Effects of Model Salivary Esterases and MMP Inhibition on the Restoration’s Marginal Integrity and Potential Degradative Contribution of Cariogenic Bacteria

By

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Faculty of Dentistry

University of Toronto

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Abstract

Enzyme-catalyzed degradation of the restoration-tooth interface compromises interfacial integrity, thereby contributing to secondary caries, which is a major cause of resin-based restoration failure.

It is hypothesized that in addition to salivary esterases, the cariogenic bacterium *Streptococcus mutans* has specific esterases that degrade the resin-dentin interface, releasing biodegradation by-products (BBPs) such as bis-hydroxy-propoxy-phenyl-propane (BisHPPP). In turn, BisHPPP affects *S. mutans* by stimulating the expression of esterases. Another hypothesis is that the biostability of the resin-dentin interface is affected by simulated salivary esterases, dentinal matrix metalloproteinase (MMP) inhibition, and restorative materials.

To test the first hypothesis, putative esterase genes in *S. mutans* UA159 were identified, purified, and characterized. SMU_118c was identified as the dominant esterase in *S. mutans* UA159 and showed a similar hydrolytic activity profile to salivary esterases. BisHPPP upregulated expression of the SMU_118c gene and related protein in a concentration-dependent manner. This positive feedback process could accelerate the degradation of the restoration-tooth interface and lead to premature restoration failure. To test the second hypothesis, an *in vitro* model was established to
evaluate the effects of salivary esterases, MMP inhibition and restorative materials on interfacial integrity. It was confirmed that interfacial integrity was compromised with time and was further deteriorated by simulated salivary esterases, as indicated by the greater depth of bacterial ingress and more bacterial biomass of biofilm along the interface. However, this process could be modulated by using different restorative materials and MMPs inhibition.

This project elucidated the mechanistic interaction between oral bacteria and restorative materials and established a new, in vitro, and physiologically relevant model to assess the effect of material chemistry, properties, and application modes on bacterial penetration and biofilm formation. These findings offer the oral health community practical ways to reduce secondary caries by altering material composition and restorative procedures.
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATR</td>
<td>Acid tolerance response</td>
</tr>
<tr>
<td>BBP</td>
<td>Biodegradation by-product</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid assay</td>
</tr>
<tr>
<td>BisEMA</td>
<td>Ethoxylated bisphenol A dimethacrylate</td>
</tr>
<tr>
<td>BisGMA</td>
<td>Bisphenol A glycidyl methacrylate</td>
</tr>
<tr>
<td>BisHPPP</td>
<td>Bis-hydroxy-propoxy-phenyl-propane</td>
</tr>
<tr>
<td>BTC</td>
<td>Butyrylthiocholine iodide</td>
</tr>
<tr>
<td>CDM</td>
<td>Chemically defined medium</td>
</tr>
<tr>
<td>CE</td>
<td>Cholesterol esterase</td>
</tr>
<tr>
<td>CLSM</td>
<td>Confocal scanning laser microscopy</td>
</tr>
<tr>
<td>Cm</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>CQ</td>
<td>Camphorquinone</td>
</tr>
<tr>
<td>CSP</td>
<td>Competence stimulating peptide</td>
</tr>
<tr>
<td>DEGDMA</td>
<td>Diethylene glycol dimethacrylate</td>
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<tr>
<td>EGDMA</td>
<td>Ethylene glycol dimethacrylate</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EPS</td>
<td>Extra cellular polymeric substances</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence <em>in situ</em> hybridization</td>
</tr>
<tr>
<td>FTF</td>
<td>Fructosyltransferase</td>
</tr>
<tr>
<td>GTF</td>
<td>Glucosyltransferase</td>
</tr>
<tr>
<td>GTF-I</td>
<td>Glucosyltransferase-Insoluble</td>
</tr>
<tr>
<td>GTF-S</td>
<td>Glucosyltransferase-Soluble</td>
</tr>
<tr>
<td>GTF-SI</td>
<td>Glucosyltransferase-Soluble/Insoluble</td>
</tr>
<tr>
<td>GBPB</td>
<td>Glucan binding protein B</td>
</tr>
<tr>
<td>HEMA</td>
<td>Hydroxyl ethyl methacrylate</td>
</tr>
<tr>
<td>HK</td>
<td>Hidtidin kinase</td>
</tr>
<tr>
<td>KDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-out</td>
</tr>
<tr>
<td>LC-ESI-MS/MS</td>
<td>Liquid chromatography electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>MA</td>
<td>Methacrylic acid</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-Morpholino)-ethanesulfonic acid</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-Morpholino) propanesulfonic acid</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>oNPB</td>
<td>o-nitrophenyl butyrate</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCE</td>
<td>Pseudo cholinesterase</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethyl sulfonyl fluoride</td>
</tr>
<tr>
<td>pNPA</td>
<td>p-nitrophenyl acetate</td>
</tr>
<tr>
<td>pNPB</td>
<td>p-nitrophenyl butyrate</td>
</tr>
<tr>
<td>pNPL</td>
<td>p-nitrophenyl laurate</td>
</tr>
<tr>
<td>PTS</td>
<td>Phosphotransferase system</td>
</tr>
<tr>
<td>QRT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RR</td>
<td>Response regulator</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SE</td>
<td>Self-etch</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SHSE</td>
<td>Simulated human salivary esterase</td>
</tr>
<tr>
<td>SIEVE</td>
<td>a label-free mass spectrometry protein quantification software</td>
</tr>
<tr>
<td>TCS</td>
<td>Two-component system</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>TCSTS</td>
<td>Two-component signal transduction system</td>
</tr>
<tr>
<td>TE</td>
<td>Total-etch</td>
</tr>
<tr>
<td>TEG</td>
<td>Triethylene glycol</td>
</tr>
<tr>
<td>TEGDMA</td>
<td>Triethylene glycol dimethacrylate</td>
</tr>
<tr>
<td>THYE</td>
<td>Todd-Hewitt-Yeast extract</td>
</tr>
<tr>
<td>TYEG</td>
<td>Tryptone yeast extract supplement with 0.1% glucose broth</td>
</tr>
<tr>
<td>UDMA</td>
<td>Urethane dimethacrylate</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
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Chapter 1

Introduction

1.1 Introduction

Increasing concerns about possible adverse health and environmental effects of mercury, growing aesthetic demands, and improved adhesive technology in dental materials have increased the popularity of resin composite restorations, making resin composite the most commonly used dental restorative material (Arenholt-Bindslev 1998; Frankenberger et al. 2013; Murray et al. 2002b; Simecek et al. 2009; Wakefield and Kofford 2001). However, the service length of resin-composite restorations has been limited due to their high fracture rates, bacterial proliferation, and prevalence of secondary caries which have been the focus of research for several decades (El-Mowafy et al. 1994; Kopperud et al. 2012; Taylor et al. 1994; Turssi et al. 2003; Ástvaldsdóttir et al. 2015). The occurrence of secondary caries at a compromised resin-dentin interface by bacterial penetration has been a major concern, since it is a primary cause (31-70%) of resin composite replacements (Beazoglou et al. 2007; Murray et al. 2002b; Simecek et al. 2009). To prevent secondary caries and increase service length of resin composites, studies have focused on (1) the physical and chemical integrity of a restoration’s adhesive bonded layer (the interface) and (2) cariogenic bacterial behaviors, such as their adaptation mechanisms and microleakage within the interface (Donmez et al. 2005; Gerdolle et al. 2005; Kermanshahi et al. 2010; Khalichi et al. 2004; Khalichi et al. 2009; Sadeghinejad et al. 2017).

Resin composites are bonded to tooth structures by adhesives, resulting in the formation of the restoration-tooth (resin-dentin) interface, also known as the hybrid layer. The hybrid layer is characterized by a 3-dimensional polymer/collagen network that provides continuous and stable
bonding between the bulk of restorative materials and the dentin substrate (Donmez et al. 2005; Van Meerbeek et al. 1998). It is generally accepted that adequate adhesive bonding can be achieved by wetting of the dentin substrate by components of the adhesive system (Erickson 1992) and micromechanical interlocking by resin penetration and entanglement of exposed collagen fibrils in the demineralized dentin (Hashimoto et al. 2000; Wang and Spencer 2003). The bonding mechanism is based on two fundamental processes: (1) removal or modification of the mineral phase from the dentin substrate and (2) filling of the voids that are left by the modification or removal of the mineral phase with adhesive resin.

Two main types of resin-based adhesive systems are currently available: total-etch and self-etch systems. Total-etch (“etch and rinse”) systems are two- or three-step procedures that involve conditioning with an acid, usually 37% phosphoric acid, followed by rinsing, priming, and application of adhesive resin. In some cases, two-step adhesives combine the primer and the adhesive resin in one bottle. These systems involve complete removal of the smear layer by the etchant during the conditioning step. Phase separation between the hydrophobic (2.2-bis [4(2-hydroxy-3-methacryloxypropoxy)-phenyl] propane bisphenol glycidyl dimethacrylate, BisGMA) and hydrophilic (hydroxyl ethyl methacrylate, HEMA) moieties of the adhesive system components results in incomplete penetration of the primer and adhesive into the demineralized dentin and reduced monomer contents and conversion within the hybrid layer. As well, the collapse of exposed collagen fibrils in the demineralized dentin reduces porosity and prohibits resin penetration through the demineralized layer, compromising the formation of a structurally integrated resin-dentin interface. The above processes result in exposed collagen fibrils in the hybrid layer (Spencer et al. 2010).
The two-step original self-etch systems utilize an acidic primer and adhesive that modify the mineral and smear layers, eliminating the rinsing phase. In an effort to increase the efficiency of clinical procedures and reduce technique sensitivity, manufacturers developed all-in-one single-step system where the etching primer and adhesive are combined into a single bottle using non-rinse acidic monomers that simultaneously condition and prime dentin. Since these systems only modify the mineral phase of the dentin, they may prevent collagen collapse and incomplete penetration of the adhesive into the etched dentin (Babb et al. 2009; Hebling et al. 2005a). However, there is still a concern that the hydrophilic nature of the components within the self-etch systems enhances water sorption and intra-oral hydrolytic breakdown, since the bonded interface lacks a non-solvated hydrophobic resin coating (Bouillaguet 2004; Matharu et al. 2001; Sano et al. 1999; Sano et al. 1995b; Santerre et al. 2001a; Zivković et al. 2001). The resultant hybrid layer behaves like a semi-permeable membrane because it permits water movement throughout the bonded interface even after adhesive polymerization, causing reduced structural integrity of the resin-dentin interface (Ateyah and Elhejazi 2004; Manhart et al. 2001; Serkies et al. 2016).

Due to the aforementioned inherent drawbacks in both adhesive systems, perfect interfacial sealing cannot be achieved. As a result, water movement in the interface leads to the hydrolysis of the resin-dentin interface, which is further catalyzed by endogenous and exogenous enzymes, referred to as interfacial biodegradation. And, the hydrolysis and biodegradation processes compromise the overall longevity of the restoration (Delaviz et al. 2014; Spencer et al. 2010).

Two major mechanisms are involved in interfacial biodegradation. One mechanism involves the breakdown of water-rich, resin-sparse collagen fibrils within hybrid layers by the host-derived dentinal matrix metalloproteinase (MMPs) (Liu et al. 2011). MMPs are known as zinc- or calcium-dependent proteolytic enzymes capable of degrading exposed collagen fibrils within the hybrid
layer (Breschi et al. 2010; Ingman et al. 1994; Pashley et al. 2004). Dentin matrix has been shown to contain at least four MMPs: stromelysin-1 (MMP-3) (Boukpessi et al. 2008), true collagenase (MMP-8) (Sulkala et al. 2007), and gelatinases A and B (MMP-2 and MMP-9, respectively) (Niu et al. 2011). Once activated by etchant during the bonding procedure, these peptidases are responsible for the intrinsic auto-degenerative process of collagen fibrils in the demineralized dentin that could lead to the break-down of the interfacial margins (Arola and Reprogel 2005; Boushell et al. 2008; Mazzoni et al. 2009; Nishitani et al. 2006b; Van Strijp et al. 2000). It has been suggested that inhibition of MMP may slow down this process (Montagner et al. 2014). In the current study, galardin was selected as the MMP inhibitor of choice since it has inhibitory activity specifically against MMP-1, -2, -3, -8 and -9 (Grobelny et al. 1992; Hao et al. 1999) at a low concentration and does not have toxic effects toward bacteria. Therefore, its anti-degradative effects can be measured more exclusively under experimental conditions in the current project, which includes bacterial viability as an outcome measure. It is anticipated that application of an MMP inhibitor will reduce the auto-degradation of the collagen fibers within the hybrid layer and enhance the quality of the interface.

The second mechanism of interfacial biodegradation is the hydrolysis of typical methacrylate-based resin moieties: BisGMA and triethylene glycol dimethacrylate (TEGDMA). As in all methacrylate monomers, BisGMA is an universal monomer contained in most matrices of adhesives and composites (Ferracane 2011). Due to the high viscosity of BisGMA caused by its high molecular weight, phenol rings, and hydroxyl groups, a co-monomer TEGDMA is often required as a diluent to reduce viscosity and enhance the efficiency of polymerization by mixing and blending the constituents (Ferracane 2011). These monomers are inherently prone to hydrolysis because of the presence of unprotected ester linkages (Finer and Santerre 2004a).
Hydrolysis can be further catalyzed by salivary and bacterial esterases (Kermanshahi et al. 2010; Shokati et al. 2010).

Esterases (EC 3.1.1.X) are a diverse group of hydrolases that catalyze the hydrolysis of ester bonds. They are widely distributed in human tissues (Finer and Santerre 2004a) and microorganisms (Bornscheuer 2002). In human saliva, cholesterol esterase (CE)-like and pseudocholinesterase (PCE) activities showed strong degradative ability toward resin composite components (Finer and Santerre 2004a; Jaffer et al. 2002), contributing to the resin-dentin interface biodegradation (Kermanshahi et al. 2010; Serkies et al. 2016; Shokati et al. 2010). A recent study confirmed that esterase activity from *S. mutans* UA159 contributes to the biodegradation of resin composites and adhesives (Bourbia et al. 2013). Biodegradation results in the disruption of resin-dentin interface and the release of biodegradation products such as methacrylic acid (MA), triethylene glycol (TEG) and bishydroxypropoxyphenylpropane (BisHPPP), which have been reported to modulate growth, gene expression, and protein synthesis of cariogenic bacteria (Khalichi et al. 2009; Sadeghinejad et al. 2017; Sadeghinejad et al. 2016). The interfacial biodegradation not only weakens the hybrid layer and enlarges gaps between teeth and restorations, allowing for bacterial microleakage to take place (Matharu et al. 2001; Sano et al. 1999; Sano et al. 1995b; Zivković et al. 2001), but it also provides a unique micro-environment containing the biodegradation products, which can affect bacteria present in the interfacial gap (Kermanshahi et al. 2010; Khalichi et al. 2004; Sadeghinejad et al. 2017; Sadeghinejad et al. 2016).

*S. mutans*, as a primary cariogenic bacterium, is associated with caries formation and progression with three well-established virulence factors: biofilm formation, aciduricity (acid tolerance), and acidogenicity (acid production) (Hamada et al. 1984). Although the degradative activity of *S. mutans* towards resin composites demonstrates clear clinical relevance as a contributor to
restoration failure (Bourbia et al. 2013), no studies have fully investigated the specific degradative activity of *S. mutans* as a virulent factor associated with the progression of secondary caries. Thus, the first aim of the current investigation was to further expand on the work by Bourbia et al. and to identify and characterize the specific esterases from *S. mutans* with degradative activity toward methacrylate-based resin composites. While previous studies have reported on the impact of BBPs on the growth and virulence gene expression in *S. mutans* (Khalichi et al. 2004; Khalichi et al. 2009; Sadeghinejad et al. 2017; Sadeghinejad et al. 2016), the investigations utilized invasive techniques (RNA extraction and RT-PCR) that disrupted the biofilm structure and thus prohibited spatial and temporal analysis of gene expression. Therefore, the second aim of this project was to establish a non-invasive methodology based on the fluorescence *in situ* hybridization (FISH) technique to analyze the esterase gene response to BBPs in biofilm with intact and complex 3D structure, which would reveal the bacterial adaptation to environmental changes under physiologically relevant conditions.

It is currently accepted that the breakdown of resin-dentin interface by bacterial and salivary esterases is inevitable (Kermanshahi et al. 2010; Serkies et al. 2016; Shokati et al. 2010) and that the infiltrated *S. mutans* increase its cariogenicity in response to accumulated BBPs in the interface (Sadeghinejad et al. 2017; Sadeghinejad et al. 2016). Therefore, another aim of this study was to find practical ways to increase the longevity of resin-based restorations by investigating the effect of biological and chemical factors on the initial integrity and overall biostability of the hybrid layer. In this study, galardin, an MMP inhibitor, was introduced as part of the restorative procedure to assess its ability to reduced auto-degradation of the marginal interface. Traditional resin composite or antimicrobial material, polyacid modified composite resins (PMCR or compomers) were bonded to dentin with either total-etch or self-etch bonding system, and were used to study the
biostability of the marginal interface and the anti-microbial effect of PMCR. The penetration and biofilm biomass of the cariogenic bacterium *S. mutans* along the interface were used as indicators of the restoration’s interfacial margin quality.

The characterization of the specific esterases from *S. mutans* will allow for mechanistic exploration of the biological effect of oral bacteria on dental materials. In turn, the reciprocal effect of these materials and their biodegradation products on the bacteria will guide manufacturers to design materials that are more resistant to these effects. The interaction between this bacterial esterase and resin composite will elucidate processes that contribute to the progression of secondary caries. The assessment of material chemistry, properties, and application modes on bacterial penetration, biofilm formation, and bacterial viability will guide future design of new restorative materials and adhesive systems, and contribute to the modification of clinical procedures to reduce degradation of bonded interfaces, ultimately enhancing the performance and longevity of resin composite restorations.
1.2 Hypotheses

1.2.1 Central Hypotheses: Specific esterases produced by cariogenic bacteria degrade resin-based materials and bacteria respond to the accumulated biodegradation by-products by modulating the expression of esterases. Bacterial penetration, biofilm biomass, and bacterial viability along the compromised resin-dentin interfacial margins are modulated by SHSE, MMP inhibition, and properties of resin composites and adhesives.

1.2.2 Specific Hypotheses:

- Esterases produced by *S. mutans* are stable and active in a simulated oral environment and capable of hydrolyzing resin monomers and polymerized resin composites.
- *S. mutans* respond to the accumulated biodegradation by-product (BisHPPP) by up-regulating the expression of esterases, which are associated with the biodegradation of resin composites.
- The biodegradation of resin-dentin interfaces is accelerated by simulated human salivary esterases.
- The resistance to biodegradation of resin-dentin interfaces, as measured by bacterial penetration, biofilm biomass, and bacterial viability along the interfaces, is affected by the restorative material and adhesive, as well as the application of an MMP inhibitor.

1.3 Objectives

1.3.1 To identify and characterize esterases from *S. mutans* UA159 that degrade methacrylate-based resin monomers, polymerized composites, and adhesives.

**Approach:**

- Putative esterase genes were searched in *S. mutans* UA 159 genome database at The National Center for Biotechnology Information ([http://www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)).
• Putative esterase gene (SMU_118c) was PCR amplified from S. mutans UA159 genomic DNA, then cloned into p15Tv-LIC vector.

• The esterase enzyme, SMU_118c, was expressed in E. coli BL21 (DE3), then isolated and purified.

• The hydrolase activity and kinetics of SMU_118c toward nitrophenyl-esters substrates p-nitrophenylbutyrate (p-NPB), o-nitrophenylbutyrate (o-NPB), p-nitrophenylacetate (p-NPA), or butyrylthiocholine iodide (BTC), were measured.

• The stability of SMU_118c in the presence of BisGMA and TEGDMA at pH 7.0 and 5.5, was assessed.

• The ability of SMU_118c to degrade resin monomers and polymerized resin composite was measured by incubating BisGMA, TEGDMA or light-cured resin composite (Z250) in PBS with or without the esterase. Quantification of the biodegradation by-product, BisHPPP, from the monomers and polymerized resin composites was analyzed by high performance liquid chromatography (HPLC).

• The catalytic site of SMU_118c, including a serine, an aspartate, and a histidine, was verified by measuring its enzymatic activities towards pNPB using irreversible serine proteases inhibitor, phenylmethyl sulfonyl fluoride (PMSF).

1.3.2 To verify the association of the specific esterase gene to the whole bacterial esterase activity.

**Approach:**

• SMU_118c knockout mutant (ΔSMU_118c) was constructed through PCR-ligation mutagenesis.
• *SMU_118c* complemented strain (ΔSMU_118cC) was constructed using pIB166 plasmid.

• Resin composites biodegradation were conducted using the specific gene knockout (ΔSMU_118c), SMU_118c complemented strain (ΔSMU_118cC) and *S. mutans* UA159 wild strain.

1.3.3 To quantitatively measure the level of the expression of esterase gene *SMU_118c* associated with biodegradation of the resin monomers and polymers in response to the biodegradation by-product (BisHPPP) using FISH.

**Approach:**

• Biofilms of *S. mutans* UA 159 and ΔSMU_118c (control) were incubated with relevant *in vivo* concentrations of BisHPPP (0.0, 0.01, 0.1 and 1 mM) for 18 hours.

• After fixation and dehydration, the biofilms were hybridized with green-fluorophore-labeled oligonucleotide probes targeting on mRNA of *SMU_118c* and red-fluorophore-labeled oligonucleotide probes targeting on 16s rRNA (control).

• The biofilms were then stained with nuclear dye 4’ ,6’-diamidino-2-phenylindole (DAPI) (blue) for further biomass analysis.

• Changes of gene expression in the presence of various concentrations of BisHPPP were analyzed by confocal scanning laser microscopy (CSLM) and quantification software IMARIS. Changes of gene expression presented and calculated in IMARIS as different fluorescence intensities relative to biofilm biomass.
1.3.4 To fabricate standardized resin-dentin specimens using traditional or polyacid-modified (anti-microbial) resin composites bonded to dentin with self-etch or total-etch adhesive systems, the latter with or without the application of MMP inhibitor (galardin).

**Approach:**

- Dentin blocks from fully intact and unrestored human molars were obtained. Standardized resin-dentin specimens were made from traditional or polyacid-modified composites bonded to human dentin using self-etch or total-etch adhesives. The latter specimens were prepared with or without the application of MMP inhibitor (galardin) after the etching step.

1.3.5 To induce biodegradation of the resin-dentin interfacial margin and analyze the bacterial microleakage along the compromised interface.

**Approach:**

- Resin-dentin specimens were incubated in phosphate-buffer (PBS) or simulated human salivary esterases (SHSE) media for up to 180 days.

- Biofilm of *S. mutans* UA159 were generated and sustained over the degraded resin-dentin specimen by suspending specimens in a chemostat-based biofilm fermentor (CBBF).

- The level of bacterial penetration, biofilm biomass, and bacterial viability along the resin-dentin interfacial margin were quantified using vital fluorescent staining combined with CLSM and IMARIS software analysis.
Chapter 2

Literature Review

2.1 Composite Restorations

The increasing concern about mercury exposure, combined with growing aesthetic demands and improved dental material adhesive technologies have resulted in resin composites becoming the most commonly used restorative material for direct restorations in modern dentistry, with 13 million annual procedures in United States and over 40 million worldwide (Murray et al. 2002b; Simecek et al. 2009; Wakefield and Kofford 2001).

2.1.1 Resin Composite System

The formulations of resin composites can be modified for specific applications as sealants, cements, and restorative and provisional materials, among others (Ferracane 2011). Resin-based dental materials are generally composed of four major components: polymer matrix, filler particles, polymerization promoters, and coupling agents (Santerre et al. 2001a).

Polymeric matrix: The polymer matrix is usually comprised of methacrylate-based monomers containing reactive carbon double bonds (C=C) undergoing polymerization and ester linkages (-COO) linking the central chain of the monomer to functional groups (Fig. 2.1). The resin monomers commonly used in dentistry 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) (Fig. 2.1) (Santerre et al. 2001b). BisGMA is the predominant resin monomers used in commercial resin composites, first introduced by Bowen in late 1950s (Bowen 1956). The two hydrophobic aromatic rings in the backbone of BisGMA provide better deformation resistance upon mechanical loading relative to linear non-
aromatic monomers (Delaviz et al. 2014). However, the rigidity of these aromatic rings reduces the mobility of methacrylate molecules. As a result, one methacrylate molecule reacts to extend the length of the linear polymer chain, thereby determining the degree of polymerization, whereas the second one forms a branch engaging in cross-linking with adjacent polymer chains (Anusavice and Phillips 2003). Due to the rigid aromatic rings, hydrogen bonds formed between hydroxyl groups in the alkyl chain and its high molecular weight (Sandner et al. 1997), BisGMA performs at a high viscosity that prevents the incorporation of high filler loading and reduces the degree of conversion when used alone. To improve materials performance and clinical handling, low molecular weight and high flexibility diluent monomers such as TEGDMA, EGDMA, DEGDMA, and UDMA are usually mixed with BisGMA to reduce viscosity and enhance of polymerization efficiency (Craig 2002; Delaviz et al. 2014; Sandner et al. 1997; Smith 1985). Among the diluent monomers, TEGDMA is the most extensively used in current resin restorations (Delaviz et al. 2014).

**Inorganic fillers:** Filler particles are major constituents of resin composites by volume and weight, and plays an important role in improving the mechanical properties of resin composites, namely increasing the strength and modulus of elasticity while reducing water sorption and polymerization shrinkage after curing (Santerre et al. 2001a). Different fillers can be classified based on chemical composition (glass, quartz, or ceramic oxid), average particle size (macrofiller, microfiller, nanofiller, or hybrid), and manufacturing technique (pyrogenic or grinding) (Table 2.1). Additionally, the fillers are usually used to classify resin composites based on their size (Fig. 2.2).
Structure of 2, 2 – bis [4(2-hydroxy methacryloxypropoxy)-phenyl] propane (BisGMA)

\[
\begin{align*}
\text{CH}_3 & \quad \text{CH}_3 \\
\text{CH}_2=\text{C} & \quad \text{C}=\text{O} \\
\text{O} & \quad \text{CH}_2\text{CH}_2\text{O} \\
\text{CH}_3 & \quad \text{O} \\
\end{align*}
\]

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

Structure of Urethane dimethacrylate (UDMA)

Structure of Ethoxylated bisphenol A based dimethacrylates (BisEMA)

**Figure 2.1: Common methacrylate based di-vinyl monomers used in dental resin materials.**

**Table 2.1: Type of fillers and filler size used in dental composites.** Adopted from Klapdohr and Moszner. *Monatshefte für Chemie/Chemical Monthly* 136 (2005) 21-46. Used with permission from the publishers.

<table>
<thead>
<tr>
<th>Filler composition</th>
<th>Particle size</th>
</tr>
</thead>
<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>0.7μm, 1.0 μm, 1.5 μm or larger</td>
</tr>
<tr>
<td>Radiopaque, finely ground Ba/Sr</td>
<td>1.0 μm, 1.5 μm or larger</td>
</tr>
<tr>
<td>Fluoro silicate glasses</td>
<td>1.0 -1.5 μm</td>
</tr>
<tr>
<td>Ground quartz glasses</td>
<td>1.0 -1.5 μm</td>
</tr>
<tr>
<td>YbF3, YF3</td>
<td>0.10 - 3.0 μm</td>
</tr>
<tr>
<td>Si/Zr mixed oxide</td>
<td>250 - 500 nm</td>
</tr>
<tr>
<td>Ti, Zr, and Al oxide used as opacifier</td>
<td>250 - 500 nm</td>
</tr>
<tr>
<td>Splinter polymerisate mainly based on SiO2</td>
<td>10 - 100 nm</td>
</tr>
</tbody>
</table>
Polymerization system: The viscous resin composites have to turn into rigid materials in order to provide appropriate hardness and strength as restorative materials (Santerre et al. 2001b). To do so, a free radical polymerization process is required for resin monomers to form a cross-linking network by adding free molecules to terminal free radical reaction sites. Different initiator and accelerators are used depending on the various polymerization reactions. For the chemically initiated self-cured composites, peroxide initiator and amine accelerator are utilized for polymerization reactions. For commonly used light-cure composites, the photo-initiator camphorquinone (CQ) is first activated by blue light (400-450 nm) and converted to an excited triplet state that reacts with a co-initiator to form free radicals. These newly formed radicals interact with a monomer molecule forming an active center, whereby the propagation of this reaction starts the polymerization process (Schneider et al. 2010). In addition, some dual-cured composites use a combination of chemical and light activation to carry out the polymerization reaction. Regardless the initiation system used, polymerization rates of only 50% to 70% of are achieved (Cook et al.)
Unreacted monomers may be released into the oral cavity resulting in adverse effects in local tissues and cells including dental pulp irritation, proliferation of cariogenic bacteria, and estrogenic impact (Kwon et al. 2015; Pameijer 2012; Sadeghinejad et al. 2017). In addition, insufficient polymerization will compromise the physical properties of resin composites by increased water absorption, which leads to increased hydrolysis of resin matrix and dentin collagen (Finer and Santerre 2004a; Pashley et al. 2004).

**Coupling agent:** To improve the bonding of the filler particles to the polymeric matrix, coupling agents such as silane are often used. The coupling agents should contain vinyl groups at one end to polymerize with the resin matrix and hydroxyl groups at another end to bind to the filler surface (Fig. 2.3). The strong bonding among coupling agents, monomers, and fillers decreases the water sorption and hydrolysis process of resin matrix in the presence of water (Santerre et al. 2001b, Bowen, R. L. 1963)).

![Figure 2.3: γ-methacryloxypropyltrimethoxy silane. Adopted from Dr. Yoav Finer’s thesis. Used with permission.](image)

**2.1.2 Resin Adhesive Systems**

Adhesive systems are designed to bond composite resins to tooth structure (enamel and dentin), retain composite restorations in place, and seal the interface with the tooth structure. Traditionally, adhesives contain similar components as composite resin, including methacrylate-based resin
monomers such as BisGMA and TEGDMA, organic solvents, initiators, inhibitors, and sometimes fillers.

