Biodegradation of Dental Resin Composites and Adhesives by

*Streptococcus mutans*: An *in vitro* Study

By

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Biodegradation of Dental Resin Composites and Adhesives by
Streptococcus mutans: An in vitro Study

Masters of Applied Science, 2013

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ABSTRACT
A major cause for dental resin composite restoration replacement is secondary caries attributed to Streptococcus mutans. Salivary esterases were shown to degrade resin composites. Hypothesis: S. mutans contain esterase activities that degrade dental resin composites and adhesives. Esterase activities of S. mutans were measured using synthetic substrates. Standardized specimens of resin composite (Z250), total-etch (Scotchbond-Multipurpose, SB), and self-etch (Easybond, EB) adhesives were incubated with S. mutans UA159 for up to 30 days. Quantification of a bisphenol-glycidyl-dimethacrylate (BisGMA)-derived biodegradation by-product, bishydroxy-propoxy-phenyl-propane (BisHPPP) was performed using high performance liquid chromatography. Results: S. mutans were shown to contain esterase activities in levels comparable to human saliva. A trend of increasing BisHPPP release throughout the incubation period was observed for all materials and was elevated in the presence of bacteria vs. control for EB and Z250
(p<0.05) but not SB. Conclusion: biodegradation by cariogenic bacteria could compromise the resin-dentin interface and reduce the longevity of the restoration.
Acknowledgements:

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# Table of Contents

Acknowledgements: iv

List of Abbreviations: vii

List of Figures: x

List of Tables: xi

Chapter 1 – Introduction: 1
  Hypothesis: 3
  Objectives: 3
  References: 4

Chapter 2 – Literature review: 6
  2.1 Preamble: 6
  2.2 Resin composites: 6
    2.2.1 The matrix: 7
    2.2.2 Filler systems: 9
    2.2.3 Polymerization systems: 12
  2.3 Dental resin adhesives: 12
    2.3.1 Total-etch systems: 13
    2.3.2 Self-etch systems: 14
  2.4 Degradation of dental resin composites and adhesives: 15
    2.4.1 Degradation by human salivary esterase activity: 18
    2.4.2 Degradation by model esterases (cholesterol esterase and pseudocholinesterase): 21
    2.4.3 Degradation of resin-dentin interfacial margins: 23
  2.5 Interactions between bacteria and dental resin composites and adhesives: 27
  2.6 Summary: 31
  2.7 References: 32

Chapter 3 – Cariogenic Bacteria Degrades Dental Resin Composite and Adhesives: 36
  3.1 Introduction: 36
  3.2 Materials and methods: 37
    3.2.1 Bacterial esterase activity assay: 37
    3.2.2 Preparation of composite and adhesive resin specimens: 38
    3.2.3 Degree of vinyl group conversion at the surface: 38
    3.2.4 X-ray photoelectron spectroscopy: 38
    3.2.5 Contact angle measurements: 38
    3.2.6 Biodegradation experiments: 39
    3.2.7 Scanning electron microscopy: 39
    3.2.8 Statistical analysis: 39
  3.3 Results: 39
Chapter 3 – Biodegradation Studies: 

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3.1 Bacterial esterase activity assay:</td>
<td>39</td>
</tr>
<tr>
<td>3.3.2 Material characterization:</td>
<td>40</td>
</tr>
<tr>
<td>3.3.3 Biodegradation:</td>
<td>40</td>
</tr>
<tr>
<td>3.4 Discussion:</td>
<td>41</td>
</tr>
<tr>
<td>3.5 Acknowledgements:</td>
<td>45</td>
</tr>
<tr>
<td>3.5 References:</td>
<td>46</td>
</tr>
<tr>
<td>3.6 Figures:</td>
<td>49</td>
</tr>
</tbody>
</table>

Chapter 4 – Cariogenic bacteria studies: 

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1 Extended materials and methods:</td>
<td>53</td>
</tr>
<tr>
<td>4.1.1 Bacterial strains:</td>
<td>53</td>
</tr>
<tr>
<td>4.1.2 Bacterial CE-like activity assay:</td>
<td>53</td>
</tr>
<tr>
<td>4.1.3 Bacterial PCE-like activity assay:</td>
<td>54</td>
</tr>
<tr>
<td>4.1.4 Bacterial esterase stability assays:</td>
<td>56</td>
</tr>
<tr>
<td>4.1.5 Construction of ΔUA159_SMU.118c:</td>
<td>56</td>
</tr>
<tr>
<td>4.1.6 Construction of UA159_SMU.118c+:</td>
<td>58</td>
</tr>
<tr>
<td>4.1.7 Bacterial esterase-like activity profile assay:</td>
<td>60</td>
</tr>
<tr>
<td>4.1.8 Monomer degradation:</td>
<td>60</td>
</tr>
<tr>
<td>4.1.9 High performance liquid chromatography (HPLC):</td>
<td>61</td>
</tr>
<tr>
<td>4.2 Extended results and discussion:</td>
<td>63</td>
</tr>
<tr>
<td>4.2.1 CE-like and PCE-like activity assays:</td>
<td>63</td>
</tr>
<tr>
<td>4.2.2 Bacterial esterase stability assays:</td>
<td>65</td>
</tr>
<tr>
<td>4.2.3 Bacterial esterase-like activity profile assay:</td>
<td>67</td>
</tr>
<tr>
<td>4.2.4 Monomer degradation:</td>
<td>68</td>
</tr>
<tr>
<td>4.3 References:</td>
<td>71</td>
</tr>
</tbody>
</table>

Chapter 5 – Conclusions and recommendations: 

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1 Conclusions:</td>
<td>73</td>
</tr>
<tr>
<td>5.2 Recommendations:</td>
<td>74</td>
</tr>
<tr>
<td>5.3 References:</td>
<td>76</td>
</tr>
</tbody>
</table>
List of Abbreviations:

BHI  Brain heart infusion

BisEMA  Bisphenol A polyethylene glycol diether dimethacrylate

BisGMA  Bisphenol glycidyl dimethacrylate

BisHPPP  Bishydroxypropoxyphenylpropane

BTC  Butyrylthiocholine iodide

CE  Cholesterol esterase

CSP  Competence stimulating peptide

DEGDMA  Diethylene glycol dimethacrylate

DTNB  5,5-dithio-bis (2-nitrobenzoic acid)

EB  Easybond

E-BPA  Ethoxylated Bisphenol A

EGDMA  Ethylene glycol dimethacrylate

Erm  Erythromycin

gtfb  glucosyltransferase B

HEMA  Hydroxyethyl methacrylate

HPLC  High performance liquid chromatography

HSDEA  Human salivary derived esterases
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>LTA</td>
<td>Lipoteichoic acid</td>
</tr>
<tr>
<td>MA</td>
<td>Methacrylic acid</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>PCE</td>
<td>Pseudocholinesterase</td>
</tr>
<tr>
<td>o-NPA</td>
<td>o-nitrophenolacetate</td>
</tr>
<tr>
<td>o-NPB</td>
<td>o-nitrophenolbutyrate</td>
</tr>
<tr>
<td>p-NPA</td>
<td>p-nitrophenolacetate</td>
</tr>
<tr>
<td>p-NPB</td>
<td>p-nitrophenolbutyrate</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>SB</td>
<td>Scotchbond</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SHSE</td>
<td>Simulated human saliva esterases</td>
</tr>
<tr>
<td>TEG</td>
<td>Triethylene glycol</td>
</tr>
<tr>
<td>TEGDMA</td>
<td>Triethylene glycol dimethacrylate</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>THYE</td>
<td>Todd-Hewitt supplemented with 0.3% yeast extract</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>TYEG</td>
<td>Tryptone yeast extract supplement with 0.2% glucose broth</td>
</tr>
<tr>
<td>UDMA</td>
<td>Urethane dimethacrylate</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
</tr>
</tbody>
</table>
List of Figures:

Chapter 2 – Literature Review

Fig 2.1: Common dimethacrylate monomers

Fig 2.2: Classification of resin-based filling composites

Fig 2.3: Structural formula of γ-methacryloxypropyltrimethoxy silane

Fig 2.4: The bonding mechanism of a dentin-composite interface based on the formation of a resin-infiltrated interface zone (hybrid layer) (10).

Fig 2.5: Biodegradation of BisGMA and TEGDMA

Fig 2.6: HPLC chromatographic profile of biodegradation products following incubation of Z250 and TPH with HSDEA and PBS

Fig 2.7: SEM analysis of Z250 samples prior to and following 16 days of incubation with HSDEA and PBS

Fig 2.8: Z-stack image series captured at interfacial regions of interest of 2 90-day PCE+CE incubated resin-dentin specimens

Chapter 3 – Biodegradation of Dental Resin Composites and Adhesives by Streptococcus mutans: An in vitro Study

Fig 3.1: Activity profile for S. mutans strains

Fig 3.2: Amount of BisHPPP production after 30 days of incubation of Z250, SB, and EB with BHI + S. mutans UA159 or with BHI alone

Fig 3.3: Scanning electron micrographs of Z250, SB, and EB at day 0 and following 30 days of incubation with BHI, and with S. mutans UA159

Chapter 4 – Cariogenic bacteria studies

Fig 4.1: CE-like activity of S. mutans UA159 at lag, log and stationary phases

Fig 4.2: Relative CE-like activity of S. mutans incubated in chemically defined media, and with the addition of BisGMA or TEGDMA monomers

Fig 4.3: Activity profile for S. mutans strains

Fig 4.4: Relative % of TEGDMA and BisGMA remaining in solution after 72 hours of incubation with BHIS + S. mutans, or with BHIS alone
List of Tables:

Chapter 2 – Literature Review

Table 2.1: Typical composition of dental composites

Table 2.2: Type of fillers and filler size used in dental composites

Table 2.3: Degradation products, their retention times and chemical formula

Chapter 3 – Biodegradation of Dental Resin Composites and Adhesives by Streptococcus mutans: An in vitro Study

Table 3.1: Surface properties of composite resin (Z250), total-etch (SB) and self-etch (EB) adhesives. Also, the composition (% by weight) of the materials according to manufacturing company (3M Canada Inc., Material Safety Data Sheet).

Chapter 4 – Cariogenic bacteria studies

Table 4.1: Primers for PCR ligation mutagenesis to delete S. mutans SMU.118c gene

Table 4.2: HPLC gradient method for separation of biodegradation products
Chapter 1 – Introduction:

In the United States, 166 million dental restorations were placed in 2005 (1), and clinical studies suggest that nearly 70% were replacements for failed restorations (2). Replacement dentistry costs $5 billion/year in the US alone (3). Studies have shown that dental resin composites have an average replacement time of 5.7 years, mainly due to secondary caries and fracture of the restoration (4). Recurrent or secondary caries is one of the primary causes (31-70%) for composite restorative replacement and occurs at the compromised restoration-tooth interface margin (5,6).

The choice between resin composite and amalgam restorations has been widely driven by aesthetic and health concerns. Over the past decades, concerns with respect to the possibility of adverse health effects from exposure to mercury in dental amalgams, and the desire for improved esthetic dental restorations have lead to the steady and rapid increase of the use of composite resin restorations (7). However, concern over higher fracture rates, reduced longevity, prevalence of secondary caries, and bacterial proliferation associated with biodegradation of resin composites, have been an issue and a focus of research for several years (8,9).

Most research on resin materials has focused on physical process (wear, mechanical studies and effect of diet) that lead to degradation. These physical processes are classified either; under material loss and uptake (sorption, extraction, dissolution and mineralization) or physical changes (softening, stress cracking, fatigue fracture, etc…) (10). On the other hand, biochemical processes leading to degradation have seldom been
discussed in literature; however, attention to the issue has increased in the last decade (11,12). Resin composites contain ester linkages that are vulnerable to hydrolysis by esterase activity present in the oral cavity (7). The results of biodegradation are the deterioration of the bulk structure in resin composites, the composite-tooth interface, and the release of degradation products such as methacrylic acid (MA), triethylene glycol (TEG) and bishydroxy-propoxy-phenyl-propane (BisHPPP) just to name a few (11,13). These products have been shown to affect bacterial growth and gene expression (14,15). The compromised composite-tooth interface allows oral saliva and bacteria to infiltrate the spaces between the tooth and the composite, exacerbating the effects of biodegradation, undermining the restoration and is believed to contribute to recurrent caries, hypersensitivity and pulpal inflammation (2,16,17).

