 ● 0.65 and 5.83 ± 0.16 μg/hour/ml for TEGDMA, bisGMA and bisEMA (Figure 3.33). Accordingly, the PCE catalyzed hydrolysis rate for TEGDMA was 3.8 fold higher than that of CE, while CE catalyzed the hydrolysis of bisGMA and bisEMA at respectively 12.79 and 10.23 fold higher that that for PCE.

In addition to the different degradation activities, CE and PCE show different activities with the soluble unnatural substrates, *o*- and *p*-nitrophenyl esters (Figure 3.12). The approach taken to further observe the different specificities of the two enzymes was to use four water-soluble synthetic nitrophenyl ester substrates with different lengths and position of the fatty acid side chain. When a factorial analysis for the length and position of the side chain was performed, only the length of the side chain was a significant variable for CE activity ($p<0.001$), with a higher hydrolysis rate for the longer side chain nitrophenyl esters. This agrees with previous reports that CE hydrolyzes the non-water soluble long chain fatty acid esters of cholesterol when these are solubilized by bile salts

in the digestive tract [Labow, 1983]. For PCE, only the location of the side chain was a significant variable for the enzyme activity, with a higher hydrolysis rate for the ortho positioned side chains. However, when the side chain was located in the ortho position, a higher hydrolysis rate was found for the butyrate as compared with the acetate side chain ($p<0.05$). Williams [1985] reported similar results for this enzyme. On the other hand, a possible explanation for the PCE higher preferences for the ortho location of the side chain was not found in the literature.

The greater specificity of CE to hydrolyze water-insoluble substrates, such as bisGMA, was also demonstrated by its ability to cleave bisEMA (ethoxylated bisphenol-A dimethacrylate) to form E-bisPA in the u-bis system, while no such product was detected for the PCE incubated groups. Furthermore, the net increase in the amount of a radiolabeled urethane-containing molecule, originating from the ^{14}C-urethane-modified bisGMA, following incubation in CE is 3 fold higher than that released following incubation in PCE (Figure 3.49). These data further emphasize the specific differences between CE and PCE.

The similarities among bisGMA, bisEMA and the urethane-modified bisGMA (Figures 1.3, 1.6 and 1.7) as well as the natural substrate of CE (Figure 4.19) is that they all have a rigid ring structured domain linked to a long chain via an ester-type bond. Since CE appears to be more active for this type of structure [Labow, 1994], it is perhaps not surprizing that CE is more active with respect to bisGMA, bisEMA and the urethane-modified bisGMA relative to PCE. On the other hand, the similarities (i.e. the presence of

ethylene oxide in a relatively low molecular weight substrate) between butyrylcholine (Figure 4.20), TEGDMA and TEGMA (Figures 1.3, 4.2 and 4.3) could explain the preference of PCE for the latter molecules.

In summary, CE and PCE show different activities with respect to different monomers, and these activities bear some resemblance to their interaction with natural substrates. Therefore, the hypothesis "that the different esterases have distinct specificities for defined composite resin components"- is shown to be true.

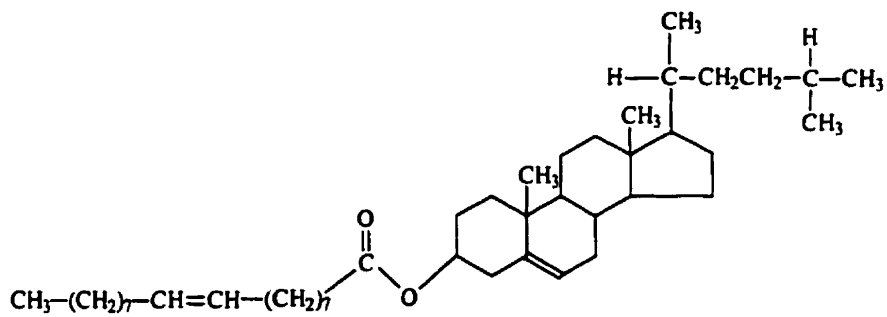


Figure 4.19 The structure of cholesteryl oleate, the most common natural substrate of CE [Labow, 1994]

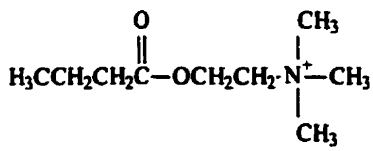


Figure 4.20 Structure of butyrylcholine

4.7 HYDROLASE ACTIVITY IN HUMAN SALIVA

Numerous studies have reported on the presence of enzymatic activity in human saliva [Chauncey, 1961; Nakamura, 1983a; Ryhänen, 1983a and 1983b; Zambon, 1985; Yamalik, 1990], which has been associated with composite resin material degradation [Freund, 1990; Munkgaard, 1990; Larsen, 1991; Larsen, 1992]. Hence, it is of interest to consider which hydrolase activities co-exist in human saliva and can they degrade composites in a similar manner to PCE and CE. Therefore, it can be hypothesized that saliva contains hydrolase activity, CE and/or PCE-like, within the defined limits of activity that affect ester containing composite resin materials.

The saliva analysis data show a CE-like hydrolase activity in different human subjects [Figures 3.13]. All subjects show activities with the different substrates and the average activity level, measured using *p*-NPA as a substrate was 0.19 ± 0.02 unit/mL. This value is well within the activity range used for this study (0.01 to 1 unit/mL) (Figure 3.13). Lower stock CE activity levels, as measured with *p*-NPA, have been shown to significantly degrade the composite resin samples (Figures 3.34 and 3.36).

The approach taken to compare the salivary esterase activity with that of the stock enzymes was to use four water soluble synthetic substrates that exhibit different lengths and positions (*i.e. o* or *p*-nitrophenylesters) of the fatty acid side chain so that the activity profile could be described and compared with that of the stock CE. Butyrylthhiocholine (BTC) was used to assess the activity compared with the stock PCE. The effect of the

ester position on the substrate was similar for both stock CE enzyme and the saliva; *i.e.* the para position shows higher activity in all cases. The selectivity of *p*-NPB over that of the other substrates suggests a similarity for all subjects with the stock CE enzyme [Labow, 1994].

As well, the presence of PCE-like activity in human saliva was confirmed, with an average of 0.011 ± 0.001 unit/mL (Figure 3.14). This activity level is slightly above the lower limit of stock PCE used in this study (Figures 3.35 and 3.37). Similar results, with respect to the PCE activity levels in saliva, were found by others [Ryhänen, 1983a and 1983b; Yamalik, 1990 and 1991]. The fact that the stock CE enzyme does not show any activity with the BTC substrate (Figure 3.12) while the saliva does, is not surprising and indicates that saliva has a broader activity spectrum than does the stock CE. It can be suggested that the kinetics of composite degradation in saliva may vary substantially from that with the individual enzymes.

The current thesis has demonstrated levels between 0.09 to 0.26 unit/mL for CE-like and 0.004 to 0.018 unit/mL for PCE-like activities in human saliva (Figures 3.13 and 3.14), which were within the range used in this study for CE and PCE (Figures 3.34-3.37). Since the degradation process illustrated a dependence on the enzymes' activity levels, this study suggests that the level of degradation product generated for a material will depend on the esterase make-up for an individual's saliva. It is therefore inferred that dental restorative products may be selected based on a patient's saliva activity profile.

The results show that CE and PCE are suitable models for esterase induced composite resin degradation in the oral cavity. Furthermore, the broad activity of human saliva, as demonstrated by its ability to hydrolyze BTC in addition to the nitrophenyl esters, suggests that it is capable of degrading composite resins *in vivo*. In fact, that was documented in a recent study with commercial composites [Jaffer, 2000].

In addition, the literature reports that particulate matter in saliva can contribute significantly to the overall enzymatic activity of human saliva [Chauncey, 1961; Lindqvist, 1977; Nakamura, 1983a]. In the study by Jaffer et al [2000], only the supernatant activities of the saliva were assessed. As a consequence, *in vivo* degradation processes are suspected to be higher than that projected based on the supernatant activities reported in the current thesis and used by Jaffer [2000]. Therefore, the hypothesis "that saliva contains hydrolase activity, CE and/or PCE-like, within the defined limits of activity that affect ester containing composite resin materials"- is shown to be true.

4.8 MUTUAL INFLUENCE BETWEEN CE AND PCE ON THE BIODEGRADATION OF THE COMPOSITES

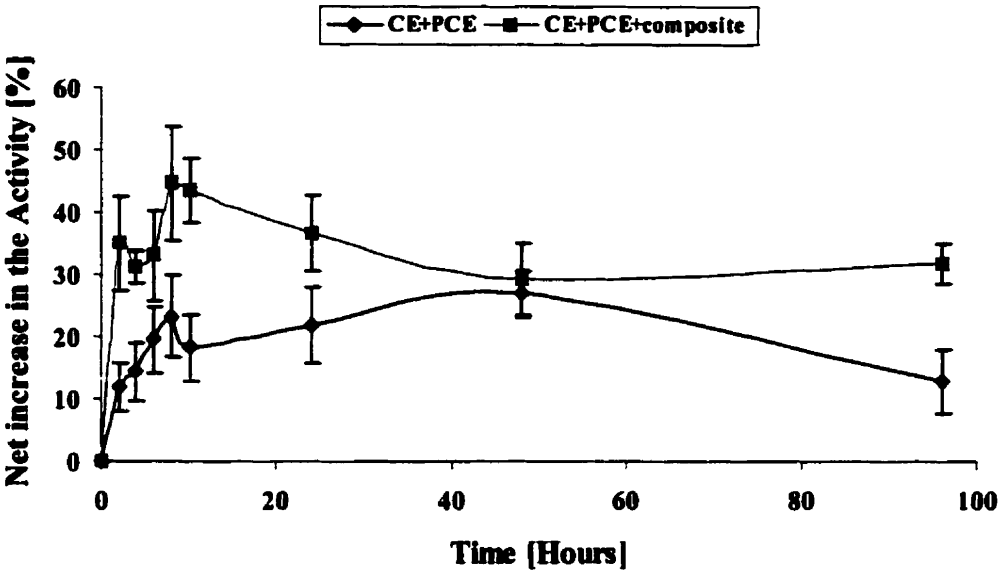
It has been shown in this thesis and is well documented that human saliva contains different enzymatic activities [Chauncey, 1961; Pershad, 1999] and these activities may originate from different sources including oral micro-organisms, salivary glands and gingival crevicular fluid [Nakamura, 1983a]. Since multiple activities are present in

saliva, it was of interest to determine if there could be a mutual influence between different esterases, and more specifically, between CE and PCE.

The results of the biodegradation experiments show that when both enzymes are present in incubation solutions with bis or u-bis composites, the amount of composite degradation is higher than that compared with the arithmetic sum of the degradation produced by each enzyme alone (Figures 3.50-3.53). However, it is difficult to determine if this effect was due to a synergistic mechanism on the enzymes' activity, maintenance of enzyme stability, access to sites on the material or other factors.