Based on application procedures and interaction with the smear layer (Wang and Spencer 2002), resin-based adhesive systems are classified into two major categories in current dentistry: the total-etch and self-etch systems. The compositions and application procedures of each system are listed in Fig. 2.4. Total-etch systems (etch and rinse), either three- or two-step procedure, involve conditioning with an acid, usually 37% phosphoric acid (pH 0.17), for 15-30 seconds to completely remove the smear layer, followed by rinsing, priming, and application of adhesive resin. The three-step etch and rinse adhesive system is regarded as the gold standard in terms of durability (De Munck et al. 2005). However, it is highly technique sensitive and deviations in clinical application could result in rapid de-bonding and degradation of adhesive-tooth interface (Van Meerbeek et al. 2011). The two-step total-etch adhesive combines the primer and the adhesive resin into one bottle (De Munck et al. 2005). Although the total-etch systems claimed to be able to completely remove the smear layer with the etching step, the incomplete penetration of the monomers in the primer and adhesive into demineralized dentin as well as phase separation between the hydrophobic (BisGMA) and hydrophilic (hydroxyl ethyl methacrylate, HEMA) moieties of the adhesive system components may result in reduced monomer conversion and increased water sorption, which prohibits the formation of an impervious, structurally integrated resin-dentin interface leading to restorations with compromised mechanical properties (Spencer et al. 2010). Self-etch systems, either one- or two-step procedure, combine etching and priming into a single procedure by using non-rinse acidic monomers that simultaneously condition and prime dentin, thereby eliminating the rinsing phase. It modifies the mineral phase of the dentin, thus potentially preventing collagen collapse and incomplete penetration of the adhesive into the etched dentin (Babb et al. 2009;
Hebling et al. 2005b). However, the self-etching adhesive-dentin interface is initially appeared to create a thin and uniform hybrid layer without long resin tags, which has been shown to quickly lose their structural integrity during aqueous aging (Bouillaguet 2004; Matharu et al. 2001; Sano et al. 1999; Sano et al. 1995b; Santerre et al. 2001a; Zivkovic et al. 2001). It is likely that the relative hydrophobicity of the bonding resin limits the penetration into deeper parts of the dentinal tubules where more hydrophilic conditions prevail, resulting in incompletely infiltrated zones within the hybrid layer (Sano et al. 1995a). Water within the dentinal tubules inhibited the polymerization of acidic monomers in the self-etch adhesives due to adhesive phase separation, resulting in incomplete polymerization (Wang and Spencer 2005).

![Chemical composition of dental adhesives: total-etch vs. self-etch.](image)

**Figure 2.4:** Chemical composition of dental adhesives: total-etch vs. self-etch. Adopted from Van Landuyt KL, et. al. *Biomaterials* 28(26):3757-3785. Used with permission from *Biomaterials*.

### 2.1.3 Antimicrobial Agents in Resin Composites

As the most commonly used dental restorative materials (Beazoglou et al. 2007), resin composites have higher failure rate compared to amalgam mainly due to the development of secondary caries.
attributed to the increased accumulation of oral bacteria on resin composites relative to materials. Although several conventional filling materials possess antimicrobial properties, such as amalgam, glass ionomer, and calcium hydroxide, their physical and esthetic properties have limited their clinical applications. Therefore, effective antimicrobial dental materials were introduced in early 1980s to prevent secondary caries and improve the longevity of resin-based restorations. Since composites possess little to no antimicrobial properties, the addition of antimicrobial components to resin composites or adhesive systems can be achieved by modifying the filler particles or resin matrix. Based on the properties of incorporated agents and modification strategies, the antimicrobial compounds in resin composites can be classified as released antimicrobial or non-released antimicrobial agents (Table 2.2).

**Table 2.2: Antimicrobial agents incorporated into resin composites.** Adopted from Klapdohr and Moszner. *Monatshefte für Chemie/Chemical Monthly* 136 (2005) 21-46. Used with permission from the publishers.

<table>
<thead>
<tr>
<th>Released antibacterial agents</th>
<th>Resin matrix modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strontium fluoride (SrF2)</td>
<td>Acrylic-amine-HF salts [17]</td>
</tr>
<tr>
<td>Silver ions [8]</td>
<td>Methacryloyl acid-fluoride [18]</td>
</tr>
<tr>
<td>Zinc Oxide (ZnO) [14]</td>
<td>Chlorhexidine [19]</td>
</tr>
<tr>
<td>Nonreleased antibacterial agents</td>
<td>Benzalkonium chloride [23]</td>
</tr>
<tr>
<td>Silver supported fillers [9]</td>
<td>Cetylpyridinium chloride [21]</td>
</tr>
<tr>
<td>12-Methacryloyloxydodecylpyridinium bromide (MDPB) [16]</td>
<td>Chitosan [22]</td>
</tr>
<tr>
<td>Quaternary ammonium polyethyleneimine (QPEI) [41]</td>
<td></td>
</tr>
</tbody>
</table>

In general, released antimicrobial agents have ability to diffuse into the surrounding environment and penetrate through cell walls and membranes. However, the release usually leaves voids in matrix that compromises the structural integrity. In addition, the release of antimicrobial agents is
a non-controllable process. As a result, after the initial burst release, the antimicrobial agents leach from the resin composite at a very low rate for a very limited period of time, which may not be effective to inhibit bacterial growth (Beyth et al. 2014). The commonly used releasable agent is fluoride in both filler particles and resin matrix, which has ability to interfere with biofilm formation and enhance remineralization (Wiegand et al. 2007). However, it has been claimed that most of the fluoride has been released during the setting reaction without long-term antimicrobial effects (Beyth et al. 2014; Hicks et al. 2003; Papagiannoulis et al. 2002; Yap et al. 1999). To overcome the drawback of the short-term and uncontrollable release, polyacid modified composite resins (PMCR or compomers) were introduced as a new class of anti-microbial dental materials, which is claimed to combine the mechanical and esthetic properties of composites with the fluoride-releasing advantage of glass-ionomer cements (Nicholson 2007). In contrast to the traditional composites that contained virtually inert filler, PMCR contains reactive fluoro-alumino-silicate glass particles that are susceptible to acid attack and provide the source of fluoride ions that are used as an antimicrobial treatment. In addition, PMCR has unique matrix monomers that react simultaneously with the dimethacrylate monomers and the cations liberated from the glass particles (Hes et al. 1999; Nicholson 2007). PMCR is mostly shown to maintain levels of fluoride over time without an initial fluoride “burst” effect (Attar and Turgut 2003; Forsten 1998; Shaw et al. 1998; Wiegand et al. 2007; Yap et al. 2002). However, the effect of PMCR on recurrent caries is controversial and dependent on the types of cavities, material brands, and investigation models (Van Dijken 1996; Van Dijken 2003; Welbury et al. 2000).

The non-released antimicrobial agents inhibit bacterial growth by direct contact without releasing compounds, which has the advantage of being nonvolatile, chemically-stable, and sustaining long-time activity (Beyth et al. 2014; Wicht et al. 2005). However, due to some limitations, this kind of
antimicrobial restorative material is still under investigation (Aydin Sevinç and Hanley 2010; Fan et al. 2011; Imazato et al. 2003). For example, the bactericide, 12-methacryloyloxy-dodecylpyridinium bromide (MDPB) can be immobilized on filler particles to perform contact killing against surface-adherent bacteria, which is considered more stable for a long-term inhibition effect. However, since the immobilized agent is unable to penetrate, the antibacterial effect of the filler-modified MDPB is not as robust as the free particles (Imazato et al. 2003).

2.2 The Resin-dentin Interface

2.2.1 The Creation and Characteristics of Resin-Dentin Interface

The resin-dentin interface is created from resin composites bonded to dentin using adhesives. The bonding capacity of dental adhesives is based on two-fold adhesion: its ability to bind to dentin and to the lining composite. The latter has been shown to be a process of co-polymerization of methacrylate monomers in the composite and adhesive. As for the bond to dentin, micromechanical adhesion is assumed to be the prime bonding mechanism (Van Meerbeek et al. 2001). This is achieved by an exchange process where the mineral phase of tooth substrate is replaced by resin monomers (Eick et al. 1993; Erickson 1992). The first step of this process is tooth conditioning that demineralizes dentin and exposes dentin collagen fibril, followed by priming and application of adhesive resin that infiltrates the voids left by the mineral phase of the dentin structure and entangles with collagen fibril, forming a micromechanical interlocking network between resin and tooth substrate (Hashimoto et al. 2000; Spencer and Swafford 1999; Wang and Spencer 2003). Microscopically, this process is like a “hybridization” (Nakabayashi et al. 1982), so this biological interface between composite and dentin is also called a hybrid layer (Fig. 2.5). The hybrid layer is characterized as a 3-dimensional polymer/collagen network that is supposed to provide a continuous and stable bonding between the bulk adhesive and dentin substrate (Donmez
et al. 2005; Gerdolle et al. 2005; Maupome and Sheiham 1998; Murray et al. 2002a; Van Meerbeek et al. 1998). However, neither total-etch nor self-etch adhesives can form a perfect hybrid layer to seal the interface. The total-etch (etch and rinse) system has the problem of incomplete penetration of the primer and adhesive into the demineralized dentin, as well as phase separation between the hydrophobic and hydrophilic moieties of the adhesive system components, which may result in reduced monomer conversion. Additionally, the collapse of exposed collagen fibrils occurs in the dentinal interface, which reduces the porosity in the demineralized dentin, severely compromising the resin-dentin interlocking (Spencer et al. 2010). The self-etch systems use non-rinse acidic monomers that simultaneously condition and prime dentin, which is unable to completely remove the mineral phase of the dentin. Although this procedure potentially prevents collagen collapse, it leads to incomplete penetration of adhesive monomers into the etched dentin (Babb et al. 2009; Hebling et al. 2005a). In addition, there is another concern that the hydrophilic nature of the components of self-etch system enhances water sorption, which accelerates the hydrolysis of resin matrix (Bouillaguet 2004; Matharu et al. 2001; Sano et al. 1999; Sano et al. 1995b; Santerre et al. 2001a; Zivkovic et al. 2001). As a result, the resultant hybrid layer behaves more like a semi-permeable membrane that permits water movements throughout the bonded interface even after adhesive polymerization (Ateyah and Elhejazi 2004; Manhart et al. 2001).
Figure 2.5: Image of resin-dentin interface acquired from Confocal Laser Scanning Microscopy (CLSM). Unpublished data from Bo Huang.

2.2.2 Biodegradation of Resin-dentin Interface

The hybrid layer, as a permeable membrane, allows for the movement of water that initiates hydrolysis involving the scission of condensation type bonds in both resin matrix and dentin collagen (Chersoni et al. 2004a; Tay and Pashley 2003). This process can be further catalyzed by enzymes present in the oral cavity, often referred to as biodegradation (Delaviz et al. 2014; Finer and Santerre 2003; Shokati et al. 2010). The biodegradation of resin-dentin interface is considered as the main contributor that compromises the integrity of the resin-dentin interface leading to the limited overall longevity of the resin-based restorations (Spencer et al. 2010).

The Biodegradation of Resin-based matrix by salivary and bacterial esterases

Methacrylate-based materials are inherently prone to hydrolysis due to the presence of unprotected ester linkages in their major matrix monomers such as bis-phenyl glycidyl dimethacrylate (BisGMA) and triethylene glycol dimethacrylate (TEGDMA), yielding degradation by-products
such as methacrylic acid (MA), triethylene glycol (TEG), and bis-hydroxy-propoxy-phenyl-propane (BisHPPP) (Fig. 2.6) (Finer and Santerre 2004a). This process can be affected by materials properties such as chemistry, crosslinking, hydrophobicity, and solubility (Finer and Santerre 2004b). Esterases are the most associated enzymes for catalyzing the hydrolysis of adhesives and resin composites (Finer and Santerre 2004a). Previous studies reported that human saliva contains cholesterol esterase (CE)-like and pseudocholinesterase (PCE) activities with a strong degradative ability toward resin composite components (Finer and Santerre 2004a; Jaffer et al. 2002). Based on these findings, simulated human salivary esterase was formulated with 16 units/mL and 0.01 units/mL of CE-like and PCE-like activities, and used as an ideal analog to investigate the biodegradation process, which further verified the effect of esterase activity on the resinous interfacial deterioration (Finer and Santerre 2004a; Serkies et al. 2016; Shokati et al. 2010). More recently, the cariogenic bacterium *S. mutans* UA159 was found to hydrolyze resin composite and adhesives (Bourbia et al. 2013), which is considered as another major contributor to resinous interface degradation.

The esterase-catalyzed resin composite degradation not only enlarges gaps between restorations and teeth (Kermanshahi et al. 2010), compromises interfacial bond strength (Serkies et al. 2016; Shokati et al. 2010), and allows for bacterial invasion (Matharu et al. 2001; Zivković et al. 2001), but also provides a unique micro-environment containing the biodegradation by-products (BBPs) in close proximity to the bacteria that reside in this gap (Kermanshahi et al. 2010; Sadeghinejad et al. 2017).
Figure 2.6: Degradation of BisGMA (a) and TEGDMA (b) by esterases resulting in the production of biodegradation by-products BisHPPP, TEGMA, TEG, and MA.

The Biodegradation of Dentin by Collagenolytic Enzymes

Current findings indicate that the incomplete seal of the hybrid layer is susceptible to degradation and considered as the weak link that determines the longevity of the resin restorations (Spencer et al. 2010). The breakdown of water-rich, resin-sparse collagen fibrils within hybrid layers is caused by the activation of dentinal MMPs during bonding procedures (Liu et al. 2011). MMPs are known...
as zinc- or calcium-dependent proteolytic enzymes capable of degrading exposed collagen fibrils within the hybrid layer (Ingman et al. 1994; Pashley et al. 2004; Tjaderhane et al. 1998). The dentin matrix contains at least four MMPs: stromelysin-1 (MMP-3) (Boukpessi et al. 2008), true collagenase (MMP-8) (Sulkala et al. 2007), and gelatinases A and B (MMP-2 and MMP-9, respectively) (Martin-De Las Heras et al. 2000; Mazzoni et al. 2007). Once activated, these peptidases are responsible for the intrinsic auto-degenerative process of dentinal degradation (Amaral et al. 2007; Arola and Reprogel 2005; Boushell et al. 2008; Mazzoni et al. 2009; Nishitani et al. 2006b; Van Strijp et al. 2000) and breaking down components of the interfacial margin (Amaral et al. 2007). In addition, there are other endogenous proteases involved in dentinal matrix degradation (Nascimento et al. 2011; Tersariol et al. 2010). The activity of cysteine cathepsins was found in the active dentin matrix of caries contributing to collagen degradation in demineralized area (Nascimento et al. 2011). In addition, extracellular cathepsin B has been confirmed to be related to the collagenous matrix degradation in gingivitis and periodontitis (Kennett et al. 1997a; 1997b), which might be another contributor to collagen degradation in resin-dentin interface.

2.2.3 The Strategies to Improve Interfacial Bonding

Studies show that the quality and integrity of resin-dentin interface is a determinant factor in the longevity of resin-based restorations (Kermanshahi et al. 2010; Spencer et al. 2010).

**Ethanol wet bonding**

Ethanol is used to dehydrate demineralized dentin matrix and reduce the hydrophilicity of dentin collage, which facilitates the infiltration of hydrophobic monomer in adhesive systems (Nishitani et al. 2006a; Shin et al. 2009). The penetration of hydrophobic monomers with greater molecular weight into demineralized dentin matrix can decrease the polymerization shrinkage, water
absorption, and resin plasticization (Tay et al. 2007). In addition, the inhibitive effect of ethanol on collagen degradation was reported to improve the durability of interface bonding (Sadek et al. 2010; Tezvergil-Mutluay et al. 2011).

**Increasing degree of conversion of resin monomers**

The poor degree of conversion of resin monomers leads to increased monomer release and water absorption in the interface, thereby compromising interfacial integrity. Traditional photo-initiators, such as Camphoroquinone (Gerdolle et al.), was reported to have a poor degree of hydrophilic monomers conversion due to its high affinity to hydrophobic monomers (Wang et al. 2006; Ye et al. 2007; Ye et al. 2009). To improve the overall conversion of resin monomers in adhesive systems, modified photo-initiators, such as iodonium salt diphenyl iodonium hexafluorophosphate (DPIHP), are recommended to be included in adhesive systems to accelerate polymerization with improved mechanical properties (Ye et al. 2009).

**Increasing cross-linking of resin monomers**

Ester linkages anchoring the cross-linking dimetharylates are susceptible to hydrolysis catalyzed by esterases. To reduce the exposure of these vulnerable linkages, multifunctional methacrylate with branched side chains, such as trimethylolpropane mono allyl ether dimethacrylate (TMPEDMA), can be introduced into the resin matrix to form a denser polymer network due to its tri-vinyl group that contributes to the cross-linking reaction (Park et al. 2008). In addition, the incorporation of urethane functional groups in methacrylate side chains allows for an increased hydrophilicity of resin matrix and provides improved esterase resistance (Hagio et al. 2006; Park et al. 2009)
**Collagen cross-linking**

Cross-linking agents are developed to produce covalent cross-linking in dentin matrix that may inactivate the endogenous proteases by reducing the mobility of active sites or by changing the charged amides in the active sites of proteases (Tjäderhane et al. 2013a). However, none of them has been introduced into clinic to date due to some inherit disadvantages: glutaraldehyde is toxic (Bedran-Russo et al. 2008); grape-seed extract stains dentin brown (Fang et al. 2012); riboflavin requires a separate curing device that makes it less favorable for clinical application (Fawzy et al. 2012).

**Enzyme inhibition**

The effect of dentinal endogenous enzymes, matrix metalloproteases (MMPs), and cysteine cathepsins on collagen degradation has been well documented. Consequently, current research focuses on reduction of collagen degradation via inhibition of MMPs and cysteine cathepsins. In addition to endogenous inhibitors, tissue inhibitor of metalloproteinases (TIMPs) maintaining the balance of matrix destruction and generation (Niu et al. 2011), synthetic inhibitors are developed and studied. Since MMPs are zinc-dependent metalloproteinases, the synthetic inhibitors generally contain functional groups chelating with the zinc atom at the active site of the MMPs. Chlorhexidine, as an antimicrobial agent, has been proven to be an effective inhibitor against MMP and cysteine cathepsins, resulting in decreased collagen degradation (Carrilho et al. 2007; Gendron et al. 1999; Scaffa et al. 2012a). This may explain the therapeutic effect of chlorhexidine in periodontitis, a tissue-destructive inflammatory disease wherein matrix metalloproteinases play a major role (Sorsa et al. 2004). Tetracycline antibiotics are not only widely used for infection treatment, but also adopted to treat diseases caused by upregulated collagenases activity in tissues. Doxycycline has been used in various experimental trials for this purpose, such as for the
recalcitrant recurrent corneal erosions (Fisher and Mobashery 2006). Periostat (CollaGenex Pharmaceuticals Inc.), also known as doxycycline, has been approved by the FDA in 1998 and remained the only registered collagenase inhibitor used in the clinic as an adjunct to scaling and root planning (SRP) to slow the progression of adult periodontitis (Peterson 2004). Another synthetic MMP inhibitor, Galardin (GM6001 or Ilomastat), has a collagen-like backbone that facilitates binding to MMPs and a hydroxamate structure (R-CO-NH—OH; R is an organic residue) that chelates the zinc ion located in the MMPs’ catalytic domain. It is introduced as a specific MMP inhibitor against the major MMPs found in human dentin, namely MMP-1, MMP-2, MMP-8, and MMP-9. It has a comparable effect to CHX in preserving the integrity of the resin-dentin interface but at lower concentration (0.2mM) (Breschi et al. 2010).

**Biomimetic remineralization**

Biomimetic remineralization is a new technique to improve the quality of resin-dentin interface after it has been created. Biomimetic remineralization mimics the natural process of bio-mineralization by using nanotechnology principles to fill the water-left voids in resin-sparse areas of the hybrid layer with apatite crystallites occupying extra and intrafibrillar compartments of the collagen matrix. The key to dentin remineralization is to create fluidic nanoprecursors that could pass through polymerized resin matrix and fill the voids leading to self-remineralization (Gower 2008). In addition, this process limits water content in demineralized dentin matrix in the hybrid layer, preventing collage hydrolysis, which also improves the interfacial quality (Sadek et al. 2010). However, biomimetic remineralization remains a proof-of-concept approach and additional research is required to transform this concept into a clinically applicable technique.
2.3 Esterase

Esterases represent a diverse group of hydrolases catalyzing the cleavage and formation of ester bonds. The enzyme commission number assigned to these hydrolases is EC 3.1.1.X, where the X is determined by substrate (Bornscheuer 2002).

2.3.1 Esterase Introduction

Esterases are widely distributed in plants, human tissues, and microorganisms (Bornscheuer 2002). The bacterial esterases have been of interest since early 1990s as attractive biocatalyst in the synthesis of optically pure molecules for the pharmaceutical and agrochemical industries due to their wide substrate tolerance and high regio- and stereo-specificity (Alphand et al. 1995; Berglund 2006; Patel 2000). In addition, esterases do not require co-factors and are very stable in organic solvents.

There are two major classes in the hydrolases: lipases (EC 3.1.1.1; triacylglycerol hydrolases) and “true” esterases (EC 3.1.1.3; carboxyl ester hydrolases). Lipases have preference towards water-insoluble substrates with long-chain fatty acids (C>12), while esterases prefer simple esters with a short chain length (C<12) (Jaeger et al. 1999). Although there are several differences between lipases and “true” esterases (Table 2.3), they both have the similar characteristic structure of $\alpha/\beta$-hydrolase fold (Fig. 2.7), which is shared by several other enzymes including proteases, peroxidases, dehalogenases, and epoxide hydrolases (Nardini and Dijkstra 1999; Ollis et al. 1992). This structural similarity suggests that they had possibly evolved from a common ancestor. This $\alpha/\beta$-hydrolase fold is well studied and characterized as consisting of a mostly parallel 8-strand $\beta$-sheet surrounded by $\alpha$-helices at both sides (Fig. 2.7) (Nardini and Dijkstra 1999), which is able to provide stable scaffold for active sites. The catalytic triad is highly conserved and always constitute three residues: an absolute conserved histidine residue, an acidic residue (Asp for esterase and Glu
for lipase), and a nucleophile (serine for esterase and lipase) usually located in a very sharp turn between an α-helix and a β-strand as “nucleophile elbow”, providing easy accessibility for the substrate (Jaeger et al. 1999; Nardini and Dijkstra 1999).


<table>
<thead>
<tr>
<th>Property</th>
<th>Lipase</th>
<th>Esterase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preferred substrates</td>
<td>Triglycerides (long-chain), secondary alcohols</td>
<td>Simple esters, triglycerides (short-chain)</td>
</tr>
<tr>
<td>Interfacial activation/lid</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Substrate hydrophobicity</td>
<td>High</td>
<td>High to low</td>
</tr>
<tr>
<td>Enantioselectivity</td>
<td>Usually high</td>
<td>High to low to zero</td>
</tr>
<tr>
<td>Solvent stability</td>
<td>High</td>
<td>High to low</td>
</tr>
</tbody>
</table>

Figure 2.7: Secondary structure diagram of the ‘canonical’ α/β hydrolase fold. Adopted from et, al. *Current Opinion in Structural Biology* 9,1999, 732–737. Used with permission from the publishers.

2.3.2 Hydrolysis Mechanism by Esterase

The mechanism for ester hydrolysis is described in four steps (Fig. 2.10). First, the substrate is bound to the active serine, forming a tetrahedral intermediate stabilized by the catalytic His and Asp residues. Next, the alcohol is released and an acyl-enzyme complex is formed. Due to the attack of a nucleophile such as water in hydrolysis, or an alcohol or ester in (trans-)esterification,
a tetrahedral intermediate forms again. At the end, after the product (an acid or an ester) is yielded, the enzyme will be freed for the next hydrolysis reaction (Bornscheuer 2002).

![Diagram of the hydrolysis mechanism of esterase](image)

**Figure 2.8: The hydrolysis mechanism of esterase.** Adopted from UWE Bornscheuer, et, al. *FEMS microbiology rev.* 2002, 73-81. Used with permission from the publishers.

This hydrolysis process could be affected by several factors such as pH, temperature and inhibitors. The optimal pH is that in which the enzyme has the best tertiary structure to fit substrates and exhibit maximum activity. And, the pH changes could lead to unfavorable structures of enzyme or substrate for interaction. In addition, extreme high or low pH may cause protein denaturation and result in complete loss of enzyme activity. Another factor is temperature that the optimum temperature of an enzyme is at which the maximum activity of the enzyme is obtained. In most cases, the enzyme starts losing its activity as the temperature increases, with exception to thermostable enzymes. The normal range of working temperature is room temperature to 40°C and storage temperature should below 4°C. Enzyme inhibitors are critical factors that can alter the catalytic action of the enzyme decelerating reaction rate, or stop catalysis. There are three common
types of enzyme inhibition, competitive, non-competitive and substrate inhibition. The various inhibitors of esterase include phenylmethyl sulfonyl fluoride (PMSF), ions like Fe3+, Cu2+, Zn2+, Mn2+, Co2+, Ca2+, Ag2+ (Bannerman et al., 1976; Degrassi et al, 1998). The hydrolysis capacity of an esterase is usually presented as kinetic properties mainly including Michealis-Menten constant (Km) and maximum velocity (Vmax). The affinity of esterase binding to specific substrate is presented as Km, and Vmax indicates the hydrolysis rate.

2.3.3 Bacterial Esterase

Numerous esterases have been identified and characterized from various bacterial species including *Bacillus, Pseudomonas, Streptomyces*, and *Streptococcus* (Table. 2.4) (Sayali et al. 2013; Xie et al. 2008; Zhu et al. 2009). In general, there is a very wide range of Km, Vmax, pH, and temperature spread among identified bacterial esterases (Table 2.4) (Sayali et al. 2013), which indicates their high tolerance to catalysis conditions.

In this thesis, the specific interest of bacterial esterase is their contributions to the bacterial pathogenesis. It has been known for a long time that esterases are widespread in bacterial pathogens such as *Streptococcus* (Xie et al. 2008; Zhu et al. 2009) and are critical for the survival and pathogenesis of pathogens (Andersen et al. 1991; Brodin et al. 2004). *Mycobacterium tuberculosis* (*M. tuberculosis*), the causative agent of tuberculosis, produces the esterase rv2525c that is important for the virulence and survival of the bacillus and for the pathogenesis of tuberculosis due its role of in the biogenesis of cell envelope that affects the susceptibility of β-lactam antibiotics (Saint-Joanis et al. 2006; Sassetti and Rubin 2003). Additionally, the Streptococcus Secreted Esterase (SsE) produced by the human pathogen *Streptococcus pyogenes*, commonly referred to as group A Streptococcus (GAS), is involved in the invasion of skin tissue causing various of diseases including cellulitis, pharyngitis, necrotizing fasciitis, streptococcal
toxic shock syndrome, scarlet fever, and rheumatic heart disease (Liu et al. 2007; Zhu et al. 2009).

2.3.4 Possible Secretion Mechanisms for Bacterial Esterases

In order to promote virulence and manipulate target cells, many pathogens use dedicated protein secretion systems to export various virulence factors from the cytosol of bacteria into the extracellular environment or host cells (Green and Mecsas 2016).

With the average MWs ranging from 20 to 60 KDa (Sayali et al. 2013), bacterial esterases are unable to simply penetrate the bacterial membrane, so protein transport systems are required to relocate the enzymes into the extracellular environment or to target substrates (Parizad et al. 2016). Although the investigations of secretion mechanisms of identified bacterial esterases are limited, the protein secretion systems involved in protein transport in bacteria are well established (Coombes 2009; Korotkov et al. 2012; Natale et al. 2008; Parizad et al. 2016). For esterases, the specific system and mechanism required for re-localization are dependent on the bacteria type: gram positive or gram negative (Costa et al. 2015), and pathogen or non-pathogen (Coombes 2009; DeCanio et al. 2013; Parizad et al. 2016). In gram-negative bacteria, there are six secretion systems involved in protein transport and localization named as Type I Secretion System (T1SS), T2SS, T3SS, T4SS, T5SS and T6SS (Fig. 2.8). To translocate proteins through both the inner and outer bacterial membranes, gram-negative bacteria can transport their substrates in an either one- or two-step secretion process. The one-step transportation is carried out by the T1SS, T3SS, T4SS, or T6SS, which contains periplasm-spanning channels secreting proteins from the cytoplasm directly into extracellular space (Green and Mecsas 2016). In addition, the T3SS, T4SS, and T6SS also can transport proteins across an additional host cell membrane to deliver secreted proteins directly to the cytosol of a target cell (Green and Mecsas 2016). The T2SS and T5SS secrete proteins in a two-step manner that the first step of transporting proteins across the cytoplasmic membrane is
executed by the general secretion (Sec) and twin arginine translocation (Tat) pathways in both gram-negative and gram-positive bacteria (Natale et al. 2008). In T2SS, proteins can be initially transported by either the Tat or Sec pathway (Korotkov et al. 2012). In contrast, auto-transporters of the T5SS must be unfolded prior to outer membrane transport and thus must be secreted across the inner membrane by the Sec pathway (Pugsley 1993), then, second secretion systems are used to transport protein across outer membrane (Green and Mecsas 2016).

Gram-positive bacteria also benefit from the Sec and Tat universal pathways to transport proteins through the first cytoplasmic membrane. However, due to the heavily lapidated cell wall surrounding the inner membrane, some secreted proteins remain embedded in cell wall and some proteins are often transported through this peptidoglycan layer by passive diffusion (Green and Mecsas 2016). The distinct secretion apparatus in gram-positive bacteria is T7SS, which contains core inner membrane proteins forming channel to facilitate proteins secretion (Green and Mecsas 2016). It has been proposed that this system also has a spanning channel through the cell wall through which proteins are secreted into the extracellular environment (Fig. 2.9). However, this model is still under investigation and has not been experimentally proven (Green and Mecsas 2016).

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Microbial source</th>
<th>Type of Esterase</th>
<th>Optimal pH</th>
<th>Optimal Temp (°C)</th>
<th>Molecular Weight (kDa)</th>
<th>Km (μM)</th>
<th>Vmax (μM/min/mg)</th>
<th>( p_I )</th>
<th>Reference</th>
</tr>
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<td>7.8-8.0</td>
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<td>-</td>
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<td>3.5</td>
<td>35</td>
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<td>-</td>
<td>-</td>
<td>Chen et al., 2011</td>
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<td>55</td>
<td>40</td>
<td>1540</td>
<td>360</td>
<td>4.8</td>
<td>Degrassi et al., 1998</td>
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<td>5.5</td>
<td>60-80</td>
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<td>Recombinant Escherichia coli</td>
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<td>36.7</td>
<td>14.1</td>
<td>-</td>
<td>-</td>
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<td>Upto 65</td>
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<td>-</td>
<td>6.4</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
<td>Meghj et al., 1990</td>
</tr>
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<td>Rhodospiridium toruloides</td>
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<td>5180</td>
<td>7.9</td>
<td>5.6</td>
<td>Politz et al., 1997</td>
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<td>Vibrio fischeri</td>
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<td>7.0</td>
<td>30</td>
<td>37</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Ranjitha et al., 2009</td>
</tr>
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<td>17</td>
<td>Aureobasidium pullulans</td>
<td>Fenzoyl esterase</td>
<td>6.7</td>
<td>60-65</td>
<td>210</td>
<td>-</td>
<td>-</td>
<td>6.5</td>
<td>Runbold et al., 2003</td>
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<td>18</td>
<td>Streptomyces lividans 60</td>
<td>-</td>
<td>6.0-9.0</td>
<td>45-55</td>
<td>48</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Schrepf and Haar, 1995</td>
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<td>Thermoanaero- bacterium sp. Strain JW/SL-YS485</td>
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<td>7.0</td>
<td>80</td>
<td>195</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Shao and Wiegel, 1993</td>
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<td>20</td>
<td>Thermoanaero- bacterium sp. Strain JW/SL-YS485</td>
<td>Acetyl xylan esterase II</td>
<td>7.5</td>
<td>84</td>
<td>106</td>
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Figure 2.9: Secretion systems in gram-negative bacteria. Adopted Erinet R Green, et, al. *Trends in microbiology* 2009, 89-94. Used with permission from the publishers.