Dental caries are believed to be the result of acid release from bacterial activity that leads to the demineralization of tooth structures. *Streptococcus mutans* have been identified in dental plaque found at the margins of composite fillings, and is regarded as the chief etiological agent responsible for dental caries. While there have been studies investigating the impact of composite degradation by-products on bacterial growth and virulence (14), the potential effect of bacterial degradative activity on composite resins have yet to be explored. In the current study, the effects of *S. mutans* on resin composite biostability and degradation was investigated to elucidate the impact of bacteria on resin composite. This study complements previous research on the impact of salivary esterase activities on resin composites (18-20).
**Hypothesis:**
In addition to acid production, cariogenic bacteria are hypothesized to contain esterase activities that degrade dental resin composites and adhesives.

**Objectives:**
1) To measure esterase activities from different strains of *S. mutans.*

2) To measure the hydrolytic-mediated degradation of dental resin monomers by *S. mutans.*

3) To measure the hydrolytic-mediated degradation of cured dental resin composites and adhesives by *S. mutans.*
References:


(11) Y. Finer. The Influence of Resin Chemistry on a Composite's Inherent Biochemical Stability University of Toronto; 2000.


Chapter 2 – Literature review:

2.1 Preamble:
The concerns over the negative effects of biodegradation on dental resin composites and adhesives have raised suspicion over the biocompatibility of resin composites and adhesives, and led to a host of studies on resin composite and adhesive stability using human saliva or model esterases, cholesterol esterase (CE) and pseudochoolinesterase (PCE). A review of these studies was conducted, the information summarized and organized as follows. First, composite and adhesive resins are introduced and their properties discussed, followed by an analysis of human saliva’s hydrolytic activity on resin composites and adhesives, as well as CE and PCE’s hydrolytic activity and their suitability to be used as a model for human saliva. Lastly, the effects of biodegradation and its associated products on bacteria and oral health will be discussed.

2.2 Resin composites:
The constituents of dental restorative composites are its polymeric matrix (usually methacrylate based), filler particles (usually glass, quartz, or ceramic oxide such as alumina or silica), and coupling agents, which are used to improve bonding at the filler/polymer-matrix, in addition to a photoinitiator system or in some cases other curing systems and further additives (Table 2.1) (1-4).
Table 2.1: Typical composition of dental composites (4).

<table>
<thead>
<tr>
<th>Dental Composites</th>
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<tr>
<td>Inorganic fillers</td>
</tr>
<tr>
<td>75-85% w/w</td>
</tr>
<tr>
<td>- Radiopaque silicate glasses</td>
</tr>
<tr>
<td>- Fumed oxides/mixed oxides</td>
</tr>
<tr>
<td>- etc.</td>
</tr>
<tr>
<td>Organic matrix</td>
</tr>
<tr>
<td>15-25% w/w</td>
</tr>
<tr>
<td>- Polymerizable monomers</td>
</tr>
<tr>
<td>- Initiator system</td>
</tr>
<tr>
<td>- Stabilizers, mixers</td>
</tr>
</tbody>
</table>

2.2.1 The matrix:
Dental restorative resin monomers have been primarily based on the coupling of chemical moieties via ester linkages (Figure 2.1). The predominant resin monomers consist of complexed methacrylate resins. Some of the resin monomers used in dental restorative materials, as shown in Figure 2.1, are bisphenol-glycidyl-dimethacrylate (BisGMA), ethylene glycol dimethacrylate (EGDMA), diethylene glycol dimethacrylate (DEGDMA), triethylene glycol dimethacrylate (TEGDMA), urethane dimethacrylate (UDMA), and bisphenol A polyethylene glycol diether dimethacrylate (BisEMA) (2,3).

![Chemical structure of BisGMA](image)

bisphenol-glycidyl-dimethacrylate (BisGMA)
n=1: Ethylene glycol dimethacrylate (EGDMA), n=2: Diethylene glycol dimethacrylate (DEGDMA), n=3: Triethylene glycol dimethacrylate (TEGDMA)

Urethane dimethacrylate (UDMA)

**Figure 2.1:** Common dimethacrylate monomers. Each of the structures has a common vinyl monomer group coupled to different organic molecules via an ester bond (3).

BisGMA is a very common monomer because it is relatively non-volatile, exhibits low polymerization shrinkage, hardens rapidly under oral conditions, and is compatible with current inorganic filler systems. A disadvantage of BisGMA is its high viscosity, which results from the hydrogen bonds between hydroxyl groups in the alkyl chains and the rigid aromatic ring structure. To facilitate handling and manipulation, various diluent monomers are used in conjunction with BisGMA, most commonly TEGDMA, but other monomers such as UDMA are also used. The ratios and compositions of monomers constituting resin composites vary depending on the application, location (anterior vs.
posterior) of the restoration, and on the manufacturer. Therefore, resin composites vary in characteristics and properties depending on the monomers, fillers and ratios used (2,3,5).

2.2.2 Filler systems:
The reinforcing fillers have been the major constituents of resin composites by weight and volume. Fillers provide the composite with improved physical properties such as increased strength and modulus of elasticity, as well as reduced polymerization shrinkage, coefficient of thermal expansion and water sorption. Composite restorations have been classified according to the type of filler used (Figure 2.2) (4). Fillers are characterized by different chemical composition, average particle size, and manufacturing techniques. Macrofilled particles are inorganic particles that are produced by grinding larger particles of glass, quartz, or ceramics into smaller ones and are usually splinter shaped. Macrofilled composites have an average particle size of 0.2–5 μm. On the other hand, microfilled particles such as pyrogenic silica are usually spherical with an average particle size of 5-100 nm. They are also referred to as nanoparticles because of the small particle sizes. Agglomerates are often formed from these particles and the formed agglomerates may influence the transparency of the composite. A significant thickening effect can be observed because of the large surface area of microfiller or nanofiller particles. These particles have been used in order to increase the microfiller loading in heterogeneous microfilled composites. This can be achieved by incorporating pyrogenic silica into a resin matrix, curing the mixture, and then milling the obtained microfilled composite into splinter shaped particles, with a particle size of 10-100 μm. Traditionally, the inorganic component of these hybrid composites consist of 70-80% w/w of glass fillers and 20-30% w/w of microfillers (4). Microfilled composites contain
silica microfine particles with filler concentrations approximately 38% by weight. Because of the greater percentage of resin, microfilled materials exhibit increased water sorption and a higher coefficient of thermal expansion when compared to microhybrid composites that contain a filler concentration of 74-84% w/w (6). Many contemporary dental composites use the fillers listed in Table 2.2.

**Figure 2.2:** Classification of resin-based filling composites (4).

**Table 2.2:** Type of fillers and filler size used in dental composites (4).

<table>
<thead>
<tr>
<th>Filler composition</th>
<th>Particle size</th>
</tr>
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<tbody>
<tr>
<td>Highly dispersed SiO₂</td>
<td>10-40 nm</td>
</tr>
<tr>
<td>Radiopaque, finely ground Ba or Sr silicate glasses</td>
<td>0.7 µm, 1.0 µm, 1.5 µm, or larger</td>
</tr>
<tr>
<td>Radiopaque, finely ground Ba/Sr fluoro silicate glasses</td>
<td>1.0 µm, 1.5 µm, or larger</td>
</tr>
</tbody>
</table>
In order to improve the properties of resin composites, coupling agents have been developed to make a stronger link between the hydrophobic organic matrix, and the hydrophilic inorganic fillers. Coupling agents contain the characteristics of both the filler particles and the organic matrix; they have hydrophilic hydroxyl groups at one end and hydrophobic methacrylate groups at another end. The most common coupling agents are organic silicon compounds called silanes (Figure 2.3). The silane coupling agent’s hydroxyl groups covalently bind to the hydroxyl groups found on the surface of silica based glasses on one end. Also, the silane coupling agent’s methacrylate group can covalently link to the resin matrix via carbon double bond at the other end, resulting in a strong bond between the organic matrix and the inorganic fillers (3).

![Structural formula of \( \gamma \)-methacryloxypropyltrimethoxy silane](image)

**Figure 2.3**: Structural formula of \( \gamma \)-methacryloxypropyltrimethoxy silane
2.2.3 Polymerization systems:
Resin composites are converted from a viscous resin to a rigid solid through free radical polymerization of the methacrylate monomers via photo-polymerization. During polymerization, each molecule grows by the addition of a monomer to a terminal free radical reaction site. Not all of the monomer’s double bonds have reacted once the polymerization reaction has terminated. In fact, studies suggest that degrees of conversion of the methacrylate double bonds range from 40 to 85% (1,7,8). This is in part due to a reduction in the diffusion rates of the propagating free radicals, the unreacted dimethacrylate molecules and the pendant methacrylate species as the polymerization reaction progresses (3,8).

2.3 Dental resin adhesives:
Resin composite restorations require the application of resin adhesives in order to bond efficiently to the tooth (dentin and enamel) by forming the resin-dentin interface. Dental resin adhesives are low viscosity methacrylate based liquids, which spread on the dentin surface, and solidify to bond the primed dentin and composite substrates (9). Micromechanical interlocking is believed to be the primary mechanism by which dental resin adhesives bind to tooth substrates. Micromechanical interlocking is achieved by the replacement of tooth inorganic material by adhesive resin monomers that become interlocked upon curing, forming what is known as the “hybrid layer” (Figure 2.4) (10,11). Currently, there are two types of adhesive systems; total-etch (also known as etch-and-rinse), and self-etch adhesive systems.
Figure 2.4: The bonding mechanism of a dentin-composite interface based on the formation of a resin-infiltrated interface zone (hybrid layer) (10).

2.3.1 Total-etch systems: In total-etch adhesives, the first step is the demineralization of the dentin surface (1-10 μm) by the application of phosphoric acid (30-40% phosphoric acid), followed by a rinsing step, and then the application of the primer and the adhesive agents, which will infiltrate the exposed collagen and polymerize to form the dentin-composite interface (11,12). Dental adhesive agents and primers contain resin monomers that are similar to those found in the composite resin matrix. These adhesive monomers provide the covalent link between the adhesive and the composite. Therefore they provide structural continuity and thus physical co-mechanical properties such as strength. These monomers can be classified into two categories: functional monomers and cross-linkers. Functional monomers have one polymerizable group and a particular chemical group “functional group” which imparts monomer specific functions. Cross-linking monomers have two or more polymerizable groups. As suggested by their name,
cross-linking monomers form cross-linked polymers upon curing, whereas functional monomers form linear polymers. Cross-linked polymers exhibit better mechanical properties, such as strength, and therefore they are important to reinforcing the adhesive resin. Traditionally, the primers contained hydrophilic functional monomers, whereas the adhesive agents contained hydrophobic functional cross-linkers. The application of a primer followed by the adhesive agent successively in separate steps forms the basis for the three-step etch-and-rinse technique. Simplified two-step etch-and-rinse adhesives combine the primer and the adhesive agent into one step (bottle) (12).

### 2.3.2 Self-etch systems:
A trend towards developing simpler techniques that are less practitioner-sensitive and that are more time efficient has resulted in the manufacturing of what are known as “self-etch adhesives”. Self-etch adhesives function by using non-rinse acidic monomers that simultaneously condition and prime dentin. Self-etch adhesives can be divided into two categories: two-step and one-step self-etch adhesives, depending on whether the hydrophobic adhesive resin is applied in a separate step (two-step) or combined with the hydrophilic self-etch primer (one-step) (13).