4.8.1 STABILIZATION OF ENZYME ACTIVITIES

The CE stability assays (Figures 3.8 and 3.9) showed that the addition of bis60 composites to the incubation solution reduced the stability of the enzyme over the first 6 to 24 hours (Figure 3.8). When PCE was added to the incubation solution, the effect of the composite on the stability of CE was significantly reduced. However, since PCE itself has a hydrolytic activity with respect to the substrate used to measure CE activity, *p*-NPA (Figure 3.12), it becomes necessary to take into account the activity profiles of PCE (Figure 3.10 and 3.11) in order to truly assess the possibility of enhanced CE stability by PCE in the presence of composite. This can be done by calculating the net change in enzyme activity for CE, upon adding PCE, and comparing these values for the case with and without composite samples. Data for such an analysis is presented in Figure 4.21. In the bis system, the net increase for the CE activity in the presence of composite is



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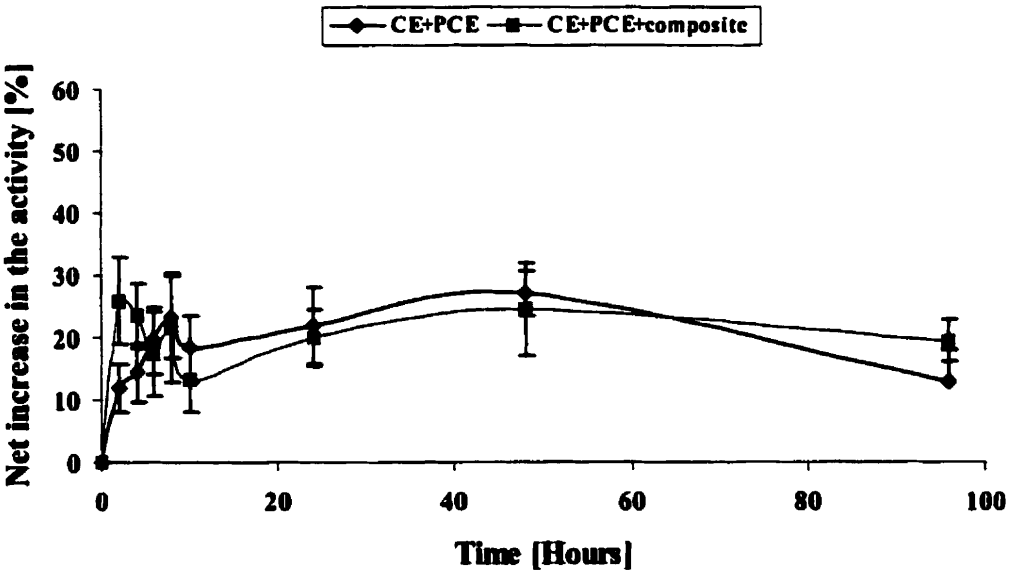

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Figure 4.21 **Comparison of the net increase of CE stability in the presence of PCE, with and without composite samples. A) bis60 system, B) u-bis60 system**

significant for several time points in the 96 hour experiment (Figure 4.21A). This suggests that the effect of PCE on the stability of CE in the presence of composite is not merely associated with PCE's inherent contribution to the hydrolysis of the substrate, but to a synergistic effect. The same effect is not observed in the u-bis system, where the data show no statistically significant differences between PCE enhancement of CE stability with and without composite (Figure 4.21B). As an aside comment, this also further highlights important differences between the two material types. It also indicates the potential existence of active complexes between composite materials and multiple biochemical agents found in saliva. Hence, in the case of CE and PCE with the bis60 system, it would appear that a mechanism of enhanced maintenance of CE activity is in part responsible for the synergistic degradation effect observed in Figures 3.50-3.53.

The PCE stability assay (Figures 3.10 and 3.11) shows that the addition of bis60 composites to the incubation solution shows a reduced stability as compared with the enzyme incubated alone but only following 32 days of incubation (Figure 3.10). When CE is added to the incubation solution, the effect of the composite on the stability of PCE is eliminated. Since CE does not show any activity with the BTC substrate that was used to measure the PCE (Figure 3.12), no further analysis is needed, and it can be assumed that CE increases the stability of PCE in the presence of composite samples.

One explanation for the reduced hydrolytic activity of enzyme in the presence of bis60 composites is related to the nature and amount of leached unreacted monomers, and/or degradation products generated during the period of the experiment. The fact that one

material (bis60) shows an effect and the other (u-bis) does not, can only be related to the resin type since this is the only differing parameter. Radic et al [1991] reported that acetyl cholinesterase is inhibited by high levels of its substrate, acetyl choline, while Augustinsson [1948 and 1963] demonstrated that PCE was inhibited by certain aromatic esters such as benzocholine and acetyl-salicylcholine. If this observation is true for both enzymes, then the reduced stability in the presence of bis60 composite may very well be related to the greater release of unreacted monomers and degradation products for this composite. As an example, the rapid release of TEGDMA during the first 48 hours (Figure 4.14) could explain the dramatic reduction in the CE activity in the first 24 hours, upon the addition of bis60 composites. Since PCE has been shown to have a higher specificity than CE for the cleavage of TEGDMA (Figure 3.33), the addition of PCE to the incubation solution may reduce the amount of TEGDMA at a faster rate (Figure 3.35) and minimize the inhibition effect of the monomer. In a similar manner, the addition of CE to incubation solutions of PCE may increase the cleavage rate of TEGDMA and TEGMA and reduce the inhibitory effect of these products. The only question that remains is how does one enzyme work to help the other in eliminating this effect? Experiments to further probe these ideas were not pursued in this thesis but would be of significant interest for future work.

4.8.2 COOPERATIVE DEGRADATION OF COMPOSITES BY ENZYMES

Although the u-bis60 composite did not show an effect on the stability of CE or PCE, a synergistic effect between the enzymes is noticed on the degradation of u-bis60 (Figure

3.53). Therefore, one must consider an alternate explanation to that of the effect on enzyme activities, in order to explain the apparent enzyme synergy with respect to the generation of polymer derived degradation products. This may be related to a cooperative effect with respect to the cleavage sites. As discussed in section 4.7, CE and PCE showed distinct specificities for defined composite resin components. For example, CE showed a greater specificity than PCE to hydrolyze bisGMA and bisEMA components of the composites, while PCE showed a greater specificity toward TEGDMA and TEGMA components. The measurement of hydrolysis rates for the monomers (Figure 3.33) demonstrated a similar trend for the enzymes. Since degradation of the resin matrix could proceed via the different monomer components, the co-existence of both enzymes should generate a more efficient hydrolase activity with respect to all these units. In doing so, one enzyme may gain faster access to its preferred sites because the other enzyme is rapidly cleaving out products for which the first enzyme was not particularly efficient at degrading. In this manner, the overall matrix would be cleaved with a greater efficiency.

In summary, both CE and PCE are suitable as models for esterase induced composite resin degradation in the oral cavity. Furthermore, the increased stability and activity of each enzyme in the presence of the other (Figures 3.8-3.11 and 4.21) suggests that an even greater degradation effect, than the sum of the individual effects by each enzyme measured alone, may occur in the oral cavity, where it is known that a variety of esterases are present together. It should be kept in mind that the *in vivo* system also contains many other co-factors which can inhibit or facilitate the action of enzymes, and these will also need to be considered in future studies.

4.9 SUMMARY

- Human saliva contains hydrolase activities, CE and PCE like, that are in levels which can degrade restorative composite resin materials, present in the oral cavity. Incubation of the composites with CE or PCE has been shown to be dose dependent. Since different subjects exhibited different hydrolase activities, it can be postulated that the extent of the degradation process will also be determined by the principal activities present in an individual's saliva.

- The u-bis composite resin materials show a significant increase in stability over that of the more common bis composite resin materials. This has significant clinical relevance since it reproduces observations made with commercially available materials representing both of these commonly used restorative materials in the dental field today (namely TPH from L.D. Caulk/DENTSPLY and Z-100 from 3M Corporation) [Santerre, 1999].

- CE and PCE show distinct specificities for defined composite resin components. The mutual enhancement of each enzyme activity in the presence of the other enzyme is very important, since saliva contains a mixture of different enzymes that may also enhance each other's stability and/or activities and increase the degradation of composite resins present in the oral cavity.

This study suggests that the level of degradation products generated for a material will depend on the esterase make-up for an individual's saliva and the specific formulation of monomer components in the composite. Since resin formulation has a significant effect on the composite chemical stability, manufacturers should consider testing biochemical stability as part of their routine product evaluation.

5.0 CONCLUSIONS

1. The enzyme catalyzed hydrolysis of dental composite resin components by cholesterol esterase and pseudocholinesterase occurs at the ester bonds of the methacrylate based resin monomeric units, in a dose dependent manner. Both enzymes increased the rate of hydrolysis for monomeric units in the methacrylate-based polymers, as evident by the increased production of methacrylic acid and bisHPPP. Their inhibition by a specific serine esterase inhibitor, PMSF, suggests that the hydrolysis of the monomers occurs via the same active site (serine) that the enzymes utilize to attack other substrates of the enzymes (i.e. nitrophenyl esters).

2. TEGMA, bisHPPP and E-bisPA were shown to be formed via enzymatic hydrolysis of the ester bonds contained in their corresponding monomeric unit, i.e. TEGDMA, bisGMA and bisEMA. These products are partially generated by the cleavage of pendant monomers and repeat segments of the monomers within the polymerized matrix as well as some leaching monomer. The hypothesis of matrix degradation is supported by the SEM analyses.

3. Methacrylic acid was shown to be formed from all methacrylate based monomers hydrolyzed via the ester linkage. The sources for methacrylic acid in composites can be from the cleavage of unreacted monomers or their pendant derived molecules, which have only reacted with the resin matrix via one vinyl group. Different levels of methacrylic acid were found, depending on the resin system and the incubation period.

4. Unreacted TEGDMA monomer released into the incubation solution was shown to be correlated with the amount of TEGDMA incorporated within the composite resin materials. This relation is probably attributed to its relatively high solubility in aqueous solutions and its relative ease of mobility to diffuse through the resin matrix. This diffusion process is dependent on the established gradients from within the resin matrix to the interface of the composite/incubation solution. With time, this gradient is diminished and less TEGDMA is released into the incubation solution.

5. The release profiles for the biodegradation products of the enzyme catalyzed hydrolysis show a trend with respect to the filler content of the composites. Following the initial enzyme attack onto the polymer matrix, the higher filler composite (bis60) was shown to be less susceptible to degradation than the lower filler content material (bis10). This would suggest that the former material's resin component has a reduced exposure to the enzymes. Accordingly, the amounts of bisHPPP generated from the higher filler materials showed a reduction in its accumulation rates between the 16 and 32 days incubation periods with CE.

6. CE and PCE show distinct specificities for defined composite resin components. The differences in the specificities are reflected in the composite's biodegradation experiments, which show a greater production of bisHPPP for the CE incubated groups as compared with the PCE incubated groups, while the elimination rate of TEGMA from the PCE incubation solutions with composites is faster than that for the CE incubated groups.