Figure 2.10: Secretion systems in gram-positive bacteria. Adopted from Erinet R Green, et, al. *Trends in microbiology* 2009, 89-94. Used with permission from the publishers.
Although protein secretion is critical for bacterial pathogenesis, several critical virulence factors are presented as intracellular proteins (Foulks et al. 2008; Nakagawa et al. 1984). The localization of these enzymes into extracellular environment can be achieved by enzyme release from autolysis process of bacteria in biofilm (Perry et al. 2009).

2.3.5 Screening Methods for Bacterial Esterase

Chromophoric substrates, such as p-nitrophenyl esters or o-nitrophenyl esters (Fig 2.11), can be used to screen esterases. By its inherent definition, active esterase catalyzes the cleavage of ester bonds in substrates generating by-products with color. Thus, the esterase activity will be indicated by a color change in the reaction solution. The reaction involved as follows:

\[ \text{p-nitrophenyl acetate (p-NPA) } + \text{H}_2\text{O} \xrightarrow{\text{esterase}} \text{p-nitrophenol (yellow)} + \text{acetic acid} \]

![Figure 2.11: Structures of nitrophenyl esters.](image)

The commonly used method to identify esterase-producing bacteria is using tributyrin-supplemented agar plates. The bacterial colonies, which produces active enzyme, will hydrolyze dispersed ester substrates in the plate forming a clearing zone referred as a halo formation. Various
substrates can be used in plates to distinguish lipase from esterase due to the different substrate spectra: p-nitrophenyl palmitate can only be hydrolyzed by lipase, while p-nitrophenyl butyrate can be effectively cleaved by esterase. In addition to these two traditional assays, DNA library expression screening is emerging due to the advanced development of modern molecular biology techniques, which allows for esterase identification in non-culturable bacteria.

2.4 The cariogenic bacterium: *Streptococcus mutans*

2.4.1 Virulence factors of *Streptococcus mutans*

Of the over one thousand bacterial species that colonize and persist in the oral cavity, *S. mutans* is one of the few species that have been consistently linked with caries formation (Loesche 1986; Seemann et al. 2005). The main virulence factors for *S. mutans* are its ability to form biofilm (dental plaque) to survive and persist in continuously changed oral environment (Li et al. 2001c), producing acid (acidogenicity) from dietary fermentable carbohydrates initiating tooth demineralization that leads to dental caries, and surviving in acidic environments (aciduricity) (Li et al. 2001b).

**Biofilm formation:** Biofilms are bacterial communities adherent to a hard surface, and their formation drives by a variety of environmental signals that lead to the development of new bacterial phenotypes distinguishing biofilm cells from planktonic cells (Burne et al. 1997; Costerton et al. 1995; O'Toole et al. 2000). Compared to life in a planktonic environment, bacteria in biofilm exhibit surviving advantages such as up to 1,000-fold more antibiotics resistances, altered gene expression patterns, and different quorum sensing responses (Burne et al. 1997; Cvitkovitch 2003; Hoyle and Costerton 1991). The virulence factors associated with adhesion of *S. mutans* within biofilm have been extensively investigated: sucrose-independent adhesion of *S. mutans* is mostly influenced by antigen I/II, a surface protein, encoded by *spaP* (House et al. 2009);
the major mechanism of sucrose-dependent adhesion is the synthesis of glucans mediated by glucosyltransferases (GTFs) encoded by \textit{gtfB}, \textit{gtfC}, and \textit{gtfD} synthesizing both water-soluble and water-insoluble glucans as extracellular polymer facilitating bacterial adhesion (Hanada and Kuramitsu 1988; Hanada and Kuramitsu 1989; Khalichi et al. 2004); in addition to GTFs, other non-enzymatic glucan-binding proteins A (Gbp A) and glucan-binding proteins D (Gbp D) has been proven to be involved in bacterial adhesion (Banas et al. 1990; Krastel et al. 2009; Shah and Russell 2002). In addition to the proteins and enzymes contributing to bacterial adhesion, there are proteins involved in the metabolism of various carbohydrates providing energy resource which are also considered as virulence factors including fructosyltransferase (Ftf), fructanase (FruA), extracellular dextranase (DexA), and proteins responsible for intracellular polysaccharide accumulation such as Dlt1-4 (Banas 2004; Burne et al. 1996; Colby and Russell 1997; Colby et al. 1995). It has also been reported that quorum-sensing systems encoded by \textit{comCDE}, have an effect on the capacity of biofilm formation (Li et al. 2002a; Li et al. 2002b; Wen and Burne 2003; Yoshida and Kuramitsu 2002).

\textbf{Acidogenicity:} \textit{S. mutans} consumes dietary carbohydrates and produces various fermentation products including lactate, formate, acetate, and ethanol. The precise distribution of fermentation products depends on growth conditions. When glucose is abundant, lactate (pKa 3.8) is the major end-product decreasing biofilm pH leading to tooth demineralization and caries development (Dashper and Reynolds 1996). Lactate dehydrogenase (Ldh), responsible for lactic acid production, is recognized as a virulence factor of \textit{S. mutans}, since reduced cariogenicity was observed on animal models inoculated with \textit{Ldh} mutant strain (Brown and Wittenberger 1972; Fitzgerald et al. 1989; Hillman 1978).
**Aciduricity:** Although other oral streptococci have the ability to produce acid, *S. mutans* is the one of a few that has the ability to maintain its function at the growth inhibitory pH levels (pH 4.4) (Bender et al. 1985), which is characterized as aciduricity or acid-tolerance. To adapt to pH challenged environments, the gene or protein expression patterns are modified by *S. mutans*, and this adaptive response is called the acid tolerance response (ATR) (Ma et al. 1997; Svensäter et al. 1997). There are two critical mechanisms that *S. mutans* use to survive at low pHs. The first one is maintaining intracellular pH to avoid external proton penetration and disruption to cytoplasmic enzymes functions. Studies showed the membrane-bound proton-translocating, F$_1$F$_0$-ATPase proton pump could be up-expressed to maintain a pH gradient across the cytoplasmic membrane (Bender et al. 1985; Dashper and Reynolds 1992; Hamilton and Buckley 1991). The second one is DNA repair, and its importance for bacterial survival of acid shock is well-established (Quivey et al. 1995). In *S. mutans*, one DNA repairing enzyme encoded by *uvrA* was not only confirmed to be responsible for the recovery of pH-induced DNA damage, but also for the bacterial growth at moderately acidic pH. In addition to these factors directly contributing to the acidurucuty of *S. mutans*, the synthesis of water-insoluble glucan and biofilm formation are considered to be beneficial for ATR due to the involvement of quorum sensing systems efficiently inducing physical characteristics of the biofilm such as extracellular polysaccharide matrix (EPS) barrier blocking acidic molecules (Hata and Mayanagi 2003; McNeill and Hamilton 2003).

2.4.2 **Regulations of Bacterial Virulence by Two-component Signal Transduction Systems (TCSTs)**

All pathogens have the ability to adapt to microenvironments in the host, and commonly, these adaptive responses increase bacterial survival capacities in challenged environments, which are considered as virulence factors associated with bacterial pathogenicity. In bacteria, two-component signal transduction systems (TCSTs or TCSs) are widely adopted to regulate its
virulent performance by sensing environmental stimuli and responding accordingly (Stock et al. 2000). A typical two-component regulatory system contains a membrane-associated, histidine kinase sensor protein, which senses the environmental conditions, and a cytoplasmic response regulator, which allows the bacteria to respond by regulation of gene expression (Stock et al. 2000). In *S. mutans*, 13 TCSTSs and one orphan regulator have been reported.

Among all these TCSTSs associated with virulence regulation, the quorum-sensing system in *S. mutans* will be discussed in detail as a perfect example to explain how TCSTSs regulate bacterial social activity and virulence (Cvitkovitch 2003; Li et al. 2001a; Li et al. 2002b; Li et al. 2008a). In the quorum-sensing system, in addition to a TCSTS including a membrane-bound histidine kinase sensor (ComD) and an intracellular response regulator (ComE), a signal peptide (CSP) is encoded by *ComC* which act as an environmental stimulus (Cvitkovitch 2003). In general, bacteria constantly secrete low levels of signals molecules (CSP) and monitor its concentration by the corresponding receptor (ComC) (Cvitkovitch 2003; Miller and Bassler 2001). The receptor is not activated until the signal concentration exceed to a critical threshold (De Kievit and Iglewski 2000). Once the receptor is activated, the “message” passes to the cognate response regulator (ComE) of *S. mutans* via phosphorylation and in turn, activates the downstream target genes that determine the bacterial responses and adaptive behaviors (Cvitkovitch et al. 2003; Senadheera and Cvitkovitch 2008). It has been verified that biofilm-associated *gtfB*, *gtfC* and *gbpB* are the target genes of *comCDE* system (Cvitkovitch et al. 2003.) As a result, the increased CSP concentration initiates *S. mutans* biofilm formation. In addition, the ComDE system is also involved in the regulation of *S. mutans* competence, acid tolerance, and bacteriocin synthesis (Li et al. 2001b; Li et al. 2002c; Li et al. 2008b; Reck et al. 2015; Van der Ploeg 2005). In summary, bacteria monitor physical or chemical signals in extracellular environment through receptors in TCSTSs, and then
regulate diverse physiological responses through the adjustment of regulator-target genes expression.

2.5 The Interaction between Resin Composites and Cariogenic Bacteria

As the most popular restorative materials, resin composites are widely used in clinics for various purposes (Murray et al. 2002b; Simecek et al. 2009; Wakefield and Kofford 2001). However, compared to amalgam, resin-based restorations have higher failure rates mainly due to the recurrent caries, also known as secondary caries, along the restoration-tooth interface. (Chrysanthakopoulos 2011a; Kopperud et al. 2012; Mjör 2005; Murray et al. 2002b; Soncini et al. 2007; Sunnegårdh-Grönberg et al. 2009; Van Nieuwenhuysen et al. 2003). In the etiology regard, there is no difference between primary caries and secondary caries, which are both bacterial infectious disease and characterized as tooth demineralization when local pH value drops below 5.4 (Loesche 1982). However, the existing restorative material is the additional determinant for the initiation and progression of secondary caries due to the interaction between cariogenic bacteria and restorative materials (Mjör and Moorhead 1998; Sunnegårdh-Grönberg et al. 2009).

2.5.1 The Biodegradation of Resin Composites by Cariogenic Bacteria

The suspected degradative effect of oral bacteria on resin composites was reported through the observation of increased surface roughness after bacterial colonization on different resin composites (Beyth et al. 2008; Willershausen et al. 1999). Further investigations based on surface topography all support this hypothesis (Delaviz et al. 2014; Gregson et al. 2011). One interesting finding is that among several oral bacteria such as *S. mutans*, *Streptococcus sanguis*, and *Streptococcus Gordonii* (*S. gordonii*), the cariogenic ones, *S. mutans* and *S. gordonii*, presented a stronger effect on resin composites degradation, which could be explained by the stronger adaptive behaviors of cariogenic bacteria to potential toxic effects from resin monomers (Gregson et al.)
Focusing on the primary cariogenic bacteria, our group verified that *S. mutans* has esterase activities at levels that can degrade polymerized commercial Z250 resin composites, total etch (Scotchbond), and self-etch (Easybond) adhesives by surface topography analysis and the quantification of degradation by-products, BisHPPP, through high performance liquid chromatography (HPLC) analysis (Bourbia et al. 2013).

### 2.5.2 Surface Properties of Resin Composites and Bacterial Adhesion

A higher number of total bacteria, significantly more *S. mutans*, and a greater microbial diversity are found on the surface of resin composites, compared to amalgams, glass-ionomers, and tooth enamel (Skjørland 1973; Skjørland and Sønju 1982; Svanberg et al. 1990). Based on current investigations, there are several surface characteristics of resin composites that contributes to bacterial adhesion and biofilm formation: surface toughness, hydrophobicity/hydrophilicity, composite surface composition, and the salivary pellicle coating (Hahnel et al. 2008; Teughels et al. 2006; Yamamoto et al. 1996).

It has been confirmed increased surface roughness facilitates bacterial adhesion (Teughels et al. 2006) due to the better protection against shear force on the rougher surface so that the irreversible bonding between the bacteria and materials surfaces are formed more easily and frequently (Hannig 1999). However, when the average surface roughness (*Ra*) is less than 0.2µm, which is considered as smooth, there is no significant effect on bacterial adhesion (Teughels et al. 2006). Bacterial adhesion is also affected by different composition of composite surface. For example, *S. oralis* was reported to adhere to composite filler particles by fimbriae (Yamamoto et al. 1996). Apart from the fillers, the chemical property of polymers in resin composites is also an important determinant of biofilm adhesion, which can be explained by monomers’ hydrophobicity (Hahnel et al. 2008; Ikeda et al. 2007). Most bacteria favor hydrophilic surfaces to form biofilm (Buergers
et al. 2009). The other factor influencing bacterial adhesion is the salivary pellicle, which determines the amount and composition of early microbial colonizers by its physiochemical properties including density, distribution, and composition (Carlen et al. 1996; Schweikl et al. 2013; Siqueira et al. 2012a). Studies revealed the physiochemical properties of the pellicle are largely dependent on the physical and chemical nature of composites such as hydrophobicity, surface roughness, and surface charge (Baier and Glantz 1978; De Jong et al. 1984; Fine et al. 1984; Lee et al. 1974; Schweikl et al. 2013).

2.5.3 Interaction Between Bacteria and Released Composite Components

The incomplete polymerization of resin composites and resin-matrix degradation allows resin monomers and degradation by-products leaching out into oral cavity. The barrier formed by the extracellular polysaccharides matrix (EPS) produced by bacteria in biofilms could restrict the solute diffusion and accumulate the leachable monomers and degradation by-products in the biofilm, particularly in the resin-dentin interface with limited space (Kawai and Tsuchitani 2000; Kuper et al. 2014).

TEGDMA not only promoted the growth of cariogenic bacteria such as *Lactobacillus acidophilus* (*L. acidophilus*) and *Streptococcus sobrinus* (*S. sobrinus*), but also enhanced the activity of glucosyltransferase in *S. sobrinus* boosting biofilm formation (Kawai and Tsuchitani 2000). Like TEGDMA, its degradation product, TEG, was also reported to promote the growth of *S. mutans* NG8 in acidic environments (pH 5.5) and up-regulate two biofilm-associated genes, *gtfB* and *yfiV*, in a concentration- and pH-dependent manner (Khalichi et al. 2009; Singh et al. 2009). These results could explain the observations that composites lead to increased biofilm formation and certain changes in the dental biofilm favoring the outgrowth of more cariogenic species (Beyth et al. 2008). However, the monomer BisGMA and its degradation products MAA and BisHPPP all
have an inhibitory effect on bacterial growth rates in a multifactor manner (Kawai and Tsuchitani 2000; Khalichi et al. 2004; Khalichi et al. 2009; Singh et al. 2009). Recent studies even explored the effect of BisHPPP on the gene and protein expression patterns of *S. mutans* by microarray and quantitative proteomics (Sadeghinejad et al. 2017). There were 137 proteins that exhibited altered expression in *S. mutans* in the presence of BisHPPP, of which 90 proteins were up-regulated while 47 protein were down-regulated (Sadeghinejad et al. 2017). Biofilm-associated GtfB, GtfC, GbpB, and virulence-associated ComDE were all up-regulated, indicating the enhancing effect of BisHPPP on the cariogenicity of *S. mutans*. In addition, other up-regulated proteins were involved in stress-response, metabolic process, and phosphoeholpyruvate:sugar phosphotransferase transport system (PEP:PTS), which allows bacteria to survive and persist in challenged oral environments (Sadeghinejad et al. 2017). In addition to the positive regulatory effect on the proteins productions, both BisHPPP and TEG could directly increase GTF enzyme activity, promoting the synthesis of exopolysaccharide glucan associated with the formation of cariogenic biofilms in the presence of sucrose (Sadeghinejad et al. 2017; Sadeghinejad et al. 2016).

Overall, the degradative effect of *S. mutans* on resin composites produces the stimuli such as BisHPPP and TEG for its growth and virulence leading to secondary caries, which could be the explanation of the high failure rate of the resin-based restorations.

### 2.5.4 Strategies to Investigate Bacterial Responses to Materials at Gene Level

Bacterial behavior or response is carried out by the activity of proteins regulated by the expression of their encoding gene. Since the gene must be transcribed into mRNA to be expressed (or turned on), the amount of corresponding mRNA transcribed is measured to reflect the gene expression level.
Gene expression analysis using qRT-PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) is the most commonly used method to investigate gene expression or expression patterns and comparing mRNA levels in various samples (Orlando et al. 1998). The \textit{in vivo} mRNA extractions are converted into cDNA by reverse transcription, which is used as a template for further PCR amplification. The amount of amplified PCR product varies based on the quantity of initial mRNA. As a result, the detected quantity of PCR products presents the specific mRNA level, indicating the gene expression level (Wittwer et al. 2013). In a recent study, qRT-PCR was used to determine the effect of clinically-relevant concentrations of BisHPPP on the expression of seven virulence genes in \textit{S. mutans} UA159 (Sadeghinejad et al. 2017).

QRT-PCR will continue to be used extensively, since it is a rapid, sensitive, and affordable procedure. Additionally, it is often the only feasible approach for investigation of rare or unique tissues (Klein 2002). However, the successful application of qRT-PCR heavily depends on the integrity of extracted RNA and the accuracy of subsequent data analysis is affected by the reverse transcription step, which must accurately reflect RNA input amounts (Bustin 2002; Bustin and Nolan 2004). Therefore, the dynamic range, sensitivity and specificity of the enzyme are prime considerations for a successful RT-PCR assay.

Gene expression analysis using Microarray

Microarrays are emerging as a powerful and effective tool to investigate large scale gene expression in one experiment, which allows for parallel studies providing both static (in which tissue the specific gene is expressed) and dynamic information (the interaction between the gene of interest and other genes) (Moreau et al. 2003). Based on how the genes are probed, there are two array-based technologies: cDNA and oligonucleotide arrays. A typical microarray experiment
involves the hybridization of labeled mRNA pools from investigated tissues or cells to the solid substrates with immobilized DNA fragments of interested genes. For gene expression measurement, there are two principles involved: 1) hybridization of a single labeled sample from mRNA pool, for one-channel detection, in which the absolute quantification (the concentration of target) is presented as the intensity of the hybridization signal; 2) competitive hybridization of a mixed sample pool in which there are two labeled samples derived from two compared groups (reference and test group), respectively. Then, the two-channel detection is conducted which provides the ratio of fluorescence intensities from the two labeled sample pools indicating the ratio of concentrations of the same target between reference and test groups.

**Gene expression analysis using Fluorescence in situ Hybridization (FISH)**

FISH detection was first applied in 1980 using RNA labeled with fluorophore as a probe for specific DNA sequence (Bauman et al. 1980). With the development of probes synthesis and fluorophore labeling, FISH with rRNA-targeted probes is routinely used for microorganism identification (Al-Ahmad et al. 2007). In situ hybridization (ISH) of mRNA sequences is a popular technique for studying gene expression in eukaryotic cells and tissues (Wendeberg et al. 2012). In general, the procedure includes the following steps: (1) fixation of specimen; (2) pretreatment and preparation of sample; (3) hybridization with specific probes for target sequences; (4) washing steps to remove unspecific bound probes reducing background; (5) visualization using laser scanning microscopy (Moter and Göbel 2000).

Compared to qRT-PCR and microarray, FISH has an advantage in providing extra spatial and distributing information in intact samples with 3D structure. However, like the other two methods, the success of FISH for mRNAs depends strongly on the integrity of the target mRNAs in biological samples. The chief concerns in this assay are the signal irregularity, samples auto-
fluorescence and reproducibility (Nath and Johnson 2000; Nederlof et al. 1992). There are various factors could affect the sensitivity and reproducibility of this technique: mRNA preservation and probe accessibility influenced by fixation procedure, probe type and quality dependent on probe design, hybrid stability determined by both probe quality and post-hybridization treatments, and detection methods and background noise due to the non-specific probe binding and auto-fluorescence from biological samples (Levsky and Singer 2003; Moter and Göbel 2000).

Although there are limitations, FISH has already changed our way in visualizing and analyze genes and transcriptions (Levsky and Singer 2003; Nath and Johnson 2000). Rapidly developed signal detection techniques and labeling systems will allow FISH to be increasingly employed in microbiology where intact biofilm structure is critical to address questions such as the detection of bacterial responses to environmental changes at the transcription level. Ultimately, FISH will be the favorable approach to analyze the complicated components of gene expression (Levsky and Singer 2003).
Chapter 3

Isolation and Characterization of an Esterase from *Streptococcus mutans* that Hydrolyzes Dental Resins

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3.1 Abstract

**Objectives:** The aim of this study was to identify and characterize the specific esterases from *S. mutans* with degradative activity toward methacrylate-based resin monomers. **Methods:** Out of several putative esterases, an esterase, encoded in the open reading frame SMU_118c (The National Center for Biotechnology Information, NCBI) was found to have true hydrolase activities. SMU_118c was cloned, expressed, purified, and characterized for its respective hydrolytic activity towards ester-containing substrates and the universal resin monomers bis-phenyl-glycidyl-dimethacrylate (BisGMA) and triethyleneglycol dimethacrylate (TEGDMA) at neutral (7.0) or cariogenic (5.5) pH. The active site of SMU_118c was verified using a specific serine-esterase inhibitor, phenylmethylsulfonyl fluoride (PMSF). In addition, mass spectrometry (MS) analysis was used to verify the expression of SMU_118c protein in *S. mutans* UA159. **Results:** Similar to the whole cell activity of *S. mutans*, SMU_118c showed the highest affinity toward p-nitrophenyl acetate (pNPA) and p-nitrophenyl butyrate (pNPB) \( \text{vs.} \) o-nitrophenyl butyrate (oNPB) and butyrylthiocholine iodide (BTC) \( p < 0.05 \). The esterase retained 60% of its activity after 21 days
and hydrolyzed BisGMA at a higher rate than TEGDMA at both neutral and cariogenic pH ($p < 0.001$), which is similar to the predominant human salivary esterase degradative activity. PMSF induced a similar reduction of the enzyme’s hydrolytic activity towards pNPB and BisGMA, indicating that SMU_118c has a functional mechanism that is similar to other enzymes in the esterase-lipase superfamily. MS confirmed that SMU_118c is an intracellular protein in *S. mutans* UA159 and expressed under pathogenic (pH 5.5) growth conditions. **Significance:** The similarity in the activity profile to the whole *S. mutans* bacterial cell, the stability over time at cariogenic pH, the preference to hydrolyze BisGMA, and confirmed expression profile suggest that SMU_118c could be a significant contributor to the whole bacterial degradative activity of *S. mutans* toward the biodegradation of resin composites, adhesives, and the restoration-tooth interface, potentially accelerating restoration failure.

**Keywords:** *Streptococcus mutans*, esterase, resin monomers, hydrolysis, biodegradation, dental caries

### 3.2 Introduction

Resin composites are the most popular restorative materials in dentistry (Beazoglou et al. 2007). However, compared to amalgam, resin composites suffer from high failure rates (Opdam et al. 2014; Ástvaldsdóttir et al. 2015). According to published data, nearly 70% of resin composite restorations are replacements of failed restorations (Mjör 2005; Murray et al. 2002b). The primary reason for composite restoration replacement is secondary caries, defined as the progression of decay at a compromised restoration-tooth interface (Brunthaler et al. 2003b; Ferracane 2011; Opdam et al. 2007). Previous studies have shown that biostability of restorative materials has a significant impact on the quality of the restoration-tooth interface that could impact the prevalence of secondary caries (Kermanshahi et al. 2010; Serkies et al. 2016; Spencer et al. 2014).
Resin composites are inherently prone to hydrolysis due to the presence of unprotected ester linkages in their major matrix monomers bis-phenyl glycidyl dimethacrylate (BisGMA) and triethylene glycol dimethacrylate (TEGDMA), generating degradation by-products bishydroxypropoxy-phenyl-propane (BisHPPP) and triethylene glycol (TEG) and methacrylic acid (MA), respectively (Finer and Santerre 2004a; Shokati et al. 2010). And, this hydrolysis process can be further catalyzed by esterase activities from human saliva and cariogenic bacteria in the oral cavity, which is often referred to as biodegradation (Kermanshahi et al. 2010; Shokati et al. 2010). In turn, these by-products negatively affect host cells and the metabolism and function of oral microorganisms (Khalichi et al. 2004; Khalichi et al. 2009; Stanley 1993). It has been reported that bisphenol-A-derived species are estrogenic (Olea et al. 1996), and the degradation by-products of TEGDMA, TEG, modulate the growth of oral bacteria (Khalichi et al. 2004). These degradation processes compromise the resin-tooth interface (Serkies et al. 2016; Shokati et al. 2010), allowing for the ingress of cariogenic bacteria along the interface, which leads to initiation and progress of secondary caries (Kermanshahi et al. 2010; Spencer et al. 2010).

Esterases (EC 3.1.1.X) are a diverse group of hydrolases that catalyze the hydrolysis of ester bonds. They are widely distributed in human tissues (Finer and Santerre 2004a) and microorganisms (Bornscheuer 2002). In the oral environment, the sources of esterases include human gingival epithelium, salivary glands, inflammatory responses, and microorganisms (Lindqvist et al. 1976). Previous studies reported that human saliva contains cholesterol esterase-like (CE) and pseudocholinesterase (PCE) activities at levels that degrade resin-based composites (Cai et al. 2014; Delaviz et al. 2014; Finer and Santerre 2004a). It has been known for a long time that esterases are widespread in bacterial pathogens such as Streptococcus species (Xie et al. 2008; Zhu et al. 2009). However, to date, the identification of specific esterases from cariogenic bacteria and
their association with the biodegradation process of resin composites and adhesives have not been investigated.

We previously reported on hydrolytic activity from the cariogenic bacteria *Streptococcus mutans* (Bourbia et al. 2013). This activity profile was compared to hydrolytic activity in human saliva and was shown to degrade dental composite and adhesives in a material-dependent manner, demonstrating clear clinical relevance of these processes as major contributors to restoration failure. However, the aforementioned study did not identify the specific esterases from the bacteria that were responsible for the hydrolysis. Therefore, the aim of this study was to further expand the work of Bourbia et al and to identify and characterize the specific esterases with degradative activity toward methacrylate-based resin monomers from *S. mutans*, the primary cariogenic species associated with caries formation and progression (Hamada et al. 1984). The characterization of these specific esterases will allow for mechanistic exploration of the biological effect of oral bacteria on dental materials, the reciprocal effect of these materials and their biodegradation products on the bacteria, and guide manufacturers to design materials that are more resistant to these effects.

### 3.3 Materials and Methods

#### 3.3.1 Bioinformatic Analyses

Putative esterase genes were searched in the *S. mutans* UA159 genome database at The National Center for Biotechnology Information (NCBI) ([http://www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). Nucleotide and deduced amino-acid sequences were analyzed using MacVector software. The signal peptide was analyzed by SignalP 4.0 server ([http://www.cbs.dtu.dk/services/SignalP/](http://www.cbs.dtu.dk/services/SignalP/)).
3.3.2 Cloning, Expression and Purification of Bacterial Esterases

Basic Local Alignment Search Tool (BLAST) identified several putative esterase genes in the *S. mutans* UA159 genome database. The gene candidates (*SMU_118c, SMU_400, SMU_643, SMU_1443c, SMU_1678*) were PCR amplified from *S. mutans* UA159 genomic DNA by using designed primers (Table 3.1), then cloned into the p15Tv-LIC vector as previously described (empty vector was used as a control) (Eschenfeldt et al. 2009), providing an N-terminal His6-tag fusion followed by a TEV protease cleavage site. The esterases were expressed in *E. coli* BL21 (DE3) and then harvested for further protein isolation and purification (Stogios et al. 2015). Cells were resuspended in binding buffer [50 mM Hepes (pH 7.5), 100/300 mM NaCl, 10 mM imidazole and 2% glycerol (v/v)] and lysed using a sonicator. Cell debris was removed via centrifugation at 30,000G. Cleared lysate was loaded onto a 5-mL Ni-NTA column (QIAGEN, Dusseldorf, Germany) pre-equilibrated with binding buffer and washed. Proteins were eluted using the above buffer with 250 mM imidazole. The His6-tag was removed by cleavage with TEV protease overnight at 4 °C in dialysis with buffer 0.3 M NaCl, 50 mM Hepes (pH 7.5), 5% glycerol and 0.5 mM tris [2-carboxyethyl] phosphine, followed by binding to Ni-NTA resin and capture of flow-through. Fractions containing the protein of interest were identified by SDS-polyacrylamide gel electrophoresis and further purified via gel filtration on a HiLoad 16/60 Superdex75 prep-grade column [10 mM Hepes (pH 7.5) and 50 mM KCl].

3.3.3 Enzymatic Assays

Esterase activities shown to be relevant to biodegradation of resin composites were measured using the substrates *p*-nitrophenyl acetate (pNPA), *p*-nitrophenyl butyrate (pNPB), *p*-nitrophenyl laurate (pNPL), *o*-nitrophenyl butyrate (oNPB) and butyrylthiocholine iodide (BTC) (Sigma Chemical Co. St. Louis, MO, USA) as described previously (Finer and Santerre 2004a; Serkies et al. 2016;
All experiments were performed in triplicate using a spectrophotometer (Beckman Coulter DU 800, Brea, CA) at 37°C, and corrected for the effects of non-enzymatic hydrolysis of substrates. One unit of CE-like activity was defined as the amount of enzyme producing 1 µmol p-nitrophenol from p-nitrophenyl esters per minute ($\varepsilon_{401}=1.6 \times 10^4$ M$^{-1}$cm$^{-1}$) (Finer and Santerre 2004a; Lin et al. 2005) and specific activity was expressed as units per mg of protein. For PCE-like activity, one unit was defined as the amount of enzyme activity producing 1 µmol butyrate per minute ($\varepsilon_{405}=1.36 \times 10^4$ M$^{-1}$cm$^{-1}$) and specific activity as units per mg of protein (Finer and Santerre 2004a; Jaffer et al. 2002; Lin et al. 2005).