Unlike total-etch adhesives, self-etch adhesives do not completely expose collagen when forming the hybrid layer at dentin surfaces, resulting in lower levels of micromechanical interlocking (10,13,14). Clinical studies have shown that three-step total-etch adhesives are the most effective approach to achieve efficient and stable bonding. The clinical effectiveness of two-step etch-and-rinse adhesives was less favorable when compared to its three-step counterpart (12,14). Meanwhile, one-step self-etch adhesives result in an “inefficient bond” clinically and perform the worst (12,14). Two-step self-etch adhesives were found to be clinically reliable because the application of a separate hydrophobic
adhesive resin that makes the interface more hydrophobic and therefore seals it better resulting in a better bond durability (10,12,14). Therefore, the drive towards simpler techniques that are less susceptible to practitioner variability has resulted in a self-etch approach that needs more development to reach a satisfactory clinical performance.

2.4 Degradation of dental resin composites and adhesives:
The degradation of dental resin composites and adhesives in the oral cavity is a result of many complex interactions, physical, chemical, and biochemical. Most research has focused on physical processes (food, chewing, etc) that lead to degradation. These physical processes are classified either under material loss and uptake (sorption, extraction, dissolution and mineralization) or physical changes (softening, stress cracking, fatigue fracture, etc…). On the other hand, chemical processes leading to degradation have seldom been discussed in literature until the last decade. Chemical processes that lead to degradation are thermolysis, oxidation, solvolysis, photolysis and radiolysis. Solvolysis, and more specifically hydrolysis, is the most investigated and relevant chemical degradation process because of the presence of unprotected ester linkages in the resin monomers, polymer, and coupling agents. By definition, hydrolysis is a chemical reaction during which water is used to cleave a molecule into two parts. One fragment of the parent molecule gains a hydrogen ion, and the other a hydroxyl group from the water molecule. This process can be catalyzed by enzymes present in the oral cavity and is often referred to as biodegradation. Figure 2.4 illustrates the biodegradation of dental resin monomers BisGMA and TEGDMA (3).
a)-

\[
\text{bisphenol-glycidyl-dimethacrylate (BisGMA)}
\]

\[
\text{Enzyme} \downarrow \text{H}_2\text{O}
\]

\[
\text{Bis-hydroxy-propoxy-phenyl-propane (BisHPPP)} \quad \text{Methacrylic acid (MA)}
\]

b)-

\[
\text{Enzyme} \downarrow \text{H}_2\text{O}
\]

\[
\text{Triethylene glycol dimethacrylate (TEGDMA)}
\]

\[
\text{Triethylene glycol methacrylate (TEGMA)} \quad \text{Methacrylic acid (MA)}
\]
Figure 2.5: Biodegradation of BisGMA (a) and TEGDMA (b) by salivary esterases resulting in the production of biodegradation by-products BisHPPP, TEGMA, TEG, and MA.

Fillers are chemically inert, but their associated coupling agents intended to improve the link between the inorganic filler and the organic matrix of the composite resins are vulnerable to hydrolysis via ester linkages within the coupling agents or siloxane links that are formed with the filler particle (Figure 2.3) (3).

Generally, material discoloration is a sign of chemical changes. Studies by Seung-Heon et al. (15) and Yong-Keun et al., (16) who incubated resin composite materials with porcine liver esterase for 9 weeks and compared them to controls incubated in PBS for the same period, showed that esterase influence on dental resin composite color was negligible. This does not mean that chemical change is negligible and that biodegradation should not be looked at as a contributing factor to resin degradation. Rather, another study conducted by Yong-Keun et al. (17) showed that sequential immersion of composite resin specimens in porcine liver esterase, organic substances and chemical agents resulted in various discolorations of resin composite specimens. This latter experiment is one that parallels the real world scenarios, where resin composites are subjected to various agents in conjunction with biodegradation. The results of the latter experiment signal the existence of chemical activity as a factor in resin composite degradation, since there was no physical loading in the study.
2.4.1 Degradation by human salivary esterase activity:
The potential for enzymes to interact with resin composites and adhesives is significant and accomplished via salivary enzymes, tissue inflammatory responses and bacterial activity. In the oral cavity, the enzymes most associated for aiding in the hydrolysis of resin composites and adhesives are esterases. Salivary esterase origins include human gingival epithelium, salivary glands, inflammatory responses and microorganisms (8). Studies have shown that human saliva contains esterase activities that hydrolyze resin monomers such as BisGMA and TEGDMA (8), as well as matrix polymers (2,3,5,8). Amongst these studies is one conducted by Jaffer and colleagues (8), who showed that human saliva degrades commercial composite resins (Z250 from 3M Inc, and Spectrum TPH from L.D. Caulk), which contain BisGMA, TEGDMA and urethane modified BisGMA. Standardized commercial photopolymerized composites were incubated with buffer and human saliva under standard conditions (pH 7.0 and 37°C) for 2, 8 and 16 days. The incubation solutions revealed that human saliva catalyzed the biodegradation of both commercial composites. Biodegradation products were identified, isolated and quantified via high performance liquid chromatography (HPLC) in combination with UV spectroscopy and mass spectrometry (MS) (3). A typical HPLC chromatographic profile is shown in Figure 2.5, where retention times of 7 min, 10.5 min, 19 min, 20 min and 21 min are highlighted; each peak represents a biodegradation product (8).
Figure 2.6: HPLC chromatographic profile of biodegradation products following 2 days incubation; a) Z250 incubated in HSDEA, b) TPH incubated in HSDEA, c) TPH incubated in D-PBS and d) HSDEA in the absence of composite samples (3).

The identity of the degradation products associated with the various retention times were determined (MA, TEGMA, BisHPPP, TEGDMA and Ethoxylated Bisphenol A (E-BPA)) and are summarized in Table 2.3.

Table 2.3: Degradation products, their retention times and chemical formula (8).

<table>
<thead>
<tr>
<th>Product</th>
<th>Retention time (min)</th>
<th>Chemical formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methacrylic acid</td>
<td>7</td>
<td>(\text{CH}_2=\text{CCH}_2\text{COOH})</td>
</tr>
<tr>
<td>TEGMA(^a)</td>
<td>10.5</td>
<td>(\text{CH}_2=\text{C(CH}_3\text{CO}_2\text{(CH}_2\text{CH}_2\text{O})_2\text{H}})</td>
</tr>
<tr>
<td>BisHPPP(^b)</td>
<td>19</td>
<td>(\text{HOCH}_2\text{CH}_2\text{OH})</td>
</tr>
<tr>
<td>TEGDMA(^c)</td>
<td>20</td>
<td>(\text{CH}_2=\text{C(CH}_3\text{CO}_2\text{(CH}_2\text{CH}_2\text{O})_2\text{OC(CH}_3\text{C}=\text{CH}_2})</td>
</tr>
<tr>
<td>E-BPA(^d)</td>
<td>21</td>
<td>(\text{HOCH}_2\text{CH}_2\text{O})</td>
</tr>
</tbody>
</table>

\(^a\) Triethylene glycol methacrylate.  
\(^b\) Bis(hydroxypropyl)phenylpropane.  
\(^c\) Triethylene glycol dimethacrylate.  
\(^d\) Ethoxylated Bisphenol A.
The nature of degradation products was similar in both composite resins, but differences existed in the amounts of degradation products released. Both BisHPPP and methacrylic acid were produced in significantly greater values as a result of the biodegradation of Z250 composite resin when compared to TPH degradation. However, E-BPA was produced in greater amounts as a consequence of TPH degradation, albeit in less quantity than BisHPPP and methacrylic acid. These differences can be explained by the variability of composite resin composition with respect to monomer types, monomer ratios, filler content and filler/resin ratios (1,2,18). Also, the surface of the final cured structure can offer easier access to human salivary esterases and therefore access to more binding sites, and this can also lead to discrepancies in degradation product profile. Another factor may be the fact that urethane modified BisGMA resin associated with TPH is more stable and resilient to hydrolysis and thus results in less degradation products released from TPH (2). Therefore, degradation product profiles vary in terms of identity and amount of degradation products released, depending on the identity of the composite resin under investigation.

Other researchers using similar materials and methods as the ones described above to purify and quantify the degradation by-products reached similar conclusions on saliva’s ability to hydrolyze dental resin composites (19,20). Hsu et al. (19) incubated commercial dental composite resins containing different monomers (BisGMA, TEGDMA, and UDMA) with human saliva and found that saliva hydrolyzes the restorative materials at the ester bonds releasing the expected degradation by-products from these monomers (such as TEGMA and MA). Overall, studies via isolation and identification of degradation by-products, scanning electron microscopy (SEM) analysis, and fracture
toughness tests confirmed that human salivary esterases hydrolyze resin composites (2,5,8,19-21). The consensus is that human saliva degrades whole matrix and not just unreacted monomers, as displayed by SEM analysis of Z250 samples prior to incubation as compared to samples incubated in human saliva (test group) or PBS buffer (control group), which revealed the appearance of exposed filler particles (Figure 2.6). Analysis of SEM images of other resin composite products showed similar results.

![Figure 2.7](image)

**Figure 2.7**: SEM analysis of Z250 samples a) - prior to incubation in HSDEA, b) - following 16 days of incubation in D-PBS and c) - following 16 days of incubation in HSDEA (8).

### 2.4.2 Degradation by model esterases (cholesterol esterase and pseudocholinesterase):

The potential for the spread of infection when handling saliva from different donors, as well as the variability of esterase activity and the cumbersome protocol involved in collecting and treating saliva to harvest its esterase activity from different donors, has necessitated the search for model salivary esterases that are safer and more practical to use for scientific research. A study by Finer et al. (5) compared human saliva’s hydrolase activity to that of model esterases, cholesterol esterase (CE) and pseudocholinesterase (PCE), by recording data for human saliva’s ability to hydrolyze p-nitrophenolacetate (p-
NPA), p-nitrophenolbutyrate (p-NPB), o-nitrophenolbutyrate (o-NPB), o-nitrophenolacetate (o-NPA), or butyrylthiocholine iodide (BTC), and of CE and PCE’s ability to degrade the same five substrates. The synthetic substrates contain ester linkages, and the hydrolysis of these ester linkages results in the substrates’ breakdown to their respective alcohol and carboxylic acid degradation products. The findings showed that human saliva has PCE character by degrading BTC, and that human saliva also has CE character by degrading p-NPA, o-NPA, p-NPB, and o-NPB yielding a similar profile of sensitivity to the different substrates. The average CE-like activity level for human saliva was determined to be $0.19 \pm 0.02$ units/ml, and the average PCE-like activity level for human saliva was $0.011 \pm 0.001$ units/ml. Therefore, this study (5) showed that indeed human saliva contains CE- and PCE-like esterase activities that can degrade composite resin restorative materials. This study also revealed that a combination of CE and PCE could be used as a model for human salivary esterase activity in biodegradation studies.

Several studies have been conducted on the hydrolytic activity of CE and PCE (22-25). Studies showed that CE and PCE are enzymes that can catalyze the hydrolysis of BisGMA and TEGDMA monomers, as well as composite resins (22,24,25). CE is an inflammatory cell-derived enzyme (22) and pseudo-cholinesterase belongs to a family of cholinesterases, a family of esterases that hydrolyze choline esters at a higher rate than they do other esters (5). Studies revealed that the degradation of resin matrix by CE and PCE is a concentration dependent process and that CE has a greater specificity towards catalyzing the hydrolysis of BisGMA and BisEMA components, while PCE has a greater specificity towards hydrolyzing TEGDMA and TEGMA components (8,22). In another
study, Finer et al. (25) showed that CE and PCE act synergistically to increase the biodegradation of composite resin materials; i.e. each of the two enzymes functions better in the presence of the other, resulting in higher levels of degradation products released in a solution of both enzymes as opposed to when they are alone. One proposed explanation for this was that the activity of one of the enzymes can provide access for the other enzyme to sites that would have been difficult to engage and bind to otherwise, and vice versa, leading to an increased efficiency of resin matrix degradation.