This is further supported by the measurements of hydrolysis rates for the monomers. These data showed a higher rate for the cleavage of TEGDMA by PCE while CE shows a greater ability than PCE to eliminate bisGMA and bisEMA.

7. The u-bis system shows an elevated biochemical stability relative to the bis system, which is evident by the lower production of degradation products. SEM micrographs also supported this finding. Since the model composite resins differ from each other only in their resin, this increased stability must be associated with the resin chemistry. Possible explanations for the greater stability could be due to the presence of urethane/bisGMA oligomers that can hinder the hydrolysis of groups and limit diffusion. The reduced amount of TEGDMA in the u-bis system could also increase its chemical stability. E-bisPA was only produced by the u-bis system following incubation with CE but not PCE.

8. Saliva analysis shows the presence of different levels of CE and PCE-like hydrolase activities for different human subjects, and these activities were within the range used to study the degradation of composites in this thesis. The actual hydrolase activity in human saliva may be higher, since most of the hydrolase activity within saliva originates from the particulate matter of saliva, while in the current study only the supernatant was assessed. Furthermore, the synergistic interactions between the enzymes suggests that an even greater degradation effect than that observed for single enzyme systems would exist within the oral cavity, where a variety of esterases are present together.

6.0 RECOMMENDATIONS

1. Saliva analysis shows CE and PCE-like hydrolase activities for the different human subjects. The activities with the different substrates are well within the activity range used for this study, as measured by *p*-NPA and BTC for CE and PCE respectively. However, the exact nature of these activities and their origin have not been defined within this study. Therefore, it would be of a great interest to further investigate the origin and the nature of such activities in human saliva.

2. As mentioned above, human saliva from different subjects has shown the presence of CE and PCE-like activities, as measured by their corresponding substrates, *p*-NPA and BTC. However, the capability of human saliva to degrade composite resins was not investigated. A pilot study has been conducted in our laboratory for commercially available dental composites [Jaffer, 2000]. The results show different stabilities for different materials. Since the commercial materials differ in more than their resin chemistry, it would be important to investigate the hydrolytic capacity of human saliva with respect to the model dental composite resins, used in the current thesis and to determine the specific effects of resin chemistry.

3. The release of composite resin components, such as unreacted monomers is of great importance, since numerous studies have shown that they can have toxic effects *in vitro* [Hanks, 1988; Heil, 1996; Olea, 1996; Scvheweikel, 1998]. However, the effects of degradation products *in vitro* have not been thoroughly investigated. Since the saliva has

the ability to degrade the composites, it is recommended that studies, which would investigate the effect of degradation products on human cells, be carried out. Specifically, toxicity levels, mutagenic capacity and effects on cell function are particularly important.

4. As mentioned in the literature review, the polymerization contraction of composites may lead to the formation of a gap around the cavity margins, resulting in micro-leakage and passage of bacteria. These latter organisms may include mutans streptococci and lactobacilli, which have been specifically identified in plaque found at the margins of composite fillings [Svanberg, 1990; Kidd, 1993]. In addition, it has been documented that co-monomers such as EGDMA and TEGDMA may promote the proliferation of important cariogenic micro-organisms, *Lactobacillus acidofilus* and *Streptococcus sobrinus* [Hansel, 1998]. It was also noted that most enzymes were partially or entirely produced by micro-organisms, which normally inhabit the oral cavity [Chauncey, 1961; Nakamura, 1983a]. Therefore, it is of great significance to investigate the effects of the biodegradation products on oral flora as well as the effects of oral micro-organisms on the degradation of composite resins.

5. Since the oral environment exposes composite resins to mechanical forces and wear processes in addition to the chemical attack, it would be desired to study the materials in a manner that more closely resembles their oral environment. Such studies would include a combination of mechanical loading concurrently with exposure to enzymes. In previous work [deGee, 1996], the effect of an enzymatic medium on the *in vitro* wear of commercial dental composite resin materials was evaluated. However, it was difficult to

determine if the difference in the wear rates between composites wase due to resin type, filler type and concentration. As well, these studies measured material wear and did not assess the presence of degradation products. The use of mechanical wear is more representative of the *in vivo* conditions, and thus would yield a better evaluation of the clinical performance of composite resin materials. The use of model composites would enable the direct assessment of the resin and filler formulations for clinical applications. In addition, since it was noted that the lower degradation of the bis60 composite as compared to the bis10 group was attributed to the interference of the filler particles, the above wear/enzyme experiment might provide an ideal system for confirming whether the interference of particles was the true cause for the observed difference. Since the wear apparatus would remove degraded debris and filler particles from the composites and may yield higher rates of degradation for the bis60 material as compared with bis10 group, in a similar manner to that demonstrated by Shajii et al [1999].

6. All materials exhibited a significant release of resin components, unreacted monomers or degradation products up to 32 days of incubation. However, nothing is known about the process beyond this time frame. Therefore, it would be important to run the biodegradation experiments for periods beyond 32 days, so as to observe the progress of matrix degradation following the exposure of its sub surface layer to enzyme.

7. In this study, the sampling regime for the accumulation of products in the incubation solutions was only done after 16 and 32 days of incubation. This is not frequent enough to permit a detailed analysis of the degradation kinetics. Therefore, it is recommended

that future work should include more frequent sampling times for product accumulation in the incubation solutions. Due to the high anticipated number of samples, it would be expected that faster HPLC methods would need to be developed.

8. It was suggested in the discussion that the reduced activity of the enzyme in the presence of composite samples may be due to the effect of leached unreacted monomers and/or degradation products released during the period of the experiment. To further investigate this hypothesis, an activity assay for each of the enzymes, in the presence and absence of the other should be conducted. The studies would be carried out in the presence of purified monomers or degradation products to assess their effect on the enzymes activity.

7.0 REFERENCES

Adams A.M., Soames J.V., Searle R.F. Morphological changes in cultured human periodontal ligament cells exposed to dental materials, Biomaterials 1995, 16:1031-1035

Adams A.M., Soames J.V., Searle R.F. Ultrastructural changes in cultured human periodontal ligament cells exposed to dental materials, J. Biomed. Mater. Res. 1995, 29:999-1004.

Andrade J. D., Smith L. M., Gregonis D. E., "Surface and interfacial aspects of chemistry and physics", in "Biomedical polymers Vol I: Surface", Andrade J. D. ed., Plenum Pres, New York, 1985.

Anseth K.S., Goodner M.-D., Reil M.A., Kannurpatti A.R., Newman S.M., Bowman C.N., The influence of comonomer composition on dimethacrylate resin properties for dental restorations, J. Den. Res. 1996, 75:1607-1612.

Anusavice K.J., deRijk W.G., Performance of dental biomaterials: Conference Report, Dent. Mater. 1990, 6:69-72.

Arkles B., Silane coupling agent chemistry, Petrarch Systems Catalogue, Bristol PA 1987, p. 59.

Asmussen E., NMR-analysis of monomers in restorative resins Acta Odontol Scand 1975, 33(3):129-34.

Asmussen E., Factors affecting the quantity of remaining double bonds in restorative resin polymers, Scand. J. Dent. Res. 1982, 90:490-496.

Asmussen E., Softening of BISGMA-based polymers by ethanol and by organic acids of plaque, Scand. J. Dent. Res. 1984, 92:257-261.

Asmussen E., Peutzfeldt A., Mechanical properties of heat treated restorative resins for use in the inlay/onlay technique, Scand. J. Dent. Res. 1990, 98:564-567.

Asmussen E., Uno S., Adhesion of restorative resins to dentin: Chemical and physiochemical aspects, Operative Dent 1992, 5:68-74.

Asmussen, E., Peutzfeldt A., Influence of UEDMA, BisGMA and TEGDMA on selected mechanical properties of experimental resin composites, Dent. Mater. 1998, 14:51-56.

Aso C., Polymerization of ethylene glycol dimethacrylate, J. Polym. Sci 1959, 34:475-486.

Augustinsson K. B., Cholinesterases; A study in comparative enzymology, Acta Physiol. Scand., 1948, 15 Suppl. 52:1-182.

Augustinsson K. B., Classification and comparative enzymology of the cholinesterases and methods for their determonation, in: "Cholinesterases and Anticholinesterase Agents, G. B. Kolle, ed. (Springer, Berlin) pp. 89-128.

Bagis H.Y., Ruggeberg F.A., Mass loss in urethane/TEGDMA- and BisGMA/TEGDMA-based resin composites during post-cure heating, Dent. Mater. 1997, 13:377-380.

Baker s., Brooks S.C., Walker D.M., The Release of Residual Monomeric Methyl Methacrylate from Acrylic Appliances in the Human Mouth: An Assay for Monomer in Saliva, J. Dent. Res. 1988, 67(10):1295-1299.

Bausch J.R., de Lange C., Davidson C.L., The influence of temperature on some physical properties of dental composites, J. Oral Rehabil. 1981, 8:309-317.

Bayne S.C., Taylor D. F., Roberson T. M., Wilder A. D., Sturdevand J. R., Heymann H. O., Lisk M. W., Long term clinical failure in posterior composites. J Dent Res 1989, 68:185.

Bayne S. C., Dental composites/glass ionomers: clinical reports, Adv. Dent. Res. 1992, Sep;6:65-77.

Beamson G., Briggs D., "Descroption of the spectrometer', in "High resolution XPS of organic polymers", pp.3-8, 1992.

Bean T.A., Zhuang W.C., Tong P.Y., Eick J.D., Yourtee D.M., Effect of esterase on methacrylates and methacrylate polymers in an enzyme simulator for biodurability and biocompatibility testing, J. Biomed. Mater. Res. 1994, 28:59-63.

Beatty M.W., Swartz M.L., Moore B.K., Phillips R.W., Roberts T.A., Effect of crosslinking agent, monomer functionality, and repeat unit chemistry on properties of unfilled resins, J. Biomed. Mater. Res. 1993, 27:403-413.

Ben-Jonathan N., Steinmetz R., Xenoestrogens: The emerging story of bisphenol A, Trends Endocrinol. Metab. 1998, 9:124-128.

Billmeyer F.W., Textbook of Polymer Science, 3rd edition 1984, New York: John Wiley and Sons, 49-58.

Bowen R.L., Use of epoxy resins in restorative materials. J Dent Res 1956, 35:360-369.

Bowen R.L. inventor (1962), Dental filling material comprising vinyl silane treated fused silica and a binder consisting of the reaction product of bis phenol and glycidyl acrylate. *US patent* 3,066,112.

Bowen R.L., Properties of silica-reinforced polymer for dental restorations. J. Am. Dent. Assoc. 1963, 66:57-64.

Bowen R.L. inventor (1965), Silica-resin direct filling material and method of preparation. *US patents* 3,194,783 and 3,194,794.