**3.3.4 Kinetic Measurements**

The kinetic parameters of the purified esterase toward pNPB, a substrate that has been shown to be a good predictor for composite biodegradation (Finer and Santerre 2004a; Jaffer et al. 2002) were determined by measuring the enzyme activities with different substrate concentrations, from 0.0625 mM to 16 mM, in D-PBS (pH 7) at 37°C for 5 min. Kinetic parameters, the Michaelis-Menten constant ($K_M$), and maximum velocity ($V_{max}$) were calculated using the Lineweaver-Burke plot (Berg JM et al. 2002).

**3.3.5 The Effects of pH and Resin Monomers on Esterase Activity and Stability**

The effect of pH on enzyme activity towards pNPB was measured by adding lactic acid (37°C) to create conditions with pH ranging from 3 to 7 (n = 3/pH), using methods described in section 3.3.4. The effect of resin monomers on enzyme stability was evaluated by incubating the purified enzyme (0.5 mg/ml) in 5 ml of 10 mM D-PBS (pH 7 and pH 5.5) with or without 100 µM BisGMA or TEGDMA at 37°C up to 21 days (n = 3) as previously described (Finer and Santerre 2004a). During incubation, 0.20 ml of samples were taken at different time points (0, 1, 2, 3, 5, 14 and 21 days) for esterase activity measurement as described above.
3.3.6 Monomers Biodegradation

Monomer biodegradation was performed in 2 ml of 10 mM PBS (pH 7.0 and pH 5.5) containing 100 µM BisGMA or TEGDMA incubated with or without 16 U/ml of purified esterase at 37°C for 125 hours (n = 3) (Finer and Santerre 2004a; Lin et al. 2005). During incubation, 0.25 ml of samples were taken at 0, 4, 8, 24 and 125-hour time points then mixed with equal volume of pre-chilled methanol (Sigma Chemical Co. St. Louis, MO, USA) to quench the reaction immediately (Finer and Santerre 2004a; Jaffer et al. 2002; Serkies et al. 2016; Shokati et al. 2010). For HPLC analysis, 0.4 ml of the mixed incubation solutions were filtered with Millipore centrifuge filters (UltrafreeTM-C UFC3LG00; Millipore) to remove molecules with a molecular weight > 5000 Da in a centrifuge (H-25FI, Silencer) at 1,000 G. The processed samples were refrigerated until required for further analysis.

An HPLC system (Waters, Mississauga, ON, Canada) equipped with photodiode detector was used for the chromatographic separation and quantification of BisGMA, TEGDMA, and the derived biodegradation products bisHPPP and MA, as described previously (Bourbia et al. 2013). The retention time of each product was confirmed by HPLC chromatographic profile of standard monomers and mass spectrometry (MS) analysis (Finer and Santerre 2003; 2004b; Serkies et al. 2016; Shokati et al. 2010).

3.3.7 Inhibition Assays

The inhibitory effect of phenylmethyl sulfonyl fluoride (PMSF) (Sigma, St. Louis, MO, USA) on the enzyme was investigated by adding the inhibitor to the enzyme solution, and then measuring the enzymatic activities towards pNPB as previously described (Finer and Santerre 2004a). The carrier solvent of the inhibitor, ethanol, was assessed as a control for its effect on the enzymatic activity. A PMSF and ethanol mixture was used as a non-enzymatic control. The final
concentration of PMSF was adjusted to 0.5 mM in the enzyme solution (16 U/ml) to provide partial inhibition.

To further verify the reduction of enzymatic activity by PMSF, monomer biodegradation was carried out by incubating 100 µM BisGMA in 3 ml of either PBS (negative control), esterase (16 U/ml), or mixture of enzyme (16 U/ml) and PMSF (0.5 mM) solution at 37°C for 72 hours (pH 7.0). A PMSF and ethanol mixture was used as a non-enzymatic control. Samples from incubation solutions were analyzed by HPLC to quantify the biodegradation by-product, BisHPPP.

3.3.8 The Verification of SMU_118c Expression in S. mutans UA159

Biofilms of S. mutans UA159 (n = 3) were cultured in 3 mL of ¼ TYEG medium for 18 hours (pH 5.5, 37°C, 5% CO₂). Adhered biofilm cells were then collected and disrupted as described previously (Sadeghinejad et al. 2016). The intracellular components were collected and separated in aliquots for the total protein concentration assessment by the Micro Bicinchoninic Acid (Micro BCA) assay (Siqueira et al. 2012b). Protein contents (20 µg) were dried, denatured, and reduced for 2 hours as described previously (Sadeghinejad et al. 2017). Then, peptide separation and MS analyses were carried out as described previously (Sadeghinejad et al. 2016; Siqueira et al. 2012b). The obtained MS/MS spectra were searched against streptococci protein database (Swiss Prot and TrEMBL, Swiss Institute of Bioinformatics, Geneva, Switzerland, http://ca.expasy.org/sprot/) using SEQUEST algorithm in Proteome Discoverer 1.3 software (Thermo Scientific, San Jose, CA, USA) (Sadeghinejad et al. 2017; Sadeghinejad et al. 2016; Siqueira et al. 2012b).

3.3.9 Statistical Analysis

All experiments were conducted in triplicate. One-way analysis of variance (ANOVA) and Tukey’s multiple comparison tests were performed to determine differences in esterase activity profiles, pH profile, enzyme stability, and inhibition. Student’s t-test was used to determine
3.4 Results

3.4.1 Genes Sequence Analysis and Expression of Esterases of S. mutans UA 159

Based on gene sequence analysis, out of ten putative esterase genes found in S. mutans UA 159, five genes might be expressed as soluble proteins. Out of these five, three genes were successfully expressed as soluble intracellular enzymes (SMU_118c, SMU_400, and SMU_643; Fig. 3.1A). The empty vector had no measured activity. Activity assays confirmed that only one esterase, SMU_118c, functioned as a true hydrolase toward the nitro-phenyl substrates. The deduced protein consisted of 280 amino-acid residues (Fig 3.1B). The N-terminal did not contain a predicted secretion signal peptide.

3.4.2 Expression and Purification of the Esterase SMU_118c

SMU_118c was expressed in E. coli BL21 as a soluble intracellular enzyme. Homogeneity of the protein was checked by SDS-PAGE and was confirmed to be a molecular subunit of 33.8 kDa (Fig. 3.1A), which is similar to the molecular mass calculated from the deduced amino-acid sequence.

3.4.3 Substrate Specificity and Kinetic Parameters of SMU_118c

SMU_118c showed preference towards para-nitrophenyl esters, pNPA and pNPB than ortho-nitrophenyl, oNPB ($p < 0.001$). Among the para-nitrophenyl substrates, SMU_118c showed preference towards pNPA containing two carbon acyl-chain rather than pNPB containing four carbon acyl-chain ($p < 0.05$); the highest activity was 1620 U/mg towards pNPA at pH 7.0, followed by 1189 U/mg to pNPB and 80 U/mg to oNPB (Fig. 3.2). The enzyme had limited activity towards pNPL and BTC substrates. The $K_m$ and $V_{max}$ towards pNPB were 13.25 mM and 1.88mM/min/mg protein at pH 7.0, respectively.
3.4.4 The Effect of pH and Resin Monomers on the Activity and Stability of SMU_118c

SMU_118c exhibited optimal activity toward pNPB at pH 7.0, and activity was reduced significantly with pH values lower than 6.8 ($p < 0.05$; Fig. 3.3A).

After 21 days of incubation at pH 7.0 and pH 5.5, SMU_118c showed a non-significant reduction of its activity to $71.8 \pm 2.4\%$ and $74.4 \pm 13.8\%$ of its initial activity, respectively ($p < 0.05$; Fig. 3.3B and 3.3C). The addition of BisGMA or TEGDMA did not significantly affect the activity and stability of the enzyme at either pH (Fig. 3.3B and 3.3C).

3.4.5 Hydrolysis of the Monomers by SMU_118c

The amount of degraded monomers and subsequently produced by-products are presented as percentage of the initial amount of each monomer. The results are normalized to the buffer control. After 125 hours, $92.5 \pm 2.5\%$ and $94.1 \pm 2.9\%$ of BisGMA was hydrolyzed by SMU_118c at pH 7.0 and 5.5, respectively (Fig. 3.4A). The production of BisHPPP was similar to the incremental loss of BisGMA (Fig. 3.4A). For TEGDMA, $17.6 \pm 4.4\%$ and $11.9 \pm 4.7\%$ of its original amount were degraded at pH 7.0 and pH 5.5, respectively (Fig. 3.4B). MA increased to $27.6 \pm 2.2\%$ and $28.1 \pm 2.4\%$ at pH 7.0 and pH 5.5, respectively (Fig. 3.4B). The reaction rate of SMU_118c for BisGMA ($0.53 \pm 0.03 \mu M/h$.) was 5.4 times higher than that of TEGDMA ($0.098 \pm 0.05 \mu M/h$) at pH 7.0 and 7 times higher ($0.49 \pm 0.02 \mu M/hr.$ vs. $0.07 \pm 0.03 \mu M/hr.$) at pH 5.5 ($p < 0.001$).

3.4.6 SMU_118c Inhibition

The addition of ethanol (PMSF carrier solvent) had no significant effect on enzyme activity (Fig. 3.5A). The addition of PMSF to SMU_118c solution decreased the relative enzymatic activity of SMU_118c to $58.3 \pm 1.2\%$ vs. the enzyme alone ($p < 0.05$). PMSF dissolved in ethanol was used as a non-enzymatic control and exhibited no activity toward p-NPB.

In monomer biodegradation experiments, the addition of PMSF to the buffer solutions had no
effect on the hydrolysis rate of BisGMA when compared to the buffer control (Fig. 3.5B). The addition of PMSF to SMU_118c significantly reduced the relative amount of BisHPPP production to $49.3 \pm 6.9\%$ ($p < 0.05$; Fig. 3.5B).

3.4.7 The Verification of SMU_118c Expression in S. mutans UA159

The base-peak chromatogram for reversed-phase chromatography monitored by MS showed a consistent elution of protein/peptides in the range of 20 to 50 min, in which the esterase SMU_118c was identified from intracellular components of S. mutans UA159 biofilm cells.

3.5 Discussion

Although there are potentially several sources of hydrolytic enzymes (e.g., saliva, bacteria, and leukocytes) in the oral cavity, only salivary esterases and simulated salivary esterase have been extensively investigated as possible contributors to the biodegradation of the matrix component of resin composites, adhesives, and the restoration-tooth interface (Cai et al. 2014; Delaviz et al. 2014; Finer et al. 2004; Finer and Santerre 2004a; Kermanshahi et al. 2010; Spencer et al. 2010). More recently, it has been shown that, similar to the degradative activities of human saliva, the cariogenic bacterium S. mutans also degrades resin composites and adhesives (Bourbia et al. 2013). In other bacteria, esterases have been linked to virulence and pathogenesis (Lun and Bishai 2007; Wall et al. 2007). The above studies suggest a potential role of bacterial esterases in the biodegradation of the resin composite and the restoration-tooth interface, and emphasize the need to study the role of S. mutans esterases as virulence factors that contribute to the progression of secondary caries (Kermanshahi et al. 2010; Spencer et al. 2014; Spencer et al. 2010).

The current investigation is the first to identify and characterize an esterase, SMU_118c, from S. mutans that hydrolyzes dental resins and verify its expression in S. mutans biofilm cells grown under an acidic condition. With a molecular mass of 34 kDa, which is similar to other reported
microbial esterases (20 to 60 kDa; Xie et al. 2008; Zhu et al. 2009), SMU_118C is unlikely to simply permeate the cell membrane. However, even if this enzyme is not actively secreted, it would still be available to degrade dental materials, since it has been reported that a significant portion of the cells in bacterial biofilms are lysed and their contents released (Perry et al. 2009). Furthermore, the ability the whole cell bacteria to hydrolyze resin composites and adhesives (Bourbia et al. 2013) suggests that this enzyme is indeed released from bacteria and is available to degrade dental materials and as such represents a significant virulence factor of the S. mutans, thereby warranting further mechanistic investigation.

Kinetic assessment of SMU_118c was done with pNPB, BisGMA, and TEGDMA monomers as it allows for accurate characterization of enzyme activity toward pure and well-defined substrates (Cai et al. 2014; Finer and Santerre 2004a). Furthermore, the affinity toward these substrates has been previously found to be a good predictor of an enzyme’s ability to degrade methacrylate-based polymers (Cai et al. 2014; Finer and Santerre 2004a). Compared to other bacterial esterases, SMU_118c has a relatively high $K_m$ toward pNPB, indicating low affinity to such substrates (Sayali et al. 2013). However, its high $V_{max}$ value suggests a rapid hydrolytic rate (Sayali et al. 2013), which is critical for initiating resin composite biodegradation and providing accessibility for other salivary enzymes to the deeper surface of resin composite.

Substrate specificity of SMU_118c was determined using p-nitrophenyl-linked esters (pNPA, pNPB, and pNPL) of various alkyl chain lengths (C2 to C12), ortho-nitrophenyl butyrate (oNPB) with a different side chain location and BTC, a specific substrate for PCE-like activity assay (Finer and Santerre 2003; 2004a; Jaffer et al. 2002; Lin et al. 2005). The enzyme was most active with p-nitrophenyl-linked esters with a short chain length (C2 and C4), whereas low activity was detected for substrates with longer chain lengths (C12), indicating that SMU_118c is a typical esterase.
rather than a lipase (Jaeger et al. 1999). In addition, SMU_118c showed preference for substrates with chains in the para-position (pNPB) vs. those with the ortho-chains (oNPB) of the same length (C4), indicating that its enzyme activity is substrate-structure dependent. Compared to human salivary esterases that show both CE and PCE-like activity (Finer and Santerre 2004a), SMU_118c has CE-like activity towards nitrophenyl esters, but not PCE-like activity towards BTC. The above findings are consistent with the activity profile previously reported for the whole cell of S. mutans (Bourbia et al. 2013), suggesting that SMU_118c is a major contributor toward the hydrolase activity of this species.

In the oral cavity, the pH balance is mainly influenced by cariogenic bacteria, such as S. mutans, due to their ability to produce lactic acid (acidogenicity) by carbohydrate fermentation, which reduces the local pH and initiates tooth demineralization and caries (Ajdić et al. 2002; Spencer et al. 2014). Therefore, it is important to assess the ability of SMU_118c to retain its activity under the relevant intra-oral pH range, adjusted by lactic acid. It is interesting to note that despite reduced activity toward nitrophenyl esters in acidic pH vs. pH 7.0, SMU_118c showed the same degradative effect toward BisGMA and TEGDMA at pH 5.5 and 7.0. This apparent discrepancy could be explained by partial activity loss of SMU_118c due to protein precipitation caused by the combination of acidic pH (isoelectric precipitation) and the presence of acetonitrile (miscible solvent precipitation) in the enzyme activity assays but not in the monomer biodegradation assays (Scopes 2013). Furthermore, compared to the p-nitrophenyl esters, the reaction sites of BisGMA and TEGDMA have distinct properties: BisGMA (pKa 31.4) and TEGDMA (pKa 32.7) exhibit weak base property at the reaction site compared to pNPA (pKa 25.0) and pNPB (pKa 26.1), so the former compounds may locally neutralize the acidic pH of the media and provide favorable pH for enzyme activity. In addition, the ester linkages in the p-nitrophenyl esters are directly linked to
an aromatic structure, which may cause unfavorable steric hindrance between the enzyme and the substrates after structural conformation in an acidic environment. Conversely, the carbon chains linked to the ester linkage in BisGMA and TEGDMA are more flexible, allowing for steric interactions.

Although a protein’s activity is important for its biological functions, its stability is also critical for maintaining its structural integrity, which is necessary to carry out its function (Talley and Alexov 2010). SMU_118c showed considerable stability over 21 days at both acidic and neutral pH, indicating that it could hydrolyze dental resin composite over a significant length of time under pathogenic growth conditions of the biofilm, potentially compromise the restoration-tooth interface, facilitate bacterial invasion and, as a result, further promote secondary caries (Kermanshahi et al. 2010; Spencer et al. 2014; Spencer et al. 2010). In addition to the acidic pH in oral environment, other factors may affect enzyme activity and stability. Previous studies demonstrate that human salivary derived esterases (HSDE) are more stable than the model degradative enzyme cholesterol esterase (CE) (Jaffer et al. 2002; Lin et al. 2005; Shokati et al. 2010). However, both showed reduced activity and stability in the presence of resin-based materials or monomers (Jaffer et al. 2002; Shokati et al. 2010). The current findings demonstrate that in contrast to HSDE and CE, the presence of monomers TEGDMA and BisGMA did not have a significant effect on the activity and stability of SMU_118c at both pH 7.0 and 5.5 over 21 days. This suggests that the bacterial esterase maintains its ability to degrade resinous moieties for a long time after it is released, even in the absence of continues replenishment, thus enhancing its deleterious effect on the material and restoration-tooth interface (Jaffer et al. 2002; Shokati et al. 2010; Smith et al. 2001).

Biodegradation of BisGMA was assessed by measuring the amount of its final by-product,
BisHPPP, in the incubation media. However, since the final product of TEGDMA, TEG, cannot be detected by PDA detector of the HPLC system used in this study due to the lack of double bonds in this compound, the analyses of the biodegradation of TEGDMA was done by measuring the rate of its elimination and formation of MA in the media (Finer and Santerre 2003; 2004a). Our findings show that SMU_118c was able to degrade the methacrylate-based monomers BisGMA and TEGDMA, which are commonly used in the production of commercial resin composite and/or adhesives (Ferracane 2011). The use of these well-defined monomers allows for an accurate characterization of the enzyme’s affinity toward methacrylate-based materials that is not possible with the complex whole composite material, since the latter material’s multiple ingredients confound true degradative activity and preclude mechanistic exploration and generalizing the results (Cai et al. 2014; Finer and Santerre 2003; 2004a).

SMU_118c enzymatic kinetics was specific for each monomer. BisGMA was almost completely hydrolyzed by SMU_118c after 125 hours, and corresponding amounts of its biodegradation product, BisHPPP, were produced during that time frame, indicating that SMU_118c catalyzed almost a complete hydrolysis of BisGMA without any measurable formation of the monomethacrylate intermediate BBP. In contrast, only 17.6 ± 4.4% and 11.9 ± 4.7% of the monomer TEGDMA were hydrolyzed at pH 7.0 and pH 5.5 after 125 hours, respectively, suggesting lower affinity toward this monomer. Since, in theory, one molecule of TEGDMA monomer should result with the release of two MA molecules and one molecule of TEG when completely cleaved, the molar ratio of MA detectable in the HPLC system vs. the rate of elimination by hydrolysis of TEGDMA could provide a good estimate of the rate of biodegradation of the TEGDMA monomer (Finer and Santerre 2004a; 2004b). For example, 17.6 ± 4.4% (pH 7.0) of TEGDMA original amount were degraded, and 35.2% (pH 7.0) of MA should be produced. However, the isolated
MA levels were lower than the theoretical amount.

This difference between the theoretical vs. measured MA indicates that TEGDMA was not completely hydrolyzed by SMU_118c to form one molecule of TEG and two molecules of MA, and that the middle mono-methacrylate biodegradation product of TEGDMA, TEGMA, was generated (Finer and Santerre 2003; 2004b). This finding is in agreement with a previous study investigating resin monomer biodegradation by HSDE (Jaffer et al. 2002). In addition, the faster biodegradation of BisGMA vs. TEGDMA by SMU_118c suggests that SMU_118c has higher affinity towards the phenol-containing monomer than the water-soluble monomer. This is similar to the predominant salivary esterase CE-like activity, which has been shown to degrade both composites and adhesives (Finer and Santerre 2004a; Lin et al. 2005; Yourtee et al. 2001). Since BisGMA is a universal ingredient in current adhesive systems (Ferracane 2011), the preferences of SMU_118c toward this substrate indicates high potential of the bacteria to compromise the resin-dentin interface not just by the production of acid, but also through the hydrolysis of a main component of the adhesive in the interface (Bourbia et al. 2013; Finer and Santerre 2004a; Kermanshahi et al. 2010; Serkies et al. 2016; Shokati et al. 2010).

As CE and PCE (Finer and Santerre 2003; 2004a; Jaffer et al. 2002; Lin et al. 2005), SMU_118c belongs to the esterase-lipase superfamily with a highly conserved arrangement of serine (Ser), histidine (His), and aspartic acid (Asp) residues in the catalytic site (Nardini and Dijkstra 1999; Oakeshott et al. 2005). PMSF is an irreversible inhibitor of serine proteases that alkylates the hydroxyl of serine residue in the active site (Sekar and Hageman 1979). PMSF’s similar reduction of the hydrolysis of BisGMA and pNPB by SMU_118c suggests that the conserved active site of SMU_118c is involved in the biodegradation and supports the hypothesis that the hydrolysis of nitrophenyl substrates and matrix monomers occurs at this same active site. Since the active sites
are highly conserved in the esterase family and the active sites of human CE are highly hydrophobic (Bencharit et al. 2006; Bencharit et al. 2003), it is assumed the active site of SMU_118c is also hydrophobic, which could explain the selective activity of SMU_118c towards the hydrophobic monomer BisGMA (Finer and Santerre 2003; 2004a).

To date, several studies have shown that degradative activities exist in human saliva (Cai et al. 2014; Delaviz et al. 2014; Finer and Santerre 2003; 2004a) and that human and simulated salivary esterases are capable of degrading resin composite and adhesives (Finer and Santerre 2003; 2004a; Kermanshahi et al. 2010). More recently, salivary and simulated salivary activities were shown to degrade the resin-tooth interface, comprising its integrity and increasing interfacial bacterial ingress (Kermanshahi et al. 2010; Serkies et al. 2016; Shokati et al. 2010). The current study suggests that esterase produced by the cariogenic bacterium S. mutans has potential to degrade and compromise the interface and produce biodegradation by-products that increase the bacteria virulence (Sadeghinejad et al. 2017; Sadeghinejad et al. 2016). Since the bacteria is a natural inhabitant in the interfacial gap that is formed by acids, human, and bacterial enzymes, future studies should further evaluate the ability of bacterial esterase to degrade polymerized resin composites and the restoration-tooth interface. In addition, the biofilm structure of S. mutans could encapsulate esterases over the restoration’s surface and within a limited volume of the resin-tooth interfacial gap, resulting with increased effective esterase concentration and resultant biodegradation by-products (Kermanshahi et al. 2010; Sadeghinejad et al. 2017; Sadeghinejad et al. 2016). These processes could lead to a higher level of biodegradation, which can compromise the interfacial seal and increase in the risk of recurrent caries (Finer and Santerre 2003; Kermanshahi et al. 2010), potentially reducing the longevity of the restoration (Spencer et al. 2014; Spencer et al. 2010).
3.6 Conclusion

The esterase SMU_118c is expressed by the caries-causing bacterium *S. mutans* under acidic conditions and can hydrolyze methacrylate-based resin monomers, BisGMA and TEGDMA, which are widely used in resin composites and adhesives, while retaining its activity in an acidic pH for an extended period. As such, SMU_118c may contribute to the biodegradation of the restoration-tooth interface and accelerate the restoration failure rate.

3.7 Acknowledgements

The authors wish to thank Dr. A. Savchenko and Dr. D. Ma for protein synthesis and purification.
3.8 References


B.A. Lin, F. Jaffer, M.D. Duff, Y.W. Tang, J.P. Santerre, Identifying enzyme activities within human saliva which are relevant to dental resin composite biodegradation, Biomaterials 26(20) (2005) 4259-64.


3.9 Figures and Tables

Figure 3.1: Protein gel analysis of expression and purification of SMU_118c, SMU_400 and SMU_643(A) and the nucleotide and deduced amino-acid sequences of SMU_118c (B). The empty vector was used as control and there is no protein expressed (not included in the final gel image).
Figure 3.2: Activity profile of SMU_118c towards different nitro-phenyl substrates at pH 7.0 and pH 5.5 (n = 3; data are reported as mean ± standard errors). *represents significant differences between the two pH values for each substrate ($p < 0.05$). Values with the different capital case letter denote statistically significant differences within pH 5.5 group ($p < 0.05$). Values with the different lower letters indicate significant differences within pH 7.0 group ($p < 0.05$).
Figure 3.3: The effect of different pH on the enzymatic activity of SMU_118c towards pNPB (A), and the relative effect of BisGMA and TEGDMA on SMU_118c activity at pH 7.0 (B) and pH 5.5 (C) following 21-day incubation (n = 3; data are reported as mean ± standard errors). Values with the different lower letters indicate significant differences among different pH values (p < 0.05).
Figure 3.4: SMU_118c catalyzed hydrolysis of BisGMA (A) or TEGDMA (B) at pH 7.0 and pH 5.5 following up to 125-hour incubation. Results were normalized to buffer-only incubation condition (n = 3; data are reported as mean ± standard errors). The reaction rate of SMU_118c for BisGMA (0.53 ± 0.03µM/hr.) was 5.4 times higher than that of TEGDMA (0.098 ± 0.05µM/hr.) at pH 7.0, while it was 7 times higher (0.49 ± 0.02 µM/hr. vs. 0.07 ± 0.03µM/hr.) at pH 5.5 (p < 0.001). *represents significant reduction after 21-day incubation (p < 0.05).
Figure 3.5. The inhibitory effect of PMSF (0.5 mM) on the hydrolytic activities of SMU_118c towards (A) pNPB or (B) BisGMA (n = 3; data are reported as mean ± standard errors). Values with the different lower letters indicate significant differences among groups \((p < 0.05)\).

Table 3.1: Specific primers for PCR amplification

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers</th>
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<tbody>
<tr>
<td>SMU_118c</td>
<td>5’ end 5'TTGTATTTTCAGGGCATGAAACAACTATCAAAAAATAAAATTTT3'</td>
</tr>
<tr>
<td></td>
<td>3’ end RC 5'CAAGCTTCGTCATCATGTTCTTTCTCTTAATCCCAAATAGT3'</td>
</tr>
<tr>
<td>SMU_400</td>
<td>5’ end 5'TTGTATTITCCAGGGCATGACCTATCAGAAAATTATTGATAGA3'</td>
</tr>
<tr>
<td></td>
<td>3’ end RC 5'CAAGCTTCGTCATCATGACCTATCAGAAAATTATTGATAGA3'</td>
</tr>
<tr>
<td>SMU_643</td>
<td>5’ end 5'TTGTATTITCCAGGGCATGAAACAACTATCAAAAAATAAAATTTT3'</td>
</tr>
<tr>
<td></td>
<td>3’ end RC 5'CAAGCTTCGTCATCATGTTCTTTCTCTTAATCCCAAATAGT3'</td>
</tr>
<tr>
<td>SMU_1443c</td>
<td>5’ end 5'TTGTATTITCCAGGGCATGAAACAACTATCAAAAAATAAAATTTT3'</td>
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<td></td>
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</tr>
<tr>
<td>SMU_1678</td>
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</tr>
<tr>
<td></td>
<td>3’ end RC 5'CAAGCTTCGTCATCATGTTCTTTCTCTTAATCCCAAATAGT3'</td>
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Chapter 4

Gene Expression and Protein Synthesis of Esterase from Streptococcus mutans are affected by Biodegradation By-product from Resin Composites and Adhesives

This chapter will be submitted to “Biomaterials”

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4.1 Abstract

Previous studies have demonstrated the ability of cariogenic bacteria Streptococcus mutans UA159 to hydrolyze dental resin composites and adhesives. Recently, an esterase, SMU_118c, was isolated and characterized from S. mutans UA159 and shown to be capable of hydrolyzing methacrylate resin-based dental monomers. Objective: This study sought to investigate the association of SMU_118c to the whole cellular hydrolytic activity of S. mutans toward polymerized resin composites and examine how the bacteria adapts its hydrolytic activity to environmental stresses triggered by the presence of biodegradation by-products (BBPs) by measuring the bacterial response to BBPs in terms of esterase gene (SMU_118c) expression and related protein (SMU_118c) synthesis. Materials and Methods: Photopolymerized cylindrical specimens of resin composite (Z250, 3M) (n = 3/group) were incubated with biofilms of S. mutans UA159 wild strain and SMU_118c knockout strain (ΔSMU_118c) for up to 30 days (pH = 5.5). Quantitation of a universal bis-phenyl-glycidyl-dimethacrylate (BisGMA)-derived biodegradation product from composite and adhesives, bishydroxy-propoxy-phenyl-propane (BisHPPP), was done using high performance liquid chromatography (HPLC). Biofilms of S. mutans UA159 were
cultured with different concentrations of BisHPPP, after which fluorescence \textit{in situ} hybridization (FISH) was used to measure \textit{SMU\_118c} gene expression and quantitative proteomic analysis was used to determine the production of SMU\_118c protein. \textbf{Results:} After a 30-day incubation, the levels of BisHPPP released from composite were similar for ΔSMU\_118c and media control (15.69 ± 0.27 and 15.62 ± 0.23 \(\mu g/cm^2\), respectively; \(p > 0.05\)), and were significantly lower compared to the parent stain (21.37 ± 1.55 \(\mu g/cm^2\); \(p < 0.05\)). Gene expression of \textit{SMU\_118c} and productions of SMU\_118c protein were higher for BisHPPP incubated biofilm (\(p < 0.05\)).

\textbf{Significance:} This study suggests that SMU\_118c is a dominant esterase in \textit{S. mutans} and capable of catalyzing the hydrolysis of the resinous matrix of polymerized composites and adhesives. In turn, the bacterial response to BBPs is to increase the expression of the esterase gene and enhance esterase production, potentially accelerating the biodegradation of the restoration-tooth interface and ultimately contributing to premature restoration failure.

\textbf{Keywords:} resin composite, biodegradation, \textit{Streptococcus mutans}, esterase, gene response, protein regulation

\underline{4.2 Introduction}

Increasing concerns about possible adverse health effects of mercury, growing aesthetic demands, and improved adhesive technology in dental material have led resin composites to become the most commonly used dental restorative materials (Krifka et al. 2013; Wakefield and Kofford 2001). However, nearly 70\% of restoration operations done by dentists are replacements of failed resin composite restorations (Brunthaler et al. 2003a; EI-Mowafy et al. 1994; Murray et al. 2002b; Simecek et al. 2009). The primary reason for restoration failure is recurrent or secondary caries developed along the compromised restoration-tooth interface (Bernardo et al. 2007b; Brunthaler et al. 2003a; Ferracane 2011; Opdam et al. 2007).
Methacrylate-based resin composites are prone to be hydrolyzed due to the unprotected ester linkage in the matrix monomers, the most abundant of which is the monomer 2,2-Bis [4-2(2-hydroxy-3-methacryloxypropoxy) phenyl] propane (BisGMA) (Jaffer et al. 2002), yielding the degradation product bishydroxy-propoxy-phenyl-propane (BisHPPP) (Finer and Santerre 2004a; Shokati et al. 2010). Previous studies have shown that human or model salivary and cariogenic bacteria in the oral cavity catalyze this hydrolysis, a process that is often referred to as biodegradation (Kermanshahi et al. 2010; Shokati et al. 2010). This process compromises the restoration-tooth interface, allowing for cariogenic bacterial penetration into the interface, which leads to the initiation and progression of secondary caries (Kermanshahi et al. 2010).

*Streptococcus mutans* is the chief etiological agent responsible for dental caries (Hamada et al. 1984). The main virulence factors of interest for *S. mutans* are its ability to form biofilm, survive in acidic environments, and produce acid. In addition to these well-understood cariogenic properties, *S. mutans* has been shown to contain esterase activity that degrades resin composites, which could contribute to secondary caries by compromising the restoration-tooth interface (Bourbia et al. 2013). A recent study has identified and characterized SMU_118c, an esterase from *S. mutans* UA159 that has high hydrolytic activity toward BisGMA similar to the salivary esterases activity profile (Huang et al. 2017). In other bacteria, esterases have been linked to virulence and pathogenesis (Lun and Bishai 2007; Wall et al. 2007). However, there are no reports on the role of esterases in the biodegradation of biomaterials, specifically dental methacrylates, as a contributing factor to bacterial virulence. Therefore, one aim of the current study was to investigate the association of SMU_118c to the whole cellular hydrolytic activity towards resin composite.

Esterase-catalyzed resin composite biodegradation enlarges gaps between restorations and teeth (Kermanshahi et al. 2010), compromising interfacial bond strength (Serkies et al. 2016; Shokati et
al. 2010), allowing for bacterial invasion (Matharu et al. 2001; Zivković et al. 2001), and providing a unique micro-environment containing the biodegradation by-products (BBPs) in close proximity to the bacteria that reside in the gap (Delaviz et al. 2014; Kermanshahi et al. 2010). Bacteria have a high capacity to adapt to environmental changes as a result of their robust metabolism (Ishii et al. 2007), which is controlled by a network of regulatory circuits requiring cellular programming at transcriptional and translational levels. Previous studies have reported the impact of BBPs on the growth of and virulence gene expression in S. mutans (Khalichi et al. 2004; Khalichi et al. 2009; Sadeghinejad et al. 2017; Sadeghinejad et al. 2016). However, these investigations relied on disruptive techniques (RNA extraction and RT-PCR) that destroy the biofilm structure and thus do not allow for observation of the spatial and temporal gene expression patterns. Therefore, the second aim of the current study was to establish non-destructive methodology based on fluorescence in situ hybridization (FISH) for analyzing gene responses of the esterase gene SMU_118c to BBPs in biofilm with intact and complex 3D structure. Furthermore, the effect of BBPs on the relevant SMU_118c protein production was investigated by employing label-free quantitative proteomics.

4.3 Materials and Methods

4.3.1 Resin Composite Specimen Preparation

Cylindrical pellets (4 mm diameter x 4 mm height) of resin composite (Z250; 3M, London, ON, Canada) were prepared with Teflon™ molds and Mylar™ strips, photopolymerized for 15 s from each side (Sapphire plus, DenMat, Santa Maria, CA, USA) as described previously (Jaffer et al. 2002). All instrumentation was autoclaved or disinfected by 70% ethanol solution. Specimen preparation was performed in a biosafety cabinet.
4.3.2 Resin Composite Biodegradation by SMU_118c

The composite specimens (n = 3 for each incubation condition and period) were pre-incubated in phosphate-buffered solution (D-PBS, pH 7.0, 37°C) (Gibco, Grand Island, NY) for 2 days to allow for diffusion of unreacted resin monomers (Bourbia et al. 2013), then incubated in sterile vials (Chromatographic Specialties Inc., Brockville, ON, Canada) containing 1mL of D-PBS with or without 50 U/ml of SMU_118c for up to 30 days. Incubation solutions were collected at 0, 5, 7, 17, 24 and 30 days, mixed with equal volume of pre-chilled methanol (Sigma-Aldrich, St. Louis MO, USA) to quench the reaction, filtered at 1000g with a Millipore centrifuge filter device (Ultra-0.5, 3kDa MWCO, Millipore, Bedford, MA), and then analyzed for the biodegradation product, BisHPPP, by high-performance liquid chromatography (HPLC) as previously reported (Shokati et al. 2010). A Student’s t-test was used to determine differences between BisHPPP released after the two incubation conditions (D-PBS + SMU_118c or D-PBS alone) at the same time-point (p < 0.05). One-way analyses of variance (ANOVA) and Tukey’s multiple comparison tests (p < 0.05) were performed to determine the difference in BisHPPP release in same conditions at different time points. Homogeneity of variance and normality were verified with Leven’s and Shapiro-Wilk tests, respectively.

4.3.3 Construction of ΔSMU_118c and ΔSMU_118c complemented strain

The sequence of esterase gene SMU_118c was found in S. mutans UA159 genome database as a putative esterase (http://www.genome.ou.edu/smutans.html). The SMU_118c knockout mutant (ΔSMU_118c) was constructed through PCR-ligation mutagenesis according to a previously established protocol (Lau et al. 2002; Li et al. 2001c; Senadheera et al. 2005). The SMU_118c complemented strain (ΔSMU_118cC) was made using pIB166 plasmid that contained the S. mutans recombinant SMU_118c as described previously (Li et al. 2002c). The primers used for
mutation, confirmation, and complementation constructs are listed in Table 1 (ACGT Corporation, ON, Canada). The ΔSMU_118c deletion mutation and complementation was confirmed by PCR analysis.

4.3.4 Resin Composite Biodegradation by S. mutans UA159 and ΔSMU_118c strain

Cured specimens (n = 3/group) were incubated in sterile vials containing either 3 mL of 1:4 dilution of brain-heart infusion (BHI) (Becton, Dickinson and Co., Sparks, MD, USA) with pH adjusted to 5.5 using lactic acid (control) (Sigma Lactic acid, Sigma-Aldrich, St. Louis MO, USA), or 1:4 dilution of BHI with overnight S. mutans UA159 or ΔSMU_118c (experimental groups). 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 quantitation of biodegradation product, BisHPPP after 30-day incubation by HPLC as previously reported (Shokati et al. 2010). The purity of the bacterial culture was assessed by gram stain at each media replacement under a light microscope (Olympus® BX 51; Olympus America Inc., NY, USA) and viability was assessed by colony forming unit (CFU) on agar plates at the time interval. One-way analyses of variance (ANOVA) and Tukey’s multiple comparison tests (p < 0.05) were performed to determine the difference in BisHPPP release. Homogeneity of variance and normality were verified with Leven’s and Shapiro-Wilk tests, respectively.

Resin composite specimens were collected from each biodegradation experimental condition (n = 3) and sonicated to remove bacteria from the surface for direct observation of the materials’ surfaces. Surface morphology was observed after 30-day incubation period by scanning electron microscopy (S2500, Hitachi, Mito City, Japan) at an operating voltage of 10 kV as described previously (Shokati et al. 2010).
4.3.5 Verification of the Association of SMU_118c gene on Resin Composite Biodegradation

Cured specimens (n = 3/group) were incubated in sterile vials containing either 1 mL of Fujiwara minimum medium with pH adjusted to 5.5 using lactic acid (control) (Sigma Lactic acid, Sigma-Aldrich, St. Louis MO, USA) or medium with overnight S. mutans UA159 ΔSMU_118c or ΔSMU_118c complemented strain (experimental groups). Purity of bacterial culture and viability were conducted as described above. Analyses for BisHPPP was conducted after 8-days incubation as described. One-way analyses of variance (ANOVA) and Tukey’s multiple comparison tests (p < 0.05) were performed to determine the difference in BisHPPP release for specimen incubated in different conditions and different time points.

4.3.6 The Effect of BisHPPP on SMU_118c Expression by FISH

The assay was conducted based on previously described protocol with modifications (Fröjd et al.). All water and buffers were treated with 0.1% (vol/vol) diethylpyrocarbonate (DEPC) (Sigma, Taufkirchen, Germany) and then autoclaved. Briefly, 18-hour biofilms of S. mutans UA 159 with relevant in vivo concentrations of BisHPPP (0.0, 0.01, 0.1 and 1 mM) (Sadeghinejad et al. 2017) and ΔSMU_118c were formed in 8-well chamber (Lab-Tek camber slide, NUNC, Naperville, IL, USA) containing 400 μl of chemically defined Media (CDM) at 37°C with 5% CO2, then fixed in 4% paraformaldehyde (pH 7.2) at 4°C for 12 hours. After fixation, all specimens were treated with freshly prepared 0.1% (vol/vol) active DEPC in D-PBS for 12 min at RT then rinsed with D-PBS and MilliQ water for 1 min each and subjected to cell membrane permeabilization with 200 μl of 10mg/ml lysozyme (Sigma, St Louis, MO, USA) in 100 mM Tris-HCl, pH 7.5 (Sigma, St Louis, MO, USA) containing 5 mM EDTA (Merck, Damstadt, Germany) at 37°C for 30 min. The biofilms were then dehydrated with a series of ethanol washes. Specimens were then incubated with green-fluorophore-labeled oligonucleotide probes (Table 4.2) targeting on mRNA of
SMU_118c at a concentration of 50 ng/20μl in hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 50 % (v/v) formamide and 0.01 % (w/v) sodium dodecyl sulphate (SDS). Hybridization was conducted at 38°C in a humid chamber for 12 hours. Following probe hybridization, specimens were incubated for 15 min at 42°C in wash buffer containing 20 mM Tris/HCl (pH 7.2), 5 mM EDTA, 159 mM NaCl and 0.01 % (w/v) SDS. As a control, a parallel assay was conducted to investigate the effect of BisHPPP on the expression of a housekeeping gene (16S rRNA) under same conditions except for the hybridization: red-fluorophore-labeled probes STR405 (Table 4.2) hybridized to 16s rRNA at 48°C for 3 hours to assess changes in housekeeping gene expression. All biofilms were also stained with nuclear dye 4’,6’-diamidino-2-phenylindole (DAPI; blue) for further biomass analysis. Finally, the images of biofilms (n = 3 random sites/biofilm) were acquired using confocal scanning laser microscopy (CSLM; IX81 Inverted Microscope, Olympus, Tokyo, Japan) with a water immersion objective (60x/1.2 UPlanApo Water). Each CLSM image stack had a substratum coverage field area of 211 × 211 μm and z-step interval of 0.5 μm.

4.3.7 Image Analysis

The image stacks were converted into 3D and analyzed using the quantitative software Imaris (Software version 8.3, Bitplane AG, Zurich, Switzerland) to calculate the parameters of the biofilm. Biofilm formation of each group was quantified in terms of total cell number presented from DAPI staining. Gene expression level was proportional to the respective fluorescence intensity in the biofilm, so the level was quantified using total voxel intensity of fluorescence from the specific probes in each biofilm and then standardized by dividing by the total cell number of each biofilm. The green fluorescence from ΔSMU_118c-formed biofilm was considered as baseline for the SMU_118c gene expression. One-way analyses of variance (ANOVA) and Scheffe’s multiple comparison tests (p < 0.05) were performed to validate differences in gene expression between
control (no BisHPPP) and experimental groups (different concentrations of BisHPPP for the wild and ΔSMU_118c strains).

4.3.8 Biofilm Preparation for Protein Identification and Quantification

18-hour biofilm of *S. mutans* UA 159 were grown in TYEG medium buffered at pH 5.5 with MES (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 0.1% glucose and relevant in vivo concentrations of BisHPPP (0.0, 0.01, 0.1 and 1 mM) at 37°C with 5% CO₂, then, the biofilm cells were collected and disrupted using a homogenizer (Thermo Savant, FastPrep FP 101) at setting 6 for 45 minutes and then centrifuged at 15,700 x g for 1 minute. Supernatant was carefully removed, separated in aliquots of 50 µL, and stored at -80°C. The total protein concentration in each sample was assessed by the Micro Bicinchoninic Acid (Micro BCA) assay (Siqueira et al. 2012b). Equal protein amount (20 µg) from both experimental and control groups were dried by a rotary evaporator, denatured, and reduced for 2 hours by the addition of 200 µL of 4 M urea, 10 mM dithiothreitol (DTT), and 50 mM NH₄HCO₃, pH 7.8. After four-fold dilution with 50 mM NH₄HCO₃, pH 7.8, tryptic digestion was carried out for 18 h at 37°C, following the addition of 2% (w/w) sequencing-grade trypsin (Promega, Madison, WI, USA). Peptide separation and mass spectrometric analyses were carried out as previously described (Sadeghinejad et al. 2017; Siqueira et al. 2012b).

4.3.9 Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS) and Relative Proteome Quantitation

The obtained MS/MS spectra were searched against the streptococci protein database (Swiss Prot and TrEMBL, Swiss Institute of Bioinformatics, Geneva, Switzerland, http://ca.expasy.org/sprot/) using SEQUEST algorithm in Proteome Discoverer 1.3 software (Thermo Scientific, San Jose, CA, USA).
For quantitative proteome analysis, three MS raw files from each group (control and experimental; total of 12 MS raw files) were analyzed using SIEVE software (Version 2.0 Thermo Scientific, San Jose, CA, USA) (Siqueira et al. 2012b). For the alignment step, a single MS raw file belonging to the control group (0 mM) was selected as the benchmark and the other files were adjusted to generate the best correlation to this reference file. After alignment, the feature detection and integration (or framing) process was performed using MS-level data. For statistical analyses of protein abundance, peak integrations were summarized into protein-level annotation in SIEVE using a weighted average of intensities of LC-ESIMS/MS of a protein by run. In addition, a statistical model based on an ANOVA framework with Tukey’s post hoc test was carried out. Relative abundance of \( SMU_\text{I18c} \) protein from different TEG or BisHPPP concentration groups was considered significantly different from the control group (0 mM) when the values observed were >1.5 for increased and <0.5 for decreased abundance with a \( p \)-value cut-off of < 0.05 (Siqueira et al. 2012b).

### 4.4 Results

#### 4.4.1 Resin Composite Biodegradation by \( SMU_\text{I18c} \)

A trend of increasing BisHPPP release with time throughout the incubation period was observed for both control and \( SMU_\text{I18c} \)-incubated groups \( (p < 0.05; \text{Fig. 4.1}) \). The amount of BisHPPP released was elevated significantly in the presence of \( SMU_\text{I18c} \) vs. control \( (p < 0.05) \) at each incubation period. The total amount of BisHPPP released from Z250 after 30 days of incubation with \( SMU_\text{I18c} \) \( (8.76 \pm 0.50 \mu g/cm^2) \) was 21 times higher than that released from control \( (0.41 \pm 0.03 \mu g/cm^2, p < 0.05) \).
4.4.2 Resin Composite Biodegradation by S. mutans UA159 and ΔSMU_118c strain

30-day cumulative BisHPPP amounts (21.37 ± 1.55 μg/cm²) were highest for specimens incubated with the wild strain S. mutans UA159 (p < 0.05), while the amount of BisHPPP released from ΔSMU_118c-incubated specimen (15.69 ± 0.27 μg/cm²) was similar to that released from media control without bacteria (15.62 ± 0.23 μg/cm², p > 0.05).

SEM micrographs (Fig. 4.2) demonstrated that the surfaces of the specimens incubated with S. mutans UA159 for 30 days appeared roughest, while the surface of ΔSMU_118c-incubated specimens after 30 days were similar to media-incubated and non-incubated specimens.

4.4.3 Verification of the Association of SMU_118c to the Degradative Activity of the Whole Bacterial Cell towards Resin Composite

Results for BisHPPP release are provided as a percentage of the amount of BisHPPP isolated from the resin composites incubated with S. mutans UA159 wild strain. The relative BisHPPP amounts were lowest for specimens incubated with ΔSMU_118c after an 8-day incubation (52.9% ± 8.9%, p < 0.05), while the S. mutans UA159 and ΔSMU_118c complemented strain (ΔSMU_118cC) incubated groups had similar BisHPPP release (100.0% ± 7.4% vs. 102.3% ± 6.1%, p > 0.05).

4.4.4 The Effect of BisHPPP on SMU_118c Gene Expression

To investigate the effect of BisHPPP on SMU_118c gene expression, the biofilm incorporated both DAPI and UA159_SMU118 probes presented in blue and green wavelengths, respectively, and the specificity of probe targeting mRNA of SMU_118c was demonstrated by comparing green fluorescence signal from biofilm formed by ΔSMU_118c to that by S. mutans UA159 (Fig. 4.3). For the control groups, biofilm incorporated the DAPI and STR405 probes presented in blue and red fluorescence range, respectively (data not shown). A reduced biofilm mass was observed at 1 mM BisHPPP (Fig. 4.3A; p < 0.05). There were no differences in biofilm formation of ΔSMU_118c and the parent stain, indicating that the mutation had no effect on bacterial viability
and the ability to form biofilm (Fig. 4.3A; $p > 0.05$). FISH images showed enhanced fluorescence intensity in biofilm with increased BisHPPP concentration from 0.01 mM to 1 mM, indicating possible up-regulation of $SMU_{118c}$ (Fig. 4.3A). Due to the non-specific binding and auto-fluorescence from biofilm or/culture media, fluorescence detected in Δ$SMU_{118c}$ biofilm was used as baseline (background) and subtracted from all groups for the quantitation of $SMU_{118c}$ gene expression. Quantified results were normalized by total cell number in each biofilm (Fig. 4.3B). Up-regulation of $SMU_{118c}$ was concentration dependent, and the significance was observed in biofilm with 1 mM of BisHPPP ($p < 0.05$). In all strains, the expression of the housekeeping gene 16S rRNA did not change in response to BisHPPP (data not shown; $p > 0.05$).

### 4.4.5 The Effect of BisHPPP on $SMU_{118c}$ Protein Synthesis

$SMU_{118c}$ from all four different groups ($S. mutans$ UA159+ 0.0mM, 0.01 mM, 0.1 mM and 1 mM BisHPPP) showed a consistent elution of protein/peptides. Compared to the control group (0.0 mM BisHPPP), increased levels of $SMU_{118c}$ production were observed in two experimental groups ranging from 2.21 (0.1 mM BisHPPP; $p > 0.05$) to 2.32 (0.01 mM BisHPPP; $p < 0.05$) (Table 4.3). However, a slight decrease in protein levels was observed for $S. mutans$ UA159 incubated with 1 mM BisHPPP (Table 4.3).

### 4.5 Discussion

Resin composites are the most popular dental restorative materials (Sunnegårdh-Grönberg et al. 2009), and their physical and mechanical properties have been well studied. Recent studies have reported that the biostability of resin composites can affect their biocompatibility, since the release of biodegradation by-products affects gene expression of cariogenic bacteria (Sadeghinejad et al. 2017; Sadeghinejad et al. 2016). Esterase activities in human saliva degrade the bulk of the materials and the restoration-tooth interface (Finer and Santerre 2004a; Kermanshahi et al. 2010;
Serkies et al. 2016; Shokati et al. 2010). More recently, the cariogenic bacterium *S. mutans* UA159 was found to hydrolyze resin composite and adhesives (Bourbia et al. 2013). The current study adds this knowledge by reporting on the association of a specific esterase from *S. mutans* to the biodegradation process of the resinous matrix in resin composites and the reciprocal effect of the biodegradation by-product on the oral bacteria at both gene and protein levels. These findings contribute to our understanding of the interaction between materials, host, and bacteria and could provide guidance to materials manufacturers.

In this study, the commercial resin composite Z250 was chosen as a representative for biodegradation investigation because it contains the most abundant matrix monomer used in commercial resin-based materials and adhesives, BisGMA (Peutzfeldt 1997; Zimmerli et al. 2010). BisHPPP, a BisGMA-derived degradation by-product, was chosen as an indicator of esterase-catalyzed biodegradation due to its hydrophobic nature and the un-leachable property of its precursor after polymerization (Finer and Santerre 2007). In addition to esterase-mediated hydrolysis, hydrogen ions produced by oral bacteria can catalyze the hydrolysis of the ester bonds present in the polymer matrix (Borges et al. 2011; Moszner et al. 2005; Silva et al. 2012). To preclude the latter effect on resin biodegradation, all culture media controls were adjusted to pH 5.5 by lactic acid, which is the organic acid produced by *S. mutans*.

### 4.5.1 The Role of SMU_118c in *S. mutans*-mediated Resin Composites Biodegradation

A study by Huang et al. (in submission 2017) reported that esterase SMU_118c has a similar enzymatic profile to that of human saliva (Finer and Santerre 2004a) and *S. mutans* (Bourbia et al. 2013) towards ester substrates, indicating its potential to degrade resin-based materials and thereby promote secondary caries. By knocking out and then restoring *SMU_118c*, this study confirmed that SMU_118c is able to continuously degrade cured composite materials specimens and that it
is associated with the whole bacterial esterase activity of *S. mutans*. Compared to the parent stain, ΔSMU_118c-mediated biodegradation was reduced to a level similar to the media control and the complemented stain recovered whole bacterial hydrolytic activity, demonstrating that SMU_118c is the dominant esterase in *S. mutans* responsible for esterase-mediated hydrolysis of dental resin composites.

SEM micrographs showed that the surfaces of pre-incubated specimens were smooth and covered by resin, which excludes any effect of the filler on the availability of resin surface for biodegradation. After a 30-day incubation, SEM micrographs demonstrated rougher surfaces for specimens incubated with *S. mutans* vs. media control and ΔSMU_118c. The observation for the parent strain was consistent with previous studies (Bourbia et al. 2013; Fúcio et al. 2008; Gregson et al. 2012) and corroborates the HPLC analysis of biodegradation. However, inconsistent results between HPLC and SEM analysis were observed from the media control groups where BisHPPP release from media-incubated specimens was detected by HPLC but SEM images showed that the surfaces of specimens incubated with media were as smooth as pre-incubated ones. This could be explained by the different sensitivity and resolution of two detection methods. Unlike the quantitative HPLC analysis, SEM is unable to detect minor changes and provides descriptive (qualitative) results of only the surface. Alternatively, the apparent discrepancy could be due to production of BisHPPP from unreacted BisGMA monomers that diffused from the bulk of the specimens rather than the surface of the sample. The latter process does not result in surface changes that can be observed by SEM.

BHI media has esterase-like activity (Bourbia et al. 2013), and in order to reduce its effect on the surface biodegradation of the composite, which would potentially mask the true bacterial biodegradation, a 2.5 times diluted BHI was used for the SEM study vs. the media used for the
analyses by HPLC. Therefore, it is not surprising to observe reduced degradative activity with diluted BHI. Although the exact composition of the BHI medium and the identity of the specific degradative activity from this medium are not fully defined, use of this medium demonstrated the importance of the incubation condition and the steps that needed to be taken when analyzing the impact of different cell cultures on material biostability. The findings further demonstrate the significance of the interaction between bacteria, host, and material, and the important of using *in vitro* conditions to mimic these interactions.

### 4.5.2 The Effect of BisHPPP on SMU_118c Gene Expression and SMU_118c Protein Synthesis

Previous studies confirmed that in addition to temperature, pH, and chemical agents, BBPs induced adaptation mechanisms in *S. mutans* by stimulating bacterial growth and up-regulating key virulence genes (Khalichi et al. 2009; Sadeghinejad et al. 2017; Sadeghinejad et al. 2016). The effect of BisHPPP on the expression of bacterial virulence genes was reported to be concentration dependent only at optimum conditions for the development of dental caries (cariogenic pH and in the form of biofilm) (Sadeghinejad et al. 2017). Hence, in this study, experiments were conducted under conditions deemed optimum for the development of dental caries.

To analyze the effect of BisHPPP on *SMU_118c* gene expression, clinically relevant BisHPPP concentrations were tested (Jaffer F et al. 2004; Jaffer et al. 2002; Sadeghinejad et al. 2017). However, while concentrations up to 0.1 mM BisHPPP are considered clinically relevant, these values are based on analysis of BisHPPP content in saliva after restorations (Jaffer F et al. 2004; Sadeghinejad et al. 2017). Considering the restrained local space in the restoration-tooth interface, even higher concentrations of BisHPPP (1 mM) could be expected due to accumulative BisHPPP release (Sadeghinejad et al. 2017). In addition, considering that all detections were conducted *in situ* in intact biofilm without planktonic counterparts, higher concentrations (1 mM) of BisHPPP
were employed to test its adverse effects on biofilm biomass, which could affect gene expression and protein.

*In situ* hybridization (ISH) of mRNA sequences is a popular technique for studying gene expression in eukaryotic cells and tissues (Wendeberg et al. 2012). In microorganism, FISH with rRNA-targeted probes is routinely used for bacterial identification (Al-Ahmad et al. 2007). With the development of probe designs and detection techniques, FISH is able to detect bacterial responses to environmental changes at the transcription level. Compared to the more commonly used qRT-PCR method for investigating gene expression, the design and labeling of specific probes in FISH might be challenging, but the results are more informative due to the undisrupted biofilm structure that allows for spatial analyses and more complex biological growth conditions.

The inhibitive effect of BisHPPP on *S. mutans* UA159 biofilm formation in terms of reduced total cell numbers was observed at 1 mM, a much higher concentration than what has previously been reported for the NG8 strain (0.1 and 0.25 mM; Singh et al. 2009). However, previous studies reported these two concentrations based on the growth rates of planktonic cells, which are more vulnerable to unwanted extracellular agents (Jefferson 2004). Based on FISH images, more mRNA copies of *SMU_118c*, presented as higher intensity of fluorescence from mRNA probes, were produced in biofilm with 1 mM of BisHPPP. Further quantitation of gene expression confirmed this observation. This concentration-dependent up-regulation of *SMU_118c* may be explained by the accumulative effect of BisHPPP on the bacterial growth environment. Since BisHPPP is hydrophobic and weakly charged in an acidic environment, the accumulation of BisHPPP around bacterial cells at high concentration could change microenvironments, leading to pH fluctuations, fluid flow interruption, and nutrient blockage (Fux et al. 2005; Olson 1993; Rohde et al. 2007). Previous studies reported that *S. mutans* could sense these changes and regulate gene expression
for adaptation via two-component signal transduction systems, which are comprised of surface sensors and response regulators (Burne et al. 1997; Hudson and Curtiss 1990; Li et al. 2002c). In addition, the direct interaction between BisHPPP and bacteria might create a new bacterial/environmental interface inducing auto-phosphorylation of sensor kinases on bacterial surface, which could activate SMU_118c expression by the intracellular response regulator (Stock et al., 2000).

The effective BisHPPP concentration that triggered an increase in gene was higher in this study than has previously been reported (Sadeghinejad et al. 2017). This could be explained by different biofilm biological conditions employed in each study. In this study, chemically defined media with limited nutrients was employed for FISH to form thin biofilm, to facilitate cell permeabilization and probe penetration, and to reduce unnecessary interactions between compounds in the media and FISH working solutions. As reported previously, nutrients can influence bacterial physiochemical properties in biofilm development (Bowden and Li 1997; Moller et al. 1997; Sauer et al. 2004), which may explain the differential gene regulation associated with different culture media used for FISH and qRT-PCR. Different pH conditions may also contribute to differential gene responses. Previous studies investigated gene responses in biofilm with controlled pH (5.5) by adding organic acid. In this study, the acidic environment, which had a similar endpoint pH (5.2), was created by S. mutans metabolism with natural pH fluctuation during biofilm formation. Changes in pH can induce a bacterial acid tolerance response associated with changes in expression of over 30 proteins (Hamilton and Svensäter 1998; Wilkins et al. 2002), which may affect SMU_118c gene expression. The differences of in situ detection vs. qRT-PCR analysis highlight the importance of using relevant in vitro models to reflect in vivo environments and to understand the precise effects of biomaterials in a biological environment.
Bacterial biological characteristics and activities are directly determined by the expression of various functional proteins. Gene responses to environmental fluctuations must be reflected at the protein level to carry out bacterial adaptation to a wide range of conditions (Chubukov et al. 2014). In the current study, BisHPPP was explored at the protein level to show that its effect on the hydrolytic activity of *S. mutans* directly contributes to resinous restoration biodegradation. Proteomic analysis showed significant increase of SMU_118c production in groups with low concentrations of BisHPPP (0.01 and 0.1 mM). The greatest increase was found in *S. mutans* biofilm incubated with 0.001 mM of BisHPPP, which increased 2.32-fold, followed by 0.01 mM of BisHPPP, which increased 1.34-fold. A significant statistical but not physiological decrease of 0.89-fold was found in the group exposed to 1 mM BisHPPP, at which level the *SMU_118c* gene expression was up-regulated 3.2-fold. These conflicting results are not surprising as previous studies have highlighted the weak correlation between mRNA levels and protein abundance, with greater variations observed for the latter (Dressaire et al. 2010; Lu et al. 2007; Nie et al. 2006a; Nie et al. 2006b). In bacteria, mRNA concentration alone can explain less than 50% of total variation in protein quantity (Corbin et al. 2003; Dressaire et al. 2010; Lu et al. 2007) due to translational regulation by which bacteria are able to fine-tune their protein expression levels (Picard et al. 2012). Bacteria regulate translation as a stress response mechanism (de Sousa Abreu et al. 2009; Nie et al. 2006b; Picard et al. 2012). For instance, *Escherichia coli* can rearrange mRNA structures to facilitate protein synthesis in response to environmental changes such as pH and temperature (Bingham et al. 1990; Giuliodori et al. 2010; Jiang et al. 1997; Nechooshtan et al. 2009; Weixlbaumer et al. 2013). In addition, *Bacillus subtilis* and *E. coli* can conduct translational selectivity by altering and modifying rRNA when encountering extracellular stresses.

The exact reason for the lowest BisHPPP concentration inducing the greatest SMU_118c protein
production by the bacteria is not completely clear. However, the bacteria have evolved global protein-mediated translational regulation mechanisms, which allow it to modulate the translation leading to a large variation in cellular mRNA levels in response to environmental stressors (Duval et al. 2015; Picard et al. 2012; Romeo et al. 2013). Therefore, it can be hypothesized that even without up-regulation of \textit{SMU\_118c} gene, the bacteria could have still promoted \textit{SMU\_118c} synthesis in response to BisHPPP by enhancing the mRNA stability and/or increasing translational efficiency/selectivity (Picard et al. 2012). It may also be the case that \textit{S. mutans} was not be stressed by the lowest concentration of BisHPPP, so \textit{SMU\_118c} protein synthesis was selected and promoted instead of other critical proteins associated with bacterial survival under harsh conditions.

As BisHPPP concentrations increased to a toxic level, the bacteria selectively reduced the translation of \textit{SMU\_118c} or enhanced the biodegradation of \textit{SMU\_118c} to facilitate expression of other critical proteins, which is agreed with the observation of reduced protein production at 1 mM. The findings indicate that mRNA levels do not determine total protein expression levels and that the overall regulation is highly dependent on bacterial stresses, such as the possible toxicity of high concentrations of BisHPPP on biofilm formation.

In summary, unlike the limited effect of BisHPPP on \textit{SMU\_118c} up-regulation requiring high concentrations of BisHPPP, \textit{SMU\_118c} protein synthesis can be significantly enhanced by BisHPPP at a wider range of concentrations. With constant BisHPPP release due to biodegradation of resin composites by both salivary and bacterial esterases, \textit{SMU\_118c} production is likely to be continuously accelerated, which further increases the biodegradation of the restoration and restoration-tooth interface and potentially promotes the premature failure of restorations.
4.6 Conclusion

This study presents a significant finding of *S. mutans* virulence factors related to the prevalence of secondary caries due to the vulnerability of current restorative materials. SMU_118c is the dominant esterase in *S. mutans* that acts synergistically with salivary esterase to degrade resin composite and release BBPs such as BisHPPP, which in turn increase SMU118c production, resulting in elevated hydrolytic activity of the bacteria. The reciprocal effect of BBPs on the bacteria creates a positive feedback loop that accelerates resin composites degradation, potentially resulting in more secondary caries and more frequent replacement of resin-based restorations. Future studies should examine the mechanisms of interaction between bacteria and dental materials to better understand the development of secondary caries *in vivo* and improve design of dental materials.
4.7 References


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Saint-Joanis B, Demangel C, Jackson M, Brodin P, Marsollier L, Boshoff H, Cole ST. 2006. Inactivation of rv2525c, a substrate of the twin arginine translocation (tat) system of
mycobacterium tuberculosis, increases β-lactam susceptibility and virulence. Journal of bacteriology. 188(18):6669-6679.


4.8 Figures and Tables

Figure 4.1: Cumulative amounts of isolated BisHPPP after incubation of resin composites (Z250) in D-PBS (gray) or with SMU_118c (black). Data are plotted as mean ± standard error. Values with the same lower case letter denote statistically non-significant differences ($p > 0.05$).

Figure 4.2: Scanning electron micrographs of resin composite at day 0 (A), or following 30 days of incubation with BHI (pH 5.5) (B), S. mutans UA159 (C) and ΔSMU_118c (D) ($10^4$ X original magnification). Scale bar applies to all figures and represents 3 μm. Note the rougher surfaces for S. mutans UA159 (wild stain) vs. ΔSMU_118c and BHI media control.
Figure 4.3: A) CLSM images of the gene expression in *S. mutans* biofilm formed with different concentrations of BisHPPP (A) and *SMU_118c* gene expression quantification analysis (B). Gene expression level is proportional to the fluorescence intensity in the biofilm. Blue signal by 4', 6-diamidino-2-phenylindole (DAPI) staining indicates the biofilm biomass. Green signal from the UA159_SMU118c probe represents levels of mRNA of *SMU_118c* expression. Increasing concentrations of BisHPPP resulted in an increase in the expression of *SMU_118c* gene in the wild strain of *S. mutans* UA159. Negative control (ΔSMU_118c) confirms specificity of the probe. Gene expression is normalized to number of cells in biofilm. For *SMU_118c* gene expression, data from ΔSMU_118c groups were subtracted as background. * represents significant differences between groups with different BisHPPP concentration (p < 0.05).
### Table 4.1: Primers for SMU_118c gene deletion and complementation

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erm-PA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5' GGCGCGGCCCCGGGCCCCAAAAA TTTGTTTGA T 3'</td>
</tr>
<tr>
<td>Erm-PB&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5' GGCCGGCCAGTCGGCAGCGACTCATAAGAT 3'</td>
</tr>
<tr>
<td>SMU_118c-P1</td>
<td>5' AAGAAGTCTCTTGTGCTGG 3'</td>
</tr>
<tr>
<td>SMU_118c-P2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5' GGCGCGGCTTGACGAATGGCTGTAGCG 3'</td>
</tr>
<tr>
<td>SMU_118c-P3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5' GGCCGGGCCGAACCATAGAAAGTTGAGG 3'</td>
</tr>
<tr>
<td>SMU_118c-P4</td>
<td>5' CCCTATTAAAACAGCACCC 3'</td>
</tr>
<tr>
<td>SMU_118c-Comp1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5' GGATCTTTGAAATTGTTGTCCCTCAACT3'</td>
</tr>
<tr>
<td>SMU_118c-Comp2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5' CTCGAGTCATTCATTACTGAGGGACGAGGGAGG 3'</td>
</tr>
</tbody>
</table>

Primers in roman (non-italic) font were used for gene deletion and primers in italics were used for gene complementation. <sup>a</sup>: AscI restriction sites are in italic boldface; <sup>b</sup>: FseI restriction site are in boldface and underlined; <sup>c</sup>: BamHI restriction sites are in boldface; <sup>d</sup>: XhoI restriction site are in boldface and double underlined.

### Table 4.2: Probes for FISH

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence</th>
<th>Target</th>
<th>Labeling</th>
</tr>
</thead>
<tbody>
<tr>
<td>UA159_SMU118c</td>
<td>5'-GACAAGGAGGCGUUGUUUUUAUCUAAACGCAACUCAGAAUCCUUUGUCGAACAUUACCAACA-3'</td>
<td>mRNA of SMU_118c in S. mutans UA 159</td>
<td>5'-fluorochrome, Alexa Fluor 488 fluorescence labeled (Ex/Em: 490/525 nm)</td>
</tr>
<tr>
<td>SRT 405</td>
<td>5'-TAGCCGTCCCTTCTTCTGGT-3'</td>
<td>Streptococcus spp. 16s rRNA</td>
<td>5'-fluorochrome, Alexa Fluor 594 fluorescence labeled (Ex/Em: 590/617 nm)</td>
</tr>
</tbody>
</table>
Table 4.3: SMU_118c protein abundance ratios from BisHPPP-incubated biofilms

<table>
<thead>
<tr>
<th>BBP</th>
<th>Gene name</th>
<th>Protein function</th>
<th>Ratio 1mM/0mM</th>
<th>P</th>
<th>Ratio 0.1mM/0mM</th>
<th>P</th>
<th>Ratio 0.01mM/0mM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>BisHPPP</td>
<td><em>SMU_118c</em></td>
<td>putative esterase</td>
<td>0.89</td>
<td>0.00</td>
<td>2.21</td>
<td>0.30</td>
<td>2.32</td>
<td>0.04</td>
</tr>
</tbody>
</table>


Chapter 5

Esterase-Catalyzed Degradation of Resin-Dentin Interfaces is Dependent on the Restorative Material and MMP Inhibition

This chapter will be submitted to “Dental Materials”

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5.1 Abstract

\textbf{Objective}: The restoration-tooth interface undergoes degradation due to the enzyme-catalyzed hydrolysis of resin composites and adhesives as well as the digestion of collagen fibrils by dentinal matrix metalloproteinases (MMPs). This study aims to measure the combined and opposing effects of simulated human salivary esterases (SHSE) and MMP inhibition on the restoration-tooth interface integrity for two different adhesives and two different restorative materials, using bacterial biofilm formation and their viability as end point measures.

\textbf{Methods}: Standardized resin-dentin specimens, made from traditional (Z, Z250) or polyacid-modified (D, Dyract-eXtra) composites were bonded to human dentin using total-etch (TE-Scotchbond) or self-etch (SE-EasyBond) adhesives. TE was applied with or without the MMP inhibitor galardin. Specimens were incubated in phosphate-buffer or SHSE (37°C/pH=7.0) for up to 180 days, then suspended in a continuous flow biofilm fermenter cultivating biofilms of \textit{Streptococcus mutans} UA159 for 3 days. Interfacial bacterial penetration, biofilm biomass and viability were assessed by confocal laser scanning microscopy in combination with biomarker dyes.

\textbf{Results}: SHSE increased bacterial penetration in all experimental samples after 180 days of incubation (p<0.05). MMP inhibition reduced interfacial bacterial ingress \textit{vs.} non-MMP-inhibited
TE-bonded specimens (p<0.05). TE+Z interfaces showed lower interfacial bacterial biomass vs. SE+Z after a 90-day incubation period (p<0.05). Dyract-eXtra specimens showed lower bacterial cell viability within the interface vs. Z250 (p<0.05).

**Significance:** This study demonstrates that the biodegradation of resin-tooth bonded interfaces is highly dependent on the material’s chemistry and mode of adhesion, and that the process is enabled by salivary esterases. When the matrix interfacing with the resin is protected with an MMP inhibitor there is a partial reduction in interfacial breakdown, even in the presence of esterases. The *in vitro* bacterial growth model used in the current study facilitated the elucidation of differences in interfacial integrity and biostability between different materials and restoration techniques and can be used to predict restorations’ performance.

**Keywords:** resin composite, dental adhesive, secondary caries, biodegradation, *Streptococcus mutans*, biofilm, MMPs, MMP inhibition, esterase

### 5.2 Introduction

Resin-based restorations are the most popular restorative materials in dentistry in large part due to their aesthetic properties, handling characteristics and modern adhesive technologies. While providing several beneficial properties, higher failure rates and more frequent replacements have been reported for resin-based restorations over amalgam (Brunthaler et al. 2003; Opdam et al. 2007). One of the primary reasons for composite restoration replacements is recurrent or secondary caries that develop at the compromised restoration-tooth interfacial margins (Bernardo et al. 2007; Chrysanthakopoulos 2011; Kopperud et al. 2012; Opdam et al. 2007; Palotie and Vehkalahti 2012; Rho et al. 2013; Soncini et al. 2007).

Resin composites require to be bonded by adhesives to the tooth structure. This results with the formation of the restoration-tooth (resin-dentin) interface, which is characterized as a 3-D
interlocking network consisting of resin polymer penetration and entanglement within the exposed collagen fibrils in the tooth dentin, also refererred to as the hybrid layer (Spencer et al. 2010). The integrity of this interface becomes compromised with time due to several processes, including incomplete adhesive seal, combined with the effect of biological degradative factors (Goldman 1983; Kermanshahi et al. 2010; Pashley et al. 2011; Spencer et al. 2010). The latter is thought to involve two main mechanisms: the hydrolysis of resinous components in both the adhesives and resin composites which is catalyzed by salivary and bacterial esterases (Bourbia et al. 2013; Delaviz et al. 2014; Finer and Santerre 2004a; Xie et al. 2008), and the digestion of collagen fibrils within an incompletely resin-infiltrated collagen by dentinal matrix metalloproteinase (MMPs) (Breschi et al. 2010; Pashley et al. 2004).

The degradation of resin-based materials is largely a result of the hydrolysis of methacrylate-based resin monomers, such as the universal monomer 2.2-bis [4(2-hydroxy-3-methacryloxypropoxy)-phenyl] propane (BisGMA) and triethylene glycol dimethacrylate (TEGDMA) due to the presence of unprotected ester linkages in the monomers, leading to the release of biodegradation by-products (BBPs). This process is often referred to as biodegradation (Bourbia et al. 2013; Cai et al. 2014; Finer and Santerre 2004a), and can be further catalyzed by salivary and bacterial esterases (Bourbia et al. 2013; Delaviz et al. 2014; Finer and Santerre 2004a). Human saliva contains cholesterol esterase (CE)-like and pseudocholine esterase (PCE) activities that show strong degradative ability toward resin composite and adhesives (Finer and Santerre 2004a; Jaffer et al. 2002). Accumulated BBPs in the resin-dentin interface promote bacterial growth and up-regulate expression of virulence genes and proteins that are associated with biofilm formation, acid production and acid tolerance, contributing to caries formation and progression (Khalichi et al. 2004; Sadeghinejad et al. 2017; Sadeghinejad et al. 2016; Xie et al. 2008).
Collagen degradation involves the breakdown of water-rich, resin-sparse collagen fibrils within the hybrid layer due to the activation of host-derived matrix metalloproteinase (MMPs) during bonding procedures (Liu et al. 2011). MMPs are known as zinc- or calcium- depended proteolytic enzymes capable of degrading exposed collagen fibrils within the interface (Ingman et al. 1994; Pashley et al. 2004; Tjaderhane et al. 1998). Dentin matrix has been shown to contain at least five MMPs: stromelysin-1 (MMP-3) (Boukpessi et al. 2008), true collagenases (MMP-1 and MMP-8) (Randall and Hall 2002; Sulkala et al. 2007) and gelatinases A and B (MMP-2 and MMP-9 respectively) (Niu et al. 2011). Once activated, these peptidases are responsible for the intrinsic auto-degenerative process of dentinal degradation (Arola and Reprogel 2005; Boushell et al. 2008; Mazzoni et al. 2009; Nishitani et al. 2006; Pashley et al. 2004; Van Strijp et al. 2000) and act in concert with other host-derived enzymes in breaking down components of the interfacial margin (Finer and Santerre 2004a; Tersariol et al. 2010; Tjäderhane et al. 2013). The MMP inhibitor, galardin, has been suggested for use as an inhibitor against MMP-1, -2, -3, -8 and -9 (Grobelny et al. 1992; HAO et al. 1999) at low concentration (0.2 mM) while not having toxic effects toward bacteria (Serkies et al. 2016).

As a result of the above enzymatic processes, the interface becomes compromised and contributes to allowing for the passage of cariogenic bacteria such as Streptococcus mutans (S. mutans) (Kermanshahi et al. 2010), a major species associated with the initiation and progression of dental caries (Seemann et al. 2005). The size of the interfacial gap was reported as the major influencing factor on the development of caries lesion (Lu and Li 2012), since larger-sized marginal gaps provide the necessary space and access to the nutrients necessarily for cariogenic bacterial colonization (Totiam et al. 2007). Studies to date have demonstrated the effect of restorative materials (Cocco et al. 2015; Finer and Santerre 2004b; Kuper et al. 2012) and adhesives (Ateyah
and Elhejazi 2004; Brackett et al. 2009; Gu et al. 2010) on the biostability of bulk material and interfacial degradation (Borges et al. 2011; Bourbia et al. 2013; Breschi et al. 2010; Mazzoni et al. 2015), which could affect bacterial behavior the development of secondary caries in the interface (Khalichi et al. 2004; Sadeghinejad et al. 2017; Sadeghinejad et al. 2016). As well, strategies to improve the interfacial integrity and prevent cariogenic bacterial biofilm proliferation, including the application of MMP inhibitors (Breschi et al. 2010; Montagner et al. 2014) and antimicrobial restorative materials, were proposed (Cocco et al. 2015; Jandt and Sigusch 2009). However, the above studies have not concurrently investigated the effect of both endogenous and exogenous enzymes on the interfacial integrity of different restorative materials as they may exist in the oral environment.

Recently, Serkies et al. (Serkies et al. 2016) investigated the combined effect of simulated human salivary esterase (SHSE) and MMP inhibition on the integrity of the restoration-tooth interface. The authors showed a mild modulating effect of MMP inhibition on the esterase-catalyzed degradation for self-etched and total-etched interfaces with the end-points being changes in the mode of fracture and/or fracture toughness values over time. The objective of the current study was to further explore the combined effect of SHSE and MMP inhibition on the biodegradation of the restoration-tooth interface which contained different adhesive and restorative materials, with the end-points being the direct observation of bacteria invasion, biofilm formation and viability of interfacial cariogenic bacteria.

5.3 Materials and Methods

5.3.1 Preparation of Resin-dentin Specimens and Interfacial Degradation

Standardized specimens (3x3x6mm) were prepared from either traditional resin composite (Z, Filtek™ Z250 Shade A1, Z250, 3M™ESPE™, St. Paul, MN, USA) or polyacid-modified
composite Dyract-eXtra (D, Dyract® eXtra Universal Compomer Restorative, Dentsply Caulk) bonded to human dentin (University of Toronto Human Ethics Protocol #25793) using total-etch (TE, Adper™ Scotchbond™ Multi-Purpose Plus, 3M™ ESPE™, St. Paul, MN, USA) or self-etch (SE, Adper™ Easy Bond, EB, 3M™ ESPE™, St. Paul, MN, USA) adhesives under sterile conditions, as described previously (Kermanshahi et al. 2010). Total-etch bonded specimens were prepared with (TE+G) or without (TE) the application of 0.2 mM of the MMP inhibitor galardin (USBiological, Swampscott, MA, USA) following the etching step associated with the total-etch adhesive application, for 30 seconds as previously described (Serkies et al. 2016). In total, there were 6 different experimental groups: TE+G+Z, TE+Z, SE+Z, TE+G+D, TE+D, SE+D (defined in Table 1). The dentin adjacent to the marginal interface was sealed with nail varnish to block access of bacteria and exogenous enzymes to the resin-dentin interface through exposed dentinal tubules (Kermanshahi et al. 2010). The specimens were then incubated in phosphate buffer (PBS) (D-PBS, 21600-010, Gibco, Grand Island, NY, USA) or simulated human salivary esterases (SHSE) at 37°C for 0, 30, 90, and 180 days with media replenishment or replacement to maintain esterase activity. SHSE solution was prepared by mixing 16 units/mL of cholesterol esterase (CE, COE-313, Lot# 86621, Toyobo Co., Ltd., Osaka, Japan) and 0.01 units/mL of pseudochoolinesterase (PCE, C7612-6KU, Lot# 078K7015V, Sigma, St. Louis, MO, USA) in PBS (Serkies et al. 2016). The SHSE activity level was verified using nitrophenyl butyrate (pNPB) and butyrylthiocholine (BTC) substrates as reported before (Bourbia et al. 2013; Kermanshahi et al. 2010; Serkies et al. 2016).

5.3.2 In vitro Biofilm Model

The biofilm culturing device is based on a chemostat-based biofilm fermenter (CBBF) as described previously (Li and Bowden 1994), that was modified to be used in bacterial microleakage studies.
(Kermanshahi et al. 2010; Roth et al. 2012). The CBBF system is equipped with pH and temperature controllers set to pH 7.0 and 37.0°C. A solution of 0.5 M KOH or HCl was used to maintain neutral pH within the vessel.

5.3.3 Biofilm Formation along Resin-dentin Interfaces

Post-incubation and non-incubated (control) specimens from the different groups were aseptically inserted into the CBBF with S. mutans UA159 inoculated cultures. Fresh medium (1/4 X Todd Hewitt yeast extract supplemented with 10 mM sucrose and 0.01% hog gastric mucin) was pumped into the vessel at a flow rate of 5.7 L/day (dilution rate of D=0.6/hour), mimicking the resting flow rate of human saliva (Humphrey and Williamson 2001; Jonathan Pratten 2000). The culture in the vessel was used to examine and ensure that the optimal biofilm state has been reached (10⁶ CFU/cm²) (Li and Bowden 1994). After 3-day incubation, specimens were removed and rinsed with PBS for further analysis.

5.3.4 Confocal Laser Scanning Microscopy (CLSM) Analysis and Images Quantification

Specimens (N=3/group) were stained by Live/Dead Backlight Bacterial Viability Kit (Molecular Probes, Eugene, Oregon, USA). Biofilm images were acquired individually by using CLSM (Olympus FluoView 1000 Laser Scanning Confocal Microscope, Tokyo, Japan) as described before (Kermanshahi et al. 2010). Briefly, one Z-stack series/side were captured along 3 sides of each resin-dentin interface through an UPlanApo 60x (water-immersion)/NA 1.2 objective lens. All regions of interest were standardized for orientation and the marginal interface was defined as that which occurred between the restorative materials and dentinal regions. CLSM analysis started at the immediate subsurface of the sample, captured images at 0.5 μm intervals, then ended where no bacteria were detectable.
Z-stack images were analyzed by quantitative software (Imaris version 8.3, Bitplane AG, Zurich, Switzerland) for bacterial penetration, biofilm biomass and viability. This software allows for the quantification of bacterial cells based on bacterial size and fluorescence intensity. Background fluorescence and baseline levels of bacterial penetration and biofilm biomass were produced by using unstained and stained non-incubated and/or non-inoculated specimens.

5.3.5 Data Analyses

All experiments were conducted in triplicate. Homogeneity of variance and normality were verified with Leven’s and Shapiro-Wilk tests, respectively. One-way analysis of variance (ANOVA) and Tukey’s multiple comparison tests were performed to determine the effect of various incubation time points of the same experimental groups on the depth of bacterial penetration or biofilm biomass (p<0.05). Student’s t-tests were applied to analyze individual comparisons for the depth of bacterial penetration or biofilm biomass between incubation conditions (SHSE or D-PBS), or adhesive type (total-etch or self-etch), or treatment (±MMP inhibitor) or restorative materials (Z or D) for same groups (p<0.05), and to compare interfacial cell viability between restorative materials (Z or D) for same groups (p<0.05).

5.4 Results

5.4.1 The Effect of Incubation Period, SHSE, Galardin, Dental Restorative Material and Adhesive Type on the Depth of Bacterial Penetration in the Interface (Fig. 1)

All groups showed increased bacterial penetration for longer incubation periods in both PBS and SHSE (p<0.05). SHSE incubated Z250 specimens (TE+G+Z, TE+Z and SE+Z) showed increased bacterial penetration depth vs. PBS incubated specimens after 90-day and 180-day incubation (p<0.05), while SHSE incubated Dyract-eXtra specimens (TE+G+D, TE+D and SE+D) showed increased bacterial penetration depth vs. PBS incubated specimens only after 180-day incubation (p<0.05). Galardin-treated Z250 groups showed reduced bacterial penetration depth along the
interfaces vs. non-galardin-treated specimens (TE+G+Z vs. TE+Z) after 30-day and 90-day incubation (p<0.05), while the same reduction effect was observed for Dyract-eXtra specimens (TE+G+D vs. TE+D) after 90-day and 180-day incubation (p<0.05). TE bonded Dyract-eXtra groups (TE+G+D, TE+D) showed reduced bacterial penetration depth than that of Z250 groups (TE+G+Z, TE+Z) at 90-day and 180-day incubation in both PBS and SHSE (P<0.05), while SE bonded Dyract-eXtra groups (SE+D) showed reduced bacterial penetration than Z250 groups (SE+Z) at 90-day in both PBS and SHSE. No significant differences were observed between the different adhesive groups (SE vs. TE) from same incubation period, materials and incubation conditions (p>0.05).

5.4.2 The Effect of Incubation Period, SHSE, Galardin, Dental Restorative Material and Adhesive Type on Bacterial Biofilm Biomass (Fig. 2) and Viability (Fig. 3) within the Interface

All groups show increased bacterial biofilm biomass as a function of incubation time in both PBS and SHSE (p<0.05). SHSE incubated SE+Z specimens had higher bacterial biomass vs. PBS incubated specimens at 180-day period (p<0.05). Galardin-treated Z250 specimens showed reduced bacterial biofilm biomass when compared to non-galardin-treated specimens (TE+G+Z vs. TE+Z) from 30-day till 180-day period, but for the Dyract-eXtra specimens (TE+G+D vs. TE+D), the reduction in biofilm biomass was significant only at 30-day (p<0.05). Self-etched Z250 specimens (SE+Z) had more interfacial bacterial biofilm biomass when compared to their total-etch counterparts (TE+Z) (p<0.05) at 90-day incubation in PBS and SHSE, and 180-day incubation in SHSE. The Dyract-eXtra specimens (TE+G+D and TE+D) had more biofilm biomass than the total-etch Z250 specimens (TE+G+Z and TE+Z) at 90-day incubation in both PBS and SHSE (p<0.05), however, SE+Z specimens showed increased biofilm biomass compared to SE+D after 90-day and 180-day incubation in SHSE (p<0.05).

Overall, Dyract-eXtra specimens showed lower bacterial cell viability within the interface (Fig.3).
The ratio of live cells to dead cells (live/dead) for traditional composite was around 2.5±0.1 throughout the incubation period, while the live/dead ratio for Dyract-eXtra was significantly lower at 1.1±0.1 at 30-day, 1.5±0.3 at 90-day and 1.5±0.23 at 180-day (p<0.05).

5.5 Discussion

The results of the current study demonstrated that the integrity of resin-dentin interface is substantially compromised over incubation time in aqueous medium, and can be further deteriorated in the presence of salivary esterase activity, as indicated by the greater depth of bacterial ingress and more bacterial biomass of biofilm along the interface. However, the latter process can be somewhat reduced by the utilization of defined restorative materials or partially inhibited by the application of an MMP inhibitor.

5.5.1 Continuous Interfacial Degradation

Regardless of incubation media, MMP inhibition, restorative material, and mode of adhesion, it was found that longer incubation periods in aqueous media resulted in an increased bacterial penetration and biofilm biomass along the interface. This indicated that the degradation of restoration-tooth interface enabled by hydrolytic activity was a continuous process that compromised the interface, and could ultimately result in the restoration’s failure to secure a stable seal. The underlying mechanism for this marginal gap enlargement is believed to be an inherit drawback of methacrylate-based materials due to the unprotected ester linkage that is susceptible to hydrolysis (Finer and Santerre 2004a). This cleaves polymer chains, leading to the loss of resin integrity at the margins (Ehrenberg et al. 2006). In addition, dentinal MMPs play an important role in digesting exposed collagen fibrils within the interface (Mazzoni et al. 2015), which has been confirmed by previous studies showing that the interfacial degradation can be modulated by MMP inhibition (Breschi et al. 2010; Serkies et al. 2016; Zheng et al. 2014). Although the degradation
process is inevitable, different resin chemistries showed slight variations in the extent of bacterial microleakage and biomass generation, suggesting that this process can be affected by other factors including salivary and bacterial esterase activity, water sorption, polymerization shrinkage, monomer elution, and temperature fluctuations (Bourbia et al. 2013; Ehrenberg et al. 2006; Ehrenberg and Weiner 2000). This indicates the importance of further investigations of the mechanisms of interfacial degradation in order to define strategies to improve interfacial quality and biostability, and ultimately to enhance the longevity of resin-based restorations.

5.5.2 The Effect of SHSE on Interfacial Integrity

This study showed that interfacial bacterial penetration depth was increased with SHSE incubation relative to PBS with both bonded interfaces made from traditional and polyacid-modified composites regardless of adhesive type. The current study corroborates previous reports regarding the effect of SHSE on the degradation of TE interfaces by Kermanshahi et al., Shokati et al (Shokati et al. 2010) and Serkies et al (Kermanshahi et al. 2010; Serkies et al. 2016). The findings emphasize the importance of SHSE with respect to the loss of margin interfacial integrity with time and indicates the clinical alignment of directly measuring bacterial ingress and growth with the ultimate loss of fracture toughness, as measures of interfacial integrity that could predict the status of the critical restoration’s interface and longevity of the restoration (Spencer et al. 2010).

Serkies et al. (Serkies et al. 2016) observed that SE interfaces demonstrate no changes in their fracture toughness values over the first 6 months of incubation with SHSE and PBS, yet the current study, based on using bacteria as biomarkers demonstrates that these interfaces are compromised at much earlier time points. Therefore, the utilization of oral bacteria (rather than chemical dies) as indicators of the lost integrity, rather than mechanical testing alone, appears to be the more sensitive and early predictor of interfacial degradation in the clinical environment.
Overall, SHSE had a less significant effect on biofilm biomass production than bacterial penetration since increased bacterial penetration was observed at higher level than PBS alone in most SHSE incubated groups, but only one group (SE+Z vs. SE+D) showed more bacterial biomass after 180 days. This also suggests that there was little correlation between the depth of marginal gaps as indicated by bacterial ingress and interfacial biofilm formation as indicated by biomass. This was not surprising, since biofilm formation is a multifactor-dependent process, relying not only on sufficient space, but also the interfacial surface morphology, content (nutrients), and environmental signals (Cvitkovitch 2003; O'Toole et al. 2000). Due to the positive relationship between cariogenic bacterial abundance and caries formation (Loesche 1982; Zinner et al. 1965), it could be hypothesized that although the depth of interfacial margins is a good indicator of marginal breakdown, bacterial adhesion and biofilm formation is more important to predict the restoration longevity. Hence, multiple indicators should be employed to evaluate the effect of margin interfacial integrity on restoration longevity.

5.5.3 The Effect of MMP Inhibition on the Interfacial Integrity

Previous studies have reported on the use of chlorhexidine as an inhibitor of MMP activity in order to explore the preservation of interfacial structure, however showing only short-term positive results (Osorio et al. 2011). Further, chlorhexidine is both a protease inhibitor and a powerful antimicrobial agent, and hence it has been challenging to use it as a probe for assessing MMP activity alone. In the present study, galardin was selected as a broad based MMP inhibitor due to its inhibitory activity against multiple dentinal MMP-1, -2, -3, -8 and -9, at a low concentration (0.2 mM) (Breschi et al. 2010; Sulkala et al. 2007) and does not have toxic effects toward bacteria. Therefore, its anti-degradative effects are exclusive of inhibiting other dentin matrix enzymes and it can be used to measure more precisely the potential influence of MMPs in the current...
experimental conditions that include viable bacteria as one of the outcome measurements. And, galardin only applied on TE samples based on a previous negative result from SE samples in terms of fracture toughness (Serkies et al. 2016). Although MMPs are not the only endogenous protease that can degrade dentin matrix and their effect on the collagen degradation is controversial (Almahdy et al. 2012; Buzalaf et al. 2012; Chaussain et al. 2013; Luhrs et al. 2013; Nascimento et al. 2011; Tersario et al. 2010), in this study the application of the galardin to TE interfacial dentin enhanced marginal biostability for both restorative materials up to 90 days and the Dyract-eXtra up to 180 days, as indicated by the reduced interfacial bacterial ingress. While this provides evidence of MMPs’ contribution in marginal degradation, the effect did not mitigate the continued deterioration of the interface by SHSE, particularly for the Z-250 restorations, which continued to manifest itself out to 180 days. This positive effect may result from the immediate inhibition of galardin towards human collagenases MMP-1 and MMP-8 (Supplemental file) and the inhibitory effect of galardin on SHSE activity used in the current study (Supplemental File). Although an overall inhibitory effect of galardin on SHSE was observed, the interfaces of the two restorative materials, Z250 and Dyract-eXtra responded differently to MMP inhibition over time. The effect on bacterial biofilm biomass was observed but less broadly than that of the bacteria ingress. This finding emphasizes the fact that the interfacial degradation is not a simple process that leads to the deepening of the marginal gap (Kermanshahi et al. 2010), but it also involves a changing interfacial geometry, morphology, and implies that even the chemical content of the immediate environment housing the bacteria must be changing, reflected by the accumulation of degradation by-products that could impact bacterial growth and metabolism (Khalichi et al. 2004; Khalichi et al. 2009; Sadeghinejad et al. 2017; Sadeghinejad et al. 2016; Singh et al. 2005).
5.5.4 The Effect of the Dental Materials on Interfacial Integrity

When considering the interfacial space resulting from the degradation of both resin and dentin matrix, it could be summarized that a TE bonded interface may have more capacity for bacteria due to the thicker hybrid layer generated by the resin-matrix network as compared to SE bonded specimens (Hegde et al. 2012). However, in this study, the self-etch bonded Z250 interface showed increased biofilm biomass when compared to total-etch counterparts. This may be an influence of the resin formulation for the self-etch adhesive that is inherently hydrophilic (Bourbia et al. 2013). The presence of residual water within the hybrid layer may lead to void formation and yield a lower degree of monomer conversion, thereby facilitating degradation and providing adequate space between dentin and restorative materials for biofilm formation (Chersoni et al. 2004; Miyazaki et al. 2003). In addition, the hydrophilic nature of the monomers in self-etch adhesive facilitates their elution into resin-dentin interface, thereby forming a vesicular-structure polymer which provides the scaffold for establishing the biofilm (Khalichi et al. 2004). This indicated that the chemical formulation and properties of the adhesive materials had an influential effect on bacterial adhesion and biofilm formation in addition to increasing the size of the interfacial space.

As discussed elsewhere, Serkies et al. (Serkies et al. 2016) reported that while SE interfaces show no changes in their fracture toughness values over a 6-month incubation period with SHSE and PBS, the fracture planes for this adhesive group transitioned over this period from the bottom of the hybrid layer to the adhesive-resin composite. It was hypothesized that this was due to the plasticizing effect of the media on the hydrophilic adhesive. The transition in the fracture plane could provide access to salivary and bacterial esterases, needed to degrade the resin-based substrates, and thereby producing degradation by-products that up-regulate genes associated with
growth and biofilm formation (Sadeghinejad et al. 2017; Sadeghinejad et al. 2016). This could explain the increase in bacterial abundance that was found for the SE-bonded interfaces.

The Dyract-eXtra is one of few antimicrobial materials claimed to combine the mechanical and esthetic properties of composites with the advantage of fluoride-releasing glass-ionomer cements (Nicholson 2007). The current study showed that the polyacid-modified Dyract-eXtra had a positive effect on the prevention of bacterial penetration and reduced bacterial viability when compared to traditional resin composites, but its antimicrobial effects on biofilm biomass was controversial when applied with different adhesives. Fluoride release from restorative materials reduce bacterial metabolism and proliferation (Marquis et al. 2003), thus inhibiting bacterial penetration and decreasing bacterial viability, but could be incapable of preventing the process of initial bacterial adhesion, which explains the positive effect of Dyract-eXtra on biofilm biomass (Auschill et al. 2002). In addition, there were variable effects of Dyract-eXtra on biofilm biomass when utilizing different adhesive systems or incubating in SHSE media (Fig 2). This could be the result of the altered environmental conditions within the Dyract-dentin interface, reported as one of several factors that affect fluoride release (Wiegand et al. 2007), by the different compositions and hydrophilicity of the adhesives materials and their susceptibility to the incubation medium.

5.5.5 The Effect of Galardin and Dental Materials on SHSE Activity (Supplemental Material)

An inhibitory effect of galardin on CE activity was observed after 12 hours of incubation. Since galardin could undergoes hydrolysis in an aqueous environment (Fisher and Mobashery 2006), it is assumed that the derivative from the hydrolysis interacted with CE and changed the enzyme conformation or environmental conditions leading to activity loss with time. However, this statistically significant, but small overall reduction of CE activity by galardin is unlikely to be sufficient to clinically influence SHSE-induced degradation due to the saturation effect of esterases
in oral cavity (Finer and Santerre 2003). The reduced PCE activity was mainly caused by the galardin carrier solvent DMSO which was reported to form DMSO-water bonds in the active sites of enzymes that influences their activity (Faulds et al. 2011). As well, this organic solvent could cause dehydration of enzymes that could lead to structural disruption and inactivation (Griebenow and Klibanov 1995). In addition to the impact of reagents on enzymes, the presence of these reagents could also influence substrate properties and alter their biostability (Faulds et al. 2011). Overall, it is unlikely that galardin could affect interfacial degradation by inhibiting PCE activity due to the non-dominant degradative effect of PCE on resin composites (Finer and Santerre 2004a).

In this study, big deviations of activity values were noticed, indicating the detecting methods for CE or PCE activity may not be suitable. To further clarify the interaction between galardin and SHSE or other enzymatic activity in oral cavity, additional testing approaches should be explored. Compared to the groups without restorative materials, short-term reductions were observed for CE activity when incubated with either restorative material and PCE activity with Z250. However, the inhibitory effect of Z250 was significantly higher than that of Dyract-eXtra. The temporal activity loss may be the result of initial enzymatic reaction with unreacted monomers, leading to distinct activity CE and PCE activity responses (Finer et al. 2004). Although the activities recovered and maintained after a short period of time, slight activity loss was still sustained for CE with both materials and for PCE with Z250. This could be explained by attachment of the enzyme to the surface of the materials due to electrostatic attraction as a part of the initial non-specific enzyme-substrate encounter (Calef and Deutch 1983; Shafferman et al. 1994), thus in effect removing some of the enzyme and associated activity from the solution. Overall, there was no significant effect of CE activity between Z250 and Dyract-eXtra after 5 days. Since CE activity is the dominant factor influencing resin-based materials degradation (Finer et al. 2004), the material-SHSE
interactions has little contributing role to explain long-term effects of different materials on interfacial degradation rates. This investigation is still however, valuable since it shows that dental materials can inhibit enzymes decelerating degradation process and clarifies and emphasizes the importance of interactions between materials and oral environmental enzymes.

5.5.6 Experimental Methods

Reports on the effect of materials on interfacial integrity have been controversial. While in vitro studies have reported on the potential impact of material chemistry on resin composite degradation (Ferracane 2016; Finer and Santerre 2004b; Krämer et al. 2015), some clinical studies have reported only a minor influence of material properties on the restoration longevity (Demarco et al. 2012; Ferracane 2016). This raises the question about the relevancy of current laboratory methodologies and their correlations with clinical performance of the restorations.

In this study, a comprehensive in vitro model was established based on continuous media-based chamber (CBBF) mimicking intraoral pathogenic growth conditions, to investigate the restoration-tooth interfacial integrity based on bacterial biomarkers. This CBBF system maintains chemical and biological components such as pH, temperature, carbohydrate source, and bacterial species composition to reproduce factors that are associated with interfacial cariogenic bacterial growth (Drake and Brogden 2002; Kermanshahi et al. 2010; Novick and Szilard 1950; Roth et al. 2012). The device and parameter settings are well-controlled, so the acquired results are highly reproducible and representative of the oral cavity (Drake and Brogden 2002). These advantages make this model suitable to reproduce relevant conditions to those that exist in the oral cavity for future dental materials investigations. The biofilm formed in resin-dentin interfaces was used to indicate interface integrity and predict future caries potential by measuring both the interfacial bacterial penetration and biofilm biomass, since it has been confirmed that a positive relationship
between the presence and quantity of *S. mutans* and caries formation (Loesche 1982; Zinner et al. 1965).

It is considered more appropriate and clinically relevant to use bacterial penetration and biofilm formation as biomarkers to indicate interfacial integrity instead of using wall lesion or dentin mineral loss as reported in most of the current studies (Kuper et al. 2014; Kuper et al. 2015; Nassar and González-Cabezas 2011; Totiam et al. 2007), since the word “wall lesion” is not uniformly defined in the literature and the detection methods have various sensitivities and specificities (Kuper et al. 2014; van de Sande et al. 2014). In addition, unlike mechanical testing, the current study demonstrated that that a bacterial detection mode is more sensitive in the detection of early interfacial changes by using a combination of bacterial penetration, biofilm formation, and live/dead parameters. In order to standardize and maintain stable and equal levels of salivary esterases, SHSE was used to replicate biomolecular processes occurring in the intraoral environment (Finer et al. 2004; Finer and Santerre 2004a; Jaffer et al. 2002; Serkies et al. 2016; Shokati et al. 2010). Resin-dentin specimens were fabricated following relevant clinical procedures, representing gingival margins of proximal restorations, the most susceptible area for recurrent caries (Kermanshahi et al. 2010).

Unlike Scanning Electric Microscopy (SEM), the CLSM does not require the destructive preparation steps prior to imaging such as dehydration and fixation which alters biofilm morphology (Kermanshahi et al. 2010). Therefore, the CLSM is considered more suitable for imaging biological components within the resin-dentin margin. The current study is the first to quantify both the biofilm formed in the restoration-tooth interface as a measure of interfacial integrity in combination with bacterial ingression, by using reconstructed 3D images and calculating cell numbers based on bacterial fluorescence intensity and cell size. This image
analysis method will be a valuable tool for adoption in the future investigation of bacterial performance in the interface, looking at bacterial ingress, biofilm formation and vitality of single or multiple bacterial systems.

In summary, resin-dentin interfaces are considered the “weak link” of the restorations and as such determining the restorations’ longevity (Spencer et al. 2010), which is undergoing continuous and inevitable hydrolysis that is catalyzed by esterases and MMP activities. So, it is important to assess the effects of material chemistry, mode of adhesion, MMP inhibition and incubation conditions on the resin-dentin interfacial integrity.

To improve interfacial quality, future studies should attempt to determine whether galardin exerts its function in a dose-dependent manner in the interface, and if so, to establish the most effective concentrations. Other non-specific inhibitors could also be explored under simulated clinical conditions, since there are other proteases such as cysteine cathepsins that contribute to dentinal collagen degradation (Carrilho 2012; Scaffa et al. 2012).

Furthermore, more studies should be carried out to clarify the combined impact from adhesive and restorative materials, in order to elucidate the interactions between materials and oral conditions on bio-burden resulting from materials design.

5.6 Conclusions

The deterioration of resin-dentin interfaces was promoted by SHSE, and was modulated by MMP inhibition and the use of antimicrobial materials. Different combinations of adhesives and restorative materials resulted in different bacterial ingress, proliferation and viability within the interface, indicating a material- and adhesive-mode depended process of interfacial degradation. The interaction among SHSE, materials and galardin, to some extent, provides a potential
explanation for the interaction of bacterial with biomaterials in the oral cavity. The described *in vitro* model will provide information about bacterial performance in a more relevant manner for the prediction of caries formation and progression than other measures currently in use, and will be important for informing on new material evaluation.
5.7 References


Drake DR, Brogden KA. 2002. Continuous-culture chemostat systems and flowcells as methods to investigate microbial interactions.


5.8 Supplemental File

5.8.1 Materials and Methods

5.8.1.1 The Effects of the MMP Inhibitor Galardin on SHSE Activity

SHSE with 0.2 mM/ml of galardin (USBiological, Swampscott, MA, USA) or galardin carrier solvent, 0.2 mM/ml of dimethyl sulfoxide (DMSO) as control were incubated at 37°C for 12 hours. SHSE solution without any reagents was used as baseline. SHSE activity was measured by assessing individual CE and PCE activity as described above (Bourbia et al. 2013; Kermanshahi et al. 2010; Serkies et al. 2016) at 1-hour, 6-hour and 12-hour time points (N=3/group).

5.8.1.2 The Effects of Dental Restorative Materials on SHSE Activity

Cylinder composite specimens of Z250 or Dyract-eXtra (4x4x4 mm) with a surface area of approximately 2.26±0.05cm² were prepared (Bourbia et al. 2013; Finer and Santerre 2004a). The specimens were pre-incubated in PBS (D-PBS, 21600-010, Gibco, Grand Island, NY, USA) for 48 hours to remove soluble unreacted monomers, then individually (N=3/group) incubated in 1 ml of SHSE in 4 ml sterile amber vials at 37°C for 5 days. The SHSE solution without any dental materials was used as benchmark. SHSE activity was measured daily (N=3/group) by assessing individual CE and PCE activity as described above (Bourbia et al. 2013; Kermanshahi et al. 2010; Serkies et al. 2016).

5.8.1.3 Statistical Analysis

One-way analysis of variance (ANOVA) and Tukey’s multiple comparison tests were performed to determine the effect of galardin or the dental materials on the activity of SHSE (p<0.05).
5.8.2 Results

5.8.2.1 The Effects of MMPs Inhibitor (Galardin) on SHSE Activity

Among groups at same time point, a statistically significant inhibition of galardin on CE activity was found only at the 12-hour time point (p<0.05) and there was no significant activity change in the same group over time (p>0.05) (Supplemental Fig.1A). Among groups at same time point, DMSO decreased PCE activity from 1-hour till 12-hour time point, and galardin showed inhibitory effect on PCE at 12-hour time point (p<0.05) (Supplemental Fig.1B). Among different time points of the same group, after 12 hours inhibition, galardin reduced PCE activity significantly (p<0.05) (Supplemental Fig.1B).

5.8.2.2 The Effects of Dental Materials on SHSE Activity

CE and PCE enzymatic activities were reduced by both Z250 and Dyract-eXtra (p<0.05), but the extent of inhibition over time was quite different for the two materials. The inhibitory effect of Z250 was at maximum on day 1, reducing the CE and PCE activity to 23.0±1.7% and 20.0±3.9%, respectively, then the inhibition was gradually diminished over the 5-day experimental period (p<0.05) (Supplemental Fig. 2 A). Unlike Z250, the inhibitory effect of Dyract-eXtra was much lower, and decreased both CE and PCE activity to 75.1±1.0% and 89.8±5.7%, respectively at day 1 (p<0.05), remained relatively stable for CE, but was completely diminished for PCE at day 5 (Supplemental Fig. 2B).
5.9 Tables & Figures

Table 5.1: The definition of specimens’ acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>TE+G+Z</td>
<td>Total-etch bonded to Z250 specimens prepared with the application of galardin</td>
</tr>
<tr>
<td>TE+Z</td>
<td>Total-etch bonded to Z250 specimens</td>
</tr>
<tr>
<td>SE+Z</td>
<td>Self-etch bonded to Z250 specimens</td>
</tr>
<tr>
<td>TE+G+D</td>
<td>Total-etch bonded to Dyract-eXtra specimens prepared with the application of galardin</td>
</tr>
<tr>
<td>TE+D</td>
<td>Total-etch bonded to Dyract-eXtra specimens</td>
</tr>
<tr>
<td>SE+D</td>
<td>Self-etch bonded to Dyract-eXtra specimens</td>
</tr>
</tbody>
</table>
Figure 5.1: The effect of incubation period, MMP inhibition by galardin, SHSE, adhesives and restorative materials on the depth of bacterial penetration in the resin-dentin interfaces of the specimens (N=9/group; data are reported as mean ± standard deviation): *** > ** > * indicate greater values between groups from different time points (p<0.05). Horizontal bars indicate significant difference between SHSE and PBS incubated specimens from same incubation period and material (p<0.05). Bars with vertical lines indicate significant difference between galardin-treated and non-galardin-treated specimens from same incubation period and material (p<0.05). Different letters indicate significant difference between Z250 and Dyract-eXtra specimens from same incubation conditions (Greater invasion depth was marked as capital letter A, while the lesser one was marked as low case letter a) (p<0.05).
Figure 5.2: The effect of incubation period, MMP inhibition by galardin, SHSE, adhesives and dental materials on bacterial biofilm biomass within the resin-dentin interfaces of specimens (N=9/group; data are reported as mean ± standard deviation: ***>***>*** indicate greater values between groups from different time points (p<0.05). Horizontal bars indicate significant difference between SHSE and PBS incubated from same incubation period and material (p<0.05). Bars with vertical lines indicate significant difference between galardin-treated and non-galardin-treated specimens from same incubation period and material (p<0.05). Dotted lines indicate significant difference between total-etch- and self-etch-bonded specimens from same incubation period and material (p<0.05). Different letters indicate significant difference between Z250 and Dyract-eXtra specimens from same incubation conditions (Greater biomass was marked as capital letter A, while the lesser one was marked as lower case letter a) (p<0.05).
Figure 5.3: Representative confocal laser microscopy images of *S. mutans* biofilm formed within the interface of TE+G+Z (A, B & C) and TE+G+D (a, b & c) after 90 days in SHSE (Cyto 9 stained live cell in green & Propidium Iodide stained dead cell in red). A) image projection of z-stacks acquired on the surface of the interface of TE+G+Z; B) image projection of stacks acquired in the middle of the TE+G+Z interface; C) image projection of stacks acquired at the bottom of the TE+G+Z interface; a) image projection of stacks acquired on the surface of the TE+G+D interface; b) image projection of stacks acquired in the middle of the TE+G+D interface; c) image projection of stacks acquired at the bottom of the TE+G+D interface.
Supplemental Figure 5.1: The effect of galardin on CE (A) and PCE (B) activity. Data are presented as percentage of CE or PCE enzymatic activity without galardin nor DMSO at each time point (N=3/group; data are reported as mean ± standard deviation). Values with different letters indicate statistically significant differences (p<0.05).
Supplemental Figure 5.2: The effect of dental materials on CE (A) and PCE (B) activity. Data are presented as percentage of either CE or PCE enzyme activity without the materials at each time point (N=3/group; data are reported as mean ± standard deviation). Values with the different lower case letters indicate statistically significant differences (p<0.05).
Chapter 6

Discussion

6.1 Summary

Resin composites are the most popular restorative materials in dentistry because of their aesthetic properties, good handling characteristics and improved adhesion technology (Murray et al. 2002b; Simecek et al. 2009; Wakefield and Kofford 2001). While providing several beneficial properties over amalgam, resin composites suffer from higher failure rates and frequently require replacements compared to amalgam (Brunthaler et al. 2003b; Opdam et al. 2007). A major contributor to the frequent replacements of resin-based restorations is secondary caries caused by the penetration and biofilm formation of cariogenic bacteria along the compromised resin-dentin interface (Bernardo et al. 2007c; Kermanshahi et al. 2010; Kopperud et al. 2012; Opdam et al. 2007).

This dissertation is the first study focused on the role of S. mutans esterases as a virulence factor that contributes to the progressing of secondary caries by investigating its effect on interfacial degradation and demonstrating the reciprocal effect of a degradation by-product on the oral bacteria at the gene and protein level. In addition to salivary esterases (Finer and Santerre 2004a), an oral bacterium, S. mutans, was reported to have degradative activity towards adhesives and resin composites. Therefore, it was hypothesized that S. mutans produces specific esterases that degrade the resin-dentin interface and release biodegradation by-products (BBPs) such as bis-hydroxy-propoxy-phenyl-propane (BisHPPP) which, in turn, affect S. mutans esterase activity by stimulating adaptive gene expression. Since the deterioration of the resin-dentin interface is a multifactor process (Breschi et al. 2010; Kermanshahi et al. 2010), it was hypothesized that overall
interfacial biostability is affected by simulated salivary esterases, dentinal matrix metalloproteinase (MMPs) inhibition and the restorative materials.

In this study, putative esterase genes in *S. mutans* UA159 were identified, purified and characterized. One esterase, SMU_118c identified as the dominant esterase in *S. mutans* UA159, showed a similar hydrolytic activity profile to salivary esterases, contributing to resinous materials degradation. The degradation by-product BisHPPP upregulated gene expression of SMU_118c in a concentration-dependent manner. This positive feedback process could continuously accelerate the degradation of the restoration-tooth interface and lead to premature failure of the restoration.

While investigating the mechanistic reasons for resin-based restoration failure, this dissertation also describes an *in vitro* model utilizing a chemostat-based biofilm fermenter (CBBF) in combination with confocal laser microscopy and biomarkers to explore the biological and chemical effects of SHSE, MMP inhibition, the adhesive and restorative materials on the initial integrity and overall biostability of the restoration-tooth interface in a more clinical relevant environment than previous studies to date (Ateyah and Elhejazi 2004; Gerdolle et al. 2005; Kuper et al. 2015; Matharu et al. 2001). In this model, the biofilm formed in compromised resin-dentin interface was used to indicate interface integrity and foresee future caries potential by measuring interfacial bacterial penetration and biofilm biomass and vitality based on the confirmed positive relationship between the presence and quantity of *S. mutans* and caries formation (Loesche 1982; Zinner et al. 1965). It was confirmed that the interfacial integrity is compromised with time and can be further deteriorated by simulated salivary esterase activity, as indicated by the greater depth of bacterial ingress and more bacterial biomass of biofilm along the interface. However, this process can be modulated by utilization of different restorative materials and decelerated by MMP inhibition.
The results of this thesis provide a detailed explanation of the possible molecular mechanisms responsible for the increase in recurrent caries around resin composite restorations compared with dental amalgam (Ferracane 2013) by elucidating the interactions between oral bacteria and restorative materials. Understanding the molecular mechanisms underlying these phenomena provides a better evaluation of the consequences associated with restoration procedures using resin composite materials, and could lead to innovative therapeutic strategies and improved material engineering. The results also accredited specific practical approaches such as application of an MMP inhibitor and an antimicrobial material for improving interfacial quality, and revealed interactions between restorative materials and intraoral enzymes providing a profound understanding of biochemical processes in the interface. Furthermore, the newly established in vitro continuous culture device, which simulates in vivo conditions but excludes other complex interactions in vivo, has the potential to be employed as a comprehensive approach to test biochemical properties of newly designed materials in the future.

6.2 General Discussion

6.2.1 Isolation and Identification of a Specific Esterase from S. mutans

An esterase from S. mutans UA159, SMU_118c, was isolated and identified as a typical intracellular esterase that belongs to the esterase-lipase superfamily with a conserved arrangement of residues, serine (Ser), histidine (His) and aspartic acid (Asp) in its catalytic site (Nardini and Dijkstra 1999; Oakeshott et al. 2005). The preference of SMU_118c towards p-nitrophenyl ester substrates with a short chain (C2 and C4) at the para-position is consistent with the activity profile previously reported for the whole cell activity of S. mutans (Bourbia et al. 2013), suggesting that SMU_118c is a major contributor to the hydrolase activity of Streptococcus species. Further characterizations of SMU_118c verified its degradative activity towards both commonly used resin
monomers BisGMA and TEGDMA at neutral (7.0) and cariogenic pH (5.5). A faster degradation rate was observed toward BisGMA vs. TEGDMA, suggesting that SMU_118c has higher affinity towards phenol-containing hydrophobic monomers than to water-soluble monomers, which could be explained by the hydrophobic property of its conserved active site (Bencharit et al. 2006; Bencharit et al. 2003; Finer and Santerre 2003; 2004a). Since BisGMA is a universal ingredient in current adhesive and resin composite systems (Ferracane 2011), the preference of SMU_118c toward this substrate indicates a high capability of oral bacteria such as S. mutans to compromise the resin-dentin interface by hydrolyzing adhesives and resin composites (Bourbia et al. 2013; Finer and Santerre 2003; 2004a; Serkies et al. 2016). In addition, unlike human salivary derived esterases (HSDE) and the model degradative enzyme cholesterol esterase (CE) that showed reduced activity and stability in the presence of resin-based materials or monomers (Jaffer et al. 2002; Shokati et al. 2010), SMU_118c maintains its activity and stability in the presence of resin monomers at both pH 7.0 and pH 5.5 over 21 days. This indicated that SMU_118c could hydrolyze resinous moieties for a long time after being released, even when not continuously released under pathogenic growth conditions of the biofilm, and likely to remain active within the confined restoration-tooth interface for a significant period (Sadeghinejad et al. 2017). Furthermore, the ability of SMU_118c to continuously degrade polymerized resin composite was confirmed by both SEM observation and HPLC analysis of biodegradation product, BisHPPP, thus confirming its deleterious effect on degrading the material and restoration-tooth interface (Jaffer et al. 2002; Shokati et al. 2010; Smith et al. 2001).
6.2.2 Verification of the Association of SMU_118c to the Degradative Activity of the Whole Bacterial Cell toward Resin Composites

Once the mechanistic exploration of the biological effect of SMU_118c on dental materials was completed, the next step was to verify the role of SMU_118c in the overall degradative potential of S. mutans UA159. The role of gene SMU_118c in the whole bacterial degradative activity was confirmed using a gene knock-out (ΔSMU_118c) and complemented (ΔSMU_118cC) strains to induced resin composite degradation: reduced surface roughness and amount of BisHPPP was nullified in the knock-out strain vs. the wild strain, while the complemented strain recovered the degradation to similar levels of the wild strain. The above results confirmed the major role of SMU_118c in the the overall degradative activities of S. mutans UA159.

6.2.3 The Reciprocal Effect of the Biodegradation Product BisHPPP on the Expression of SMU_118c at Gene and Protein levels

Previous studies reported that the accumulated degradation by-products could act as stresses that induce the adaptation mechanisms in S. mutans by stimulating bacterial growth and up-regulating key virulence genes (Khalichi et al. 2009; Sadeghinejad et al. 2017; Sadeghinejad et al. 2016), Therefore, in the current study, the effect of BisHPPP on SMU_118c gene expression was analyzed to explore the reciprocal effect of materials on bacterial degradative activity.

Previous studies employed the qRT-PCR (quantitative real-time polymerase chain reaction) method to investigate gene expression. However, this study employed Fluorescence in situ hybridization (FISH) of mRNA sequences for studying gene expression. This methodology is frequently used in studies involving eukaryotic cells and tissues (Wendeberg et al. 2012), since the FISH results are more informative due to the undisrupted tissue structure, allowing for spatial analyses and more complex biological growth conditions. The improved sensitivity of FISH approaches has promoted its applications in microbiology to detect bacterial responses to
environmental changes at the transcription level. In this study, based on the analysis of BisHPPP content in saliva after immediate restorations (Jaffer F et al. 2004; Sadeghinejad et al. 2017), BisHPPP concentrations ranging up to 0.1 mM were considered clinically relevant and adopted for the investigations of SMU_118c gene responses. In addition, a higher concentration of BisHPPP (1mM) was also included in this study, since this level is expected in the interface due to accumulative BisHPPP release within the restrained interfacial space and due to limited diffusion within the biofilm (Sadeghinejad et al. 2017). The up-regulation of SMU_118c by BisHPPP was observed in a concentration-dependent manner, which could be explained by an accumulative effect of BisHPPP on the bacterial growth environment. Since BisHPPP is hydrophobic and weakly charged in an acidic environment, the accumulation of BisHPPP around bacterial cells could change the microenvironments and lead to pH fluctuations, fluid flow interruption and even nutrient blockage which act as stressors to regulate genes expression for adaptation by Two-Components Signal Transduction Systems (TCSTS) (Burne et al. 1997; Fux et al. 2005; Hudson and Curtiss 1990; Li et al. 2002b). In addition, the direct interaction between BisHPPP and bacteria might create a new bacterial/environmental interface inducing autoprophosphorylation of sensor kinases on bacterial surface, which activates the SMU_118c expression by an intracellular response regulator (Stock et al. 2000).

Another finding in this study was that the effective BisHPPP concentration (1 mM) triggering significant gene response was higher than that reported previously (0.001- 0.1 mM) (Sadeghinejad et al. 2017). This could be explained by different biofilm biological conditions employed in each study. Chemically defined media employed in this study could influence bacterial physiochemical properties in biofilm development due to the limited nutrients which itself could act as a stressor that stimulates global regulation of gene expression (Bowden and Li 1997; Moller et al. 1997;
Sauer et al. 2004). As well, the natural pH fluctuations during biofilm formation that induce bacterial acid tolerance response is associated with changes in the expression of over 30 proteins (Hamilton and Svensäter 1998; Wilkins et al. 2002), that could also affect SMU_118c gene expression. The differences between in situ detection and qRT-PCR analysis highlights the importance of using the clinically relevant model to reflect the in vivo environment and to investigate the precise effects of biomaterials on oral bacteria. It should be noted however, that despite differences in exact concentrations between the two studies, the overall trend of dose-dependent effect of BisHPPP on gene expression was similar.

Gene responses to environmental fluctuations must be reflected at the protein level to carry out bacterial adaptation to a wide range of conditions (Chubukov et al. 2014). The effect of BisHPPP needs to be further explored at the protein-response level to reveal its effect on the hydrolytic activity of S. mutans, directly contributing to the degradation of resinous restoration. Based on Dr. Lida Sadeghinejad’s proteomic analyses, a significant increase of SMU_118c production was observed in the groups with low concentrations of BisHPPP (0.01 and 0.1 mM), while a statistically significant but physiologically small decrease was found in biofilms exposed to 1 mM of BisHPPP, in contrast to the results of the gene expression for this concentration of BisHPPP, where the level the SMU_118c was up-regulated. The contradicting results highlighted the somewhat weak correlation between mRNA levels and protein abundance where greater variations were observed for the latter (Dressaire et al. 2010; Lu et al. 2007; Nie et al. 2006a; Nie et al. 2006b). In bacteria, mRNA concentration alone can only explain less than 50% of total variation of protein quantity (Corbin et al. 2003; Dressaire et al. 2010; Lu et al. 2007) due to translational regulation by which bacteria are able to fine-tune their protein expression levels (Picard et al. 2012). Therefore, it can be hypothesized that even without up-regulation of the SMU_118c gene, the
bacteria could have still promoted SMU_118c synthesis in response to BisHPPP by enhancing the mRNA stability, increasing translational efficiency and selectivity (Picard et al. 2012). It also can be hypothesized that S. mutans might not be challenged by the lowest concentration of BisHPPP, so SMU_118c protein synthesis was promoted instead of expressing critical proteins associated with bacterial survival under harsher conditions. As BisHPPP concentration increases, the bacteria selectively reduced the translation of SMU_118c or enhanced the degradation of SMU_118c to facilitate other critical protein expression.

In summary, unlike the effect of BisHPPP on SMU_118c up-regulation that required high concentrations of BisHPPP, SMU_118c protein synthesis can be significantly enhanced by more than 2-fold when exposed to BisHPPP at a wider range of concentrations. With constant BisHPPP release due to biodegradation of resin composites by both salivary and bacterial esterases, SMU_118c production is likely to be continuously accelerated by the degradation product it produces, accelerating the degradation of the restoration and restoration-tooth interface resulting with an increased rate of interfacial degradation and potentially premature failure of restorations.

6.2.4 The Effects of Incubation Period, Condition, MMP Inhibition and Materials on Interfacial Degradation

In addition to the exploration of mechanistic reasons for resin-based restorations failure to improve future materials design, this dissertation established an in vitro model based on chemostat-based biofilm fermenter (CBBF) to explore the biological and chemical effects of SHSE, MMP inhibition, the adhesive and restorative materials on the initial integrity and overall biostability of the restoration-tooth interface in a more clinical relevant environment. In this model, the bacterial penetration and biofilm formed along compromised resin-dentin interface was used to indicate interface integrity and foresee future caries formation due to the confirmed positive relationship
between the presence and quantity of *S. mutans* and caries formation (Loesche 1982; Zinner et al. 1965).

### 6.2.4.1 Time Effect on Degradation

Regardless of incubation media, MMP inhibition, restorative material, and mode of adhesion, longer incubation periods resulted in increased bacterial penetration and biofilm biomass along the interface. This indicated that the degradation of restoration-tooth interface caused by hydrolytic activity and plasticizing effect of water on the adhesive is a continuous and inevitable process due to an inherit drawback of methacrylate-based materials that the unprotect ester linkage is susceptible to hydrolysis (Finer and Santerre 2004a). In addition, dentinal MMPs play an important role in digesting exposed collagen fibrils within the interface (Breschi et al. 2010; Mazzoni et al. 2015; Serkies et al. 2016; Zheng et al. 2014). Although the degradation process is inevitable, restoration-tooth specimens that were fabricated with different materials, adhesives and application of galardin showed various degradation rates, suggesting that this degradative process can be affected by other factors such as salivary and bacterial esterase activity, water sorption, and monomer elution (Bourbia et al. 2013; Ehrenberg et al. 2006; Ehrenberg and Weiner 2000). This indicated the importance of further investigations of the mechanisms of interfacial degradation and methods to improve interfacial quality and biostability in order to develop strategies to enhance the longevity of resin-based restorations.

### 6.2.4.2 The Effect of SHSE on Interfacial Degradation

This study showed that interfacial bacterial penetration depth was increased with SHSE incubation relative to PBS with both bonded interfaces made from traditional and polyacid-modified composites regardless of adhesive type. The current study corroborates previous reports regarding the effect of SHSE on the degradation of TE interfaces by Kermanshahi et al., Shokati et al.
(Shokati et al. 2010) and Serkies et al (Kermanshahi et al. 2010; Serkies et al. 2016). The findings emphasize the importance of SHSE with respect to the loss of margin interfacial integrity with time and indicates the clinical alignment of directly measuring bacterial ingress and growth with the ultimate loss of fracture toughness, as measures of interfacial integrity that could predict the status of the critical restoration’s interface and longevity of the restoration (Spencer et al. 2010). In addition, the current study utilizing bacteria as indicators for the quality of the interface was more sensitive than the mechanical assessment used previously (Serkies et al. 2016), as indicated by its ability to detect changes in interfacial degradation of the SE interface, while the previous study by Serkies et al. did not show any changes in the mechanical properties during the same time frame.

Overall, SHSE had less effect on biofilm biomass vs. bacterial penetration: increased bacterial penetration was observed in most SHSE incubated groups, but only one group (SE+Z vs. SE+D) showed more bacterial biomass after 180 days, indicating that there is little correlation between the depth of marginal gaps as indicated by bacterial ingress and interfacial biofilm formation as indicated by biomass. This is not surprising, since biofilm formation is multifactor-dependent process, relying not only on sufficient space, but also the interfacial surface morphology, contents (nutrients), and environmental signals (Cvitkovitch 2003; O'Toole et al. 2000). Due to the positive relationship of the presence and abundance of cariogenic bacteria and caries formation (Loesche 1982; Zinner et al. 1965), it could be hypothesized that although the depth of interfacial margins is a good indicator of marginal breakdown, bacterial adhesion and biofilm formation is more important to predict the restoration longevity. It can be concluded, therefore that in future studies, multiple indicators should be employed to demonstrate the effect of SHSE on interfacial integrity, strengthening the correlation of interfacial integrity with restoration longevity.
6.2.4.3 The Effect of MMP Inhibition on Interfacial Degradation

Since dentinal MMPs were shown to degrade dentinal collagen and compromise the restoration-tooth interface, it was hypothesized that MMP inhibition will reduce this degradation and enhance the biostability of the interface. Galardin, as a broad MMP inhibitor against dentinal MMP-1, -2, -3, -8 and -9 (Breschi et al. 2010; Sulkala et al. 2007), enhanced marginal biostability, as indicated by the reduced interfacial bacterial ingress and reduced bacterial biofilm biomass, providing evidence of the contributing role of MMPs in marginal degradation and accrediting the application of MMP inhibitor to interfacial preservation. This positive effect may result from the immediate inhibition after the activation of MMPs by acid etching in total-etch adhesive application (Breschi et al. 2010) and the inhibitory effect of galardin on SHSE activity used in the current study. Although an overall inhibitory effect of galardin on SHSE was observed, the interfaces of the two restorative materials, Z250 and Dyract-eXtra responded differently to MMP inhibition over time. It could be hypothesized that the different materials' chemical and physical properties have particular impact on the conformational changes of galardin and on the interaction between galardin and MMPs (Serkies et al. 2016). In addition, the inhibitory effect of galardin on bacterial ingress was not consistent with the effect on biofilm biomass. This finding emphasized the fact that the interfacial degradation is not a simple process causing deepening of the marginal gap, but also changing interfacial geometry, morphology and even chemical content such as the accumulation of degradation by-products which has impact on bacterial growth and metabolism (Khalichi et al. 2004).

6.2.4.4 The Effect of Restorative Materials on Interfacial Degradation

In addition to biological factors such as esterases activities and MMPs, the chemical properties of restorative materials also play important roles in the biostability of resin-dentin interfacial integrity.
Self-etch bonded Z250 interface showed increased biofilm biomass compared to the total-etch counterparts. This may result from the formulation of self-etch adhesive that is inherently hydrophilic (Bourbia et al. 2013). The presence of residual water within the hybrid layer may lead to void formation and yield a lower degree of monomer conversion, thereby facilitating degradation and providing adequate space between dentin and restorative materials for biofilm formation (Chersoni et al. 2004; Miyazaki et al. 2003). In addition, the hydrophilic nature of the monomers in self-etch adhesive facilitates their elution into resin-dentin interface, thereby forming a vesicular-structure polymer which provides the scaffold for establishing the biofilm (Khalichi et al. 2004). This indicated that the chemical formulation and properties of the adhesive materials had an influential effect on bacterial adhesion and biofilm formation in addition to increasing the size of the interfacial space. Serkies et al. (Serkies et al. 2016) reported the facture planes of self-etch created interface transitioned over this period from the bottom of the hybrid layer to the adhesive/resin composite. It was hypothesized that this was due to the plasticizing effect of the media on the hydrophilic adhesive. The transition in the fracture plane could provide access to salivary and bacterial esterases, needed to degrade the resin-based substrates, and thereby producing degradation by-products that up-regulate genes associated with growth and biofilm formation (Sadeghinejad et al. 2017; Sadeghinejad et al. 2016). This could explain the increase in bacterial abundance that was found for the SE-bonded interfaces.

Dyract-eXtra, as one of few antimicrobial material claimed to combine the mechanical and esthetic properties of composites with the fluoride-releasing advantage of glass-ionomer cements (Nicholson 2007), showed positive effect on the prevention of bacterial penetration and reduced bacterial viability compared to traditional resin composites. However, it did not reduce interfacial biofilm biomass. Fluoride release from restorative materials reduce bacterial metabolism and
proliferation (Marquis et al. 2003), thus inhibiting bacterial penetration and decreasing bacterial viability, but could be incapable of preventing the process of initial bacterial adhesion, which explains the positive effect of Dyract-eXtra on biofilm biomass (Auschill et al. 2002). In addition, there were variable effects of Dyract-eXtra on biofilm biomass when utilizing different adhesive systems or incubating in SHSE media. This could be the result of the altered environmental conditions within the Dyract-dentin interface, reported as one of several factors that affect fluoride release (Wiegand et al. 2007), by the different compositions and hydrophilicity of the adhesives materials and their susceptibility to the incubation medium.

6.2.4.5 Interactions between SHSE, Galardin and the Restorative Materials

To fully understand the biological and chemical factors of resin-dentin interfacial integrity, the interactions among the MMP inhibitor galardin, esterase activities and the dental materials were also investigated. The inhibitory effect of galardin on CE activity was observed after 12 hours. However, while this was statistically significant, the relatively small overall reduction of CE activity by galardin is unlikely to be sufficient to clinically influence SHSE-induced degradation since the level of CE used was in the saturation level of this enzyme (Finer and Santerre 2003). The reduced PCE activity was mainly caused by the galardin carrier solvent DMSO due to the DMSO-water bonds in the active sites of enzymes that influences their activity (Faulds et al. 2011), dehydration of enzymes (Griebenow and Klibanov 1995) and the influence on substrate properties (Faulds et al. 2011). Overall, it is unlikely that galardin could affect interfacial degradation by inhibiting PCE activity due to the non-dominant degradative effect of PCE on resin composites (Finer and Santerre 2004a).

Short-term reductions were observed for both CE and PCE activities when incubated with both restorative materials. Overall, there was no significant effect of CE activity between Z250 and
Dyract-eXtra after 5 days. Since CE activity is the dominant factor influencing resin-based materials degradation (Finer and Santerre 2004a), the material-SHSE interaction has little contributing role to explain long-term effects of different materials on interfacial degradation rates. This investigation is still however, valuable since it shows that dental materials can inhibit enzyme decelerating degradation process and clarifies and emphasizes the importance of interactions between materials and oral environmental enzymes.

6.2.5 Experimental Model
The other important contribution of this dissertation is the establishment and validation of a new in vitro model to assess the resin-dentin interfacial integrity. Reports on the effect of materials on interfacial integrity have been controversial. While in vitro studies have reported on the potential impact of material chemistry on resin composite degradation (Ferracane 2016; Finer and Santerre 2004b; Krämer et al. 2015), some clinical studies have reported only a minor influence of material properties on the restoration longevity (Demarco et al. 2012; Ferracane 2016). This raises the question about the relevancy of current laboratory methodologies and their correlations with clinical performance of the restorations. This CBBF-based continuous culture device established in this study strengthened the relevancy of current laboratory methodologies and their correlations with clinical performance of the restorations by maintaining clinical-relevant chemical and biological components such as pH, temperature, carbohydrate source, and bacterial species composition to reproduce factors that are associated with interfacial cariogenic bacterial growth (Drake and Brogden 2002; Novick and Szilard 1950; Roth et al. 2012).

In addition, the device and parameter settings are strict and well-controlled, so the acquired results are highly reproducible and representative of the oral cavity (Drake and Brogden 2002). These advantages make this model suitable to reproduce conditions that exist in the oral cavity for future
dental materials investigations. It is considered more appropriate and clinically relevant to use biofilm formation to indicate the interfacial integrity instead of wall lesion or dentin mineral loss as reported in most of the current studies (Kuper et al. 2014; Kuper et al. 2015; Nassar and González-Cabezas 2011; Totiam et al. 2007), since the word “wall lesion” is not uniformly defined in the literature and the detection methods have various sensitivities and specificities (Kuper et al. 2014; van de Sande et al. 2014). In addition, unlike mechanical testing, the current study demonstrated that this detection method is more sensitive in the detection of interfacial changes by using a combination of bacterial penetration, biofilm formation, and live/dead parameters that provide more sufficient information for future material testing.

Furthermore, the analysis and quantification methods of CLSM included in this model is considered more suitable for imaging biological components within the resin-dentin margin, compared to the traditional SEM, the requires destructive preparation steps prior to imaging such as dehydration and fixation which alters biofilm morphology (Serkies et al. 2016; Shokati et al. 2010). Overall, the current study is the first one to quantify the biofilm formed in the restoration-tooth interface as a measure of interfacial integrity, by employing reconstructed 3D images and sophisticated quantification software. This image analysis method allows for future use to investigate bacterial performance in the interface from different perspectives presented as bacterial ingress, biofilm formation and vitality.

6.2.6 Conclusive Remarks

This dissertation investigated factors that could influence the biostability of resin-dentin interfaces, considered the “weak link” of the restorations and as such determining the restorations’ longevity (Spencer et al. 2010). These interfaces undergo continuous and inevitable degradation that is catalyzed by salivary and bacterial esterases as well as dentinal MMP activities. This dissertation
also elucidated on the mechanistic relationship between oral bacteria and resin-based materials, specifically on the interaction between bacterial enzymatic activity and restorative materials and the reciprocal effect of the materials on the bacteria, both play a determinant role in resin-based restoration’s failure. In addition, since the *in vitro* model established in this study, provided information about bacterial performance in a more relevant manner for the prediction of caries formation and progression than other measures currently in use, it has the potential to be employed in future investigations of a comprehensive approach to enhance the performance of resin-based materials by enhancing their biostability, anti-degradative and antimicrobial properties, as well as providing clinicians with practical means to enhance the performance of these restorations.
Chapter 7

Conclusions and Recommendations

7.1 Conclusions

- In addition to virulence factors associated with biofilm formation, acid production and acid tolerance, *S. mutans* produces an esterase, SMU_118c that hydrolyzes methacrylate-based resin monomers and polymerized resin composite, while retaining its activity at cariogenic pH, potentially contributing to the degradation of the restoration-tooth interface and acceleration of the restoration’s failure rate.

- The biodegradation by-product BisHPPP up-regulated the esterase at both mRNA (*SMU.118c*), and the encoded esterase protein, SMU_118c. These findings suggest that BBP from the universal component of dental composites and adhesives, BisGMA activates the bacterial esterase gene and protein in a positive feedback loop, which could result in more rapid degradation of resin composites and adhesives, compromising the restoration-tooth interface and potentially increasing the rate of secondary caries and failure of resin-based restorations.

- Regardless of incubation media, MMP inhibition, restorative material, and mode of adhesion, longer incubation periods resulted in increased interfacial penetration and bacterial cell count. This indicated that the degradation of the restoration-tooth interface is a continuous multi-factorial dependent process that could ultimately result in the restoration’s failure.

- Bacterial penetration was increased following incubation with SHSE vs. PBS along both traditional composite and PMCR boned interfaces, confirming the contribution of salivary esterases activities to the degradation of resin-dentin interfaces, for both adhesion modes.
• SE interfaces bonded to traditional composites (SE-Z) showed an increase in interfacial bacterial cell-count vs. their TE (TE-Z) and PMCR counterparts (SE-D). TE interfaces bonded to traditional composite (TE-Z) showed reduced bacterial biofilm cell-count vs. their PMCR counterparts (TE-D). Further, only SE interfaces bonded to traditional composites showed an increase in interfacial bacterial biofilms after incubation in SHSE vs. PBS after 180 days. These observations indicate a material- and adhesion-mode dependence of interfacial degradation.

• The application of MMP-inhibitor (galardin) to TE interfacial dentin after the etching step in total-etch adhesive application enhanced marginal biostability, as indicated by the reduced interfacial bacterial ingress and reduced bacterial biofilm cell-count, providing evidence to the contributing role of dentinal MMPs in marginal degradation.

• The reduced interfacial bacterial ingress and bacterial viability at the PMCR/dentin interface vs. the traditional composite/dentin interface could be associated with the PMCR’s antimicrobial chemistry and highlight the potential benefit of this approach.

7.2 Recommendations

• Manufacturers and material scientists should employ the in vitro model established in this study to investigate new materials performance in a more physiologically-relevant manner for the prediction of materials biostability and caries formation and progression.

• Future studies should use multi-species biofilms to test the effects of BBPs on the virulence gene expression in S. mutans to simulate the bacterial community in the oral environment and to explore the possible effect of bacterial interactions on S. mutans UA159 genotypic and phenotypic response to the BBPs.
• Although there is no up-regulated trend found in 16S rRNA expression, it’s hard to normalize the expression of interested genes to that of a housekeeping gene. To solve this, in the future, the probe targeting mRNA of gene of interested should be designed with similar length and GC content as probes for control group enabling simultaneously hybridization under the same hybridization condition in the same biofilm.

• Dentinal collagen degradation by endogenous MMPs have been reported on as having strong correlation to caries formation (Buzalaf et al. 2012; Hedenbjork-Lager et al. 2015; Mazzoni et al. 2015). Based on NCBI search through S. mutans genome database, there are putative collagenase or gelatinase genes (Ajdić et al. 2002), which could be contributors to resin-dentin interfacial degradation. Future studies should investigate the whole bacterial collagenolytic activity of S. mutans to the collagen degradation in the interface and further explore the collagenase or gelatinase properties and potential impact on caries formation and development. This is important because the ability of the bacteria to degrade dentinal collagen would results with compromised margins even when more biostable materials are used.

• The above highlight the importance of developing materials that are not just biostable, but also have antidegradative and antimicrobial properties.
Appendices A

Biofilm Quantification by Imaris

Two methods have been developed for biofilm quantification: Iso-surfaces counting and Spot counting. Both process image and calculate cell numbers depend on fluorescence intensity and cell size. Biofilm was stained with Live/Dead Backlight Bacterial Viability Kit (Molecular Probes, Eugene, Oregon, USA). High quality image acquired by 63x water immersion lens with NA/1.2 (Fig. A.1A) and the detailed parameters of original image are listed in Figure A.1B. The accordingly processed images are presented in Figure A.1A and A.1B, separately. The results of these two different counting methods are shown in table A.1. The parameters of surfaces counting method and spots counting are separately listed in table A.1 and A.2. The accordingly processed images are presented in Figure A.2 and A.3, separately. The results of these two different counting methods are shown in table A.3.

Figure A.1: Original image of \textit{S.mutans} biofilm (A) and detailed parameters of image acquisition (B).
Table A.1: Parameters of surface counting method.

<table>
<thead>
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<th>RED SURFACE SEGMENTATION</th>
<th>GREEN SURFACE SEGMENTATION</th>
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</thead>
<tbody>
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</tr>
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</tr>
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<td>[Source Channel]</td>
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<td>[Classify Surfaces]</td>
</tr>
<tr>
<td>&quot;Number of Voxels&quot; above 10.0</td>
<td>&quot;Number of Voxels&quot; above 10.0</td>
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</tbody>
</table>
Figure A.2: Processed image of *S. mutans* biofilm by surface segmentation.

Table A.2: Parameters of spots counting method.

<table>
<thead>
<tr>
<th>RED SPOTS SEGMENTATION</th>
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<tbody>
<tr>
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<tr>
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<td>[ROI]</td>
</tr>
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<td>Region 1: XYZT from [257 257 1 1] to [768 768 37 1]</td>
<td>Region 1: XYZT from [257 257 1 1] to [768 768 37 1]</td>
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</table>
Figure A.3: Processed image of *S.mutans* biofilm by spots segmentation. Red = bacteria segmented as surfaces; yellow = green bacteria within 1 um of red bacteria; cyan = green bacteria further than 1 um from red bacteria

Table A.3: Cell count comparison between surfaces & spots.

<table>
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<tr>
<th>Object</th>
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<th>%Of total cell count</th>
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<tr>
<td>green spots</td>
<td>8161</td>
<td>86</td>
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<tr>
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<tr>
<td>green surfaces</td>
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<td>84</td>
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<tr>
<td><strong>Total Cells</strong></td>
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Appendices B

Protocol for FISH

Protocol for 16s rRNA FISH
(Gram-positive +, *S. mutans* UA 159)

Probe STR 405:

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<tr>
<th>Sequence</th>
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<th>5’-fluorochrome</th>
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</thead>
<tbody>
<tr>
<td>5′-TAGCCGTCCCTTCTGGT-3′</td>
<td><em>Streptococcus</em> spp 16s rRNA</td>
<td>Alexa Fluoro 350 Ex/Em: 343/442 nm</td>
</tr>
</tbody>
</table>

**Biofilm Formation:** *S. mutans* UA 159 overnight culture, 1:100 inoculate to 400μl media (Chemically-defined Media: CDM) in 8-well chamber (Lab-Tek camber); incubate in 5% CO₂ at 37°C 4 hours to form biofilm.

**Live/Dead Stain:** The 4-hour biofilm is stained by Live/Dead staining kit (two components: Cyto 9 and propidium iodide).

**Fixation:** remove culture fluid from chambers. Add 400μl of 4% PFA solution in the chamber. Leave for at least 12h at 4°C.

The 4% PFA solution should be made every week: dilute in sterile mqH₂O from the stock solution (20% PFA) which is stored in anaerobic conditions and protected from light. The stock solution can be kept for 1 year.

**Washing#1:** remove fixating solution. Inoculate 200μl of cold sterile PBS at room temperature.

**Permeabilization#1:** remove PBS, inoculate 200μl 0.5% SDS and incubate at 37°C for 15 min; discard the SDS solution, then inoculate 200μl of 10mg/ml lysozyme solution and incubate at 37°C for 30 min.

Lysozyme solution should be prepared every week: dissolve 15mg lysozyme powder in 1ml mqH₂O + 250ul 1M TRIS Buffer + 250ul 50mM EDTA, filter sterilize. Final concentration of Lysozyme solution (10mg/ml).

**Washing#2:** remove lysozyme solution. Inoculate 300μl of Mili-Q at room temperature.

**Dehydration:** Inoculate 200μl of ethanol at concentrations of 50% and 99%; for 3 min each at room temperature. Let slide dry for 10min in vertical position.

**Preparation of probes:** Probe: STR405, Alexa Fluoro 350 fluorescent tag labeled; Ex/Em: 343/442; stored at -20°C at concentration of 1000ng/μl.

**Hybridization:** inoculate 3ul probe in 57ul hybridization buffer (50ng/μl) in each well, then place
in humid incubator at 48°C for 3 hours.

**Removal of unbound probe:** remove hybridization buffer. Inoculate 300μl of washing buffer. Place slides in a humid chamber and incubate at 48°C for 30min.

**Washing#3:** remove washing buffer. Inoculate 200μl of Mili-Q at room temperature.

**Microscopy:** LEICA Confocal Laser Scanning Microscope
White Light Laser
40X (NA 1.2) water immersion objective lens

### Protocol for mRNA of *smu_118c* FISH
*(Gram-positive +, *S.*mutans UA 159)*
**SMU_118 probe:**

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<table>
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<tbody>
<tr>
<td>mRNA of <em>SMU_118c</em> in *S.*mutans UA 159</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>5′ –fluorochrome</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa Flou 488 fluorescence labeled <strong>Ex/Em: 490/520 nm</strong></td>
</tr>
</tbody>
</table>

**Biofilm Formation:** *S.*mutans UA 159, *SMU_118c* K/O stain and *SMU_118c* OE strain overnight culture, 1:100 inoculate to 400μl media (Chemically-defined Media: CDM) in 8-well chamber (Lab-Tek camber); incubate in 5% CO₂ at 37°C 18 hours to form biofilm.

**Live Cells Stain:** The 18-hour biofilm is stained by dihydroethidium.

**Fixation:** remove culture fluid from chambers. Add 400μl of 4% PFA solution in the chamber. Leave for at least 12h at 4°C.
The 4% PFA solution should be made every week; dilute in sterile mqH₂O from the stock solution (20% PFA) which is stored in anaerobic conditions and protected from light. The stock solution can be kept for 1 year.

**Washing#1:** remove fixating solution. Inoculate 200μl of cold sterile PBS at room temperature.
**Permeabilization#1:** remove PBS, inoculate 200μl 0.5% SDS and incubate at 37°C for 15 min; discard the SDS solution, then inoculate 200μl of 10mg/ml lysozyme solution and incubate at 37°C for 30min.
Lysozyme solution should be prepared every week: dissolve 15mg lysozyme powder in 1ml mQH₂O + 250ul 1M TRIS Buffer + 250ul 50mM EDTA, filter sterilize. Final concentration of Lysozyme solution is 10mg/ml.

**Washing#2:** remove lysozyme solution. Inoculate 300μl of Mili-Q at room temperature.

**Dehydration:** Inoculate 200μl of ethanol at concentrations of 50% and 99%; for 3 mins each at room temperature. Let slide dry for 10min in vertical position.

**Preparation of probes:** Probe: SMU 118c, Alexa Fluoro 488 fluorescent (green) tag labeled; Ex/Em: 490/520 nm; stored at -20°C at concentration of 1000ng/μl.

**Hybridization:** inoculate 3ul probe in 57ul hybridization buffer (50ng/μl) in each well, then place in humid incubator at 38°C for 12 hours.

**Removal of unbound probe:** remove hybridization buffer. Inoculate 300μl of washing buffer. Place slides in a humid chamber and incubate at 48°C for 30min.

**Washing#3:** remove washing buffer. Inoculate 200μl of Mili-Q at room temperature.

**Solutions for FISH**
All solutions should be stored at 4°C, except SDS (Room Temperature)

**FIX (60 ml)**
Work in fume hood when handling paraformaldehyde!
Mix 1.8 g of 100% (or 1.9 g of 95%) paraformaldehyde with MiliQ water and 0.5 ml 1M NaOH (depolymerize) to give 45ml, 4% paraformaldehyde solution. Stir while heating the solution to 60°C (depolymerize) until paraformaldehyde has completely dissolved. **Do not** heat above 60°C. Mix 3 parts of 4% paraformaldehyde solution (45ml) with 15 ml sterilized PBS, and then adjust pH to 7.5 to get Fixation Solution.

**1M Tris-HCl (50ml)**
Mix 7.88g Tris-HCl into 50ml UHQ-water and adjust pH to 7.5 with NaOH.

**0.1% SDS (100ml)**
Mix 0.1g SDS powder in 100ml UHQ-water. (0.1%: W/V)

50mM EDTA (50ml)
Mix 0.93g EDTA disodium salt in 50ml water. Adjust pH to 8.0 with NaOH.

**Lysozyme solution (50ml)**
Lysozyme solution should be prepared every week.
Option 1:
70U/μl lysozyme (Fluka) 36.3g
100mM Tris-HCl 5ml of 1M Tris-HCl, pH 7.5
5mM EDTA 5ml of 50mM EDTA, pH 8.0
Option 2:
Dissolve 60mg lysozyme powder in 4ml mqH$_2$O + 1ml 1M TRIS Buffer + 1ml 0.5M EDTA, filter sterilized.
Final concentration of Lysozyme solution is 10mg/ml.

**Ethanol Dilutions in PBS**

<table>
<thead>
<tr>
<th>Percentage</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>99.5%</td>
<td></td>
</tr>
<tr>
<td>80%</td>
<td></td>
</tr>
<tr>
<td>50%</td>
<td></td>
</tr>
</tbody>
</table>

**Hybridization Buffer (50ml)**
Work in fume hood while handling formamide!

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9M NaCl</td>
<td>2.63g</td>
</tr>
<tr>
<td>50% formamide (toxic)</td>
<td>25 ml (V/V)</td>
</tr>
<tr>
<td>20mM Tris-HCl</td>
<td>1ml of 1M Tris-HCl, pH 7.5</td>
</tr>
<tr>
<td>0.01%SDS</td>
<td>5ml of 0.1% SDS</td>
</tr>
<tr>
<td>UHQ-water</td>
<td>31.5ml</td>
</tr>
</tbody>
</table>

**Washing Buffer (50ml)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>20mM Tris-HCl</td>
<td>1ml of 1M Tris-HCl, pH 7.5</td>
</tr>
<tr>
<td>5mM EDTA</td>
<td>5ml of 50mM EDTA, pH 8.0</td>
</tr>
<tr>
<td>159mM NaCl</td>
<td>0.465g</td>
</tr>
<tr>
<td>0.01%SDS</td>
<td>5ml of 0.1% SDS</td>
</tr>
<tr>
<td>UHQ-water</td>
<td>40ml</td>
</tr>
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Appendices C

Esterase Activity of Different Fractions of *S. mutans*

Materials and Methods

**Acquisition of Supernatant, membrane and intracellular component of *S. mutans***

1. Overnight culture of *S. mutans* was 1:100 inoculated in 50 ml of THYE medium (N=4).
2. Stop culture growth in the mid-log stage (OD=0.6)
3. Concentrate *S. mutans* cells by centrifugation at 10,000 x g for 15 min at 4 °C.
4. Collect the supernatants and keep the pellets for further acquisition of cell membrane and intracellular components.
5. Add three volumes of ice cold PBS to the washed cell pellet (begin with approximately 10–40 mg dry weight cells) and vortex to re-suspend cells. Then, add 20U endonuclease and appropriate phosphatase and protease inhibitors.
6. Subject cell mixture to three times of tip-probe sonication (leval 8, 15sec/round) (MicroPulser, Bio-RAD)
7. The membranes were then deposited by centrifugation at 0°C for 30 min at 20000 × g in a super-speed centrifuge.
8. The supernatant fractions were collected as intracellular components
9. and the firmly packed pellets of membranes were washed three times at 0°C with cold PBS then re-deposite at 45000 × g for 30 min.
10. The packed membrane fractions were washed three times at 0°C with cold PBS then finally re-suspend in 2 ml of PBS for further esterase activity analysis.

**Esterase Activity Analysis**

1. Esterase activity was determined by incubating 1 ml of the suspension of three different fractions (N=3/group) and 500 μl of the prepared p-NPB substrate.
2. Esterase-like activity was normalized to dry weight of each fraction.
3. 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 (OD) 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 (OD/min). CE activity was calculated to the following equation:

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

Where:
- \(\text{O.D}\) = change in absorbance per minute at 401 nm
- \(\text{T.V}\) = Total Volume (0.0015L)
- \(\text{E.C}\) = molar absorptivity of p-nitrophenol at 401 nm (16000 M\(^{-1}\)cm\(^{-1}\))
- \(\text{L.P}\) = Length Path (1cm)
- \(\text{D.W}\) = Dry Weight of bacterial cells

**Results and Discussion**

Based on a previous report *S. mutans* UA159 had no PCE-like activities (no activity towards BTC substrate) but highest CE-like activity towards pNPB (Bourbia et al. 2013), as a result, pNPB was selected to test esterase activity of different bacterial fractions (Supernatant, membrane and intracellular). One-way ANOVA followed by Tukey’s post-hoc analysis demonstrated significant differences among the three bacterial fractions: the highest activity was found from intracellular components that is in an agreement with SMU_118c protein structure analysis which predicted SMU_118c is an intracellular protein without secretion signal peptide; there is low activity observed from supernatant while membrane showed no activity compared to the PBS control (p<0.01) (Figure C.1).
Figure C.1: CE-like activity of bacterial fractions: supernatant, membrane and intracellular (N=3/group; data are reported as mean ± standard deviation). Values with different letters indicate statistically significant differences (p<0.05).
Reference


Drake DR, Brogden KA. 2002. Continuous-culture chemostat systems and flowcells as methods to investigate microbial interactions.