It has been suggested that CE and PCE catalyze the hydrolysis of resin composites in a mechanism that is similar to their common function (i.e. using the same active site) (5). This conclusion was reached by observing a decrease in CE and PCE activity by 63±0.5% (p<0.05) and 58±4.7% (p<0.05) respectively when incubated with phenylmethyl sulfonyl fluoride (PMSF). PMSF is a serine esterase inhibitor, which alkylates the hydroxyl of the active serine site in the esterases. More kinetic studies need to be conducted to elucidate the exact mechanism by which CE and PCE degrade composite resins.

2.4.3 Degradation of resin-dentin interfacial margins:
In addition to the degradation of the resin composite bulk, the dentin-resin interface also faces the challenges of biodegradation. Shokati et al. (26) incubated adhesive resin (Scotchbond Multi Purposes), resin composite (Z250) and mini short-rod specimens in buffer and human saliva derived esterases (HSDE) for up to 180 days (pH 7.0 and 37°C). The adhesive resin and resin composite specimens were incubated with either D-PBS or HSDE for 8 days (pH 7.0, 37°C). BisHPPP as a marker of resin matrix and polymerized adhesive resin degradation was identified and measured using HPLC. BisGMA is a high
molecular weight molecule that has rigid phenyl rings, and hydrogen bonding capacity. When taking this into consideration it is realized that BisGMA is a molecule that has limited diffusion through the resin matrix and out to the surface to interact with the enzymes. Therefore BisHPPP, a BisGMA derived degradation product, is a good indicator of resin matrix degradation (1). This analysis revealed that HSDEA degraded both adhesive resin and resin composite to produce BisHPPP in comparable amounts. The mini short-rod specimens were tested for fracture toughness. Results of the fracture toughness test showed that mini short-rod specimens incubated for 180 days in HSDEA had the lowest fracture toughness \( (0.55 \pm 0.13 \text{ MPa/m}^{1/2}) \), while non-incubated mini short-rod specimens had the highest fracture toughness values \( (0.80 \pm 0.16 \text{ MPa/m}^{1/2}) \). This result emphasized the effect of biodegradation on the structural integrity of resin composites. The combination of BisHPPP production, fracture toughness results, and SEM images showed that alongside surface composite resin degradation, the dentin-resin interface also faces degradation by HSDEA (26,27). Other researchers also investigated the effects of biodegradation on the resin-dentin interface (28-31). Jung et al. (32) evaluated the effect of esterase activity on resin-dentin interface by transmission electron microscopy (TEM) and found an increased tendency of nanoleakage in the bounded interface after storage in an esterase solution as compared to a PBS buffer solution after 4 weeks. Also, Sirichan et al. (31) investigated the effect of collagenase and acetylcholinesterase on the resin dentin interface. The above-mentioned enzymes were selected to simulate oral salivary enzymes. Sirichan et al. (31) incubated resin composite (Z350, 3M) bonded to human dentin by four different adhesive systems; a total-etch adhesive (Single Bond 2, 3M, St. Paul, MN, USA), a two-step self-etch adhesive (Clearfil
SE Bond, Kuraray, Tokyo, Japan), and two one-step self-etch adhesives (Clearfil tri-S Bond, Kuraray, Tokyo, Japan; and G-Bond, GC, Tokyo, Japan). The prepared restorations were incubated for three months in water, collagenase, or acetylerase. Microtensile bond strengths (μTBS) were measured immediately after restorations were placed, or after 3 months of incubation in either medium. The specimens were also analyzed by SEM. The researchers found that μTBS were significantly lower in groups incubated in enzymes when compared to incubation in water or non-incubated specimens. They also found that the self-etch adhesives exhibited water-tree patterns within the adhesive layer, and the total-etch adhesive exhibited nanoleakage within the hybrid layer and the adhesive. These findings further provide evidence of the effects of biodegradation on the resin-dentin interface caused by salivary enzymes (31).

The effects of biodegradation at the resin-dentin interface are aided and amplified by polymerization shrinkage, thermal changes, mastication stresses, and chemical attacks by acids. These effects result in microleakage, and in the creation and expansion of marginal gaps at the resin-dentin interface that may lead to the colonization and propagation of bacteria at the interface as illustrated by previous research (27,31,33). Kermanshahi et al. (27) investigated the idea that biodegradation on resin composite restorations accelerates marginal microleakage. Kermanshahi and colleagues incubated resin composite (Z250, 3M) bonded to human dentin (Scotchbond MP, 3M, St. Paul, MN, USA) in either buffer or dual esterase media (PCE + CE) with activity levels resembling those of human saliva for 90 days. Analysis of incubation solutions for BisHPPP was performed, and as anticipated significant amounts of it were produced (BisHPPP is a marker of resin matrix and polymerized adhesive resin degradation). Post-incubation,
specimens were suspended in a chemostat-based biofilm fermentor cultivating *Streptococcus mutans* NG8 for 7 days. After, bacterial microleakage was assessed by confocal laser scanning microscopy. *S. mutans* is a species that is low G+C Gram-positive, non-haemolytic, non-spore forming, and non-motile. It is regarded as the main etiological agent in dental caries (34). Dental caries are the result of acid release from carbohydrate metabolism that leads to demineralization of tooth structures. Cumulated data showed that there was greater bacterial surface adherence and penetration along the resin-dentin marginal interface in CE + PCE incubated specimens. Precisely, CE + PCE incubated specimens had nearly 4 times more bacteria, and the maximum interfacial depth penetration was nearly 4 times more in CE + PCE incubated specimens than in buffer incubated specimens. Confocal laser scanning microscopy Z-stack images, Figure 2.8, showed bacterial invasion and growth along the marginal gap. The bacteria displayed characteristics indicative of three-dimensional biofilm growth. These findings confirm that biodegradation is a relevant issue at the resin-dentin interface and they prove that biodegradation contributes to microleakage and bacterial invasion of the marginal gap. The significant results obtained by Kermanshahi et al. (27) are for *in vitro* experiments that only lasted 90 days, whereas dental restorative composite resins are under the constant stresses of the oral environment for a period of time that is much longer than 90 days. Therefore, the real life impact of biodegradation and microleakage are expected to be much more substantial than those observed in *in vitro* experiments.
Figure 2.8: Z-stack image series captured at interfacial regions of interest of 2 90-day PCE+CE incubated resin-dentin specimens. (A) Interfacial void spanning approximately 4-5 μm in height. (B) Interfacial void spanning over 20 μm in height. Specimens were stained by means of a Live/Dead Baclight Viability Kit. The bacteria displayed characteristics indicative of three-dimensional biofilm growth (27).

2.5 Interactions between bacteria and dental resin composites and adhesives:
Research exploring the topic of bacterial adhesion and viability on dental resin composite surfaces has focused on the influences of material hydrophobicity/hydrophilicity, surface free energy, and surface roughness on bacterial adhesion and survival on these materials (35-41). Some researchers propose that hydrophobic resin composites lead to resistance of attacks by water-soluble species (40,41). Other researchers propose that adhesive forces may arise for hydrophobic materials because water is easily removed from the areas between cell surface and hydrophobic materials than from the cell surface and
hydrophilic materials, enabling a closer approach and therefore stronger adhesion (42-44). Inconclusive evidence for the effects of surface free energy and surface roughness on bacterial adhesion to resin composite surfaces has also been reported. A study by Stefan et al. (35) investigated the adhesion and viability of oral bacteria on the surfaces of resin-based dental restorative materials. The researchers postulated that modifying resin composite materials with low-surface tension active agents (hydroxyfunctional polydimethylsiloxane and polydimethylsiloxane, or silicone polyether acrylate) would result in lower bacterial count or bacterial viability. The hypothesis was tested by incorporating the above-mentioned active agents into the composition of standardized resin composite materials, with a non-modified resin as the control. The total count and viability of *Actinomyces naeslundii*, *Actinomyces viscosus*, *Streptococcus mitis*, *Streptococcus oralis*, and *Streptococcus sanguinis* on human saliva pellicle-coated specimens was analyzed using fluorescence microscopy after 8 and 24 h. The researchers found that all test materials had significantly fewer vital cells after 8 or 24 hours compared to the control. However, they found no difference in total bacterial count on test surfaces except in the group modified with silicone polyether acrylate that showed lower total bacterial count after 8 and 24 h. The researchers also concluded that contact angle did not influence bacterial adhesion, but no conclusive evidence for the effects of low total surface free energy resulting in fewer bacteria was found. Therefore, the researchers concluded that in addition to hydrophobicity/hydrophilicity and surface free energy, material chemistry (i.e: monomer mixtures) is an important factor that has to be considered when analyzing for bacterial adhesion and viability on dental resin composites.
When analyzing for the impact of resin composite chemistry on bacterial adhesion and viability, it is important to understand how the resin monomers and biodegradation by-products interact and influence bacterial cells. Studies have revealed that biodegradation products, such as TEG, influence bacterial growth and virulence gene expression (45,46). Research by Kalichi et al. (47) showed that TEG, at levels found in vivo modulated the expression levels of glucosyltransferase B (gtfB) (involved in biofilm formation) and yfiV (a putative transcription regulator). This finding directly links biodegradation to bacterial proliferation in the oral cavity, which is significant because it implies that resin composites, in their current form, are not only structurally vulnerable and not suitable for long term use, moreover they contribute to oral disease. On the other hand, another study found that BisGMA degradation products (BisHPPP and MA) slightly inhibits S. mutans growth (45). This suggests that different degradation products have different effects on oral bacteria. Therefore, to convincingly reach a conclusion on the complete effect of degradation products on bacterial activity in the oral cavity, research must be conducted on the effect of cumulative degradation products on bacterial growth.

The mechanism of biodegradation product generation is complex since residual unreacted monomers, the polymer matrix, and adhesive resin are all undergoing degradation. Also, the products of the aforementioned degradations are themselves undergoing degradation, leaving a complex matrix of degradation products such as BisHPPP, TEGMA, TEG, E-BPA, and MA, whose cumulative effect on bacterial activity has been suggested to be a harmful one for oral health. Overall, biodegradation is an ongoing clinically relevant process that affects structural integrity of resin composites and possibly oral health. More
research needs to be conducted to determine its precise effects and how to minimize or eliminate them.

Many researchers have studied the impact that material properties have on bacterial adhesion and viability on dental resin composites (35-41). However, very little research has been conducted on the impact that bacterial cells have on dental resin restorative materials (46,48). A study by Gregson et al. (46) investigated the impact of bacterial cells on the mechanical and surface properties of dental resin materials. Gregson et al. (46) hypothesized that (1) exposure of bacteria results in chemical degradation of dental resin, (2) exposure to TEGDMA or degradation products derived from TEGDMA (MA and TEG) can influence the number of the bacteria, and (3) exposure to bacteria results in a reduction of the mechanical and surface properties of a dental resin. In order to test their hypotheses, the researchers incubated standardized resin material specimens with \textit{S. mutans}, \textit{Streptococcus gordonii}, and \textit{Streptococcus sanguis} for six weeks. The investigators used FTIR analysis before and after specimen incubation to test for material degradation. They also analyzed for microhardness and took SEM images at 1 and 6 weeks. In addition, every week a flexural strength test was performed. FTIR data revealed that the carbonyl peak at 1700 cm\textsuperscript{-1} was significantly reduced after six weeks of incubation with \textit{S. mutans} and \textit{S. gordonii} but not \textit{S. sanguis}. The researchers concluded that the reduction in the carbonyl bond peak was attributed to the biodegradation of the material caused by bacterial activity. The lack of significance in reduction of the carbonyl bond in the specimens incubated with \textit{S. sanguis} was attributed to the negative impacts that TEG and MA had on the growth of \textit{S. sanguis}. The flexural strength data were not found to be significantly different between control and test groups. The SEM data
revealed that the surface of specimens incubated with *S. mutans* and *S. gordonii* were changed, indicating degradation. Another study by Fucio et al. (48) investigated the effects of a 30-day *S. mutans* biofilm on resin composite (Filtek Supreme, 3M, St. Paul, MN, USA) surface roughness, hardness and morphology. The authors found no statistically significant differences in surface roughness and hardness after 30 days of incubation. However, the scanning electron micrographs showed an increase in surface degradation, corroborating the findings of Gregson et al. (46). Overall, the results obtained by Gregson et al. (46) and Fucio et al. (48) point towards the degradative effects that bacteria have on dental resin composites and highlight the potential of secondary caries and changes in esthetic properties seen clinically with the use of resin materials in dental restorations.