Bowen R.L., Reed L.E., Semiporous reinforcing fillers for composite materials, J.Dent. Res. 1976a, 55:738-47.

Bowen R.L., Reed L.E., Semiporous reinforcing fillers for composite materials, J.Dent. Res. 1976b, 55:748-56.

Bowen R.L., Menis D.L., Setz L.E., Jennings K.A., "Theory of polymer composites", in "Posterior composite resin dental restorative materials", ed. Smith D.C., Peter Szulc Publishing Co., Netherlands 1985, pp.95-105.

Bowen R.L., Marjenhoff W.A., Dental Composite/Glass Ionomers: The Materials, Adv. Dent. Res. 1992, 6:44-49.

Braden M., Davy K.W.M., Water absorption characteristics of some unfilled resins, Biomaterials 1986, 7(6):474-5.

Brännstrom M., Dentin and pulp in restorative dentistry, London: Wolfe Medical Publ. 1981, pp.81-90.

Brown S.S., Kalow W., Pilz W., Whittaker M., Woronick C.L., Plasma Cholinesterase, In: Advances in Clinical Chemistry, Vol. 22, New York: Academic Press 1981, pp. 1-123.

Caughman W. F., Rueggeberg F. A., Curtis J. W. Jr, Clinical guidelines for photocuring restorative resins, J. Am. Dent. Assoc. 1995 Sep;126(9):1280-2, 1284, 1286.

Chadwick R.G., McCabe J.F., Walls A.W.G., Storer R., The effect of storage media upon the surface microhardness and abrasion resistance of three composites, Dent. Mater. 1990, 6:123-128.

Chauncey H.H., Salivary enzymes, JADA 1961, 63:360-368.

Caughman F.W., Caughman G.B., Dominy W.T., Schuster G.S. Glass ionomers and composite resin cements: Effect on oral cells, J Prosthe Dent. 1990, 63:513-521.

Chowdhury N.A., Wakasa K., Priyawan R., Yamaki M., Matrix strengthening in new ternary Bis-GMA/TEGDMA/urethane resin systems, J.Mat.Sci.Letters 1996, 15:1912-1915.

Chung K.H., Greener E.H., Correlation between degree of conversion, filler concentration and mechanical properties of posterior composite resins, J. Oral. Rehab 1990, 17:487-494.

Condon J.R., Ferracane J.L., *In vitro* Wear of Composite with Varied Cure, Filler Level, and Filler Treatment, J. Dent. Res. 1997, 76(7):1405-1411.

Condon JR, Ferracane JL, Reduction of composite contraction stress through non-bonded microfiller particles, Dent Mater 1998 Jul,14(4):256-60.

Convey D.A., Tahaney S.R., Davenport J.M., Mechanical properties of heat treated composite resins restorative materials, J. Prosthet. Dent. 1992, 68:458-461.

Cook W.D., Polymerization defects in composite resins, in "Posterior composite resin dental restorative materials", ed. Smith D.C., Peter Szulc Publishing Co., Netherlands 1985, pp.273-286.

Cook W.D., Beech D.R., Tyas M.J., Resin-based restorative materials-a review, Austral. Dent. J. 1984, 29:291-295.

Cootauco C.J., Rauschenberger C.R., Nauman R.K., Immunocytochemical dictribution of human PMN elastase and cathepsin G in dental pulp, J. Dent. Res. 1993, 72(11):1485-1490.

Coury A.J., Chemical and biochemical degradation of polymers, Biomaterials Science, Introduction to materials in medicine, eds. Ratner B. D., Hoffman A. S., Schoen F. J., Lemons J. E., Academic Press 1996 , San Diego, pp.243-259.

Cox S.W., Eley B.M., Preliminary studies on cysteine and serine proteinase activities in inflamed human gingiva using different 7-amino-4-trifluoromethyl coumarin substrates and protease inhibitors, Archs. Oral Biol. 1987, 32:599-605.

Cox S.W., Eley B.M., Detection of cathepsin B- and L-, elastase-, tryptase-, trypsin-, and dipeptidyl peptidase IV-like activities in crevicular fluid from gingivitis and periodontitis patients with peptidyl derivatives of 7-amino-4-trifluoromethyl coumarin, J.Perio. Res. 1989, 24:353-361.

Cox C.F., Effects of adhesive resins and various dental cements on the pulp, Oper. Dent. 1992, 17(5):165-176.

Craig G.C., Restorative Dental Materials, ninth edition, Mosby, St. Louis 1993 pp.131.

Davy K.W.M., Kalachandra, S., Pandain M.S., Barden M., Relationship between composite resin matrix molecular structure and properties, Biomaterials 1998, 19:2007-2014.

Davy K.W.M., Braden M., Study of polymeric systems based on 2,2 bis-4(2hydroxy-3-methacryloyl-oxy propoxy)-phenyl propane, Biomaterials 1991, 12:406-410.

Deb S., Polymers in dentistry, Proc. Instn. Mech. Engrs. 1998, 212:453-464.

de Gee A.J., Pallaw P., Werner A., Davidson C.L., Annealing as a mechanism of increasing wear resistance of composites, Dent. Mater. 1990, 6:266-270.

de Gee A.J., Wendt S.L., Werner A., Davidson C.L., Influence of enzymes and plaque acids on in vitro wear of dental composites, Biomaterials 1995, 17:1327-1332.

Dionysopoulos P., Watts D.C., Dynamic mechanical properties of an inlay composite, J. Dent. 1989, 17:140-144.

Dodds E.C., Lawson W., Synthetic estrogenic agents without the phenanthrene nucleus, Nature 1936, 137:996.

Dogon I.L., Present and Future Value of Dental Composite Materials and Sealants, Int. J. Technol. Ass. Health Care 1990, 6:369-377.

Ebert R.H., Florey H.W., The extravascular development of the monocyte observed in vivo, Br. J. Exp. Pathol. 1939, 20:342.

Ehrnford L., A method for reinforcing dental composite restorative materials, Odontol. Revy. 1976, 27:51-54.

Ehrnford L., Dental composite reinforced with microporous sintered glassfiber networks, Swed. Dent. J. 1983, Supplement 18:1-45.

Ellman G.L., Courtney K.D., Andres V., Featherstone R.M., A New and Rapid Colorimetric Determination of Acetylesterase Activity, Biochem Pharmacol 1961, 7:88-95.

Ewell, M., Dentists to Post Mercury Warnings, The Toronto Star, 1993, December 18, page J13.

Feigenbaum N. L., A renaissance of freehand bonding, Curr. Opin. Cobmet. Dent. 1994, 47-51.

Ferracane J.L., Greener E.H., The effect of resin formulation on the degree of conversion and mechanical properties of dental restorative resins, J. Biomed. Mater. Res. 1986, 8:224-228.

Ferracane J.L., Condon J.R., Rate of elution of leachable components from composites, Dent. Mat. 1990, 6:282-287.

Ferracane J.L., Condon J.R., Post-cure heat treatment for composites: properties and fractography, Dent. Mater. 1992, 8:290-295.

Ferracane I.L., Elution of leachable components from composites, J. Oral Rehab. 1994, 21:441-452.

Foldes F.F., Smith J.C., The Interaction of Human Cholinesterases with Anticholinesterases Used in the Therapy of Myasthenia Gravis, Ann. NY Acad. Sci. 1966, 135:287-301.

Fotos P.G., Diaz-Arnold A.M., Williams V.D., The effect of microbial contamination and pH changes in storage solutions during in vitro assay of bonding agents, Dent. Mater. 1990, 6:154-157.

Freund M., Munksgaard E.C., Enzymatic degradation of Bis-GMA/TEGDMA polymers causing decreased micro-hardness and greater wear *in vitro*, Scand. J. Dent. Res. 1990, 98:351-355.

Fung Y. K., Ewoldsen N. O., St. Germain H. A., Miaw C., Chou H-N, Gruninger S. E., Eichmiller F. C., Siew C., Pharmacokinetics of bisphenol A from a dental sealant in humans, Abstract #1316, IADR 77th General Session, Vancouver, Canada, March 10-13, 1999.

Gazi M.I., Cox S.W., Clark D.T., Eley B.M., A comparison of cysteine and serine proteinase in human gingival crevicular fluid with tissue, saliva and bacterial enzymes by analytical isoelectric focusing, Archs. Oral Biol. 1996, 5:393-400.

Genco, R.J., Slots J., Host responses in periodontal diseases, J. Dent. Res. 1984, 63(3):441-451

Gerzina T.M., Hume W.R., Effect of dentine on the release of TEGDMA from resin composites *in vitro*, J. Oral. Rehab. 1994, 21:463-468

Geurtsen W., Investigation into the duration of the toxicity of a composite, Dtsch. Zahnärztl Z. 1986, 41:496-498.

Geurtsen W., Long term effects of a composite on cultures human gingiva cells, J. Dent. Res. 1987, 68:330 (abstract #1789).

Geurtsen W., Substances released from dental resin composites and glass ionomers cements, Eur. J. Oral Sci. 1998a, 106:687-695.

Geurtsen W., Spahl W., Leyhausen G., Resudual Monomer/Additive Release and Variability in Cytotoxicity of Light-curing Glass-ionomer Cements and Compomers, J. Dent. Res. 1998b, 77(12):2012-2019.

Geurtsen W., Lehmann F., Spahl W., Leyhausen G., Cytotoxicity of 35 dental resin composite monomers/additives in permanent 3T3 and three human promary fibroblast cultures, J. Biomed. Mater. Res. 1998c, 41:474-480.

Glenn J.F., Comments of Dr. Bowen's presentation, J.Dent. Res. 1979, 58:1504-6.

Glenn J.F., Composition and properties of unfilled and composite resin restorative materials. In: Biocompatability of Dental Materials, eds. Smith D.C., Williams D.F., Boca Raton: CRC Press, 1982; V III. Pp. 97-130.

Goldman M., Polymerization shrinkage of resin-based restorative materials, Aust Dent J. 1983, Jun;28(3):156-61.

Greener E.H., Duke E.S., Chemistry and physical properties of crown and bridge veneering resins, J. Dent. Res., 1986, 65:166, Abstract S31.

Gregory W.A., Berry S., Duke E., Dennison J.B., Physical properties and repair bond strength of direct and indirect composite resins, J. Prosthet. Dent. 1992, 68:406-411.

Güven Y., Satman I., Dinccag N., Alptekin S., Salivary peroxidase activity in whole saliva of patients with insulin-dependent (type-1) diabetes mellitus, J. Clin. Periodontol. 1996, 23:879-881.

Hamid A., Hume W. R., A study of component release from resin pit and fissure sealants *in vitro*, Dent. Mater. 1997, 13:98-102.

Hanks C.T., Craig R.G., Diehl M.L., Pashley D.H., Cytotoxicity of dental composites and other materials in a new *in vitro* device, J. Oral Pathol. 1988, 17:396-403.

Hanks C.T, Strawn, S.E., Wataha J.C., Craig, R.G., Cytotoxic effects of resin components on cultured mammalian fibroblasts, J Dent Res. 1991, 70(11):1450-1455.