2.6 Summary:

Although dental resin composite restorative materials have advantages such as esthetic appeal, and no perceived danger of mercury poisoning, they do seem to be at a disadvantage when discussing long-term stability, which comes from physical and chemical influences. The chemical influences, in terms of studies of biodegradation caused by HSDE alone seem to create conditions for the proliferation of bacteria capable of causing secondary caries and resin composite failure. In addition, dental resin composites have been shown to interact with bacteria and influence its growth and virulence gene expression. When taking into consideration that the oral cavity is much more complex and various influences are acting in concurrence, then it is evident that more research needs to be done in order to better understand the problems facing resin composites and develop better biocompatible dental resin composite restorative materials.
2.7 References:


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Chapter 3 – Cariogenic Bacteria Degrades Dental Resin Composite and Adhesives

(Note: The following was submitted to the journal of Dental Research for Publication; 28/04/2013, reviews are pending)

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3.1 Introduction:
Out of the 166 million dental restorations that were placed in the USA in 2005 (1), nearly 70\% were replacements for failed restorations (2). Recurrent or secondary caries is one of the primary reasons given for composite restorative replacement (3).

Resin composite restorations require the application of resin adhesives in order to bond efficiently to the tooth structure (dentin and enamel). Currently there are two main adhesive systems, total-etch (etch-and-rinse) and self-etch. In the total-etch adhesive systems, acid etching and priming/bonding of the dentin are a separate step, whereas self-etch adhesive systems combine etching and priming/bonding in one step (4). In order for self-etch adhesive systems to etch and prime simultaneously, they have been designed to contain hydrophilic and acidic resin monomers (5,6).

Human saliva contains esterase activities, cholesterol esterase-like (CE-like) and pseudocholinesterase that degrade bis-phenyl glycidyl dimethacrylate (BisGMA)-containing resin composites and adhesives (7), yielding the degradation product
bishydroxypropoxyphenylpropane (BisHPPP) (8). This process compromises the resin–
dentin interface allowing for cariogenic bacterial ingestion along the interface (9).

Dental caries is the result of acid production from bacterial carbohydrate metabolism that
leads to the demineralization of tooth structures. *S. mutans* is regarded as the chief
etiological agent responsible for dental caries (10). In addition, streptococcus species
were shown to contain esterases (11). While there have been studies investigating the
impact of composite degradation products on bacterial growth and virulence gene
expression (12,13), the potential effect of bacterial degradative activity on the release of
degradation products from composite resins and adhesives has yet to be explored.
Therefore, it is hypothesized that in addition to acid production, cariogenic bacteria
contain esterase activities that degrade dental resin composites and adhesives, to release
monomer derived products into the media.

### 3.2 Materials and methods:

#### 3.2.1 Bacterial esterase activity assay:

*S. mutans* strains UA159, JH1005, LT11, NG8, UA140, BM71, and GS5 were sub-
cultured on Todd-Hewitt agar plates supplemented with 0.3% yeast extract (THYE) (14).
Colonies of *S. mutans* from THYE plates were cultivated overnight in THYE broth
(37°C, 5% CO₂) and then diluted 1:10 and allowed to grow to mid-log growth phase,
washed and resuspended in phosphate buffer (pH=7.0). Esterase activities, CE-like and
PCE-like were determined by incubating 1ml of the bacterial cell suspension in 0.5ml of
either *p*-nitrophenolbutyrate (*p*-NPB), *o*-nitrophenolbutyrate (*o*-NPB), *p-
nitrophenolacetate (*p*-NPA), or butyrylthiocholine iodide (BTC) substrates (Sigma, St.
Louis, MO), as described previously (15).
3.2.2 Preparation of composite and adhesive resin specimens:
Photopolymerized (Sapphire plus, Den-Mat, Santa Maria, USA) cylindrical specimens (4mm diameter x 4mm height) were made from resin composite (Z250), a total-etch adhesive (Scotchbond Multipurpose, SB) and a self-etch adhesive (Easybond, EB), (3M Canada Inc., London, ON) (7).

3.2.3 Degree of vinyl group conversion at the surface:
Degree of vinyl group conversion for the different specimens was measured as described before (16) using a Spectrum Bx FT-IR system (Perkin Elmer, Massachusetts, USA) at the Analest laboratory, University of Toronto. Analysis of the data was carried out using the software Spectrum version 5.0.1.

3.2.4 X-ray photoelectron spectroscopy:
Elemental composition analysis of all the pre-incubated specimens was performed by X-ray photoelectron spectroscopy (XPS) at 90° take-off angle relative to the sample surface, as described previously (16). The specimens were analyzed in both high and low-resolution mode, and the spectra were obtained on a Thermo Scientific K-Alpha. XPS system (East Grinstead, UK) located at Surface Interface Ontario, University of Toronto.

3.2.5 Contact angle measurements:
Advancing water contact angle measurements were obtained using a goniometer (NRL C.A. goniometer, Ramé-Hart, Inc., Mountain Lakes, NJ). Briefly, a microsyringe was used to place a droplet of distilled/deionized water on the materials surfaces (n=3). For each droplet, the contact angle on either side was measured and the average standard deviation was reported as a single measurement (17).
3.2.6 Biodegradation experiments:
Cured specimens (N=3 per group) were incubated in sterile vials containing either 2ml of brain heart infusion (BHI) (Becton, Dickinson and Co, Spark, MD, USA) (control group), or a 1:10 dilution in BHI of overnight S. mutans UA159 grown in BHI (test group). Incubation solutions were collected every 48 hours from each group and replaced with fresh solutions. Incubation solutions were accumulated, pooled and analyzed for isolation and quantification of BisHPPP degradation product at 2, 4, 7, 14, and 30 days using high performance liquid chromatography (HPLC) as previously reported (8). The purity of the bacterial culture was assessed by gram stain at each media replacement (18).

3.2.7 Scanning electron microscopy:
Surface morphology observations for pre- and post-incubation specimens were performed using scanning electron microscopy Hitachi S 2500 SEM (Hitachi, Mito City, Japan) at an operating voltage of 10 kV as described before (8). Specimens were sonicated prior to analysis to remove bacterial cells adhering to the surface therefore allowing for imaging of the materials.

3.2.8 Statistical analysis:
Statistical analysis was performed by analysis of variance (ANOVA) and Tukey’s multiple comparison analysis, or by an independent sample t-test where appropriate. Statistical significance was reported for (p<0.05).

3.3 Results:
3.3.1 Bacterial esterase activity assay:
All strains of S. mutans had activity towards the nitrophenyl esters (Fig. 3.1). All strains had preference toward the p-NPA and p-NPB vs. o-NPB (p<0.05), with no significant difference between the para-isomers. All S. mutans strains showed no activity towards
BTC substrate. The highest activity with p-NPB was observed with *S. mutans* UA159, at 2.07±0.15 units/mg cell dry weight.

### 3.3.2 Material characterization:

The degree of vinyl group conversion ranged from 66.1±4.5 % to 74.9±5.8% with no significant differences between the materials (Table 3.1).

XPS analyses showed that the initial surface elemental composition was similar for all materials with virtually pure resin on the surface (Table 3.1). High-resolution spectra of the C1s peak (Table 3.1) indicated the C1s binding energies at 285.0, 286.5, and 289 eV corresponded respectively to the C-C, C-O, and C=O bonds. All materials had similar chemical group function, with a higher amount of C-C bonds, followed by C-O and C=O (p<0.05). The presence of C=O is assigned primarily to the ester bond of the resin and identified that ester groups were present in similar levels (approximately 10%) within the surface region of all materials and potentially available for hydrolysis.

Surface wettability of the materials was analyzed by advancing water contact angle measurements (Table 3.1). The most hydrophilic material was EB, having the lowest contact angle (55.4±4.0), followed by SB and then Z250 (p<0.05).

### 3.3.3 Biodegradation:

A trend of increasing BisHPPP release with time throughout the incubation period was observed for all three materials (Fig. 3.2). The amount of BisHPPP released was elevated in the presence of bacteria vs. control for EB and Z250 but not for SB (p<0.05) after 14 and 30 days of incubation. The amount of BisHPPP released from EB after 30 days of incubation with *S. mutans* UA159 (143.15±3.28μg/cm²) was 39 and 82 times higher than
that released from Z250 (3.71±0.24μg/cm²) and SB (1.74±0.31μg/cm²), respectively (p<0.05).

In the specimens incubated with bacteria, the total amount of BisHPPP released throughout the incubation period was significantly higher in EB (375.4±3.6μg/cm²) as compared to Z250 (13.1±0.7μg/cm²) and SB (6.1±0.3μg/cm²) (p<0.05).

SEM micrographs (Fig. 3.3) demonstrate that the surface of the specimens incubated with S. mutans UA159 for 30 days appear rougher than BHI-incubated and non-incubated specimens.

3.4 Discussion:
The results of this study support the hypothesis that cariogenic bacteria (S. mutans) contain esterase activities at levels capable of hydrolytic-mediated degradation of cured dental resin composites and adhesives. This represents a significant finding for the field and identifies a clear vulnerability of current restorative materials to one of the most prominent bacteria in oral pathology.

Human saliva has been shown to hydrolyze composite resins and adhesives (8). Human salivary esterases were previously analyzed and shown to have activity toward o- and p-nitrophenyl esters and BTC (15). In the current study, all tested strains of S. mutans had activity towards the nitrophenyl esters, but not BTC, in levels that were shown previously to degrade resin composites and adhesives (8,19). Overall, the activity patterns of S. mutans suggest that microorganisms are significant contributors to acetate-like dependent esterase activities of saliva and less to the butyrate-like dependent esterases that are characteristic of human salivary esterase activity (15).
S. mutans UA159 was selected over the other strains for the subsequent biodegradation study because it had the highest activity with respect to p-NPB, a characteristic activity previously shown to also affect composites incubated with human saliva (7). BisHPPP, a BisGMA-derived biodegradation product, was analyzed since it is a good marker of true resin biodegradation due to the hydrophobic nature of its precursor, and therefore the results provide a good indication of the biodegradation potential of S. mutans UA159 in vivo (16).

Many bacterial species express esterases, but despite this knowledge the overall function of S. mutans esterases, and more specifically their importance in contributing to the biodegradation process of dental resin composite restorations, are not well-understood. In other bacteria, esterases have been linked to virulence and pathogenesis. A cell wall-anchored carboxylesterase has been shown to be essential for the virulence of Mycobacterium tuberculosis (20). Also, a Streptococcal secreted esterase from Group A Streptococcus was identified as a virulence factor that contributes to severe invasive infection (11). In addition, increased bacterial expression of esterases in acidic conditions has been observed; Lactobacillus reuteri was found to increase the expression of a putative esterase (Ir1516) in acidic conditions. This enzyme is believed to function by changing the cell wall structure and therefore increasing the cells tolerance towards acidic conditions (21). These studies highlight the importance of esterases and point towards the need to study the potential role of S. mutans esterases in aciduricity, virulence, and pathogenesis (i.e. dental caries).

The self-etch adhesive (EB) exhibited a higher release of BisHPPP relative to the total-etch adhesive (SB), and the composite resin (Z250), after incubation with bacteria or
bacterial media. The differences ranged between 39 and 82 times higher than that released from Z250 and SB, respectively after 30 days of incubation with bacteria. Because there was no difference in the degree of conversion (FTIR) and surface elemental composition of the specimens (XPS) between the different materials, and since the amount of BisHPPP production for each material was not correlated with the BisGMA content for each material, the difference in the amounts of released products could only arise from the materials’ inherent bulk differences in chemical composition.

The incorporation of acidic monomers (phosphoric acid-6-methacryloxy-hexylesters, and copolymer of acrylic and itaconic acid) and water as a co-solvent in EB at high concentrations renders this material more hydrophilic when compared to SB and Z250, as demonstrated by the advancing water contact angles. The increased hydrophilicity amplifies water sorption, which in turn leads to greater susceptibility of the ester bonds to hydrolysis (22,23). Water sorption has also been shown to contribute to hydrolysis, plasticization of the polymer, and the lowering of mechanical properties (22,23). In addition to esterase mediated hydrolysis, hydrogen ions from acidic resin monomers, and hydrogen ions produced by oral biofilms, all have the potential to catalyze the hydrolysis of the ester bonds present in the polymer matrix, generating degradation products such as BisHPPP (5,24,25). Our findings corroborate clinical studies that showed the annual failure rates of cervical bonded restorations to be greater in one-step self-etch adhesives, as compared to three-step total-etch (etch-and-rinse) adhesives and this appears to result from the poor combination of chemistry in the material (26,27).

Advancing water contact angle values revealed that SB is more hydrophilic than Z250, which could be attributed to the inclusion of 30-40 % by weight of HEMA in SB, and the
use of different monomers in Z250. A Previous degradation study (8) demonstrated greater degradation of SB vs. Z250 by human saliva. Yet, in the current study, SB showed slightly more stability and released less BisHPPP than Z250 in the presence of bacteria. Therefore, in addition to the material’s hydrophilicity, other factors are affecting the material’s relative biostability.

The co-existence of both CE-like and PCE-like activities in in vitro systems was shown to result in a more efficient biodegradation of the resin matrix (28). The lack of activity for S. mutans toward the PCE-like substrate BTC, as compared with the broader activity of saliva which contains both CE-like and PCE activity (15) could explain the less efficient degradation of SB by bacteria, in part because SB contains water-soluble moieties such as HEMA which may show more susceptibility to PCE-like enzymes. In previous work it was demonstrated that CE has greater specificity than PCE to hydrolyze phenol-containing monomers, such as BisGMA and BisEMA, while PCE showed greater affinity than CE toward short water-soluble monomers such as TEGDMA (15, 29).

XPS data and SEM micrographs revealed that the surfaces of all pre-incubated specimens were composed mainly of resin, precluding any influence of the filler on the initial amount of resin surface available for degradation. Also, the release of BisHPPP was not correlated with filler contents for the different materials, which is in agreement with other work by the investigators (16). Following 30-days incubation, SEM analyses demonstrated the degradation of all materials, as the surfaces of all the specimens incubated with bacteria were rougher than the controls. This observation corroborates previous studies, which also showed that bacteria such as S. mutans, Streptococcus gordonii, and Streptococcus sanguis could degrade polymeric surfaces (18, 30).
Kermanshahi et al showed that exposure of dentin-composite restorations to salivary esterase-like activity resulted in the formation of micro-gaps that were infiltrated and colonized by biofilms of the cariogenic bacteria such as *S. mutans* (9). When present within the confined space of the restoration-tooth marginal interface, *S. mutans* could contribute to the deterioration of the resin-dentin interface by producing both acids (24) and esterases (Fig. 3.1), affecting the hybrid layer, tooth and composite, and ultimately compromising the integrity of the margins and reducing the longevity of the restoration. Although esterase-mediated degradation occurred in all materials used in this study, the extent of degradation was material dependent and material chemistry appeared to be a critical factor in determining a restoration’s biochemical stability. Manufacturers of dental resin composites and adhesives should test for the materials biochemical stability in order to conceive more biostable materials.

### 3.5 Acknowledgements:

The authors thank Dr. Dilani Senadheera, Ms. Martha Cordova, and Ms. Kirsten Krastel for their assistance with bacterial cultures, and Dr. Meilin Yang for his technical support. Grants: The project described was supported in part by the Canadian Institute of Health Research Operating Grant MOP115113; Award Number R01DE021385 from the National Institute Of Dental & Craniofacial Research, Canadian Foundation of Innovation and University of Toronto. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute Of Dental & Craniofacial Research and the National Institutes of Health.
3.5 References:


3.6 Figures:

**Figure 3.1:** Activity profile for *S. mutans* UA159, JH1005, LT11, NG8, UA140, BM71, and GS5 with p-nitrophenolacetate (p-NPA), p-nitrophenolbutyrate (p-NPB), o-nitrophenolbutyrate (o-NPB), and butyrylthiocholine iodide (BTC). N=3, Data are the mean ± S.E. *p<0.05.
Figure 3.2: A) Cumulative amounts of BisHPPP production after incubation of a composite (Z250) (A), total-etch adhesive (SB) (B), and self-etch adhesive (EB) (C) in BHI with S. mutans UA159 (Black), and with BHI alone (Grey). N=3, Data are the mean ± S.E, t-test or one way ANOVA, Tukey’s post-hoc test (p<0.05). * represents significant differences between the two incubation conditions (groups) for the same time point. Values with the same lower-case letter denote statistically non-significant differences within groups for each material (p >0.05). Values with the same capital letters indicate non-significant differences between materials (p>0.05).
Figure 3.3: Scanning electron micrographs of Z250 (a–c), SB (d–f), and EB (g–i) at day 0 (a,d,g), and following 30 days of incubation with BHI (b,e,h), and with *S. mutans* UA159 (c,f,i) (10 x original magnification). Scale bar applies to all figures and represents 3 µm. Note the rougher surfaces of bacteria-incubated specimens, with rougher surface for EB vs. SB and Z250.
Table 3.1: Composition (based on MSDS 3M) and surface properties of composite resin (Z250), total-etch (SB) and self-etch (EB) adhesives. Data are the mean ± S.D. Statistical analysis was done using one-way ANOVA, Tukey’s post-hoc test. Values with the same letter (a, b or c) denote statistically non-significant differences between materials for the same assay (p>0.05).

<table>
<thead>
<tr>
<th>Composition</th>
<th>Z250</th>
<th>SB</th>
<th>EB</th>
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</thead>
<tbody>
<tr>
<td>Contact angle</td>
<td>89 ± 4.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>75 ± 5.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>55.4 ± 4.0&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Degree of conversion</td>
<td>71.7 ± 2.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>66.1 ± 4.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>74.9 ± 5.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Elemental (atomic %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1s</td>
<td>66.4 ± 3.6</td>
<td>72.2 ± 3.0</td>
<td>71.4 ± 2.3</td>
</tr>
<tr>
<td>O1s</td>
<td>24.8 ± 1.8</td>
<td>24.8 ± 2.1</td>
<td>25.0 ± 1.2</td>
</tr>
<tr>
<td>Si2p</td>
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<td>1.7 ± 0.7</td>
<td>1.5 ± 0.9</td>
</tr>
<tr>
<td>Zr3d</td>
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<td>0.1 ± 0.1</td>
<td>0.05 ± 0.04</td>
</tr>
<tr>
<td>Carbon bonds (atomic %)</td>
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<td></td>
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<tr>
<td>C-C</td>
<td>66.7 ± 1.5</td>
<td>66.9 ± 2.2</td>
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<td>C-O</td>
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<td>21.8 ± 2.7</td>
<td>23.7 ± 1.6</td>
</tr>
<tr>
<td>C=O</td>
<td>9.4 ± 0.5</td>
<td>10.0 ± 3.1</td>
<td>9.8 ± 0.2</td>
</tr>
<tr>
<td>Composition</td>
<td>Z250</td>
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<td>EB</td>
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<tr>
<td>Bisphenol A diglycidyl ether dimethacrylate (BisGMA)</td>
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<td>15-25</td>
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<td>2-Hydroxyethyl methacrylate (HEMA)</td>
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<td>30-40</td>
<td>15-25</td>
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<tr>
<td>Diurethane dimethacrylate (UDMA)</td>
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<td>—</td>
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<tr>
<td>Bisphenol A polyethylene glycol diether dimethacrylate (BisEMA)</td>
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<td>—</td>
<td>—</td>
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<tr>
<td>Triethylene glycol dimethacrylate (TEGDMA)</td>
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<td>10-15</td>
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<tr>
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<td>—</td>
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<td>10-15</td>
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<td>1,6-Hexanediol dimethacrylate</td>
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<td>5-10</td>
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<tr>
<td>Copolymer of acrylic and itaconic acid</td>
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Chapter 4 – Cariogenic bacteria studies:

4.1 Extended materials and methods:

4.1.1 Bacterial strains:
*S. mutans* strains UA159, JH1005, LT11, NG8, UA140, BM71, GS5, UA159_SMU.118c+, and ΔUA159_SMU.118c were used in this study. All *S. mutans* strains were stored in 15% glycerol broths at -80 °C. Bacterial cells were sub-cultured on Todd-Hewitt agar plates supplemented with 0.3% yeast extract (THYE) (1). *E.coli* strain DH5α was grown aerobically at 37 °C in Luria-Bertani (LB) medium. Antibiotics erythromycin (erm) (10 μg/ml) and chloramphenicol (20 μg/ml) were added to the media as needed. Bacterial growth was assessed by changes in optical density at 600 nm.

4.1.2 Bacterial CE-like activity assay:
CE-like esterase activity from *S. mutans* UA159 strains was measured using p-NPB substrate (Sigma, St. Louis, MO) at the lag, log, and stationary phases. The substrate p-NPB was chosen because it was previously shown to be a good indicator of salivary esterase activity (8).

Colonies of *S. mutans* from THYE plates were cultivated overnight in THYE broth (37°C and 5 % CO₂). The following day, a 1:10 dilution in THYE broth was performed. Bacterial cells were incubated and allowed to grow to lag, mid-log, and stationary phases, and then they were washed and resuspended in PBS buffer (pH=7.0). Esterase activity was determined by incubating 1 ml of the bacterial cell suspension and 500 μl of the prepared p-NPB substrate. Esterase-like activity was normalized to dry weight of bacterial cells.
1 CE unit is defined as the release of 1 nmol of p-nitrophenol per min at 401 nm measured using an Ultrospec II spectrophotometer unit (LKB Biochrom, Cambridge, UK) (pH 7.0 and 37 °C).

Optical density (O.D) was recorded every 30 seconds for 300 seconds, with a blank cuvette containing 1000 µl of PBS buffer and 500 µl of p-NPB as a reference. The rate of absorbance per minute was graphed and the resulting slope was taken as the average optical density per minute (O.D/min) (2,3)

CE activity was calculated according to the following equation:

\[
\text{CE-like activity (units/ µg dry weight cells) } = \frac{O.D \times T.V \times 10^9}{E.C \times L.P \times D.W}
\]

Where:

O.D = change in absorbance per minute at 401 nm

T.V = Total Volume (0.0015L)

E.C = molar absorptivity of p-nitrophenol at 401 nm (16000 M⁻¹ cm⁻¹)

L.P = Length Path (1cm)

D.W = Dry Weight of bacterial cells

4.1.3 Bacterial PCE-like activity assay:

PCE activity was determined using butyrylthiocholine iodide (BTC) and 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB) as the substrate at the lag, log, and stationary phases.
Esterase-like activity was normalized to dry weight of bacterial cells. One unit of PCE activity is defined as the release of 1mmol of 5-thio-2-nitrobenzoic acid per minute at 405 nm.