Hansel C., Leyhausen G., Mai U.E.H.., Geurtsen W., Effect of Various Resin Components (Co) monomers and Extracts on Two Caries-associated Micro-organisms *In vitro*, J. Dent. Res. 1998, 77(1):60-67.

Hansasuta C, Neiders ME, Aguirre A, Cohen RE, Cellular inflammatory responses to direct restorative composite resins, J Prosthet Dent 1993 Jun; 69(6):611-6.

Heil J., Reifferscheid G., Waldmann P., Leyhausen G., Geurtsen W., Genotoxicity of dental materials, Mutation Res. 1996, 368:181-194.

Horie K., Heat-treated composite resins for a denture base, Nippon Shika Ishikai Zasshi 1976, 28(11):1163-7.

Ingman T., Tervahartiala T., Ding Y., Tschesche H., Haerian A., Kinane D.F., Konttinen Y.T., Sorsa T., Matrix metalloproteinases and their inhibitors in gingival crevicular fluid and saliva id periodontitis patients, J. Clin. Periodontol. 1996, 23:1127-1132.

Inoue K., Hayashi I., Residual monomer (Bis-GMA) of composite resins, J. Oral Rehab. 1982, 9:493-467.

Ishida H., Koenig J.L., Molecular organization of the coupling agent interphase of fiber-glass reinforced plastics, J. Polym. Sci (polym. Phys. Ed.) 1979, 17:1807-1813.

Jaarda M. J., Wang R. F., Lang B. R., A regression analysis of filler particle content to predict composite wear, J. Prosth. Dent. 1997, 77:57-67.

Jaffer F., Finer Y., Santerre J.P., Biodegradation of Commercial Composite Resin by Human Saliva, submitted to– Sixth World Biomaterials Congress, Hawaii, USA, May 15-20, 2000.

Johannson O.K., Stark F.O., Vogel G.E., Fleischmann R.M., Evidence for chemical bond formation at silane coupling agent interfaces, J. Comp. Mater. 1967, 1:278-292.

Jones D.W., The enigma of amalgam in dentistry, J. Can. Dent. Assoc. 1993 ;59(2):155-60, 163-166

Jones D.W., Rizkalla A.S., Characterization of Experimental Composite Biomaterials, J. Biomed. Mater. Res. 1996, 33:89-100.

Kakaboura A.I., Eliades G.C., Palaghias , Laboratory evaluation of three visible light cured resinous liners, J. Den. 1996, 24:223-231.

Kalachandra S., Turner D.T., Water sorption of polymethacylate networks: Bis-GMA/TEGDMA copolymers, J. Biomed. Mater. Res. 1987, 21:329-338.

Kao E.C., Influence of food stimulating solvents on resin composites and glass ionomer restorative cement, Dent. Mater. 1989, 5:201-208.

Kidd E.A., Joyston-Bechal S., Beighton D., Micro-biological validation of assessments of caries activity during cavity preparation, Caries Res. 1993, 27:402-408.

Kilian R.J., Visible light cured composites: dependence of cure on light intensity, J. Dent. Res 1979., Special Issue 243, Abstract #603.

Kinomoto Y, Torii M, Takeshige F, Ebisu S, Comparison of polymerization contraction stresses between self- and light-curing composites, J Dent 1999 Jul, 27(5):383-9.

Klimek J., Jung M., Jung S., Interindividual differences in degradation of sodium monofluorophosphatate by saliva in relation to oral health status, Archs. Oral Biol. 1997, 42(2):181-184.

Koda T., Tsuchiya H., Yamauchi M., Hoshino Y., Takagi N., Kawano J., High-performance liquid chromatographic estimation of eluates from denture base polymers, J Dent. 1988, 17:84-89.

Koda T., Tsuchiya H., Yamauchi M., Ohtani S., Takagi N., Kawano J., Leachability of denture-base acrylic resins in artificial saliva, Dent. Mater. 1990, 6:13-16.

Kula K., McKinney J.E., Kula. T.J., Effects of daily topical fluoride on resin composite degradation and wear, Dent. Mater. 1997, 13:305-311.

Labow R.S., Adams K.A.H. and Lynn K.R., Porcine Cholesterol esterase, A Multiform Enzyme, Biochim. Biophys. Acta. 1983, 749:32-41.

Labow R.S., Duguay D., Santerre J.P., The hydrolysis of a synthetic biomembrane: A new substrate for cholesterol and carboxyl esterases, J. Biomater. Sci. Polymer Edn. 1994, 6(2):169-179.

Labow R.S., Erfle D.J., Santerre J.P., Neutrophil-mediated degradation of segmented polyurethanes, Biomaterials 1995, 16:51-59.

Lang B.R., Jaarda M., Wang R.F., Filler particle and composite resin classification systems, J.Oral Rehab. 1992, 19:569-584.

Lappin D.F., Koulouri O., Tdver M., Hodge P., Kinane D.F., Relative proportions of mononuclear cell types in periodontal lesions analyzed by immunohistochemistry, J. Clin. Periodontol. 1999, 26:183-189.

Larsen I.B., Munksgaard E.C., Effect of human saliva on surface degradation of composite resins, Scand J Den Res. 1991, 99:254-261.

Larsen I.B., Freund M., Munksgaard E.C., Change in surface hardness of Bis-GMA/TEGDMA polymer due to enzymatic action, J. Dent. Res. 1992, 71(11):1851-1853.

Lee S.Y., Greener E.H., Mueller H.J., Chiu C.H., Effect of food and oral stimulating fluids on dentin bond and composite strength, J. Dent. 1994, 22:352.

Lee S.Y., Huang H.M., Lin C.Y., Shin Y.H., Leached components from dental composites in oral simulating fluids and the resultant composite strength, J. Oral Rehabil. 1998, 25:575-588.

Lefebvre C., Schuster G.S., Marr J.C., Knoernschild K.L., The effect of pH on the cytotoxicity of eluates from denture base resins, Int J Prost. 1995, 8(2):122-128.

Lehman F., Leyhausen G., Spahl W., Geurtsen W., Comparative cell culture studies of the cytotoxicity of composite, Dtsch. Zahnärztl Z. 1993, 48:651-653.

Leinfelder K.F., Using composite resin components as posterior restorative materials, J. Am. Dent. Assoc. 1991, 122(4):65-70.

Lenader-Lumikari M., Laurikainen, Kuusisto P., Vilja P., Stimulated salivary flow rate and composition in asthmatic and non-asthmatic adults, Archives of Oral Biol. 1998, 43:151-156.

Letzel H., Survival rates and reasons for failure of posterior composite restorations in multicentre clinical trial, J. Dent. 1989,17(Suppl. 1):S10-S17.

Leung B.W.H., Shajii L., Jaffer F., Santerre J.P., Role of Surfactants in the Biodegradation of Commercial Bis-GMA based Resins by Esterase, in the 26th Annual Meeting of the American Association for Dental Research (AADR), Orlando, Fl, USA, March 19-23 1997, J. Dent. Res. 76SE:321, abstract #2460.

Li T., Craig R.G., Synthesis of fluorinated Bis-GMA and its use with other fluorinated monomers to formulate hydrophobic composites, J. Oral. Rehab. 1996, 23:158-162.

Li, F., Hui, D. Y., "Modified low density lipoprotein enhances the secretion of bile salt-stimulated cholesterol esterase by human monocyte-macrophages", J. Biol. Chem., 1997, 272, 28666-28671

Lindhorst, E., Young, D., Bagshaw, W., Hyland, M., Kisilevsky, R., Acute inflammation, acute phase serum amyloid A and cholesterol metabolism in the mouse, Biochim, Biophys. Acta, 1997, 1339:143-154.

Lindqvist L., Nord C.E., Söder P.O., Origin of esterase in human whole saliva, Enzyme 1977, 22:166-175.

Loesch W. J., Bretz W. A., Lopatin D., Stoll J., Rau C. F., Hillenburg K. L., Killoy W. J., Drisko C. L., Williams R., Hujoel P. P., Multi-center clinical evaluation of a chairside method for detecting certain periodonthopathic bacteria in periodontal disease, J. Periodontol. 1990, 61:189-196.

Loesche W.J., Robinson J.P., Flynn M., Hudson J.L., Duque R.E., Reduced oxidative function in gingival crevicular neutrophils in periodontal disease, Infection and Immunity 1988, 56(1):156-160.

Lomabardo D., Guy O., Effect of alcohols on the hydrolysis catalyzed by human pancreatic carboxylic-ester hydrolase, Biochim. Biophys. Acta 1981, 657:425-437.

Lundgren T., Twetman S., Johannson I., Crossner C.G., Birkhed D., Saliva composition in children and young adults with Papillon-Lefèver syndrome, J. Clin Periodontol 1996, 23:1068-1072.

Mair, L.H., Surface permeability and degradation of dental composites resulting from oral temperature changes, Dent. Mater 1989, 5:247-255.

Mair L.H., Effect of surface conditioning on the abrasion rate of dental composites, J. Dent. 1991, 19:100-106.

McCarthy S.J., Meijs F. Gordon, Mitchell Natasha, Guantillake A. Pathiraja, Heath Greame, Bradwood Arthur and Schindhelm Klaus, *In-vivo* degradation of polyurethanes: transmission-FTIR microscopic characterization of polyurethanes sectioned by cryomicrotomy, Biomaterials 1997, 18:1387-1409.

Maffezzoli A., Della Pietra A., Rengo S., Nicolais L., Valletta G., Photopolymerization of dental composite matrices, Biomaterials 1994, 15(15):1221-1228.

McHugh H.D., Statement: Effects and side-effects of dental restorative materials, Proceeding of the NIH Technology Assessment Conference on Effects and Side-effects of Dental Restorative Materials, August 26-28, 1991. Adv. Dent. Res. 1992, 6:139-144.

Mohsen M.N., Craig R.G., Effect of silanation of fillers on their dispersability by monomer systems, J. Oral. Rehab. 1995, 22:183-189.

Munk P., "Introduction to macromolecular science", John Wiley and Sons, New York, pp.139-144 (1989)

Munksgaard E.C. and Freund M., Enzymatic hydrolysis of (di)methacrylates and their polymers, Scan. J. Dent. Res. 1990, 98:261-267.

Nagel S.C., vom Saal F.S., Thayer K.A., Dhar M.G., Boechler M., Welshons W.V., Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative in vivo activity of the xenoestrogen bisphenol A and octylphenol, Environ. Health Perspect. 1997, 105:70-76.

Nakamura M., Slots J., Salivary enzymes origin and relationship to periodontal disease, J. Perio. Res. 1983a, 18:559-569.

Nakamura M., Koda H., Kawahara H., A proposition for long-term biocompatabilty test of dental materials in a vitro, Dent. Mater. J. 1983b, 2:113-123.

Nassiri R., Hanks C.T., Cameron M.J., Strawn S., Craig R., Application of flow cytometry to determine the cytotoxicity of urethane dimethacrylate in human cells, J. Biomed. Mater. Res. 1994, 28:153-158.

Nathanson D., Lertpitayakun P, Lamkin M., Edalatpour M., Chou L.L., In Vitro Elution of Leachable Components from Dental Sealants, J. Am. Dent. Assoc. 1997, 128:1516-1523.

Nomoto R., Uchida K. and Hirasawa T, Effect of light intensity on polymerization of light-cured composite resins, Dent. Mater. J. 1994, 13(2):198-205.

Øilo G., Biodegradation of Dental Composites/Glass-ionomer Cements, Adv., Dent. Res. 1992, 6:50-54.

Olea N., Pulgar R., Perez P., Olea Serrano F., Rivas A., Novillo-Fertrell A., Pedraza V., Soto A.M., Sonnenschein C., Estrogenicity of Resin-Based Composites and Sealants Used in Dentistry, Environ. Health Perspect. 1996, 104(3):298-305.

Øysæd H., Ruyter I.E., Sjøvik K., Release of formaldehyde from dental composites, J Dent Res. 1988, 67(10):1289-1294.

Papagiannoulis L., Tzoutzas J., Eliades G., Effect of topical fluoride agents on the morphological characteristics and composition of resin composite restorative materials, j. Prosthet Dent. 1997, 77(4):405-413.

Park S.H., Lee C.S., The Difference in Degree of Conversion between Light-Cured and Additional Heat-cured Composite, Oper. Dent. 1996, 21:213-217.

Pavia D. L., Lampman G. M., Kriz G. S., Introduction to spectroscopy: A guide for students of organic chemistry, Philadelphia: Saunders College, 1979.

Payne W.A, Page R.C., Oglivie A.L., Hall W.B., Histopathologic and features of the initial and early stages of experimental gingivitis in man, J. Periodont. Res. 1975, 10:51.

Pearson G.J., Long-term water sorption and solubility of composite filling materials, J. of Den. 1979, 7:64.

Pearson G.J., Longman C.M., Water sorption and solubility of resin-based materials following inadequate polymerization by visible-light curing system, J. Oral Rehabil. 1989, 16:64.

Pederson E.D., Miller J.W., Matherson S., Simonson L. G., Chadwick D.E., Covill P.J., Turner D.W., Lamberts B.L., Morton H.E., Trypsin-like activity levels of *Treponema denticola and Porphyromonas gingivalis* in adults with periodontitis, J. Clin. Periodontol. 1994, 21:519-525.

Pershad N., Chakravarti N., Finer Y. and J.P. Santerre, Effect of Saliva-like Esterase Activities on Micro-Filled Dental Composites, Abstract #1667, IADR 77th General Session, Vancouver, Canada, March 10-13, 1999.

Peters D.G., Hayes J.M., Hieftje M.G., Chemical separations and measurements Theory and practice of analytical chemistry, Saunders Golden Sunburt Series, Philadelphia, pp.647, 1974

Peutzfeldt A., Correlation between recording obtained with a light intensity tester and degree of conversion of a light-curing resin, Scand. J. Dent. Res. 1994, 102:73-75.

Prati C., Nucci C., Davidson C.L., Montanary G., Early marginal leakage and shear bond strength of adhesive restorative systems, Dent. Mater. 1990, 6:195-200.

Quin D.M., Acetylcholinesterase: Enzyme structure, reaction dynamics, and virtual transition state, Chem. Rev. 1987, 87:955-979.

Quin D.M., Selwood T., Pryor A. N., Lee B. H., Leu L-S., Acheson S A., Silman I., Doctor B.P., Rosenberry T. L., Cryptic catalysis and cholinesterase function, In "Multidisciplinary Approaches to Cholinesterase" (Shaefferman A., Velan B., eds.) pp. 141-148, New York: Plenum Press 1992.

Radic Z., Reiner E., Taylor P., Role of peripheral anionic site on acetylcholionesterase: Inhibition by substrates and coumarin derivatives, Mol. Pharmacol. 1991, 39:998-1104.

Rallof J., That feminine touch: Are men suffering from prenatal or childhood exposures to "hormonal" toxicants?, Science News 1994, 145:56-58.

Rathbun M.A., Craig R.C., Hanks C.T., Filisko F.E., Cytotoxicity of a Bis-GMA dental composite before and after leaching in organic solvents, J. Biomed. Mater. Res. 1991, 25:443-457.

Rose E.E. et al, Direct resin filling material: coefficient of thermal expansion and water sorption of polymethyl metahcrylate. J. Dent. Res. 1955a, 34:589-596.

Rose E.E. et al, The screening of materials for adhesion to human tooth structure. J. Dent. Res. 1955b, 34:577-588.

Roulet, J.F., Walti C., Influence of Oral Fluid on Composite Resin and Glass Ionomer Cement, J. Prosthet. Dent. 1984, 52:182-189.

Roulet, J.F., Degradation of dental polymers, Basel Karger ed., New York 1987, pp.1.

Rueggeberg F.A., Hashinger D.T. and Fairhurst C.W., Calibration of FTIR conversion analysis of contemporary dental resin composites, Dental Materials 1990, 6(4):241-249.

Rueggeberg F.A., Ergle J.W., Lockwood P.E., Effect of photoinitiator level on properties of a light-cured and post-cure heated model rein system, Dent. Mater. 1997, 13:360-364.

Ruyter I. E., Sjøvik I.J., Composition of dental resin and composite materials, Acta Odontol. Scand. 1981, 39:133-146.

Ruyter I.E., Øysæd H., Conversion in different depths of ultraviolet and visible light activated composite materials, Acta Odontol. Scand. 1982, 40:179-192.

Ruyter I.E., "Monomer systems and polymerization", in "Posterior composite resin dental restorative materials", ed. Smith D.C., Peter Szulc Publishing Co., Netherlands 1985, pp.109-135.

Ruyter I. E., Øysæd H., Composite for use in posterior teeth: composition and conversion, J. Biomed. Mater. Res. 1987a, 21:11-23.

Ruyter I.E., Sjøvik Kleven I.J., Monomers and filler content of resin-based crown and bridge materials, Dent. Mater. 1987b, 3:315-321.

Ruyter I.E., Composites—characterization of composite filling materials: reactor response, Adv. Dent. Res. 1988, 2(1):122-129.

Ryhänen R., Närhi M., Puhakainen E., Häanninen O., Kontturi-Närhi V., Pseudo cholinesterase Activity and Its Origin in Human Oral Fluids, J. Dent. Res. 1983a, 62(1):20-23.

Ryhänen R., Pseudocholinesterase activity in some human fluids, Gen. Pharmacol. 1983b, 14(4):459-60.

Saboori A. M., Newcombe D. S., Human monocyte carboxylesterase- Purification and kineticsJ. Biol. Chem. 1990, 265:19792-19799.

Safe F., Is there an association between exposure to environmental estrogens and breast cancer? Envir. Health Perspect. 1997, 105(3):675-678.

Sanders B, Schreiber R., Synthesis and polymerization of epoxymethacrylates, Catalysis and kinetics of the addition reaction of methacrylic acid and 2,2-bis[4-(2,3-epoxypropoxy)phenyl]propane, Makromol. Chem 1992, 193:2763-2770.

Sanders B., Baudach S., Davy K.W.M., Braden M., Synthesis of BISGMA derivatives, properties of their polymers and composites, J. Mater. Sci. Mater. Med. 1997, 8:39-44.

Sankarapandian M., Shobha H.K., Kalachandra S., Mcgrath J.F., Taylor D.F., Characterization of some aromatic dimethacrylates for dental composite applications, J. Mater. Sci.: Mat. Med. 1997, 8:465-468.

Santerre J.P., Labow R.S., Adams G., Enzyme-biomaterial interactions: effects of biosystems on degradation of polyurethanes, J. Biomed. Mater. Res. 1993, 27:97-109.

Santerre J.P., Labow R.S., Duguay D.G., Adams G., Erfle D., Biodegradation evaluation of polyether and polyester-urethanes with oxidative and hydrolytic enzymes, J. Biomed. Mater. Res. 1994, 28:1187-1199.

Santerre J.P., Labow R.S., The effect of hard segment size on the hydrolytic stability of polyether-urea-urethanes when exposed to cholesterol esterase, J. Biomed. Mater. Res. 1997, 36:223-232.

Santerre JP, Shajii L, Tsang H, Biodegradation of commercial dental composites by cholesterol esterase, J. Dent. Res. 1999 Aug; 78(8):1459-68.

Schafer T.E., Lapp C.A., Hanes C.M., Lewis J.B., Wataha J.C., Schuster G.S., Estrogenicity of bisphenol A and bisphenol A dimethacrylate *in vitro*, J. Biomed. Mater. Res. 1999, 45:192-197.

Schuster G.S., Oral microbiology and infectious disease, 3rd Ed., B.C. Decker Inc., Toronto, Canada 1990, p. 472.

Schweikl H., Schmalz G., Rackebrandt K., The mutagenic activity of unpolymerized resin monomers in *Salmonella typhimurium* and V79 cells, Mutation Research 1998, 119-130.

Shajii L., Santerre J.P., Effect of filler content on the profile of released biodegradation products in micro-filled bis-GMA/TEGDMA dental composite resins, Biomaterials 1999, 20:1897-1908.

Shajii L., Isolation and Identification of biodegradation products released from dental composite resins with varying concentrations of resin and filler, M.Sc. Thesis, Graduate Department of Chemical Engineering and Applied Chemistry, University of Toronto 1997.

Seymour J., Dockrell H.M., Greenspan J.S., Enzyme differentiation of lymphocyte subpopulation in sections of human lymph nodes, tonsils and periodontal disease, Sci. Exp. Immunol. 1978, 32:169.

Shintani H., Tsuchiya T., Hata Y., Nakamura A., Solid phase extraction and HPLC analysis of toxic components eluted from methyl methacrylate dental materials", J Analyt Toxicol. 1993, 17:73-78.

Slots J., Subgingival microflora and periodontal disease, J Clin Periodontol 1979 Oct; 6(5):351-82.

Slots J., Rosling B. G., Suppression of the periodontopathic microflora in localized juvenile periodontitis by systemic tetracycline, J. Clin. Periodontol. 1983, 10(5):465-86.

Smith D.C., "Posterior composite resin dental restorative materials: materials development", in "Posterior composite resin dental restorative materials", ed. Smith D.C., Peter Szulc Publishing Co. 1985, Netherlands, pp. 47-60.

Söderholm, K.J.M., Leaking of Fillers in Dental Composites, J. Dent. Res. 1983, 62:126-130.

Söderholm K.J., Zigan M., Ragan M., Fischlschweiger W., Bergman M., Hydrolytic degradation of dental composites, J Dent Res. 1984, 63(10):1248-1254.

Söderholm, K.J.M., "Filler systems and resin interface", in "Posterior composite resin dental restorative materials", ed. Smith D.C., Peter Szulc Publishing Co., Netherlands 1985, pp.139-160.

Söderholm K.J.M., Shang S.W., Molecular orientation of silane at the surface of colloidal silica, J. Den. Res. 1993, 72(6):1050-1054.

Söderholm K.J.M., Mukherjee R., Longmate J., Filler Leachability of Composites Stored in Distilled Water of Artificial Saliva, J. Dent. Res. 1996, 75(9):1692-1699.

Söderholm K.J.M., Richards N.D., Wear resistance of composites: A solved problem?, Dent. Mater. 1998, 46(3):256-263.

Söderholm K.J.M., Mariotti A., BIS-GMA-based resins in dentistry: are they safe?, JADA 1999, 130:201-209.

Sonnenborn U., Eiteljorge G., Ttrzeciak W. H., Kunace W. H., Identical catalytic subunit in both molecular forms of hormone sensitive CE from bovine adrenal cortex, FEBS letters 1982, 145(2):271-276.

Soreq H., Gnatt A., Lowenstein Y., Neville L. F., Excavations into the active site gorge of cholinesterases, Trends in Biochem. Sci. 1992, 17:353-358.

Spahl W., Budzikiewicz H., Geurtsen W., Determination of leachable components from four commercial dental composites by gas and liquid chromatography/mass spectrometry, J Dent 1998, 26(2):137-145.

Stanley H. R., Bowen R.L., Folio J., Compatability of Various Materials with Oral Tissues. II: Pulp Responses to Composite Ingredients, J Dent Res. 1979, 58(5):1507-1517.

Stanley H. R., Local and systemic responses to dental composites and glass ionomers, Adv Dent Res. 1992, 6:55-64.

Stanley H.R., Effects of Dental Restorative Materials: local and systemic responses reviewed, JADA 1993, 124:76-80.

St. Pierre, T., Chiellini E., Biodegradability of Synthetic Polymers Used for Medical and Pharmaceutical Applications: Part 1- Principles of Hydrolysis Mechanisms, J. Bioactive and Compatible Polymers 1986, 1:467-497.

Sutton L. D., Stout J. S., Hosie L., Spencer P. S., Quin D. M., Phenyl-n-Butylboronic Acid is a Potent Transition State Analog Inhibitor of Lipolytic Enzymes, Biochem. Biophys Res. Com. 1986, 134(1):386-392.

Sutton L. D., Lantz J. L., Eibes T., Quin D. M., Dimensional mapping of the active site of cholesterol esterase with alkyboronic acid inhibitord, Biochim. Biophys. Acta. 1990, 1041:79-82.

Sutton L. D., Froelich S., Hendrickson H. S., Quin D. M., Cholesterol Esterase Catalyzed Hydrolysis of Mixed Micellar Thiophosphatidylcholine: A Possible Charg-Relay Mechanism, Biochemistry 1991, 30:5888-5893.

Svanberg M., Mjör I.A., Ørstavik D., Mutans streptococci in plaque from margins of amalgam, composite, and glass-ionomer restorations, J.Dent. Res. 1990, 69:861-864.

Tanaka K., Taira M., Shintani H., Wakasa K., Yamaki, M., Residual monomers (TEGDMA and Bis-GMA) of a set of visible light-cured dental composite resin when immersed in water, J.Oral Rehab 1991, 18:353-362.

Tarle Z., Meniga A., Ristic M., Sutalo J., Pichler G., Davidson C.L., The effect of the photopolymerization method on the quality of composite resin samples, J. Oral Rehabil. 1998, 25:436-442.

Taylor P., Agents acting at the neuromuscular junction and autonomic ganglia, In "The Pharmacological Basis of Therapeutics" (Gilman A. G., Goodman L.S., Murad F., eds.), pp. 110-129, New York: MacMillan 1990.

Tenovuo J., Patients with locally and generally reduced host defense, Clin. Periodontol. 1990, 17(7(Pt 2)):525-526.

Treybal R.E., Mass transfer operations, McGraw Hill, New York, 3rd edition 1987, pp.4.

Truong V.T., Tyas M.J., Prediction of in vivo wear in posterior composite resins: a fracture mechanisms approach, Dent. Mater. 1988, 4:318.

Tsang H., Biodegradation of commercial composite resins by cholesterol esterase, Summer student project report, Faculty of Dentistry, University of Toronto, Toronto 1994.

van Groeningen G., Jongebloed W., Arends J., Composite degradation *in vivo*, Dent. Mater. 1986, 2:225-227.

Vaubert V.M., Moon P.C., Love B.J., Extractable Free Monomers from Self-Cured Dental Sealants Resulting from Dispensing Errors, J. Biomed. Mater. Res. 1999, 48:5-8.

Venhoven B.A.M., de Gee A.J., Davidson C.L., Polymerization contraction and conversion of light-curing BisGMA-based methacrylate resins, Biomaterials 1993, 14:871-875.

Venz S., Dickens B., NIR-spectroscopic investigation of water sorption characteristics of dental resins and composites, J. Biomed. Mater. Res. 1991, 25:1231-1248.

Vom Saal F.S., Cooke P.S., Buchanan D.L., Planza P., Thayer K.A., Nagel S.C., Parmigiani S., Welshons W.V., A physiologically based approach to the study of bisphenol A and other estrogenic chemicals on the size of reproductive organs, daily sperm production, and behavior, Toxicol. Indust. Health 1998, 14(1-2):239-260.

Wang GB, Santerre JP, Labow RS, High-performance liquid chromatographic separation and tandem mass spectrometric identification of breakdown products associated with the biological hydrolysis of a biomedical polyurethane, J Chromatogr B Biomed Sci Appl 1997 Sep 26;698(1-2):69-80.

Wataha J.C., Hanks C.T., Strawn S.E., Fat J.C., Cytotoxicity of components of resins and other dental restorative materials, J. Oral. Rehab. 1994, 21:453-462.

Watts D.C., McNaughton V., Grant A.A., The development of surface hardness in visible light-cured posterior composites, J.Dent. 1986, 14:169-174.

Wendt S.L., The effect of heat used as a secondary cure upon the physical properties of three composite resins. I. Diametrical tensile strength, compressive strength and marginal dimensional stability, Quintessence Int. 1987, 18:265-271.

Whittaker M., "Cholinesterases", Basel Karger ed., New York 1986.

Williams D.F., Definitions in biomaterials, Eur. Soci. Biomat. 1987, Amsterdam: Elsveir 1987.

Williams F. M., Clinical Significance of Esterases in Man, Clinc. Pharmaco. 1985, 10:392-403.

Winkler M.M., Greener E.H., Lautenschlager E.P., Non linear *in vitro* wear of posterior composites with time, Dent. Mater. 1991, 7:258-262.

Wu W., McKinney J.E., Influence of Chemicals on Wear of Dental Composites, J. Dent. Res. 1982, 61(10):1180-1183.

Wu W., Fanconi B.M., Post-curing of dental restorative resin polymer, Eng. Science 1983, 23(13):704-707.

Xu J., Butler I.S., Gibson D.F.R. and Stangel I., High-pressure infrared and FT-Raman investigation of a dental composite, Biomaterials 1997, 18(24):1653-1657.

Yamalik N., Özer N., Çagayan F., Çagayan G., Determination of Pseudocholinesterase Activity in the Gingival Crevicular Fluid, Saliva, and Serum from Patients with Juvenile Periodontitis and Rapidly Progressive Periodontitis, J. Dent. Res. 1990, 69(1):87-89.

Yamalik N., Özer N., Çagayan F., Çagayan G., Akdoganli T., The Effect of Periodontal Therapy on Salivary Pseudocholinesterase Activity, J. Dent. Res. 1991, 70(6):988-990.

Yamamoto K, Noda H, Kimura K, Adherence of oral streptococci to composite resin restorative materials, J Dent 1989 Oct; 17(5):225-9.

Yoshida K, Greener E.H., Effects of two amine reducing agents on the degree of conversion and physical properties of an unfilled light-cured resin, Dent. Mat. 1993, 9:246-251.

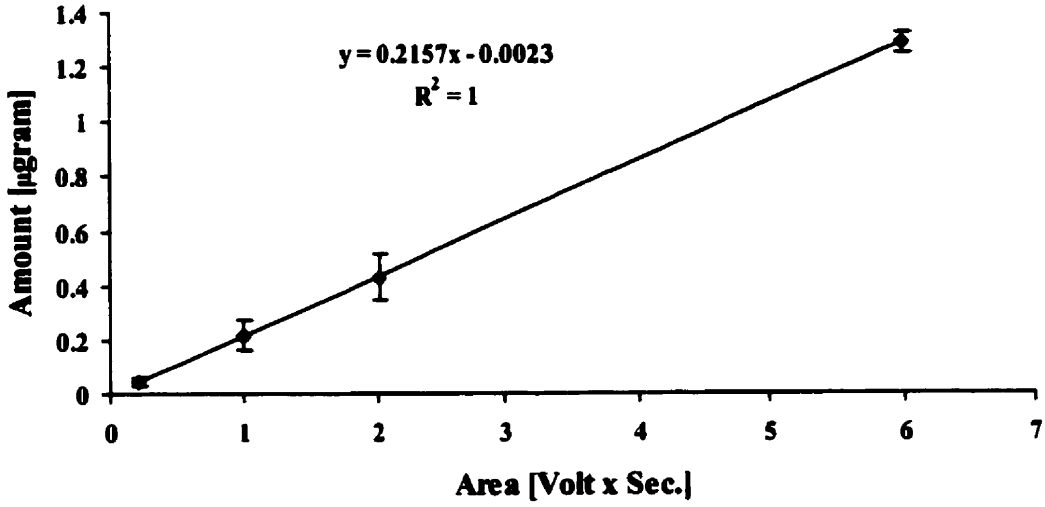
Young R.J., Lovell, P.A., Introduction to polymers, Chapman and Hall, London U.K. 1991, pp.43-51.

Zalkind M.M., Keisar O., Ever-Hadani, Grinberg R., Sela M.N., Accumulation of *Streptococcus mutans* on Light-Cured Composites and Amalgam: An In Vitro Study, J. Esthet. Dent. 1998, 10(4):187-190.

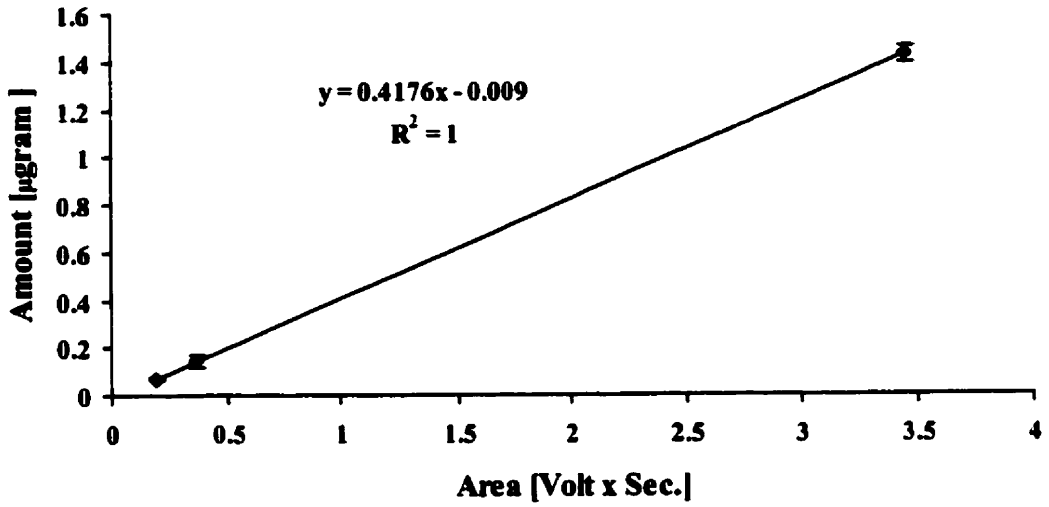
Zambon J.J., Nakamura M., Slots J, Effect of periodontal therapy on salivary enzymatic activity, J.Perio.Res 1985, 20:652-659.

8.0 APPENDICES

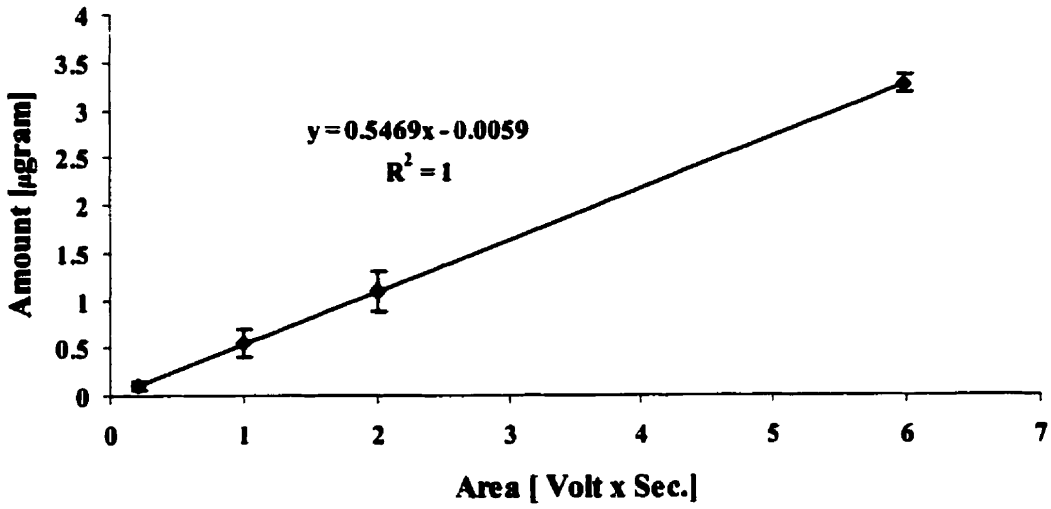
8.1 METHACRYLIC ACID CALIBRATION CURVE



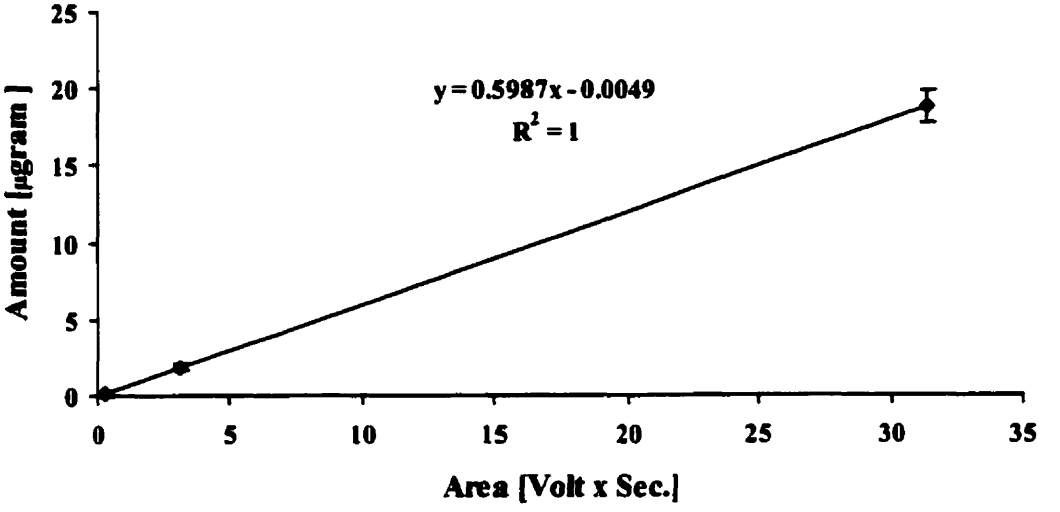
8.2 TEGDMA CALIBRATION CURVE



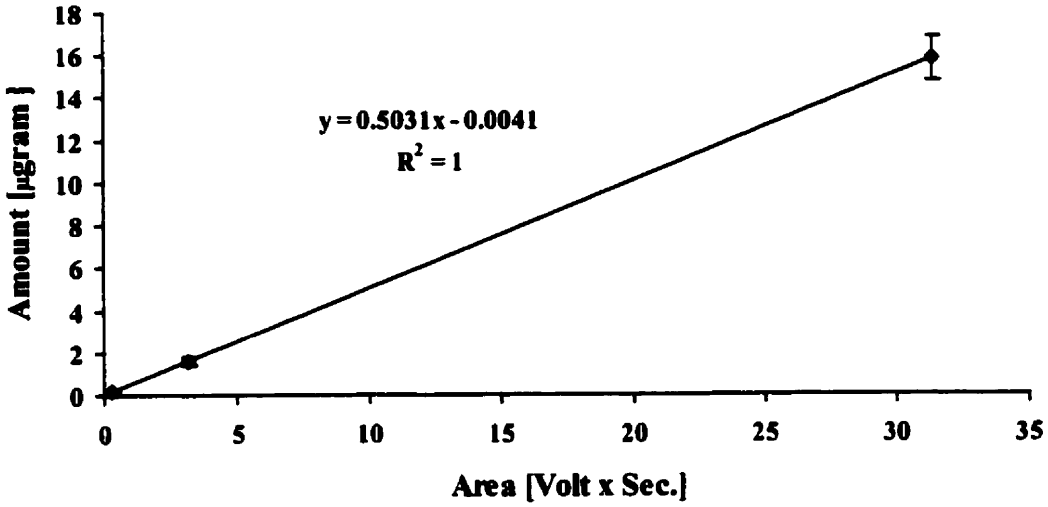
8.3 TEGMA CALIBRATION CURVE BASED ON METHACRYLIC ACID AS A MODEL PRODUCT



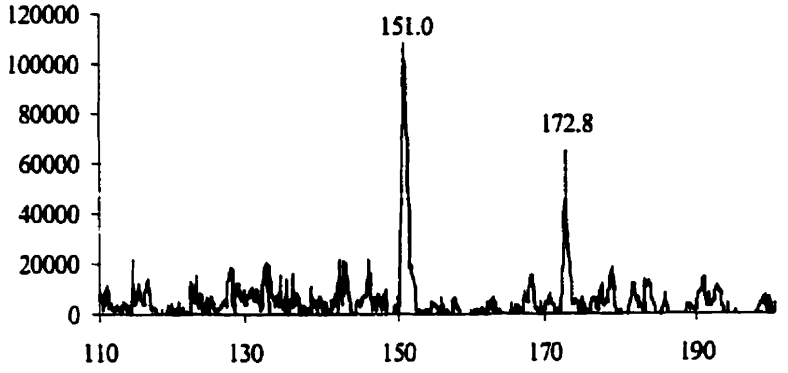
8.4 bisHPPP CALIBRATION CURVE BASED ON bisPHENOL A PROPOXYLATE AS A MODEL PRODUCT



8.5 E-bisPA CALIBRATION CURVE BASED ON bisPHENOL A PROPOXYLATE AS A MODEL PRODUCT

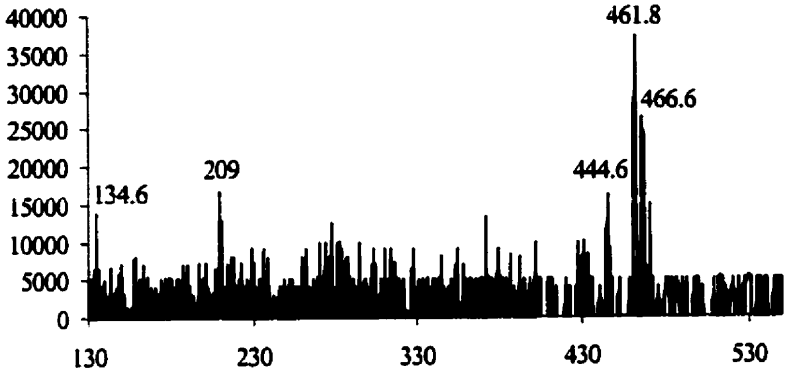


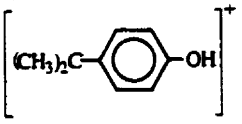
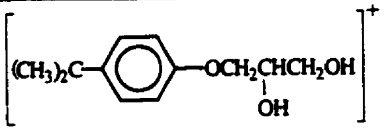
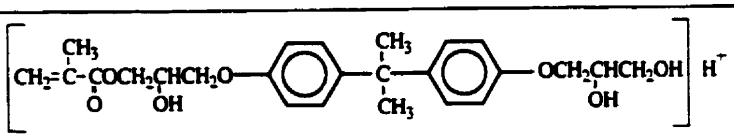
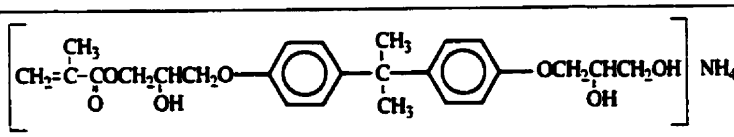
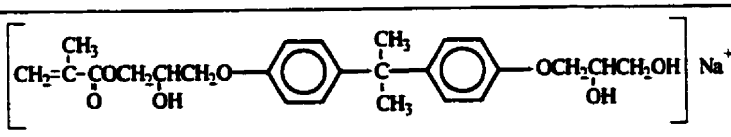
8.6 MASS SPECTRUM (MS) OF TRIETHYLENE GLYCOL



m/z	Ion chemical structure
151	$[HO\text{-}(CH_2CH_2O)_3H]H^+$
173	$[HO\text{-}(CH_2CH_2O)_3H]Na^+$

8.7 MASS SPECTRUM (MS) OF MONO-VINYL bisGMA



m/z	Ion chemical structure
135	
209	
445	
462	
467	

8.8 HPLC CHROMATOGRAM OF A STANDARD bisGMA MONOMER