An Ultrospec II spectrophotometer unit (LKB Biochrom, Cambridge, England) was used for PCE activity measurements. PCE activity was measured by adding 500 μl of BTC + DTNB solution to 1000 μl of bacterial cell suspension in a 1.5 ml plastic cuvette. Optical density measurements were obtained every 30 seconds for 300 seconds, with a blank cuvette of 500 μl of BTC + DTNB solution and 1000 μl PBS as a reference. The rate of absorbance per minute was graphed and the resulting slope was taken as the average optical density per minute (O.D/min). PCE activity was calculated according to the following formula (2,3):

$$\text{PCE-like activity (units/ml)} = \frac{\text{O.D} \times \text{T.V} \times 1000}{\text{E.C} \times \text{L.P} \times \text{D.W}}$$

Where:

- **O.D** = change in absorbance per minute at 405 nm
- **T.V** = Total Volume (0.0015L)
- **E.C** = molar absorptivity of 5-thio-2-nitrobenzoic acid at 405 nm (13600 M$^{-1}$ cm$^{-1}$)
- **L.P** = Length Path (1cm)
- **D.W** = Dry Weight of bacterial cells
4.1.4 Bacterial esterase stability assays:
CE-like esterase activities from *S. mutans* UA159 were measured using p-nitrophenolbutyrate (p-NPB) substrate (Sigma, St. Louis, MO) as described above (5.1.2). Colonies of *S. mutans* UA159 from THYE plates were cultivated overnight in THYE broth (37°C and 5% CO₂). The following day, a 1:10 dilution in tryptone yeast extract supplement with 0.2% glucose broth (TYEG) was performed. Bacterial cells were incubated and allowed to grow to mid-log, and then washed and resuspended in a chemically defined media (4) with or without TEGDMA (0.5 x 10⁻⁴ M) and BisGMA (10⁻⁴ M) (Esschem, Linwood, PA). CE-like activity measurements were carried out at times 0, 30min, 1h, 2h, 4h, and 8h.

4.1.5 Construction of ΔUA159_SMU.118c:
*S. mutans* UA159 knockout mutant for SMU.118c (ΔUA159_SMU.118c) was constructed through PCR-ligation mutagenesis according to a previously established protocol (5). Briefly, polymerase chain reaction (PCR) primer pairs SMU.118c-P1/SMU.118c-P2 and SMU.118c-P3/SMU.118c-P4 (Table 4.1) were used to amplify the 5’ and 3’ flanking regions of SMU.118c respectively from genomic DNA. The flanking regions were received from NCBI database. Erm-PA and Erm-PB primers (Table 3.1) were used to amplify the erm⁵ gene from the erm cassette. SMU.118c-P2 contains AscI restriction site and SMU.118c-P3 contains FseI restriction site, both attached to the 5’ end. Also, Erm-PA contains AscI site and Erm-PB contains an FseI site, both attached to the 5’ end.

The contents of the PCR mixtures were 10 x PCR buffer, MgCl₂, dNTPs, primers, Taq DNA polymerase, and the template DNA. Enzymes, deoxynucleotidides, buffers and solutions for PCR were supplied by MBI Fermentas (Burlington, ON, Canada), while
primers were purchased from Operon (Toronto, ON, Canada). Thermal cycling was performed using a T Gradient Thermocycler (Whatman Biometra, Goettinggen, Germany) with the following parameters: (1) 94 °C for 10 minutes, (2) 30 cycles of 94 °C for 30 seconds; 55 °C for 30 seconds; 72 °C for 1 minute, (3) 72 °C for 10 minutes, and (4) pause at 4 °C. PCR products were electrophoresed on a 1% agarose gel for visualization. PCR products were also purified using the Fermentas PCR product purification kit (Burlington, ON, Canada) according to manufacturer's instructions. The purified PCR products of the 5' and 3' flanking fragments were digested with restriction endonucleases Ascl and FseI respectively overnight at 37 °C (New Englan BioLabs, Beverly, MA, USA). The erm\textsuperscript{r} insert was digested with both enzymes. The digestion reaction was heat-inactivated at 65 °C for 10 minutes. Directional ligation was then performed by mixing the three digested PCR products, NEB 10 x buffer for T4 DNA ligase, ddH\textsubscript{2}O, and T4 DNA ligase (New England BioLabs) overnight at room temperature.

DNA from the ligation reaction was incubated with \textit{S. mutans} UA159 and competence stimulating peptide (CSP) for 90 minutes. At the end of the incubation, cells were sonicated for 15-20 seconds and plated on selective THYE-erm agar plates and incubated for 48 hours at 37 °C and 5% CO\textsubscript{2}. Clones obtained were confirmed via PCR and DNA sequencing.
Table 4.1: Primers for PCR ligation mutagenesis to delete \textit{S. mutans} SMU.118c gene

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence</th>
</tr>
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<tbody>
<tr>
<td>Erm-PA</td>
<td>5' GGC\textsubscript{CGCG}GCCC\textsubscript{CGGCC}AAAATTTGTTTGAT 3'</td>
</tr>
<tr>
<td>Erm-PB</td>
<td>5' GGC\textsubscript{CGCG}GCCC\textsubscript{AGTC}GGCAGCGACTCATAGAAT 3'</td>
</tr>
<tr>
<td>SMU.118c-P1</td>
<td>5' AAGAAGTCTGTTTGTGGG 3'</td>
</tr>
<tr>
<td>SMU.118c-P2</td>
<td>5' GGC\textsubscript{CGCG}CCTTGACGAATGGCTGTAGCG 3'</td>
</tr>
<tr>
<td>SMU.118c-P3</td>
<td>5' GGC\textsubscript{CGCG}CGCGAACCATA\textsubscript{AAGTTGAGG} 3'</td>
</tr>
<tr>
<td>SMU.118c-P4</td>
<td>5' CCCTATTAAAACACG\textsubscript{AGCACC} 3'</td>
</tr>
</tbody>
</table>

4.1.6 Construction of UA159_SMU.118c+:

\textit{S. mutans} UA159 overexpression mutant for SMU.118c (UA159_SMU.118c+) was constructed according to a previously established method (6). Briefly, PCR primer pairs SMU.118c-F (5' CAGAGCTCCTTTGTTGAAATTGTTGTCC), and SMU.118c-R (5' CGCGAATTCCTTTGTTGAAATTGTTGTCC), and SMU.118c-R (5' CGCGAATTCCTTTGTTGAAATTGTTGTCC) were used to PCR amplify SMU.118c from \textit{S. mutans} UA159 genomic DNA. SMU.118c-F contains \textit{SacI} restriction site and SMU.118c-R contains \textit{EcoRI} restriction site, both attached to the 5’ end. Thermal cycling was performed using a T Gradient Thermocycler (Whatman Biometra, Goettinggen, Germany) with the following parameters: (1) 94 °C for 10 minutes, (2) 30 cycles of 94 °C for 30 seconds; 55 °C for 30 seconds; 72 °C for 1 minute, (3) 72 °C for 10 minutes, and (4) pause at 4 °C. The SMU.118c PCR product was purified using the Fermentas PCR product purification kit (Burlington, ON, Canada) and digested with \textit{SacI} and \textit{EcoRI} (New Englan BioLabs, Beverly, MA, USA) overnight at 37°C. The enzymes in the restriction digestion were heat-inactivated at 65°C for 10 minutes before ligation. The
shuttle plasmid pIB166 was also digested with the same restriction enzymes (SacI and EcoRI). Cloning on the digested plasmid pIB166 and SMU.118c was then performed overnight at room temperature under the control of the lactococcal promoter P23. The recombinant plasmid was transferred into and purified from DH5α competent *E. coli* cells. The recombinant plasmid was confirmed by sequencing and then transformed in *S. mutans* UA159 cells using a protocol that employs CSP as described above.

Overnight cultures of *S. mutans* UA159 wild type and UA159_SMU.118c+ cells were diluted 20X in THYE and incubated (37 °C and 5% CO2) to mid-log growth phase. Total RNA was extracted using Trizol reagent (Invitrogen, Burlington, ON, Canada). After treating with DNAse, the RNA samples were subjected to reverse transcription using the First Strand cDNA Synthesis Kit (Pharmacia, Biotech). Controls included reactions with no RNA template and another in the absence of reverse transcriptase. Finally, the single stranded cDNAs were incorporated into rtPCR experiments using a Mx3000P QPCR system (Stratagene, La Jolla, CA) and Quantitect SYBR-Green PCR kit (Qiagen, Mississauga, Ontario, Canada). The primer pair used for rtPCR were: 5’ CCTGTGATACAAGTTTGCTGCTGTC3’ & 5’ GTTATGCTGCCATCTCTGTTS3’. The fold expression change was calculated according to the method of Pfaffl et al. using the following formula (7):

\[
\text{Fold change} = \frac{\text{Eff target gene } (CT_{\text{control}} - CT_{\text{experimental}})}{\text{Eff 16S rRNA } (CT_{\text{control}} - CT_{\text{experimental}})}
\]

Where E = (10-1/slope) represents the efficiency of gene amplification and CT values are the threshold cycle values of the target gene. Results were normalized against *S. mutans*
16SrRNA expression that was invariant under the experimental test conditions.

**4.1.7 Bacterial esterase-like activity profile assay:**
*S. mutans* strains UA159, JH1005, LT11, NG8, UA140, BM71, GS5, ΔUA159_SMU.118c, and UA159_SMU.118c+ were sub-cultured on Todd-Hewitt agar plates supplemented with 0.3% yeast extract (THYE) (1). Colonies of *S. mutans* from THYE plates were cultivated overnight in THYE broth (37°C, 5 % CO₂) and then diluted 1:10 and allowed to grow to mid-log, washed and resuspended in phosphate buffer (pH=7.0). Esterase activities, CE-like and PCE-like were determined by incubating 1 ml of the bacterial cell suspension in 0.5 ml of either p-nitrophenolbutyrate (p-NPB), o-nitrophenolbutyrate (o-NPB), p-nitrophenolacetate (p-NPA), or butyrylthiocholine iodide (BTC) substrates (Sigma, St. Louis, MO) (8).

The substrates o-NPB and p-NPA (4 mM for both substrates) were prepared by adding 17.75 μl of o-NPB or 18.11 mg of p-NPA to 5.5 ml of acetonitrile in a 25ml glass tube. The solutions were vortexed and diluted by 19.5 ml of PBS buffer, and stored at -80°C until needed.

**4.1.8 Monomer degradation:**
*S. mutans* UA159 was cultivated overnight in brain heart infusion supplemented with % 1 sucrose broth (BHIS, 37 °C and % 5 CO₂). The following day, a 1:10 dilution in BHIS containing either BisGMA (10⁻⁴ M) or TEGDMA (0.5 x 10⁻⁴) monomers was prepared. This mixture was incubated for 72 hours (test group). The control group was not inoculated with bacterial cells. At specific time points (0, 12h, 24h, 48h, and 72h), 200μl samples of the incubation solutions were removed and an equal volume of methanol added to denature enzyme activity and stop the hydrolysis reaction (9). The sample
solutions were then filtered using Amicon Ultra centrifugal filters (3 kDa cut-off membrane) at 14000 g for 10 minutes and 4°C to remove bacterial cells and high molecular weight proteins from resin degradation products. The resulting filtered solutions were refrigerated at 4°C until required for analysis (injection into HPLC) (2,9).

4.1.9 High performance liquid chromatography (HPLC):
High performance liquid chromatography (high pressure liquid chromatography) is a chromatographic method used to separate, isolate, identify and quantify individual components of mixtures. The HPLC apparatus consists of a reservoir, pump, injector, column, detector, a computer data station and a waste collector.

![Diagram of HPLC system](image)

**Figure 4.1:** high performance liquid chromatography system (2).
The process of separating degradation products begins with injecting a sample of the incubation solution through the injector. The injector then introduces the sample to the continuously flowing solvent (mobile phase), in which the sample dissolves. The pump provides the high pressure needed to pump the dissolved solution through the column. The column contains the chromatographic packing material (stationary phase) needed to separate sample components. The rates by which sample components are eluted are influenced by the affinity of these components to the mobile and stationary phases (hydrophobic and hydrophilic interactions). The detector senses the separated materials as they elute from the HPLC column. The time it takes for compounds to travel through the column to the detector is known as the retention time (2).

In the current study, reverse phase chromatographic process was performed, where a non-polar material will be used for the stationary phase and a polar solvent will be employed for the mobile phase. This means that polar compounds elute faster than non-polar ones because of their greater affinity to the mobile phase. Whereas non-polar compounds have a greater affinity to the non-polar stationary phase, resulting in longer retention times for them. Separation of degradation products was achieved using a gradient method set to run over a period of 30 minutes. The mobile phase consisted of a polar solvent HPLC grade methanol and a 2mM buffer solution of ammonium acetate (99.99% pure) with pH adjusted to 3.0 with HCl 6.00 N (8,10). Table 4.2 represents the optimized mobile phase method that was applied for the separation of biodegradation products.
Also in the current study, a Waters™ HPLC system (Waters, Mississauga, ON) was used, it consisted of a 600E multi-solvent delivery system and a 996 photodiode array (PDA) detector coupled with a Millennium chromatography manager, version 2.15. A Phenomenex Luna 5µm C₁₈ 4.6 x 250 (Phenomenex, Torrance, CA) column was used to separate and isolate degradation products (10).

Table 4.2: HPLC gradient method for separation of biodegradation products.

<table>
<thead>
<tr>
<th>Time (Minutes)</th>
<th>Flow Rate (ml/min)</th>
<th>Methanol (%)</th>
<th>Buffer (%)</th>
<th>Water (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.0</td>
<td>40</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>1.0</td>
<td>60</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>1.0</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>1.0</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The chromatograms were reported at a UV wavelength of 280nm (2,9).

4.2 Extended results and discussion:

4.2.1 CE-like and PCE-like activity assays:

*S. mutans* UA159 had no PCE-like activities at any of the three stages measured; lag phase, log phase, and stationary phase (no activity towards BTC substrate). CE-like activities of *S. mutans* UA159 were measured at the three different stages (activity towards p-NPB) and illustrated in Figure 4.1.

One-way ANOVA followed by Tukey’s post-hoc analysis demonstrated significant differences between the CE-like activities of the lag and log phases cells compared to the stationary phase cells (p<0.01). There was no statistical difference between the CE-like
activities of lag and log phases cells (p=0.283) (Fig. 4.1). Higher CE-like activities in the lag and log phases indicate the presence of more esterase-like activities in these phases when the bacteria are preparing to multiply (lag) and are multiplying and dividing (log).

Research has shown increased bacterial expression of esterases in acidic conditions; *Lactobacillus reuteri* (a gram positive bacterium that naturally inhabits the gut) was found to increase the expression of a putative esterase (Ir1516) in acidic conditions. This enzyme is a putative esterase belonging to a class of penicillin-binding proteins (beta-lactamase family class C) and is believed to function by changing the cell wall structure and therefore increasing the cells tolerance towards acidic conditions (20). Penicillin-binding proteins are usually involved in peptidoglycan synthesis. Another study points towards the importance of esterases in biofilm formation. *S. mutans* can utilize sucrose-dependent adhesion mediated by glucosyltransferases (Gtfs), which are enzymes that produce water soluble and insoluble glucans from sucrose (11). In order to bind to these glucans, *S. mutans* produces four glucan binding proteins (GBPs): GbpA, GbpB, GbpC, and GbpD. GbpD is a glucan binding protein that can be both secreted and cell-associated (12). Loss of GbpD has been shown to result in extremely fragile biofilms, signifying the importance of this protein (GbpD) in providing biofilm scaffolding and promoting cohesiveness between glucan and bacteria in the biofilm (13,14). In addition to binding to glucan, GbpD has been shown to contain lipase (esterase) activity by binding to a range of triglycerides in the presence of calcium and releasing free fatty acids (FFA). Because of the bi-functionality (glucan binding/lipase) of GbpD, it has been suggested that the natural substrate for this enzyme maybe a surface macromolecule consisting of carbohydrate linked to lipid. GbpD derived from *S. mutans* was found to bound to and
release FFA from lipoteichoic acid (LTA) of *S. sanguinis*, but had no effect on LTA from *S. mutans*. This result has been linked to the possibility that GbpD may be involved in direct interspecies competition within the plaque biofilm (15). Overall, when considering the potential role of esterases in cell wall modifications (20), our results are in line with expression of these activities when the bacteria are actively multiplying compared to when they are established (stationary phase).

![Graph](image_url)

**Figure 4.1:** CE-like activity (units/mg dry weight cells) of *S. mutans* UA159 at lag, log and stationary phases. Mean ± SD, One way ANOVA, N=3. *p<0.05.

**4.2.2 Bacterial esterase stability assays:**
The results for the stability assay are reported as relative CE-like activity (%) versus time (Figure 4.2). The results for CE-like stability show that *S. mutans* UA159 undergoes an initial increase in activity that reaches a maximum after 30 minutes of incubation to
approximately 118% of initial activity, and then the activity declines and diminishes after 4 hours of incubation. A similar CE-like activity trend as that of *S. mutans* UA159 is observed for *S. mutans* UA159 incubated with BisGMA and TEGDMA, with the only noticeable difference being an initial decline in activity of both *S. mutans* UA159+BisGMA and *S. mutans* UA159+TEGDMA. This reduction can possibly be attributed to the enzymes in *S. mutans* UA159 undergoing interaction with the monomers. Also, the presence of biodegradation byproducts in the incubation solution can make it more difficult for p-NPB to access the enzymes active sites (16).

**Figure 4.2**: Relative CE-like activity of *S. mutans* incubated in chemically defined media (37°C and 5% CO₂) and with the addition of BisGMA or TEGDMA monomers, (mean ± SD, N=3).
4.2.3 Bacterial esterase-like activity profile assay:

All strains of *S. mutans* had activity towards the nitrophenyl esters (Figure 4.3). All strains had preference toward the p-NPA and p-NPB vs. o-NPB (p<0.05) but there was no difference in the affinity between the 2 butyrate-isomers. All *S. mutans* strains showed no activity towards BTC substrate. A slight increase in activity of UA159_SMU.118c+ towards p-NPA and p-NPB was observed, this increase in activity is directly associated with the increased gene expression of the putative esterase SMU.118c. Also a slight decrease in ΔUA159_SMU.118c activity towards p-NPA and p-NPB compared to wild type (UA159) is observed because of the absence of the SMU.118c gene. This result indicates that SMU.118c contributes to the measured overall esterase activity, but there are other sources of bacterial esterase activity toward these substrates.

Human saliva has been shown to hydrolyze composite resins and adhesives (17). Human salivary esterases were previously analyzed to have activity toward o- and p-nitrophenyl esters and BTC (8). In the current study, all strains of *S. mutans* had activity towards the nitrophenyl esters, but not BTC, in levels that were shown previously to degrade resin composites and adhesives (17,18). Overall, the activity patterns of *S. mutans* suggest that microorganisms are a potential contributor but not the sole contributor to esterase activities of saliva (8).
Figure 4.3: a) Activity profile for *S. mutans* UA159, JH1005, LT11, NG8, UA140, BM71, GS5, UA159_SMU.118c+, and ΔUA159_SMU.118c with p-nitrophenolbutyrate (p-NPB), o-nitrophenolbutyrate (o-NPB), p-nitrophenolacetate (p-NPA), and butyrylthiocholine iodide (BTC). All strains of *S. mutans* had no activity towards BTC. N=3, Data are the mean ± S.D. *p<0.05.

4.2.4 Monomer degradation:
For BisGMA and TEGDMA monomers incubated with *S. mutans* UA159, no detectable amounts of biodegradation byproducts were observed. Therefore, the relative amounts of BisGMA and TEGDMA over time were traced (Figure 4.4). There were no differences observed between control and test groups in the amount of BisGMA and TEGDMA remaining in solution throughout the 72 hours incubation period (p > 0.05). After 72 hours of incubation, 67.1 ± 6.3 % and 66.6 ± 8.5 % of BisGMA monomer were detected.
in solution for the test and control groups respectively (p > 0.05). Similarly, 89.6 ± 5.8 % and 90.4 ± 10.8 % of TEGDMA monomer were detected in solution for the test and control groups respectively (p > 0.05). This result indicates that S. mutans interacts with the monomers differently than compared to the polymer (Chapter 3). When exposed to the polymers, the bacteria adheres to the surface of the material (Chapter 3), establishes a biofilm community and interacts with the material over a long period of time (1 month). In contrast, in the monomer study, the monomer is dissolved in solution and interacts with bacteria that is grown in planktonic phase for a shorter period of time (72 hours). These differences may explain why degradation byproducts are detected and measured in the polymer study but not the monomer study. Another factor that could explain the observed differences is the interaction between monomers and bacteria. Previous research has shown that monomers influence S. mutans growth negatively (1,13-15,19). Both BisGMA and TEGDMA have been shown to inhibit cariogenic bacterial growth (13-15). Thus, the effect of monomers on bacterial growth and gene expression must be taken into account when analyzing the observed results.
Figure 4.4: Relative % of (a) TEGDMA and (b) BisGMA remaining in solution after 72 hours of incubation with *S. mutans* and BHIS or with BHIS alone (37°C and 5% CO₂). Mean ± SD, N=3.
4.3 References:


(2) Iris Daniel. Biodegradation of Polyacid Modified Composite Resins by Human Salivary EsterasesUniversity of Toronto; 2009.

(3) S. Kermanshahi. Biodegradation of Resin-Dentin Interfaces Increases Bacterial MicroleakageUniversity of Toronto; 2009.


(16) Y. Finer. The Influence of Resin Chemistry on a Composite's Inherent Biochemical StabilityUniversity of Toronto; 2000.


Chapter 5 – Conclusions and recommendations:

5.1 Conclusions:

- *Streptococcus mutans* contain esterase activities at levels (1.02 ± 0.15 units/mg dry weight of cells for *S. mutans* NG8 to 2.07 ± 0.09 units/mg dry weight of cells for *S. mutans* UA159) that degrade dental resin composites and adhesives.

- The activity patterns of *S. mutans* suggest that microorganisms are significant contributors to acetate-like dependent esterase activities in saliva and less to the more predominant butyrate-like dependent esterases that is characteristic of human salivary esterase activity.

- Biodegradation of dental resin composites and adhesives by *S. mutans* occurred in all materials used in this study. However, the extent of degradation was material dependent, and material chemistry was the most important factor in determining its biochemical stability. The self-etch adhesive (EB) was the most degradable material followed by resin composite (Z250) and total-etch adhesive (SB).

- *S. mutans* had CE-like activity but no PCE-like activity. The lack of activity of *S. mutans* toward the PCE-like substrate BTC could explain the less efficient degradation of SB by bacteria, in part because SB contains water-soluble moieties such as HEMA which may show more susceptibility to PCE-like enzymes (1).

- Measuring esterase activities of wild type, knockout and overexpression strains of SMU.118c indicated that this gene contributes to overall esterase activities from *S. mutans* UA159.
5.2 Recommendations:

- Future investigation to identify and verify the specific source of esterase activities in *S. mutans* that is responsible for dental resin composite and adhesive degradation (in addition to SMU.118c).

- Future investigations should explore the impacts that cariogenic bacteria may have on the mechanical properties of dental resin composites and adhesives such as fracture toughness, and the impacts on the resin-dentin interface (2,3).

- In addition to the four substrates used to measure bacterial esterase activities in the current study (p-NPA, p-NPB, o-NPB, and BTC). Future investigations should explore measuring esterase activities produced by bacteria using p-nitrophenyl palmitate (which is cleaved by lipases) vs. p-NPB (which is cleaved by esterases and sometimes by lipases also). Furthermore, future investigations should also explore measuring salivary esterase activities with the same substrate (4).

- Future investigations should explore using co-cultures as a bacterial model because this better simulates the conditions in the oral cavity and bacterial genotypic and phenotypic expression are different in a co-culture system compared to mono-culture system. The choice of species of bacteria, the duration of incubation, medium of incubation, incubation under static or flow conditions are challenges that must be taken into account.

- When present within the confined space of the restoration-tooth marginal interface, *S. mutans* could contribute to the deterioration of the resin-dentin interface by producing both acids and esterases, affecting the hybrid layer, tooth
and composite, ultimately compromising the integrity of the margins and reducing the longevity of the restoration.
5.3 References:


