

December 2016

The Effects of Orthodontic Appliance Base Plate Material, PMMA, Infused with Silver and a Novel Antibacterial Compound On Biofilm Formation

Ryan D. Jolley

University of Nevada, Las Vegas, jolleyR5@unlv.nevada.edu

Follow this and additional works at: <https://digitalscholarship.unlv.edu/thesesdissertations>



Part of the [Dentistry Commons](#)

Repository Citation

Jolley, Ryan D., "The Effects of Orthodontic Appliance Base Plate Material, PMMA, Infused with Silver and a Novel Antibacterial Compound On Biofilm Formation" (2016). *UNLV Theses, Dissertations, Professional Papers, and Capstones*. 2868.

<https://digitalscholarship.unlv.edu/thesesdissertations/2868>

This Thesis is protected by copyright and/or related rights. It has been brought to you by Digital Scholarship@UNLV with permission from the rights-holder(s). You are free to use this Thesis in any way that is permitted by the copyright and related rights legislation that applies to your use. For other uses you need to obtain permission from the rights-holder(s) directly, unless additional rights are indicated by a Creative Commons license in the record and/or on the work itself.

This Thesis has been accepted for inclusion in UNLV Theses, Dissertations, Professional Papers, and Capstones by an authorized administrator of Digital Scholarship@UNLV. For more information, please contact digitalscholarship@unlv.edu.

THE EFFECTS OF ORTHODONTIC APPLIANCE BASE PLATE MATERIAL,
PMMA, INFUSED WITH SILVER AND A NOVEL ANTIBACTERIAL COMPOUND
ON BIOFILM FORMATION

by

Ryan D. Jolley

Bachelor of Science
Brigham Young University
2000

Doctorate of Medical Dentistry
University of Nevada Las Vegas
2014

A thesis submitted in partial fulfillment
of the requirements for the

Master of Science -- Oral Biology

School of Dental Medicine
Division of Health Sciences
The Graduate College

University of Nevada, Las Vegas
December 2016



Thesis Approval

The Graduate College
The University of Nevada, Las Vegas

November 4, 2016

This thesis prepared by

Ryan D. Jolley

entitled

The Effects of Orthodontic Appliance Base Plate Material, Pmma, Infused with Silver
and a Novel Antibacterial Compound On Biofilm Formation

is approved in partial fulfillment of the requirements for the degree of

Master of Science – Oral Biology
School of Dental Medicine

Katherine Howard, Ph.D.
Examination Committee Chair

Kathryn Hausbeck Korgan, Ph.D.
Graduate College Interim Dean

Karl Kingsley, Ph.D.
Examination Committee Member

James K. Mah, D.D.S.
Examination Committee Member

Jennifer Pharr, Ph.D.
Graduate College Faculty Representative

ABSTRACT

The effects of orthodontic appliance base plate material, PMMA, infused with silver and a novel antibacterial compound on biofilm formation

By

Dr. Ryan Jolley

Dr. Katherine Howard, Research Committee Chair
University of Nevada, Las Vegas

Retention is required in the majority of orthodontic patients throughout the remainder of their life. The traditional Hawley style retainer is still considered by many orthodontic professionals to be the gold standard in retention appliances. The orthodontic population, in general, is at a higher risk for caries due to plaque accumulation from poor diet, suboptimal oral hygiene, and often a lack of motivation. A Hawley retainer that reduces caries-causing oral microbes could improve oral health and be very beneficial to the post-orthodontic patient. Developing methods to safely include antibacterial products within the Hawley retainer's base plate material could help effect these improvements and prove to be an additional incentive for continued, regular wear of orthodontic retainers.

The oral bacteria most commonly associated with caries formation, *Streptococcus mutans*, was chosen to study its biofilm formation on polymethyl methacrylate (PMMA) that is used in the fabrication of traditional Hawley retainers. Biofilm formation was evaluated using a drip flow reactor system followed by staining, scanning electron microscopy (SEM) and morphology

analysis. Growth was assessed on four material types: PMMA, PMMA with silver, PMMA with a novel antibacterial agent, CZ-99, and PMMA with silver and CZ-99.

SEM images were analyzed using a combination of software products including Fiji (Image J), Matlab and MountainMaps 3D generating software (Digital Surf) to determine percentage area coverage and compared between samples. PMMA that contained silver prevented biofilm growth to the greatest degree. However, although biofilm formation was more pronounced on PMMA infused with CZ-99 a combination of CZ-99 and silver, it was found that this was likely due to poor cross-linkage of the polymer that allowed the antimicrobials to elute out fully.

In conclusion, results demonstrated significant reduction in biofilm formation in silver infused PMMA. Outcomes provided a compelling argument that the drip flow system and materials could be investigated further to determine if antibacterial PMMA can be used safely in the oral environment and explore their effectiveness in reducing biofilm formation. Future work with *in vivo* testing would be particularly beneficial.

ACKNOWLEDGEMENTS

First, I would like to thank the members of my committee for providing efforts and knowledge to this project. I would like to thank Dr. Howard, my committee chair, and Dr. Mah, my Orthodontic Department Chair, for your continuous support with this research. I would like to thank Dr. Dustin Williams for devoting much of his valuable time and resources to this project. Without the access to his ideas, laboratory, and specialized equipment, this project could never have been realized. Special thanks goes to the expert supervisors and technicians of Dr. Williams' laboratory: Tyler Epperson, Jeff DeGrauw, Li (Lousili) Cadenas, Nick Taylor and Mattias Nielson. I would like to thank Dr. Kingsley for help both in obtaining the necessary materials and interpreting the data that was collected. Additionally, I would like to thank Dr. Howard for her time and many efforts with this project. She was patient and willing to take on my ideas and help me make them a reality. Without her this project would not have been possible.

I want to thank my family for standing by my side throughout my continued education and providing me the opportunities and the time required to succeed in this project and in all of my academic endeavors. I am married to an amazing woman who is perfect for me. I have six wonderful children who have been very supportive of their dad. I am grateful for their confidence and their prayers on my behalf. I am grateful to my parents for believing in me and supporting me in my midlife career crisis. Thank you for teaching me to work as if everything depends on me but to pray as if everything depends on God.

TABLE OF CONTENTS

IRB EXEMPTION	ii
ABSTRACT	iii
ACKNOWLEDEMENTS	v
LIST OF FIGURES	vii
LIST OF DEFINITIONS	viii
CHAPTER 1: INTRODUCTION	
Purpose of the Study.....	1
Research Questions	2
Hypothesis and Aims	3
CHAPTER 2: REVIEW OF RELATED LITERATURE	
Caries	4
<i>Streptococcus mutans</i>	6
Biofilm Formation	7
Orthodontics Considerations	9
Retention's Role.....	11
The Hawley Retainer.....	13
Antibacterial Dental Materials.....	14
Silver.....	16
Biofilm Infection and Development of CZs.....	18
Biomaterial Implications	21
CHAPTER 3: METHODOLOGY	
Experimental Design Summary	26
PMMA Coupon Preparation	26
Bacterial Culture and Preparation	29
Drip Flow Reactor Setup and Materials Testing	30
SEM Imaging	31
Surface Morphology and Roughness	33
Statistical Analysis	33
CHAPTER 4: RESULTS	
Surface Morphology and Roughness	35
Primary Outcomes	40
Surface Coverage Analysis by %	43
SEM Imaging of Biofilms	45
Biofilm Plume Height.....	47
CHAPTER 5: DISCUSSION AND CONCLUSION	
Discussion	52
Limitations.....	54
Technical Setup	55
Recommendations	57
Conclusion	59
APPENDIX	60
BIBLIOGRAPHY	61
CURRICULUM VITAE	80

LIST OF FIGURES

Figure 2.1: The Hawley retainer

Figure 2.2: Progression of CZ synthetic development

Figure 2.3: AFM used to determine the method of action of CZ-99

Figure 3.1 Fabrication of PMMA coupons

Figure 3.2 Drip flow reactor chamber setup

Figure 3.3 Modified drip flow reactor setup

Figure 4.1: SEM image of PMMA control material

Figure 4.2: SEM image of PMMA with silver benzoate

Figure 4.3: SEM image of PMMA with CZ-99

Figure 4.4: SEM image of PMMA with CZ-99 and silver benzoate

Figure 4.5: Topographical imaging and Ra for control PMMA material

Figure 4.6: Topographical imaging and Ra for PMMA with CZ-99

Figure 4.7: Topographical imaging and Ra for PMMA with CZ-99 and silver benzoate

Figure 4.8: Topographical imaging and Ra for PMMA with silver

Figure 4.9: Photographic images of positive control PMMA coupons with ruthenium red

Figure 4.10: Photographic images of PMMA with CZ-99

Figure 4.11: Photographic images of PMMA with CZ-99 and silver benzoate

Figure 4.12: Photographic images of PMMA with silver benzoate

Figure 4.13: Providing contrast to perform the % surface coverage analysis

Figure 4.14: % coverage bar graph for each material type

Figure 4.15: SEM images of *S. mutans* biofilms grown on control PMMA at low and high magnifications

Figure 4.16: SEM images of *S. mutans* biofilms grown on PMMA with CZ-99 at low and high magnifications

Figure 4.17: SEM images of *S. mutans* biofilms grown on PMMA with CZ-99 and silver benzoate at low and high magnifications

Figure 4.18: SEM images of *S. mutans* on biofilms grown on PMMA with silver only

Figure 4.19: Reconstructed 3D image of control PMMA surface with no growth to act as a baseline for comparison

Figure 4.20: Reconstructed 3D image of PMMA surface with biofilm growth

Figure 4.21: Reconstructed 3D image of PMMA with CZ-99 incorporated

Figure 4.22: Reconstructed 3D image of PMMA with CZ-99 and silver incorporated

Figure 5.1: Original drip flow reactor setup

LIST OF DEFINITIONS

ADME: Absorption, distribution, metabolism, and excretion. Factors used for drug profiling

ATCC: American Tissue Type Culture Collection, a Manasa, Virginia based company that supplies bacterial isolates

BHI: Brain heart infusion

CDC: Centers for Disease Control

CFU: Colony forming units

CHG: Chlorhexidine gluconate

CZ-99: Based on the company name, Curza, a novel antibacterial product that has shown the ability disperse and kill bacterial biofilms

DMAEMA: Dimethylamioethyl methacrylate, an organic solvent

L. acidophilus: *Lactobacillus acidophilus*, a gram positive rod common in the normal flora of the oral cavity that can be associated with dental caries

PBS: Phosphate-buffered saline

PMMA: Polymethyl Methacrylate, an acrylic base plate material commonly used in Hawley retainers, denture and other dental applications

Ra: Roughness average

SEM: Scanning electron microscopy

S. mutans: *Streptococcus mutans*, gram positive cocci frequently associated with the initiation of dental caries in the oral cavity

CHAPTER 1

INTRODUCTION

1.1 The Purpose of the Study

After orthodontic treatment teeth have a tendency to revert back to their pre-treatment positions making retention a requirement for as long as the patient wishes to have straight teeth (Parker, W.S. 1989). There are many proponents who believe that the retention phase of orthodontics is a life-long commitment (Melrose, C. 1998). The Hawley retainer is a tissue-born retainer that incorporates a base plate made of polymethyl methacrylate (PMMA). On a world-wide basis, this style of retainer remains the most widely used type of orthodontic retainer (Profitt, W. 2012). Despite the extensive and prolonged use of orthodontic retainers, relatively little research has been conducted examining orthodontic retainers' close relationship with biofilm formation in a caries-prone population such as teenage orthodontic patients.

The microbial species *Streptococcus mutans* was selected for testing due to its intimate role in caries formation and for its predominant use in caries studies (Birdsall, J. 2008). Since *S. mutans* is implicated in the base formation of the polymeric surface to which other bacterial species attach and exacerbate caries formation, if bacterial biofilm formation of this isolate were decreased using an antibacterial-infused PMMA material, the antibacterial PMMA could be used to fabricate Hawley retainers for patients who are frequently at high risk for caries.

Thus, the goal of this research was to determine if the addition of antibacterial compounds to PMMA base plate material could be employed to reduce biofilm formation with a long-term objective of minimizing exposure to cariogenic bacteria during the orthodontic retention period. Specifically, this study investigated the effects of silver and a novel antibacterial compound known as CZ-99 on *S. mutans* biofilm formation. These were analyzed individually and in combination to determine the potential for synergistic activity.

Manufacturers of orthodontic materials, orthodontic laboratories, and clinicians can apply the results from this study to investigate the potential clinical implications for better orthodontic retainer fabrication and selection to improve oral health.

1.2 Research Questions

In general, what are the effects on *S. mutans* biofilm formation following incorporation of antibacterial compounds in orthodontic baseplate material? More specifically:

1. Is *S. mutans* biofilm formation reduced when grown on PMMA that incorporates silver compared to PMMA alone?
2. Does PMMA compounded with CZ-99 affect *S. mutans* biofilm formation?
3. Does CZ-99 and silver act synergistically to inhibit *S. mutans* biofilm formation?

1.3 Hypothesis and Aims

Based on the information above, the research questions posed and the clinical problem being addressed, a general hypothesis was established.

General hypothesis—The addition of antimicrobial substances to PMMA will reduce biofilm growth.

In addition to the general hypothesis, a subhypothesis was established to focus the approach.

Subhypothesis—A combination of silver and CZ-99, a unique antimicrobial, will reduce biofilm formation to the greatest degree when compared to PMMA alone or the antimicrobial agents as individual additives.

To test the hypotheses, two Aims were used.

Aim 1—Grow biofilms on the surface of PMMA alone and in combination with antimicrobial agents. This would be accomplished by using a modified drip flow reactor system to more closely model the oral cavity. More specifically, biofilms in the oral cavity are exposed to air, solid surfaces, liquid and shear force.

Aim 2—Analyze biofilm growth using a multifaceted approach. Specifically, directly image samples after being stained with Ruthenium Red to determine surface coverage of biofilms on each material type, then image with scanning electron microscopy (SEM) to determine degree of biofilm formation, purity and morphology.

CHAPTER 2

REVIEW OF RELATED LITERATURE

2.1 Caries

According to the World Health Organization, dental decay affects over 60% to 90% of young children (Marsh, P.D. 2005). Caries risk varies with age, socioeconomic status, ethnicity, and according to the individual's own immune response (Marcotte, H. 1998). Caries is considered by many to be preventable due to caries' relationship with bacterial plaque accumulation. For this reason, the majority of therapeutic efforts have been on plaque removal and prevention of plaque formation (Wilson, M. 1989). Because of the prevalence of dental caries, numerous models have been postulated to better explain the caries process. The Keyes caries model associates the host's dietary habits with relative bacterial quantities and portrays the importance of individual behaviors in predicting caries (ten Cate, J.M. 2009; Forssten S.D., Bjorklund M., Ouwehand A.C. 2010). The host contributes to caries formation based on their dietary choices, salivary and crevicular fluid composition and volume, and their personal immune response. Bacterial composition will then begin to shift with differing stages reflecting the severity of the caries. (Loesche, W.J. 1979; Cohen, B. 1980).

There are many common oral microbes which are ubiquitous in the oral cavity, yet compatible with the host. Frequently it is planktonic or exogenous bacteria that disrupt the balance of the indigenous bacteria. It is this imbalance of normal oral flora that leads to active caries and has caused researchers to adopt the ecological plaque

hypothesis (Marcotte, H. 1998). This revised hypothesis reflects that bacteria commonly associated with the disease are more environmentally driven. The host's diet affects the oral pH. Acidic foods can decrease the pH and a lower pH increases acidophilic bacteria, which contribute to caries formation (Tanner, J. 2000). This hypothesis revision appreciates the fact that without ideal conditions, otherwise pathogenic, caries-causing bacteria are of little significance (Marsh, P.D. 2005).

Clinical studies have uncovered 200-300 different microflora that inhabit the oral cavity. The oral microflora makeup shifts throughout the course of our lifetime (Struzyka, I. 2013). A study in 2011 showed that the predominant oral bacteria in adults are *Haemophilus*, *Neisseria*, *Veillonella*, *Fusobacterium*, *Oribacterium*, *Rothia*, *Treponema* and *Actinomyces* (Cephas, 2011). *S. mutans* is common in adults, but only makes up about 2% of the streptococcal species that inhabit the oral cavity and *Streptococcal* species make up about 56% of the normal oral flora (Horiuchi, M. 2009). An acidogenic environment leads to a shift in the composition of oral bacteria found in dental plaque as evidenced by an increase in the number of *S. mutans* and *Lactobacillus* species and progression of the caries formation process (Garcia-Godoy, 2008; Nasidze, 2009).

Lactobacillus acidophilus is a gram positive rod that is associated with the progression of caries. *L. acidophilus* is among the common oral microflora, but increasing levels often indicate active caries (Loesche, W.J. 1986). *L. acidophilus* is usually a secondary invader that is ubiquitous in deep, cavitated lesions where pH is the lowest (Botha, S.J. 1993; Nyvad, B. 1993). This organism is opportunist and often takes advantage of the environmental conditions and the sources of nutrients encountered in patients with orthodontic appliances (Botha, S.J. 1993). *L. acidophilus* prefers anaerobic

conditions, and along with *S. mutans* is known as a caries-associated bacterium (van Houte, J. 1994; Ollila, P.S. 2008;). As normal flora, *Streptococci* and *L. acidophilus* reach greater proportions in extremely acidic conditions (Socransky, S.S. 1971). *L. acidophilus* is usually found in areas of high carbohydrate or on surfaces that enable its preferred anaerobic conditions (Botha, S.J. 1993). Although *L. acidophilus* is a secondary colonizer and favors *S. mutans* for attachment, this bacterium produces a much stronger acid that eventually suppresses *S. mutans* growth (Lara-Carrillo, E. 2010; Smiech-Slomkowska, G. 2007; Svec, P. 2009), but it is *S. mutans* that may play the largest role in the problem of caries formation.

2.2 *Streptococcus mutans*

S. mutans is considered the principal organism in dental plaque that contributes to the initiation of caries (Nyvad, B. 1993). *S. mutans* is one of the most studied cavity-causing bacteria and has a symbiotic relationship with other caries-causing oral microbes. Despite being common in the oral cavity, *S. mutans* prevalence often indicates caries susceptibility and poor oral hygiene. *S. mutans* is a gram positive coccus, which uses nutrients not only to create a polymeric matrix that allows for its own and other bacterial adherence to a tooth, but also produces detrimental acids. This allows the bacterium to decrease the pH, eventually leading to early caries (Gizani, S. 2009; Gudkina, J. 2008). During orthodontics, *S. mutans* levels increase, but what the levels are during the retention period is controversial. One study indicated that levels of *S. mutans* during retention return to the lower bacterial levels found during pre-treatment (Rosenbloom, R.G. 1991). However, other studies have shown that the use of

removable orthodontic appliances may lead to the creation of new retentive areas and surfaces which favor the adherence of *S. mutans*. Most pertinent for this study was the indication that *S. mutans* was found at increased levels in children using removable orthodontic appliances (Bartoni, G. 2001). Also relevant to this study was work done by Lessa *et al.* which confirmed that there may be considerable retention and adherence of *S. mutans* on the surface of PMMA baseplate material of removable orthodontic appliances. The positive controls in this study demonstrated 100% contamination with many *S. mutans* colonies (Lessa, F. 2007)

2.3 Biofilm Formation

Plaque, the principal etiological agent in caries, is a stable and complex ecosystem developed on the tooth's surface (Atack, N.E. 1996; Gibbons, R.J. 1973; Socransky, S.S. 1971). It develops stepwise from inter and intra species interactions within the host, allowing bacterial adherence and colonization of the tooth's surface (Socransky, S.S. 1971). These bacteria congregate to form what is known as a biofilm. Most often this biofilm is harmless, but at times bacterial and environmental conditions can prevent equilibrium and result in caries and periodontal disease.

There are four phases to biofilm formation. During phase one, bacteria are transported to a given surface by Brownian movement and bacterial chemotaxis (Quirynen, M. 1995). Phase two comprises interactions between that surface and the bacteria (Gibbons, R.J. 1973; Quirynen, M. 1995). Initially, the adhesion is due to Vander der Waals forces and electrostatic attraction, which often is considered to be the material's surface free energy (Bollen, C.M. 1997). During phase three, a stronger

attachment with specific ionic, covalent, and hydrogen bonds link bacteria to the surface via extracellular proteins. In this phase, the increased hydrophobicity and increasing bacterial affinity for salivary proteins affects adhesion (Busscher, H.J. 1984; Wilson, M. 1989). At this point the bacteria are now attached and the final phase is initiated in which the bacteria start to grow and are matured by the shear forces of mastication and salivary flow (Gibbons, R.J. 1973). During phase four, bacteria become prolific and continue to colonize the surface by coadhesion and coaggregation (Quiryen, M. 1995).

As biofilms mature, plaque levels increase and species diversity occurs. The plaque becomes more tenacious and more pathogenic (Leung, N.M. 2006). This biofilm, which was once friable and delicate, can quickly become well-established and with increased communication between bacteria may be irreversible. Coaggregation and coadherence between cariogenic bacteria establish a sheltered and stable community, which with time tends toward gram-negativity and anaerobicity. This succession of bacterial types leads to enamel dissolution and ultimately frank caries (Kolenbrander, P.E. 2000; Kolenbrander, P.E. 2002; Nyvad, B. 1993).

The oral environment is altered when a patient is wearing removable orthodontic retainers. One case report of severe tooth demineralization has been reported in a patient using vacuum formed thermoplastic (essix style) retainers. (Birdsall, J. 2008). Orthodontic retainers introduce additional surfaces into the oral cavity upon which biofilms may form. These biofilms can harbor caries-causing bacteria that could migrate to tooth surfaces and accelerate the formation of caries. Physiochemical factors of the retainer material, such as surface free energy, hydrophobicity, and porosity could also affect bacterial adherence properties (Quiryen, M. 1995; Papaioannou, W. 2007).

Past research on bacterial adherence to PMMA and the nature of orthodontic oral flora provide a foundation for testing the adhesion of traditional PMMA in comparison to antibacterial infused PMMA. Full-time use of Hawley style orthodontic retainers may be conducive to anaerobic bacteria, especially on the intaglio or inner surface of the appliance at the interface of the retainer and the palatal mucosa of the patient.

2.4 Orthodontic Considerations

Malocclusion or dental irregularity is a common oral health problem (Glans, R. 2003). Orthodontics can help to improve alignment for better oral hygiene access and improved periodontal health, but research shows that only with average oral hygiene, does alignment help. In patients with good or even poor oral hygiene, alignment has no further effect on gingivitis (Alexander, S.A, 1991). Although studies have indicated orthodontics improves dental awareness and oral hygiene skills through frequent visits, it has been found that the oral bacteria of orthodontic patients is much different from those of healthy individuals. The difference is due to increasing levels of bacterial plaque (Batoni, G. 2001; Choi, 2009; Davies, T.M. 1991). Orthodontic appliances increase areas where food remnants can accumulate and increases the number of protected bacterial niches (Alves, P.V. 2008). An increase in opportunistic bacteria can not only cause caries, but may also lead to periodontal and fungal infections as well (Gudkina, J. 2008). It is important to note that at 6 weeks into retention, plaque levels can remain high and may not go away (Boersma, J.G. 2005).

Early caries and demineralization are often seen in orthodontic patients with sub-par oral hygiene (Petti, S. 1997; Sari, E. 2007). Whether due to the appliances' retentive nature increasing plaque levels or due to reduced clearance, it is evident that appliances can aggravate an already precarious situation (Boersma, J.G. 2005; Kitada, K. 2009;). Increased plaque accumulation, leads to an increase in bacterial number, which in turn leads to a decrease in pH that causes decalcification (Balenseifen, J.W. 1970). These developing white spot lesions have been documented in patients receiving orthodontic care and the principal bacteria responsible are *S. mutans* (Gorelick, L. 1982; Teanpaisan, R. 2007). White spot lesions, which would otherwise be nonexistent in a patient without braces, are found in 2-96% of orthodontic patients (Zachrisson, B.U. 1971). These lesions rarely end up in caries, but their progression can be quick and can leave indelible scars on the teeth post-treatment (Ogaard, B. 2001; Ogaard, B. 2006). These areas have been found to be rather resistant to normal measures of remineralization and last many years after appliances have been removed (Gorelick, L. 1982). Today most orthodontic law suits stem from patients' disapproval of their white spot lesions, despite the patient's own role in the development. Although it's been found that carious lesions are linear to plaque values, somehow it still rests on the shoulders of the orthodontic professional when a patient does not have proper oral hygiene (Zachrisson, B.U. 1971).

Orthodontics can also affect salivary flow, buffering, and oral pH, due to the fact that appliances can change the overall oral environment. Salivary flow increases with orthodontics, which in turn increases the oral pH due to elevated levels of bicarbonate.

Even though a decrease in pH can be found in an orthodontic patient due to poor oral hygiene, increased salivary flow associated with orthodontic appliances can offset this and maintain a relatively stable oral pH (Chang, H.S. 1999; Lara-Carrillo, E. 2010).

2.5 Retention's Role

Retention is necessary for nearly every orthodontic patient post-treatment. Patients are dismissed from active treatment and then supervised several times over the following years. At this point there is a shift in responsibility, from orthodontic clinician to patient, and unfortunately the amount of relapse depends on patient compliance (Sheridan,J.J. 1993). Relapse in orthodontics is most often due to forces from periodontal fibers, oral musculature and growth. Often the general dentist is the first person to observe any changes in the dentition post-orthodontics. Long-term studies investigating relapse have led clinicians to opt for retention indefinitely (Little, R.M. 1988; Little, R.M. 1990; Haruki, T. 1998). The aim of retention is to keep teeth in position, but the overall appliance design can vary depending on the demands of the orthodontist and patient. The appliance needs to be durable and comfortable, while still being easy to adapt and overall effective for the orthodontist (Cerny,R. 2001; Cerny,R. 2008). According to a review published by the Cochrane Collaboration, current retention studies have insufficient research data and are unreliable for basing clinical decisions (Littlewood,S.J. 2006). Due to this, most retention appliances are chosen based on the clinician's judgment and the patient's compliance and satisfaction.

Removable retainers were developed to address some of the negative issues that are experienced by fixed lingual retainers. Removable retainers allow for better oral hygiene by not hindering or limiting oral hygiene methods (Cerny, R. 2001; Ristic, M. 2008). Removing the appliance makes it easier for a patient and decreases their susceptibility to dental disease. The negative aspect of these appliances is that they require patient compliance. Unfortunately, it is an appliance whose retentive nature and material makeup may deteriorate in the event of poor hygiene. Researchers have demonstrated that opportunistic bacteria and fungi levels were increased in all orthodontic patients when compared to those not receiving orthodontic care. More specifically, patients with removable retainers had more dental plaque than non-orthodontic treated controls (Kitada, K. 2009). This indicates that although improvement in oral health may be seen after fixed orthodontics, the retention period may still increase risk for oral health issues.

Additional research conducted on removable appliances, found that bacterial levels in retainer patients were comparable to partial denture wearers (Addy, M. 1982). However, conflicting results do exist which implies that the oral bacterial flora of retention patients may not differ substantially from patients who are not in post-orthodontic retention. For example; in comparison to non-appliance wearers, retainer patients have less bacteria in the buccal segment of the mouth, presumably due to dislodging bacteria with appliance removal (Arendorf, T. 1985). Although bacterial levels may increase with orthodontics, patients with removable retainers are similar to healthy non-appliance wearers demonstrating no increased gingivitis or periodontitis. In one study, the only microorganism that showed exceptional differences was that of the

yeast, *Candida albicans* due to palatal coverage by PMMA (Petti, S. 1997). Due to the enormous differences in results between multiple studies, it is unclear the exact ramifications of retention appliances on oral health.

2.6 The Hawley Retainer

Developed in the 1920s, the Hawley retainer, a tissue borne retainer, represents a large proportion of retainers currently used in orthodontics. It consists of acrylic, otherwise known as Polymethyl Methacrylate (PMMA), that has low solubility and toxicity (Theroux, K.L. 2003). The Hawley type retainers are generally prescribed for those patients where settling of teeth is needed. The retainer's attributes can be seen in its design (Figure 2.1) with acrylic PMMA palatal coverage. A stainless steel wire runs along the labial surface of the teeth. This design allows for subtle adjustments to the dentition. The Hawley retainer is known for its strength and long-term durability. These retainers are adaptable and often require more time, skill and money to fabricate and maintain (Sheridan, J. 1993). Patient complaints about Hawley retainers include embarrassment due to salivary flow, speech alteration, esthetics, and the odor of the appliance (Hichens, L. 2007). The materials of the Hawley retainer can contribute to a specific set of problems. The acrylic lacks color stability and often shows shrinkage (Lewis, E.A. 1988).



Figure 2.1: Traditional Hawley retainer. The Hawley retainer viewed from the occlusal view (left) and the facial (right). It consists of both acrylic PMMA and wire clasps and bars for retention.

2.7 Antibacterial Dental Materials

Due to the clear link between dental caries and the presence of cariogenic oral bacteria, creating dental materials that may reduce the load of these oral microbes has been an area of continued research and development. The estimated quantity of *S. mutans* normally present in the oral cavity is 1×10^5 colony forming units (CFU)/mL of saliva (Dansanayake, A. 1995). Various methods have been attempted to reduce colonization of this organism. Silver has been shown to have an important antimicrobial effect against *S. mutans*. Gold and zinc have also been shown to have antibacterial effects and have thus been included in varying dental materials and dentifrices. Zinc in particular has been incorporated in many tooth pastes to help to control dental plaque formation (Phan, T. 2004). Zinc, copper, titanium and silver nanoparticles have each been added to mouthwash and tested and shown to be effective against *S. mutans* as well as other streptococcal species. (Ahrari, F. 2015). Much research has been done to assess the effects of different antibacterial products on oral microbiota in hopes that

novel antibacterial dental materials might be developed to improve oral health (Fang, M. 2006; Kim, J. 2007).

Silver is now being incorporated in bioactive glass that induces pulp cell proliferation and differentiation and may be used to achieve superior restorative results (Wang, Y. 2015). Silver containing fillers have been proposed as potential components of composite resins, glass-ionomer, and temporary cements. (Ohashi, S. 2004)

Directly related to orthodontics, researchers have coated stainless steel surfaces with silver-platinum alloy that release silver ions and showed significant antimicrobial potency against *S. mutans* and other types of oral bacteria (Hwang-Sog, R. 2012). In another study, brackets were coated with zinc oxide. Others were coated with copper oxide and some with both copper and zinc oxides, then tested against *S. mutans*. In this study, the copper oxide coated brackets had the best antimicrobial effect against *S. mutans* (Ramazansadeh, B. 2015). Researchers have also added antibacterial compounds to orthodontic cements, hoping to reduce biofilms and combat white spot lesions (Zhang, N. 2015). To varying degrees, these experiments have demonstrated success in reducing harmful oral microbiota and may provide orthodontic clinicians with new options to improve the oral health of orthodontic patients. Yet, after a thorough literature review, it does not appear that studies have specifically assessed the ability of antibacterial-loaded PMMA to resist biofilm formation using biologically relevant system that models the oral environment. This provided rationale for undertaking the current study, wherein silver would be one of the main antibacterials analyzed given its current predominance in medical device development and promising outcomes.

2.8 Silver

Silver has been one of the most extensively investigated and applied antimicrobial agents to fight infections. Classical studies demonstrating the antiseptic properties of silver date back to the times of Ambroise Pare (1517-1590), who used silver clips in facial reconstruction (Lansdown, A. 2006) Today, metallic silver is one of the most common elements used in dentistry and it has been shown that ionic silver has the highest antibacterial activity among metal ions (Kawahara, K. 2000). Silver is an electron positive element that shows a profound ability to interact with and bind proteins and anions. Silver binds receptor groups on the surfaces of bacterial and fungal cells, and ionizes in the presence of water and bodily fluids to become antibiotic in nature (Lansdown, A. 2006).

There are still limitations with silver that need to be investigated further. The exact mechanism of action of silver with bacterial cells is largely unknown and there are questions about how the surface area of the silver particles affect their antibacterial activity (Rai, M. 2009). However, there have been efforts to reveal which size of silver nanoparticles increase the antibacterial activity. One study demonstrated that smaller-sized nanoparticles show increased antibacterial activity. The reasons for this require further investigation, but some theorize that it might be related to the increased surface area of the smaller nanoparticle that leads to increased ion availability and thus an increase in antibacterial activity (Espinosa-Cristobal, L. 2009).

Different modes have been employed to incorporate silver into dental materials. Impregnation of polymers using a solution of organometallic precursors dissolved in supercritical carbon dioxide at high pressure and then exposed to hydrogen gas to

decompose the organometallic precursors has been able to produce polymer that has a homogenous distribution of silver nanoparticles. This polymer showed a continuous release of silver ions that was not blocked by conditioning film (Furno, F. 2004). Silver Zeolite has been investigated for use in dental materials and multiple studies have shown that Silver Zeolite may be a useful vehicle to provide antibacterial activity to dental materials (Kawahara, K. 2000; Hotta, M. 1998; Matsuura, T. 1997). Various forms of silver-containing fillers have been used in dentistry such as silver ion-implanted SiO₂ (Yamamoto, K. 1996), silver-containing silica glass (Kawashita, M. 1996), silver apatite (Syafiuddin, T. 1997), and silver-supported zirconium phosphate (Yoshida, K. 1999). The Fan method of silver incorporation combines nanosilver into polymers by dissolving silver benzoate into dimethylaminoethyl methacrylate (DMAEMA) and then adding this to the liquid monomer used for PMMA polymerization (Fan, C. 2010). This form of nanosilver has been shown to have low cytotoxicity and high levels of antibacterial activity when compared to other antibacterial agents (Lansdown, A. 2006; Volker, A. 2004).

Relevant to this research was a study done by Oei *et al.*, which generated silver nano particles *in situ* in PMMA. All PMMA samples with silver nano particles released silver ions *in vitro* for over 28 days. These samples inhibited 99.9% of bacteria against four different bacterial strains. Long-term antimicrobial assay showed continual antibacterial effects beyond 28 days. All of the PMMA with silver nanoparticles demonstrated lower strength due to altered polymerization (Oei, J. 2011). A study on the antibacterial effects of PMMA containing silver-TiO₂ concluded that *S. mutans* was reduced by 90.2% and that the antibacterial property of PMMA was enhanced with

silver-TiO₂ inclusion (Liu, J. 2012) Another study looked at inhibitory effects of silver loaded PMMA at varying concentrations of silver vs. *S. mutans*. A clear zone of inhibition, with 97.5% inhibition, was shown at 0.5% silver benzoate and a vague zone of inhibition, 52.4% inhibition, was shown at 0.2% silver benzoate (Fan, C. 2010).

2.9 Biofilm Infection and Development of CZ-99

Biofilm-related infections are a growing concern across the globe as is the increasing threat of antibiotic resistance. The Centers for Disease Control (CDC) has estimated that over 2 million patients are infected with antibiotic resistant organisms each year in the U.S. The NIH has further estimated that 80% of all difficult to treat infections are biofilm-related. With growing concerns of these problems, in combination with increased use of medical devices that come in contact with human tissue for extended periods of time, it is necessary to increase the pipeline of antibiotics. However, more than just antibiotic derivatives are needed, but new classes of antibiotics with emphasis on those that are specifically designed to eradicate biofilms.

To address these problems, a unique first-in-class series of antibiofilm antibiotics, referred to as CZs (named after the company, Curza), has been synthesized and is currently under development (Haussener, T. 2016). CZs were designed by merging structural characteristics of known antibiotics that interact with membranes through hydrophobic and charged interactions (e.g., cationic peptides and steroidal amines) with polyamine triggers of biofilm dispersion (e.g. norspermidine). The expectation has been that the incorporation of a norspermidine tail on hydrophobic backbones would merge the “kill” and “dispersal” attributes of these small polyamines.

A series of simple benzylic-substituted polyamines was synthesized. Key findings from this proof-of-concept study were: 1) antimicrobial activity correlated with the number of norspermidine side chains, 2) a more hydrophobic backbone increased antimicrobial activity; and 3) primary amines at the norspermidine termini conferred significant cytotoxicity that can be mitigated with sidechains. Data have indicated that our structurally similar lead compounds have broad-spectrum activity against gram-positive, gram-negative, and acid-fast gram-positive mycobacteria.

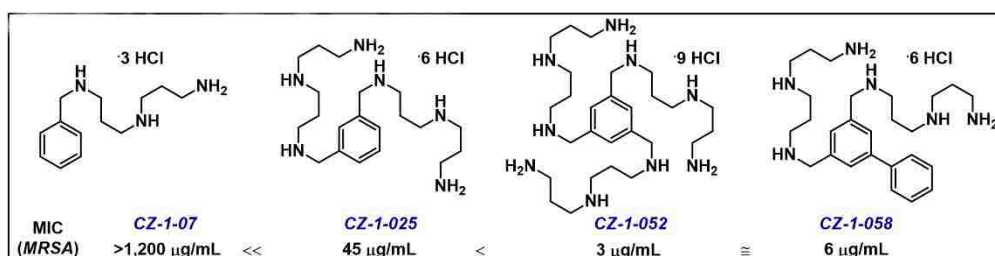


Figure 2.2: Representative progression of CZ synthetic development. Antimicrobial activity increased with the addition of side chains and increased hydrophobic nature.

According to preliminary studies, CZ compounds are targeted against biofilms and demonstrate antimicrobial activity within 5 minutes. Specific to the compound of interest in this study, CZ-99, Curza has provided proprietary data indicating that there has been no detectable resistance following passage studies that have transpired through 30 passages and it has low permeability, which is promising for oral applications as it is not absorbed well by microvilli or other endothelial cell types. CZ-99 has been shown to synergize with silver resulting in enhance activity against both planktonic and biofilm bacteria. CZ-99 is one of the safest CZs in particular for topical use or incorporation in medical devices. It has shown acceptable absorption, distribution, metabolism, and excretion (ADME) profiles, is mildly or non-hemolytic at therapeutic concentrations, and Ames negative. Method of action analysis with CZ-99

has shown that it acts on the cell wall and/or membrane of gram positive bacteria, causing it to corrugate and breakdown. This method of action likely contributes to why CZs are more active against biofilms than traditional antibiotics. It is hypothesized that CZs overcome resistance by depolarizing the cell membrane, thus allowing antibiotics to access the interior of a cell more readily.

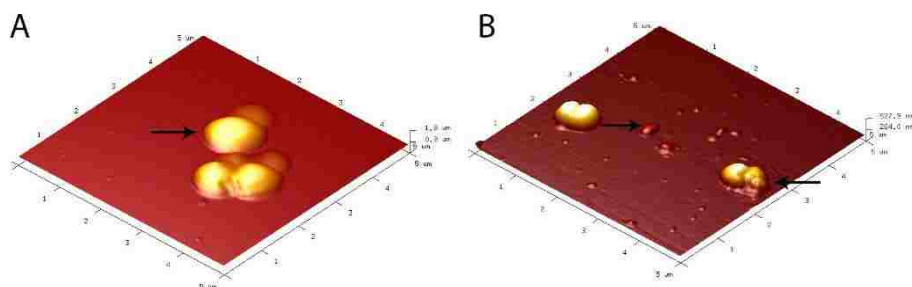


Figure 2.3: Atomic force microscopy (AFM) was used to determine method of action of CZ-99 against MRSA cells. (A) Cells that were exposed to highly pure water remained intact and had diameters of approximately 1 – 1.2 μm (see arrow). (B) Cells that were exposed to a 0.5% solution of CZs in highly pure water became corrugated (see right-most arrow) and had diameters of 0.5 – 0.8 μm . Cellular debris was also seen (left-most arrow) and confirmed to be cellular components by modulus testing. Data are being collected with CZ-1-182 against *P. aeruginosa*.

In vitro activity of CZs has been well-documented. Publications are in process, but have been reserved until intellectual property was secured surrounding these compounds. Three patents have been issued protecting composition of matter and synthesis of the compounds with two PCT and 21 provisional applications on file (Private communication with Dustin Williams, 2016).

The rationale for selecting CZ-99 was to broaden the pipeline of options for incorporating antimicrobial agents into PMMA materials. In addition, this class of compounds is being targeted toward biofilm therapy. With biofilms being the causative agents of periodontal disease and medical device-related infections, this was a good

target to investigate in addition to silver, which is being more widely accepted in clinical applications and medical device development.

2.10 Biomaterial Implications

The Hawley retainer's bacterial concern deals mostly with the acrylic base plate that is tissue born. The acrylic base can be made from a variety of materials including auto-polymerized, heat-cured, and visible-light-cured resins. Due to an extensive amount of research on the bacterial adherence to denture bases, many of the conclusions have been projected onto the Hawley retainer, since PMMA is used for both appliances. PMMA is very absorbent to saliva and bacteria so numerous attempts have been made to improve its properties. Cross-linking, adding nanofilled resins, and modifying filler content or resin structure have been tried as enhancements (Hahnel, S. 2008). Unreacted monomer and lack of full polymerization can result in cracks or craze lines that may create a safe haven for bacteria. Monomer and filler concentrations in addition to causing chemical irritation when leaching out, also attracts plaque formation. More importantly is the retainer's unique position, allowing it to rest on tissue. This is compounded by its thickness and availability for bacterial binding (Lewis, E.A. 1988). Even the retainer itself, can prevent bacteria on the intaglio surface from being interrupted. It isolates bacteria from the oral musculature and saliva, allowing the bacteria to grow under their preferred conditions, acidic and anaerobic (Pusateri, C.R. 2009).

Since a retainer can be prescribed for up to 24 hours of continuous wear, in a patient population where caries is known to be more prevalent, it is exceedingly

important that bacterial adherence of the retainer material is studied. In the case of *S. mutans*, differing amounts of monomer in PMMA have been shown to affect its adhesion (Hahnel, 2008). *S. mutans* adhesion occurs despite the materials inherently low bacterial adhesion properties. *S. mutans* are attracted to high surface energy materials that are hydrophilic, while PMMA is rather hydrophobic. However, the Hawley retainer does consist of metal portions, which could explain a higher attachment created from a slightly increased surface energy. Studies show chemical adjustments within the PMMA, such as double cross-linking, reduce streptococcal attachment (Hahnel, S. 2008). On the other hand, PMMA coated with chemicals, may somehow serve as a receptor for bacterial binding (Radford, D.R. 1998).

One study tested different types of PMMA, such as autopolymerized, heat-cured, and visible-light-cured against thermoplastic "Biocryl". SEM was used to compare surface characteristics, which are believed to affect bacterial adherence. In the study Biocryl resin was somewhat smoother in surface roughness, possibly leading to its decreased bacterial adherence. This smoother Biocryl resin had less adhered gram-positive and negative rods, which supports previous studies suggesting surface roughness leads to better bacterial adhesion. Lewis also showed that heat cured PMMA, often the kind used for making a Hawley retainer, showed the most bacterial adherence, specifically by *S. mutans*. Importantly, *S. mutans* adhered more, or to the same extent to acrylic, as it did to enamel. Regardless of increases in bacterial adhesion, this study demonstrated that the subgingival flora did not change, implying that the periodontal condition of the patient was not affected (Lewis, E.A. 1988).

Bacterial adherence is needed for growth and differs between various materials (Bollen,C.M. 1997; van Houte,J. 1994). Adherence allows continued shelter from the biological processes normally used for their removal. Often bacterial growth flourishes, making the bacteria more resistant over time. Bacteria can then communicate by increasing receptors or secreting components, which facilitates additional binding between different bacterial species (Appelbaum, B. 1979; Doyle, R.J. 1995; Gibbons, R.J. 1973). *Streptococcus* specifically, has been found to increase adhesion on prosthesis when in the presence of *Candida* (Pereira-Cenci, T. 2008). For this reason dental materials are manufactured in hopes of a low susceptibility to plaque bacteria, otherwise an additional treatment protocol of fluoride may need to be considered (Nikawa, H. 2006; Pereira-Cenci,T. 2008). Initially, bacterial adhesion is due to the elemental and molecular makeup of the material, which affects its affinity for bacteria through hydrophobicity, hydrogen bond capacity and electron potential. Therefore, surface free energy plays a large role in bacterial adhesion. Bacteria usually have high surface energy, while saliva's surface free energy remains low. In addition, bacteria tend to bind materials with surface free energy similar to their own. Therefore, materials with high surface energy attract more plaque. On the other hand, lower surface energies decrease adhesion initially, decreasing a bacterium's overall binding force (Pereira-Cenci,T. 2007). Roughness is specific to the material, and depends on the material's inherent properties as well as the impact of modifications made during fabrication by laboratory technicians (Busscher, H.J. 1984; Papaioannou, W. 2007).

In the case of PMMA, differences in material properties can vary depending on the amounts of certain chemicals and consistency of mixing (Gedik, H. 2009).

Cytotoxicity of monomer has been known to have an antibacterial effect, while fillers often used to increase wear resistance can make a material rough (Ahn, S.J. 2006; Bollen, C.M. 1997). Although there is no increased attraction for bacteria to rough materials, it is the voids, which either protect bacteria or allow additional time for growth, thereby increasing their number (Nyvad, B. 1993). These irregularities allow bacteria and biofilms to be stagnant and thicken. This increased species diversity changes to a rather irreversible binding (Thomas, R.Z. 2008). Studies have measured the effect of roughness, finding two to four times the amount of adhesion with rough materials (Quirynen, M. 1994; Quirynen, M. 1995). Roughness is measured by the spaces between irregularities. It allows the initial adhesion seen in cracks, grooves or abrasion, and is thought to be strain specific (Bollen, C.M. 1997; Quirynen, M. 1994; Quirynen, M. 1995). *S. mutans* specifically, although found on smooth surfaces, has increased adherence on rough or porous material (Pusateri, C.R. 2009).

Fabrication adjustments such as polishing, change the overall properties of a material by altering its relative roughness (Thomas, R.Z. 2008). Methods tested indicate that differences in procedures can increase surface roughness up to ten fold. But for the most part, the laboratory technique of polishing can decrease the overall roughness of a material if done using normal protocols (Bollen, C.M. 1997). Because roughness was found to speed up colonization, studies were done to standardize treatments for different materials. Through the use of various polishing techniques, it was discovered that there is a threshold for roughness that determines if a surface is plaque retentive or not. As long as the materials roughness is less than 0.2 μm , bacterial attachment differences are insignificant with change in roughness (Bollen, C.M. 1997; Quirynen, M.

1995). Most studies were done on PMMA and it can be noted that even with standardized protocols for laboratory fabrication, often a retainer would undergo damage from brushing or common cleansers (Bollen, C.M. 1997; Samaranayake, L.P. 1980). Wear over time could result in areas where roughness is above threshold roughness and bacterial attachment is increased. It should be mentioned that the edges of this retainer could possibly serve as bacterial attachment sites.

The purpose of this study is based on the fact that antibacterial additives to PMMA may reduce bacterial adhesion and improve oral health in post-orthodontic patients. A retainer placed in an orthodontic patient, allows plaque to become stagnant. This sheltered environment can change the microenvironment of the oral cavity and increase susceptibility to disease. Learning about options that may help reduce these pathogenic bacterial levels may help clinicians to better care for their patients.

CHAPTER 3

METHODOLOGY

3.1 Experimental Design Summary

An *in vitro* randomized study was performed using the most common clinically-used orthodontic base plate material, PMMA. The objective was to assess whether the incorporation of a standard antimicrobial, silver, or a unique antibiofilm antibiotic, CZ-99, into PMMA material would affect the ability of *S. mutans* to form biofilms on the material surface. These two antimicrobials were also examined in combination given the synergistic nature of CZ-99 with silver. As a negative control of growth, PMMA without bacteria on it was imaged and analyzed, whereas positive controls of growth were tested by not incorporating antibiotic into the PMMA and allowing biofilms to form. To grow biofilms, a modified drip flow reactor system, custom made by the Bone and Joint Research Lab, was used.

3.2 PMMA Coupon Preparation

Rectangular samples (referred to as “coupons” from here forward) of PMMA were fabricated to result in 12 mm x 25 mm coupons with approximate height of 3 mm. To make the coupons, dental stone molds with a 4 in x 4 in x 1/8 in depression were used to create the bulk material via the “Salt and Pepper” technique according to manufacturer’s recommendation (Procryl[®], Dentsply GAC; Figure 3.1). More specifically, PMMA was prepared using 1 part self-polymerize acrylic powder and 0.8

part monomer liquid. The monomer and polymer were added in Salt and Pepper fashion to the stone mold that had been coated twice with separating medium (Figure 3.1), with one side facing type 3 dental stone. The block was submerged in a water bath set at 45° C and cured for 20-30 minutes. Following curing, PMMA sheets were separated from their molds (Pereira-Cenci, T. 2007). The 4 in X 4 in sheets were marked and cut on a band saw to approximately 12 mm x 25 mm coupons. No polishing was done on the intaglio side of the coupons, but they were modified when needed on the upper side to allow them to lay flat in the drip-flow reactor.

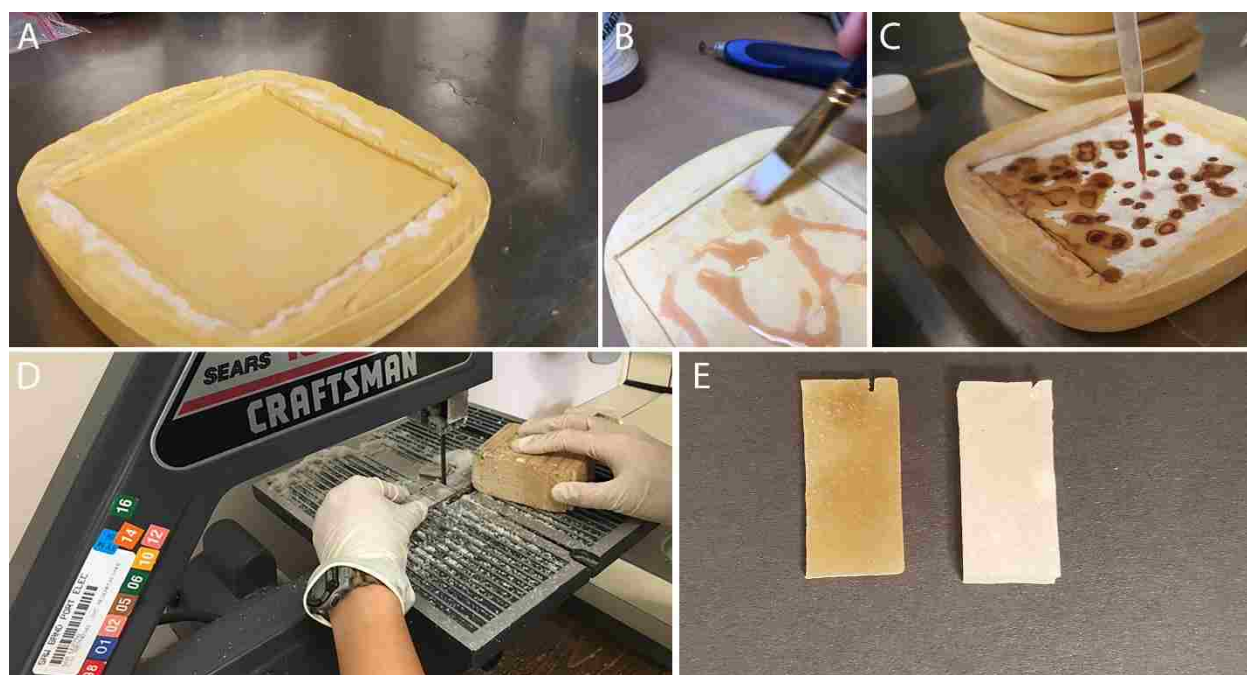


Figure 3.1: Making of the coupons that were used for analysis. (A) Dental stone mold. (B) Separating media was used to allow the PMMA materials to delaminate from the surface of the stone. (C) Salt and pepper method was used to create layered PMMA material. In this image, the brown liquid is silver-loaded polymer to provide contrast for visualization. (D) Coupons were made by cutting the bulk material on a band saw. (E) Coupons of approximately 12 mm x 25 mm were notched on the top right corner to analyze each type uniformly. On the left is a coupon that has silver in it, which is noticeably darker than the coupon on the right, which contained PMMA only.

To make the 0.4% w/w silver-based coupons, 0.1028 mg silver benzoate was added to 0.5 mL of DMAEMA and then 0.4 mL of this mixture was added to 19.6 mL of

the Procryl® monomer. It should be noted that at this stage the mixture appeared to darken in color with time (see Figure 3.1). This mixture was then used following the “Salt and Pepper” protocol with the resin powder into the dental stone molds and cut into smaller coupons as previously described.

To make the 0.4% w/w CZ-99 coupons, 294 mg of CZ compound powder was added to 49.706 g of Procryl® acrylic resin powder, then agitated and mixed for 2 minutes. This made 50 g of acrylic powder which was then mixed with 25 mL of liquid Procryl® monomer into the dental stone molds, cured, and then cut into smaller coupons as previously described.

To make combination coupons that contained a final w/w concentration of 0.4% total CZ-99 and silver, 147 mg of CZ-99 powder was added to 49.853 g of acrylic resin Procryl® powder. Then 0.1 g of silver benzoate was dissolved in 1 mL of DMAEMA. Of this solution, 0.6 mL were added to 29.4 mL monomer to make 30 mL of silver-loaded monomer. This monomer and the CZ powder were added to the dental stone mold in “Salt and Pepper” fashion, then cured and cut into coupons as previously described.

Once fabricated, each coupon was sterilized using a cold sterilization method. Initially, coupons were cleaned with tap water and a scrub brush, then immersed for 25 minutes in approximately 30 mL of 80% ethanol. Following submersion, coupons were randomly swabbed with a sterile swab and cultured to confirm sterility. Each coupon was rinsed in sterile water, then placed in sterile water and exchanged 3 x for 5 minutes each. Lastly, coupons were left in sterile for 12 hours or more to ensure that no residual alcohol remained on the surface. Notably, during each exchange, the coupons were rotated on a shaker at 150 rpm to help remove residual alcohol from the material. Once

sterilized, coupons were maintained at room temperature in sterile water until their use. No surface modification was performed after the coupons were processed (Pusateri,C.R. 2009). Defective specimens and those coupons showing any obvious surfaces imperfections were discarded (Serrano-Granger,C. 2005).

3. 3 *Bacterial Culture and Preparation*

S. mutans ATCC 25175, a common oral bacterial isolate, was purchased from the American Tissue Type Culture Collection (ATCC) (Manasa, VA). The organism (NCTC 10449) was originally isolated from a carious dentine. Upon arrival, the culture was thawed, streaked and cultured on brain heart infusion (BHI) agar plates with 2-3 day incubation at 37° C as per the supplier's recommendation. From the initial cultures, frozen glycerol stocks for long term storage were made.

To prepare *S. mutans* for biofilm growth, a sample of the agar-grown culture was collected using a sterile swab. The sample was suspended in sterile PBS and adjusted to a turbidity standard of 10% using a nephelometer. At 10% turbidity, this equated to approximately 1×10^9 CFU/mL of solution. Within approximately 15 minutes of making the standard, 1 mL of the PBS standard solution was added to each chamber of a drip flow reactor. Each chamber contained approximately 15 mL of sterile BHI. Thus, the concentration of *S. mutans* in each chamber was approximately 7×10^7 CFU/mL. The setup for the drip flow reactor is provided below.

3.4 Drip Flow Reactor Setup and Materials Testing

Drip flow biofilm reactors are one of the most common systems for growing and analyzing oral-relevant biofilms. In this reactor, a tri-phase environment exists. More specifically, three interphases (liquid, solid and air) are encountered by bacteria. This simulates the environment of the oral cavity wherein saliva is present (liquid) on teeth (solid) and air in the oral cavity. For this study, a modified and custom drip flow reactor was set up by the Bone and Joint Research Lab (Figures 3.2 & 3.3).

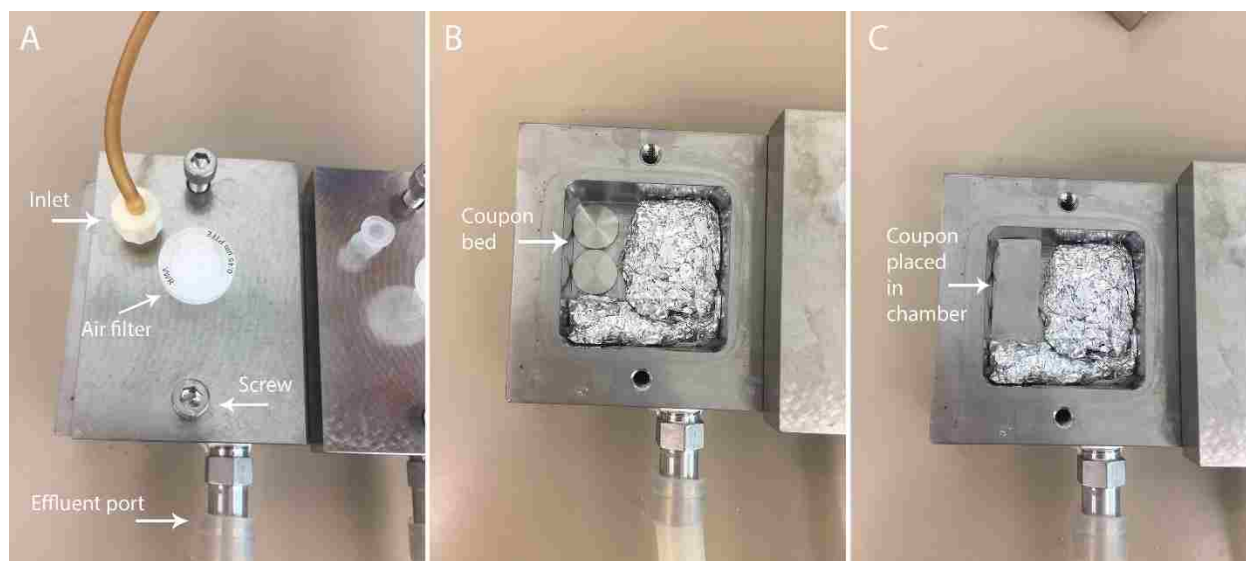


Figure 3.2: Set up of the modified drip flow reactor chambers. (A) Image showing the inlet port, which allowed broth to be flowed into the chamber, as well as an air filter, screw to secure the lid over the chamber and the effluent port that allowed broth to drain out of the chamber. As such, broth could be flowed through the chamber sterilely and provide renewable nutrients to the biofilms. (B) The inside of each chamber contained tinfoil and two circular discs to create a “bed” to secure a coupon directly underneath the incoming broth. As such, shear force and renewable nutrients were provided to optimize biofilm growth. (C) The coupon was placed on the circular discs directly underneath the incoming broth.

Prior to each run of the reactor, the unit and accompanying tubing was autoclaved for 45 minutes (using liquid cycle to reduce polymer breakdown). Once autoclaved, coupons were aseptically placed in the upper left of each chamber (see Figure 3.2). Modified drip flow reactors were placed at an angle of approximately 10° on

a customized shelf within a standing incubator (Figure 3.3). Effluent tubes were clamped closed initially so that a batch phase of growth could occur. More specifically, coupons were incubated in the bacterial solution that was prepared in section 3.3 for 6 h. This period of growth/incubation, wherein coupons were fully submerged in broth that contained bacteria, was referred to as batch phase growth. This took place in the incubator at 37° C. Batch phase referred to the time when no flow was present in the reactor chambers. This allowed bacteria to begin their growth process and begin to adhere to the coupons. If flow were present right away, the bacteria would have been diluted and not been as likely to form biofilms. Notably, the flow reactor system had been allowed to pre-warm to this temperature.

After 6 h of batch growth, the tubes were unclamped and inserted into effluent containers (see Figure 3.3). A flow of 10% BHI was flowed through each chamber using a peristaltic pump at a rate of 0.3 mL/min. This flow modeled the flow of saliva in the human mouth. Biofilms were allowed to develop over a 24 h period.

3.5 Coupon Processing, Photographs and SEM Imaging

After 24 h of growth, PMMA slides were aseptically removed from each chamber. Coupons were placed individually into approximately 10 mL of modified Karnovsky's fixative (2.5% glutaraldehyde and 4% paraformaldehyde in PBS buffered to pH 7.2) for a minimum of 2 h, then dehydrated in 95% ethanol. Notably, the modified Karnovsky's fixative (i.e., cross-linking agent) contained 0.1% ruthenium red, a dye that attaches to bacterial cells and biofilm extracellular components. This dye allowed for direct gross

photography to be performed. As will be shown in the Chapter 4, Results, these images were used to determine surface coverage of biofilms on each coupon.



Figure 3.3: Setup of the modified drip flow reactors. (A) Two reactors could be used at a time in the standing incubator. (B) Broth was flowed through each tube using a series of tubing. The effluent ports allowed broth to drain. The system allowed for shear force, fluid and air exchange to occur. (C) Peristaltic pumps were used to flow broth through each chamber. Tubes were red through a pump head that rotated, creating flow.

Following gross photography, coupons were sputter coated with plasma phase gold using a Hummer 6.2 sputter coater. This created a conductive layer of gold on each coupon with an approximately 20 nm thickness so that SEM imaging could be performed. Images were collected using a JEOL JSM6100 SEM. Secondary electron SEM imaging was performed. This mode of imaging allowed for direct observation of both surface and biofilm morphology (three-dimensional structure formation), surface coverage and made possible subsequent analysis with MountainMaps 3D software. Tests were performed with n=5 repeats.

3.6 Surface Morphology and Roughness

Secondary electron SEM images were collected to qualitatively observe surface morphology of each sample type. In order to collect quantitative surface roughness measurements, a noncontact Zygo Optical Profilometer 10x objective was used. Roughness was measured as Ra, or Roughness Average, by first determining the topography of the material (done by the Zygo Optical Profilometer). Metro-Pro® 8.3.5 software (Zygo Corp) then determined Ra by arithmetically determining the mean of peak and valley heights. Absolute values of the profile height deviations from the mean line determined Ra for the selected surface area. Notably, a single region on each material type with a surface area of approximately 1 mm² was used to determine Ra.

3.7 Statistical Analysis

Statistical analysis was performed using a one-way ANOVA comparison of means with alpha level set at 0.05. Comparisons were performed between surface

coverages of biofilms on each material type from the gross photographs. Differences were also measured for roughness outcomes.

CHAPTER 4

RESULTS

The goal of this research was to determine if any difference existed in *S. mutans* biofilm formation on standard PMMA compared to PMMA that incorporated antibacterial silver and/or CZ-99. The primary outcome measure was surface coverage of biofilms on each material. Secondary outcome measures included analysis of biofilm morphology, biofilm plume heights, surface roughness and its potential influence on growth.

4.1 Surface Morphology and Roughness

Secondary electron SEM images of material surfaces were recorded and compared (Figure 4.1). It was interesting to note the differences in the materials. First, we imaged the Procryl® PMMA material that was fabricated without additives (Figure 4.1). PMMA has a bead-like structure. When polymerized, the bead structures could be seen, but gaps between them were filled with polymer resulting in an undulating, hill and valley-like conformation. The morphology was reminiscent of smoothed elephant skin, but on a microscopic scale.

With silver benzoate added at a final concentration of 0.4% w/w, the surface morphology was rougher (Figure 4.2). This may have been a result of the crystal nature of silver benzoate and its interaction with PMMA, but to determine such was beyond the scope of this work. It was observed that the surface had a low profile, crater-like morphology (Figure 4.2).

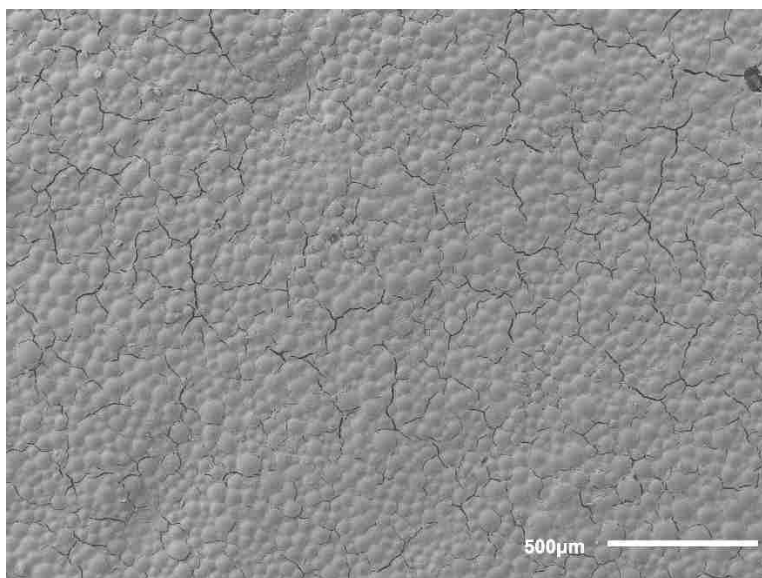


Figure 4.1: SEM image of the surface of PMMA material that did not contain antimicrobial additives. Cracks that can be seen were likely due to the dehydration process that was performed with ethanol.

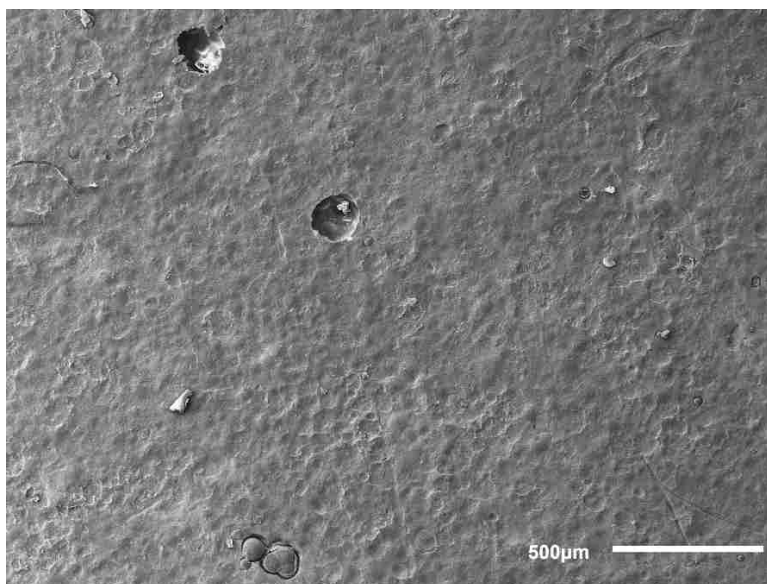


Figure 4.2: SEM image of the surface of PMMA material that had silver benzoate added to it. Less cracking was seen compared to PMMA only, but appeared to have a rougher, low profile crater-like texture. Small pockets or holes of what were presumed to be unpolymerized regions were observed on rare occasion.

When CZ-99 was added to PMMA at a final concentration of 0.4% w/w, a notable change was present in surface morphology. Large pockets of what were likely

unpolymerized regions were seen throughout (Figure 4.3). These pockets, or porous openings, may have contributed to wide scale release of CZ-99, which, as will be shown below, may have influenced biofilm formation outcomes. The pockets, or porosity of the material was presumed to occur due to a result of the powder mixture (CZ-99 was added as a powder to the acrylic powder) and lack of fully solubilized CZ-99. Thus, pockets in the material were left behind. Despite the porous nature of the surface, those regions that were not porous maintained similar morphology as PMMA only (Figure 4.3).

Lastly, the combination material, which contained additives of CZ-99 and silver each at a final concentration of 0.2% w/w (for an overall total of 0.4% w/w additives), likewise showed signs of pores having formed in the material, but to a lesser degree than the PMMA with CZ-99 (Figure 4.4). Notably, the other areas where pockets were not present likewise had similar morphology to the PMMA only material.

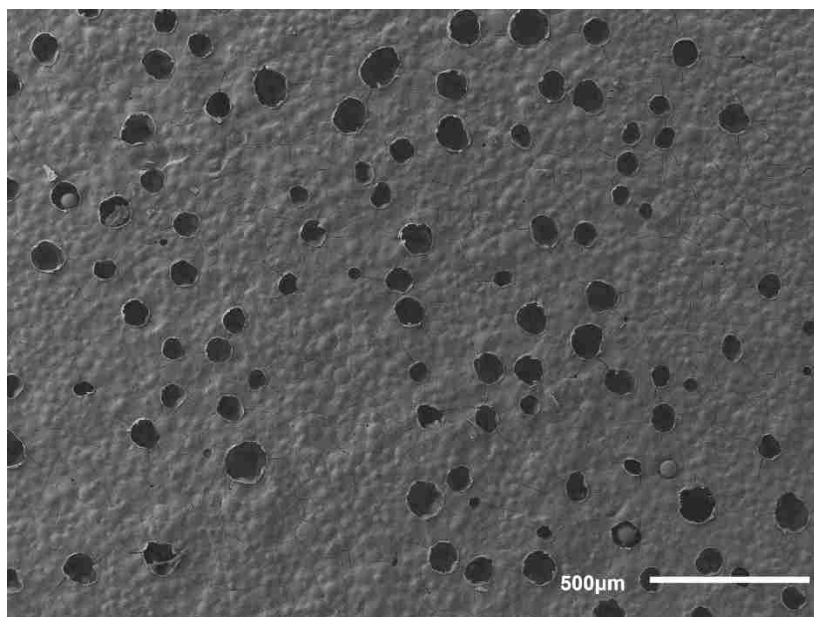


Figure 4.3: SEM image of PMMA that incorporated CZ-99. Note the pockets, or pores that were present.

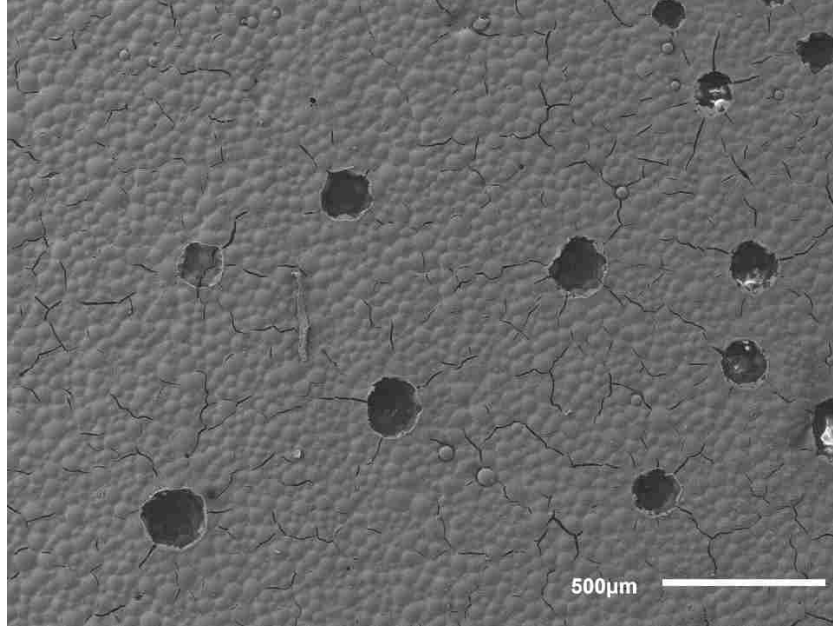


Figure 4.4: SEM image of PMMA that had CZ-99 and silver combined in the material. Note the presence of pockets/pores, but to less extent than the CZ-99 only material.

In addition to qualitatively observing the surface morphology, roughness of each material type was also determined. Data showed that the PMMA control, and both material types that contained CZ-99 had similar roughness profiles. Specifically, the Ra for the control (PMMA only) was 4.32 μm , for CZ-99 only the Ra was 3.51 μm and for the combination material the Ra was 4.54 μm (see Figure 4.5 – 4.7). In contrast, coupons that contained silver had a much rougher surface with a Ra of 8.32 μm (Figure 4.8), which was 2 – 3x rougher than control or coupons that contained CZ-99. Statistical analysis was not performed on these differences given that only one sample was used for analysis, but the trend of roughness was apparent.

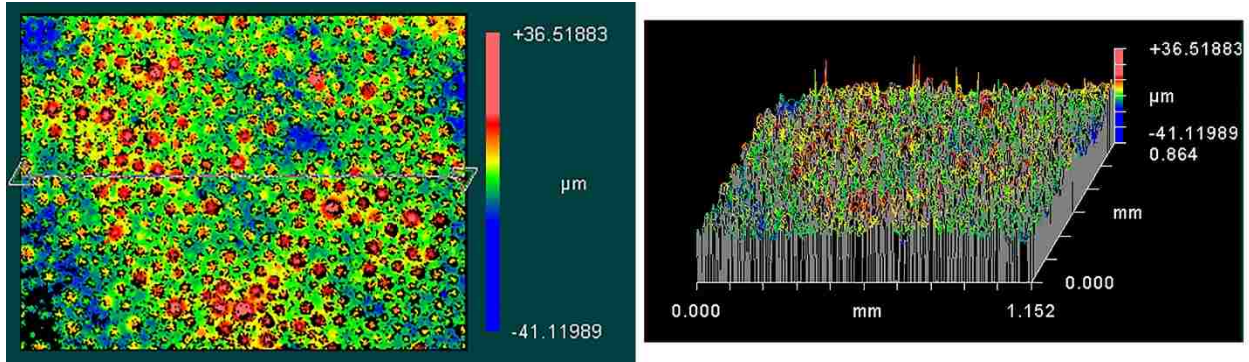


Figure 4.5: Topographical imaging and measurement of Ra for control (PMMA only) material. (Left) Overhead view of the profilometry results. The interface of green and yellow indicated the mean height with pink and blue coloring defining the peak and valley deviations, respectively. (Right) Oblique view of the profilometry results. From this image, the areas of measure including length, width and heights could be seen.

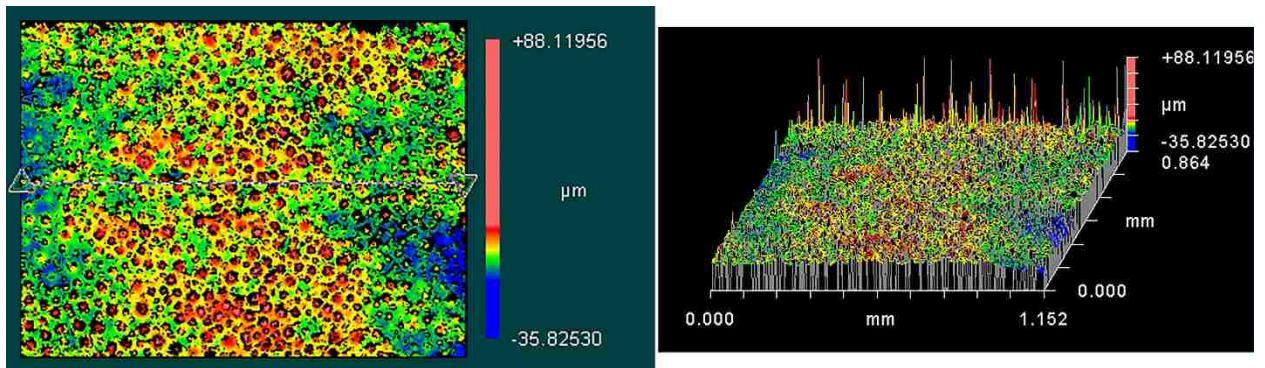


Figure 4.6: Topographical imaging and measurement of Ra for PMMA with CZ-99. (Left) Overhead view of the profilometry results. As mentioned in Figure 4.5, the interface of green and yellow indicated the mean height with pink and blue coloring defining the peak and valley deviations, respectively. (Right) Oblique view of the profilometry results. From this image, the areas of measure including length, width and heights could be seen.

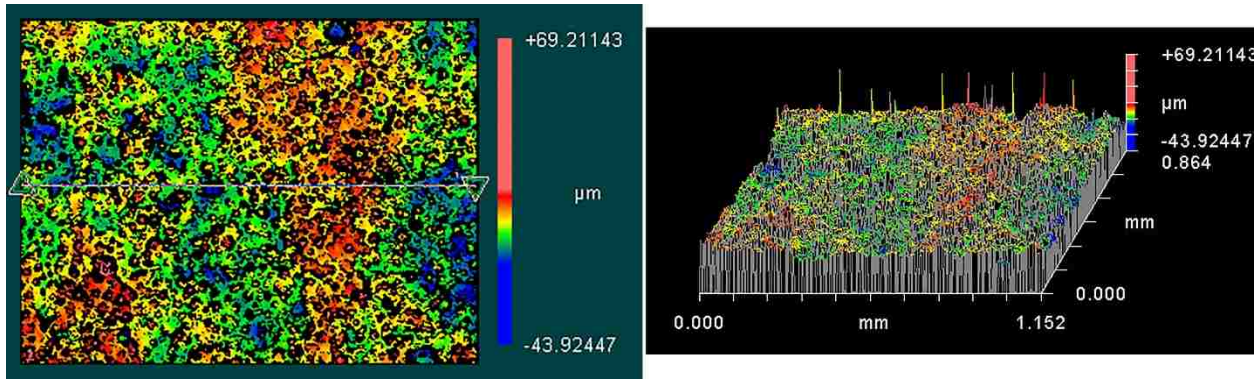


Figure 4.7: Topographical imaging and measurement of Ra for PMMA with a combination of CZ-99 and silver. (Left) Overhead view of the profilometry results. As mentioned in Figure 4.5, the interface of green and yellow indicated the mean height with pink and blue coloring defining the peak and valley deviations, respectively. (Right) Oblique view of the profilometry results. From this image, the areas of measure including length, width and heights could be seen.

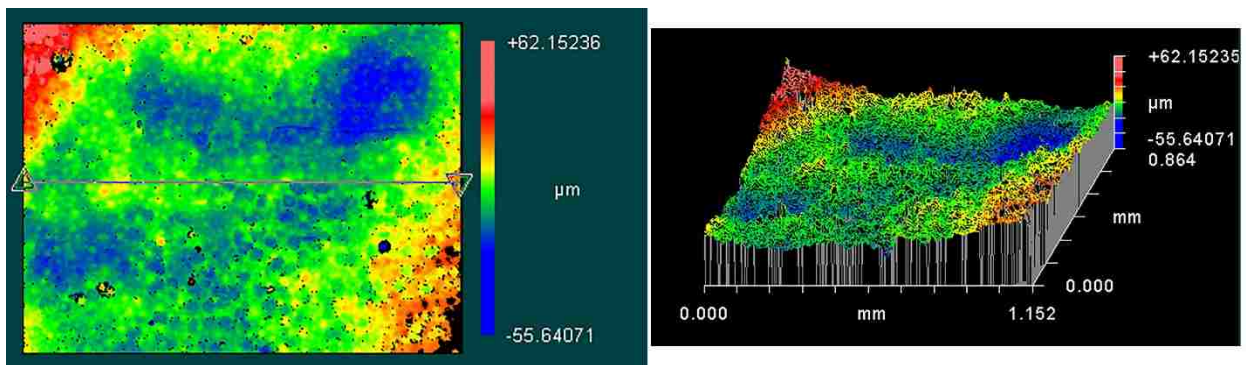


Figure 4.8: Topographical imaging and measurement of Ra for PMMA with silver only. (Left) Overhead view of the profilometry results. As mentioned in Figure 4.5, the interface of green and yellow indicated the mean height with pink and blue coloring defining the peak and valley deviations, respectively. (Right) Oblique view of the profilometry results. From this image, the areas of measure including length, width and heights could be seen.

4.2 Surface Coverage of Biofilms

The 24 h growth period was successful at forming significant and robust biofilms of *S. mutans*. Positive control samples were analyzed first. Gross photographs were taken by placing each coupon on a light box. Data showed that the reactor design and

setup allowed biofilms to form readily on the material surface with all five positive control samples showing significant biofilm growth (Figure 4.9).



Figure 4.9: Gross photographic images of positive control coupons. Red/purple areas were indicative of ruthenium red that stained the biofilms that were present. The pattern of broth flow was also highlighted by the stain. Note the speckled appearance in many regions. These were plumes of biofilms, as verified by the SEM images provided below.

In the case of CZ-99 loaded PMMA, two out of five samples had minimal growth of biofilms (Figure 4.10), but the remaining three had similar biofilm coverage compared to controls.



Figure 4.10: Gross photographic images of coupons that had CZ-99 incorporated. As with controls, red/purple areas were indicative of ruthenium red that stained the biofilms.

When silver and CZ-99 were combined in the PMMA material, outcomes were similar to what was seen with CZ-99 alone. More specifically, two out of five samples had relatively low amounts of biofilm formation (Figure 4.11). Yet the remaining three

had significant coverage. Notably, the addition of silver benzoate resulted in a darkening of the material, which was not unexpected, but observed (Figure 4.11).



Figure 4.11: Gross photographic images of coupons that had CZ-99 and silver benzoate incorporated. As with controls, red/purple areas were indicative of ruthenium red that stained the biofilms.

In the case of PMMA that was loaded with silver benzoate, biofilm formation was almost completely prevented (Figure 4.12). Relatively minimal amounts of biofilm plumes were seen on three of the coupons, with no bacteria detectable by gross photography on the two others (Figure 4.12). The silver-loaded PMMA coupons had cheetah print-like features (see Figure 4.12). This was a result of the phase separations that occurred and transferred during the Salt and Pepper method (refer Figure 3.1).



Figure 4.12: Gross photographic images of coupons that were loaded with silver only. Note the cheetah print-like features. Biofilm plumes were visible on the three right-most coupons. The graininess seen on the left hand side of the second coupon in was a result of the band saw cuts.

4.2.1 Surface Coverage Analysis by %

To determine the % of surface coverage that resulted from biofilm formation on each material type, gross photographs were first adjusted to a black & white (Figure 4.13). Because the coupons had differing widths and heights, the regions of analysis were normalized by cropping images to a 10 mm x 20 mm image. All images were further adjusted to similar brightness and contrast using Fiji software so as to normalize the data measurements. A proprietary MatLab code was used to further adjust images to blue/green (Figure 4.13) to determine surface coverage of biofilms on a % basis. The ruthenium red stain allowed for sufficient contrast to exist such that this process in general could be performed (see Figure 4.9).

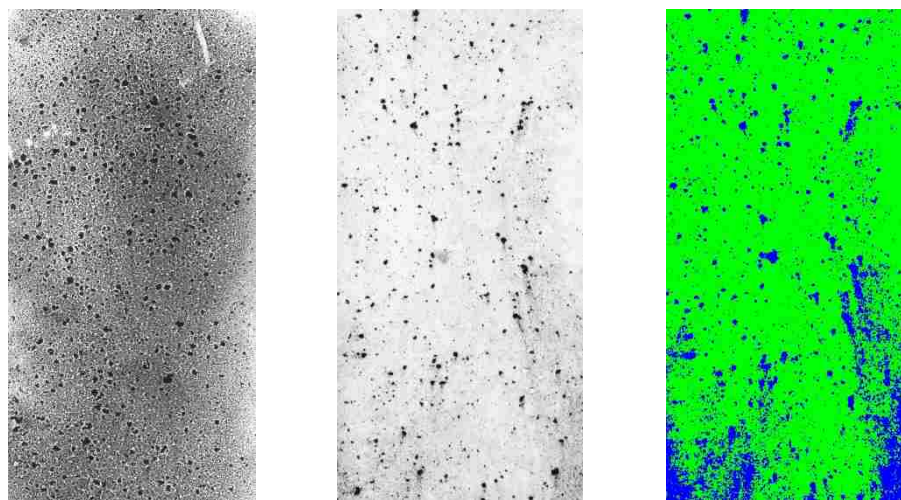


Figure 4.13: Representative images showing black/white or blue/green adjustments of gross photographs. In the images on the left and middle, the black regions (smears and dots) are biofilms and white is the PMMA surface. In the image on the right, blue was biofilm and green the material surface. These images are provided to show how contrast was provided to perform the % surface coverage analysis.

Data indicated that biofilms on positive control surfaces covered $67\% \pm 11\%$ of the surface (Figure 4.14). For coupons that had CZ-99 incorporated, biofilms were found to cover $51\% \pm 32\%$ of the surface. Coupons that had CZ-99 and silver mixed,

biofilms were found to cover $69\% \pm 26\%$ —the highest of all groups tested. Despite a trend of data showing that CZ-99 may reduce biofilm formation, no coupons that contained CZ-99 had a statistically significant difference in outcomes when compared to positive controls of growth. The sample size (n) will need to be increased in future studies and a potential method for improving polymerization determined before making conclusive suggestions for the CZ-99 materials (Figure 4.14).

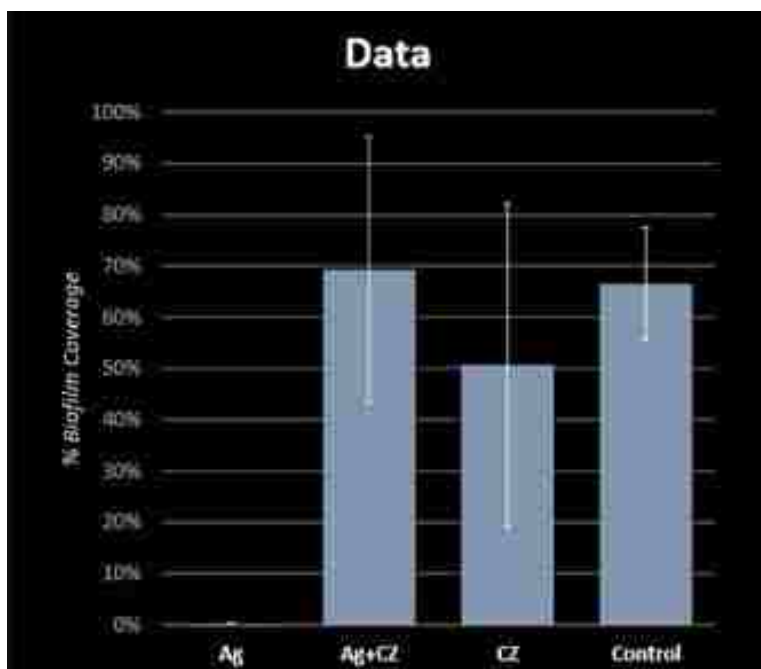


Figure 4.14: Bar graph showing the % biofilm coverage for each type of material tested. The silver infused PMMA % coverage was less than 0.3% and does not appear to register on the bar graph.

In the case of coupons that had silver incorporated, biofilms covered $0.1\% \pm 0.2\%$ of coupon surfaces (Figure 4.14). This was the only group of coupons that showed a statistically significant difference in the amount of biofilm that covered the surface between all groups. In short, data indicated that silver effectively prevented formation of *S. mutans* biofilms when exposed to an environment that modeled the human mouth.

4.3 SEM Imaging of Biofilms

SEM images of the biofilms were also used to determine the primary outcome measure, as well as provide a secondary outcome measure of biofilm morphology. Following gross photography, coupons were coated with gold as described previously. Coupons were placed on a metal stage in a JEOL JSM-6100 SEM. Positive control coupons were imaged first. Images confirmed what had been observed and measured from the ruthenium red analysis. More specifically, qualitative images showed that biofilms had covered the surface of the coupons (Figure 4.15). Plumes of *S. mutans* biofilms were observed covering the large majority of the surface. Higher magnification images showed that the classical chain-like appearance of *S. mutans* was present. This also confirmed that the isolate was pure and not contaminated.

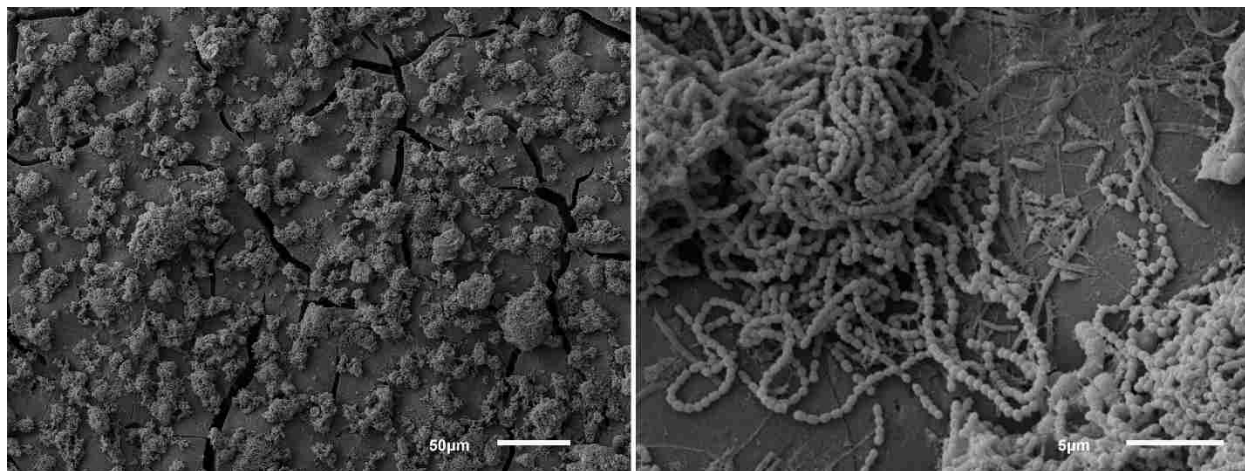


Figure 4.15: SEM images of *S. mutans* biofilms. (Left) A low magnification view of *S. mutans* biofilms grown on the surface of PMMA. Note the plumes. These developed as water flowed through the colonies, resulting in water channels that transport nutrients and molecules. Plumes were indicative that a mature biofilm had formed. (Right) High magnification view of *S. mutans* biofilms on PMMA. Note the chain-like appearance: the classic sign of streptococcal growth. In the upper right region of the image, the formation of a matrix "bed" can be seen.

Similar biofilm growth was seen on the CZ-99 PMMA material and the mixture PMMA material with silver and CZ-99 (see Figures 4.16 & 4.17, respectively).

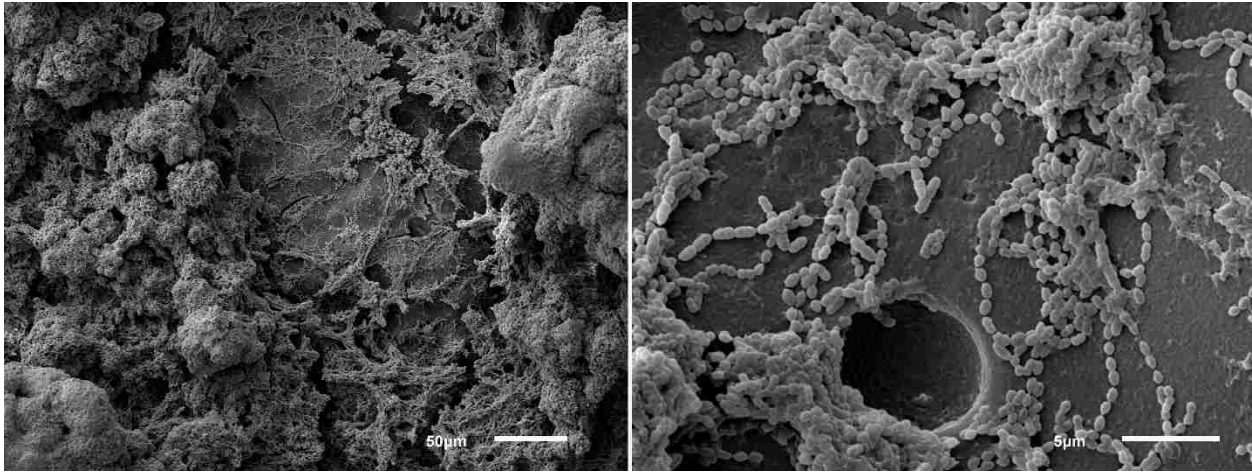


Figure 4.16: SEM images of *S. mutans* biofilms on coupons that had CZ-99 incorporated. (Left) A low magnification view of *S. mutans* biofilms. Note the polymeric nature of the biofilms and how they connect to one another. (Right) High magnification view of *S. mutans* biofilms on PMMA that contained CZ-99. As above, note the chain-like appearance: the classic sign of streptococcal growth. A bacterial cell can be seen delving into a pocket of unpolymerized material.

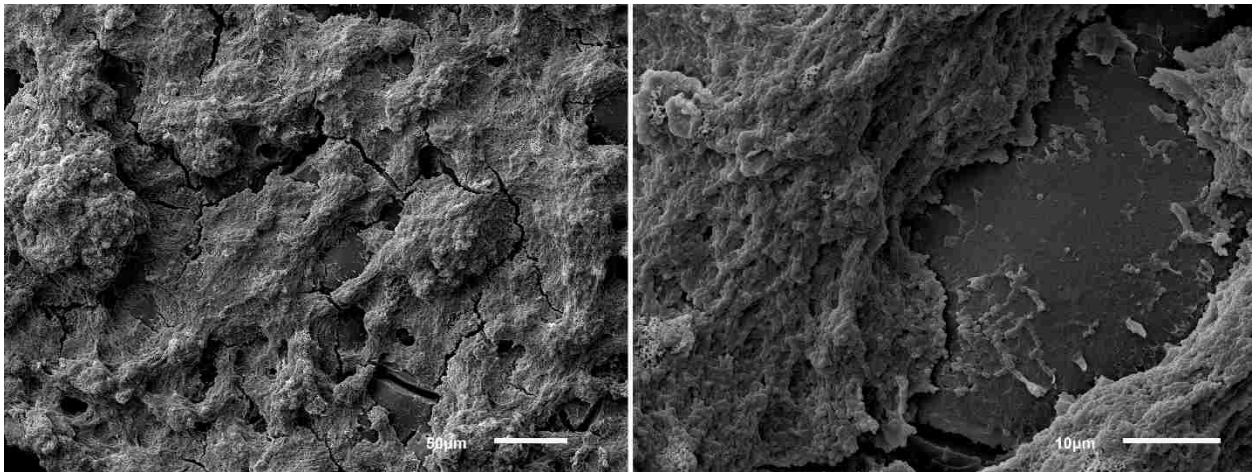


Figure 4.17: SEM images of *S. mutans* biofilms on coupons that had a mixture of silver and CZ-99 incorporated. (Left) A low magnification view of *S. mutans* biofilms. Note the extensive formation of biofilms. Between the large plumes, several pockets of unpolymerized material could be seen. It may have been that these pockets improved biofilm growth. (Right) High magnification view of *S. mutans* biofilms. The chain-like appearance was likewise present, but these biofilms seemed to produce more matrix material, giving a mucoid appearance to the biofilms.

In the case of the silver-loaded PMMA coupons, there was almost no indication of *S. mutans* biofilm formation (Figure 4.18). This was consistent with the results of gross photography and ruthenium red analysis.

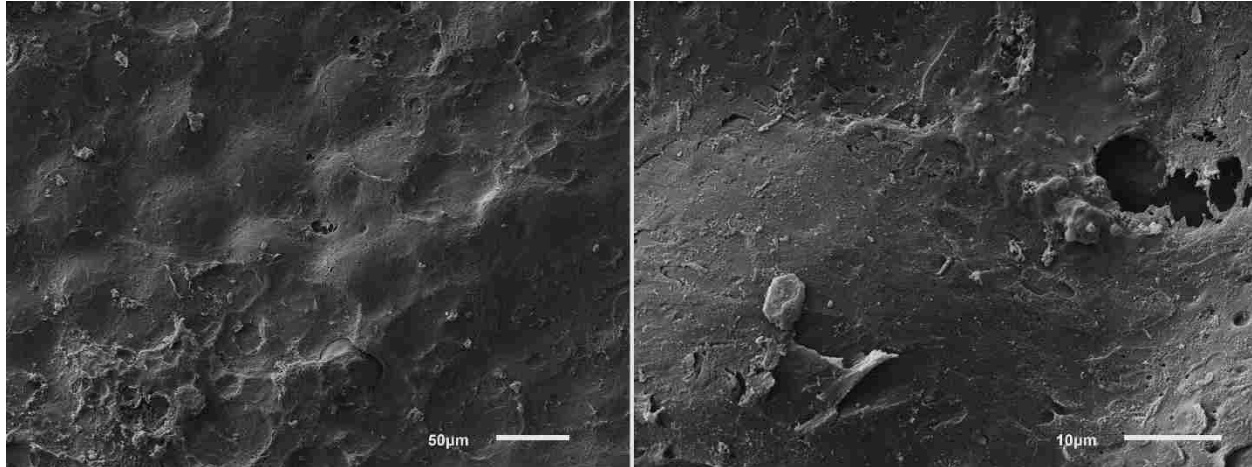


Figure 4.18: SEM images of PMMA with silver that was void of biofilm formation. (Left) A low magnification view of the surface. The rough regions were likely debris or materials from the flow reactor system. (Right) High magnification view of the surface. Debris, etc., was likely from the flow conditions.

4.3 Biofilm Plume Height

As another secondary outcome measure, Mountain Maps 3D software was used to determine plume heights of the biofilms on each of the surfaces. First, a baseline of height was established for the PMMA and silver only surfaces since these had the same profiles (i.e., no bacterial biofilms present; see Figure 4.19).

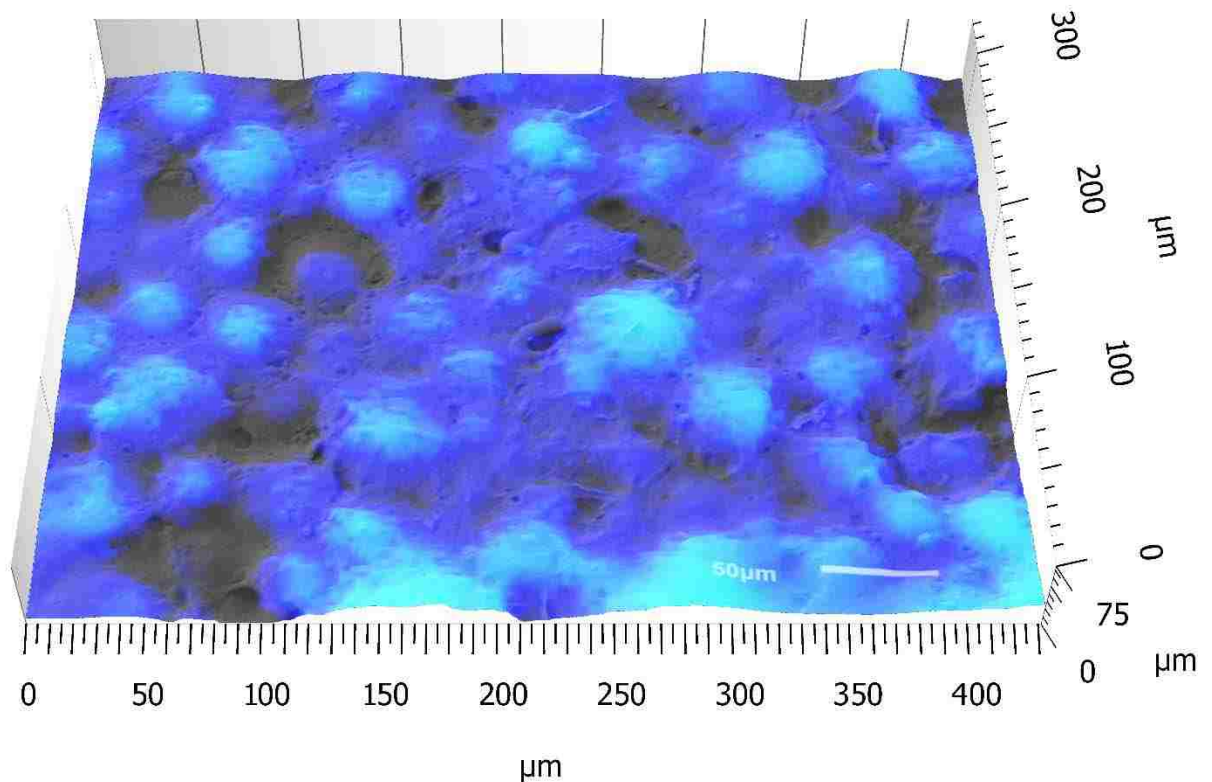


Figure 4.19: Reconstructed 3D image of the PMMA surface. This and the silver incorporated coupons had similar 3D profiles. The peaks (light blue) ranged in heights from roughly 10 – 20 μm . The gray regions are the base material. This provided the baseline height of 0.

Controls of positive growth were analyzed next. In the image provided below (Figure 4.20), relatively low profile biofilms are seen (turquoise), but reached heights of approximately 50 μm (yellow and red; see Figure 4.20).

Samples that had CZ-99 incorporated showed significant heights of plume formation with deep peak and valley formations. Plumes towered to over 75 μm in many cases (Figure 4.21). Gray coloring demonstrated the lowest height with green, yellow, red and white showing increasing height in linear fashion. The maturity and extent of biofilm formation was believed to be influenced by the presence of the pockets where it was believed unpolymerized material had resided (Figure 4.21).

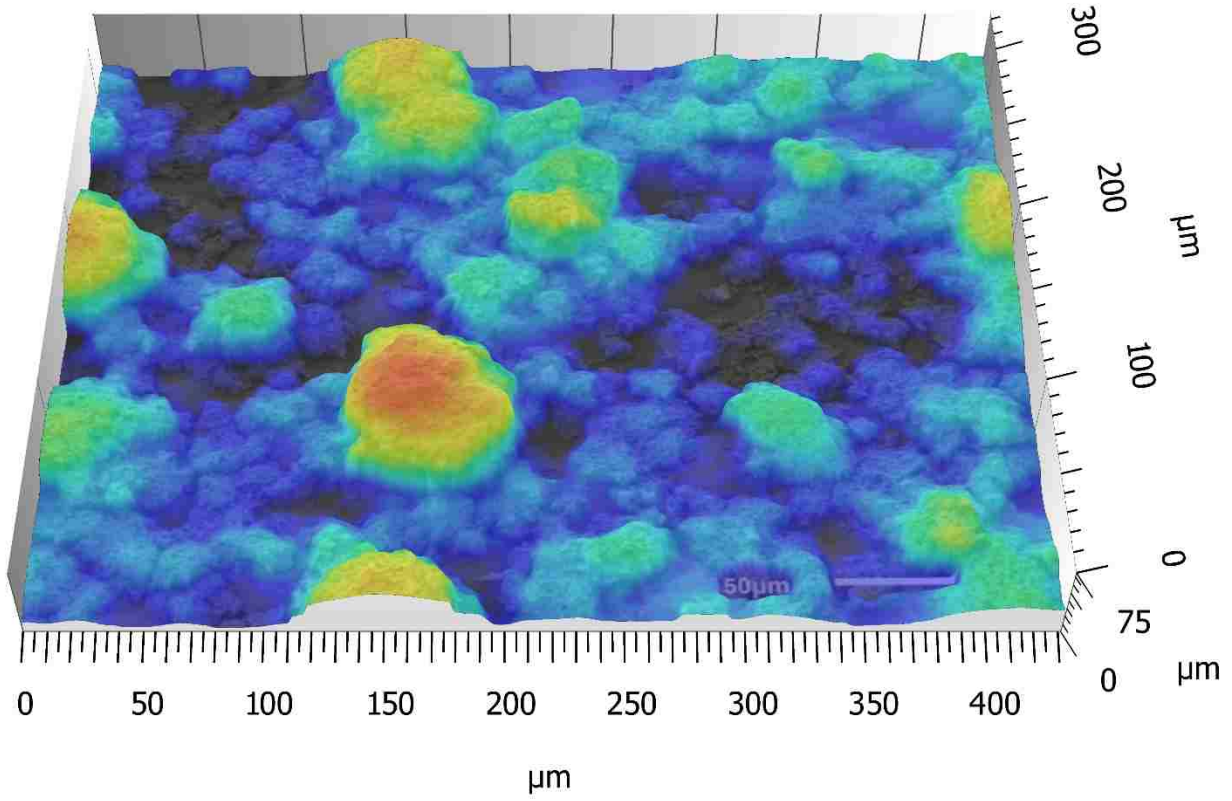


Figure 4.20: Reconstructed 3D image of a control PMMA surface that had biofilm growth. Plume heights reached approximately 50 μm. As above, the gray regions are the base material.

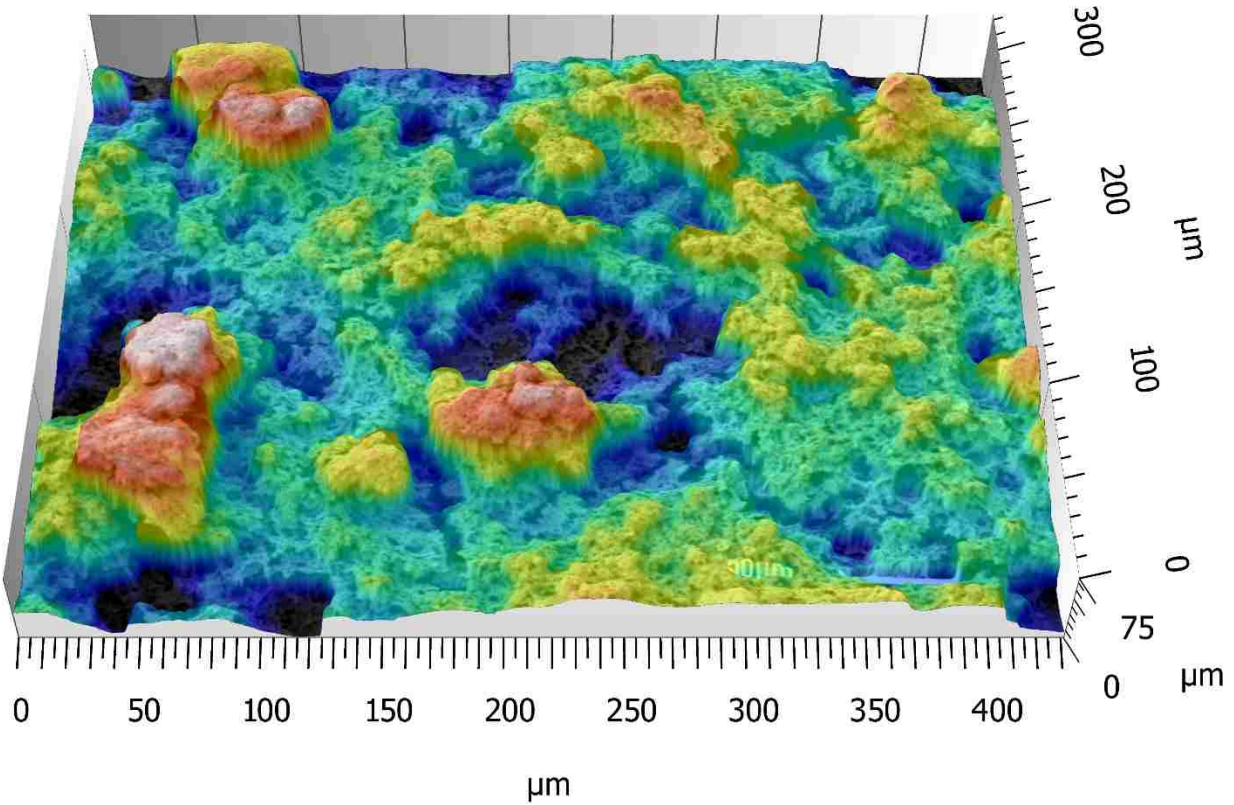


Figure 4.21: Reconstructed 3D image of PMMA material that had CZ-99 incorporated. Tall peaks (white and red) towered to more than 75 μm in many cases. Given that the diameter of a *S. mutans* cell is less than 1 μm , a biofilm plume with that height constituted more than 100 cells stacked on top of one another to create an intricate, complex community.

Similar plume heights were observed on samples that had a mixture of CZ-99 and silver (Figure 4.22).

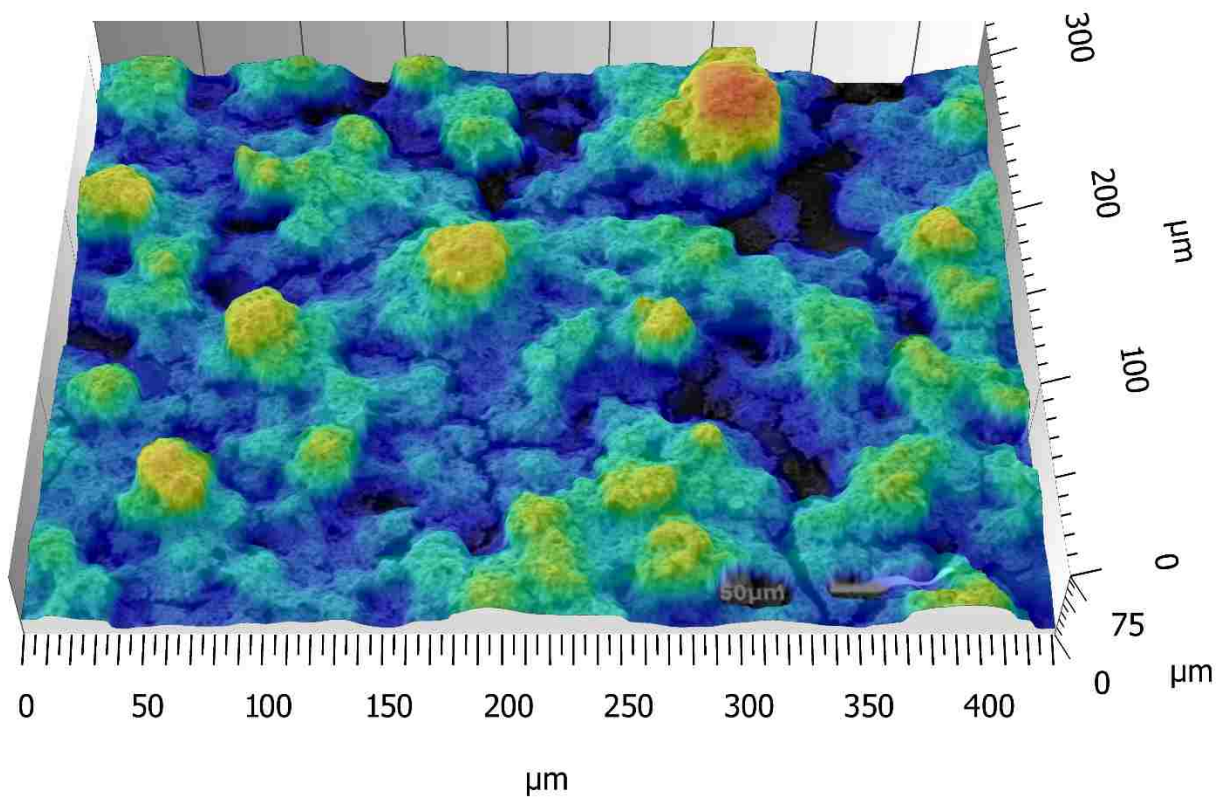


Figure 4.22: Reconstructed 3D image of PMMA material that had CZ-99 and silver incorporated. Although peak heights were not as significant as biofilms on the CZ-99 coupons, the coverage of lower height biofilms on the surface appeared to be more significant.

CHAPTER 5

DISCUSSION, LIMITATIONS, RECOMMENDATIONS, AND CONCLUSION

5.1 Discussion

One of the most promising approaches for reducing biofilm-related pathologies in a variety of medical settings is the incorporation of antimicrobial compounds into polymeric materials. The rationale is that the device has the potential to be protected and prevent formation of biofilms, which has the beneficial downstream effect of reducing risk for patient infection to occur. In the case of dentistry and orthodontics, this infection presents as caries formation. Thus, this study aimed to determine the ability of incorporating antimicrobial compounds into PMMA and assess its ability to prevent biofilm formation from occurring.

Taken together, data suggested that silver was able to effectively prevent formation of *S. mutans* biofilms under rigorous conditions for biofilm growth. This is consistent with what has been shown in the literature, but advances these data by growing biofilms of *S. mutans* under clinically-relevant conditions in a modified drip flow reactor that more closely models the human oral cavity. Data with materials that contained CZ-99 showed a trend toward biofilm formation prevention, but were inconclusive with regard to materials that contained CZ-99 as the powder that was used may have not solubilized in the monomer and thus left behind “large” pockets or craters in the materials surface that may have allowed CZ-99 to elute out of the material and thus not influence biofilm formation (see Figures 4.3 & 4.4). In addition, the pockets that

formed may have exacerbated biofilm growth given that they may have provided unique attachment points for bacterial adherence. Further testing will be needed to assess CZ-99 efficacy, but the data indicated that the general hypothesis was supported. More specifically, the addition of antimicrobial substances to PMMA did reduce biofilm growth. However, the subhypothesis—that a combination of silver and CZ-99, a unique antimicrobial, will reduce biofilm formation to the greatest degree when compared to PMMA alone or the antimicrobial agents as individual additives—was not supported. These results may be promising to advance this testing toward additional *in vitro* analyses, but most importantly toward *in vivo* testing where influence on caries formation would be more relevant.

Importantly, the question may arise as to whether the differences in surface morphology are what contributed to biofilm formation, and not the antimicrobials. Specifically, the material surface morphologies of PMMA only and both of those that contained CZ-99 were strikingly similar (with the exception of the pockets/pores). In contrast, the silver-loaded material had a much rougher surface. One might argue that this difference in morphology is what contributed to the differences in growth outcomes. However, it is well known that bacterial biofilms form to a greater degree on rough surfaces. Given that the silver-loaded material had a Ra, and thus roughness, that was much greater than PMMA only or those materials that contained CZ-99, biofilms should have developed to greater maturity on the silver. These outcomes indicated that it was indeed the silver that resulted in reduced biofilm formation on silver-loaded materials, and not the difference in surface roughness.

S. mutans is well-known and often cited as the hinge upon which bacterial attachment and biofilm formation occurs on teeth. In higher magnification images of the biofilms, polymeric structures, i.e., a “bed” of matrix was observed as it was developing, which helped to support this concept of *S. mutans* laying down an extracellular matrix to which other bacteria can attach (see Figure 4.15).

5.2 Limitations

The method for fabricating the PMMA was realized by following the manufacturer’s recommendation for the “Salt and Pepper” method, but this method is unprecise and inexact. This method of fabrication made it impossible to ensure homogeneity throughout the PMMA coupons.

Originally the coupons were cut to 3” X 1” for a nice fit in the drip flow reactor. However, the slides were later cut into smaller pieces (12mm X 24mm) to function with the custom made reactor system. Biofilm growth may have been limited as the surface area across which the bacterial broth traversed was decreased. Greater surface area may have allowed for additional biofilm growth with increased area for analysis.

Despite *S. mutans* being facultative, the cultures grew well in aerobic conditions. While other similar studies utilized a tri-gas incubator or other anaerobic conditions, American Tissue Type Culture (ATTC), which provided the bacterial cultures, confirmed that these bacteria would grow under aerobic laboratory conditions. It is possible that under preferred conditions of an anaerobic environment, more growth may have been present. This anaerobic environment may better replicate the conditions under which *S. mutans* biofilms might flourish.

While the drip flow reactor is an effective tool to model the oral environment, there are many components that cannot be duplicated *in vitro*. Saliva has many components that contribute to the salivary pellicle and ultimately affect the microbiota that exist in the oral cavity. This study did not use saliva to pretreat the PMMA samples for a closer comparison. Salivary components of an individual can vary based on diet, nutrition, and even genetics. Sheer forces that are introduced by mastication and agglutination were not duplicated or modeled in this study. The ecology of the oral cavity is complex and diverse and *S. mutans* does not grow in isolation as was studied here.

5.2.1 Technical Setup

Originally the PMMA coupons were cut to 3" X 1" sections for a nice fit in the drip flow reactor. However, the slides were later cut into smaller pieces (12 mm X 24 mm) to increase the number of available test materials. The biofilm growth was limited as the surface area across which the bacterial broth would traverse was greatly decreased. Greater surface area may have allowed for additional biofilm growth with increased area for analysis.

After months of experimental runs, the original drip flow reactor that was used was autoclaved and sterilized many times and began to break down, develop cracks and began to leak (Figure 5.1). The old reactor became inefficient due to the reduced number of samples that could be analyzed in each run. These months of testing were not a complete loss as we were able to refine and improve our study design. This testing period helped to determine the optimal batch phase growth time of 6 h and the

24 h biofilm growth period was effective and adequate. A modified drip flow reactor system was developed to increase the number of samples that could be analyzed in each experimental set (Figure 3.3). This new reactor set-up also improved the quality of the results as more samples were run under the same conditions allowing for stronger comparison.

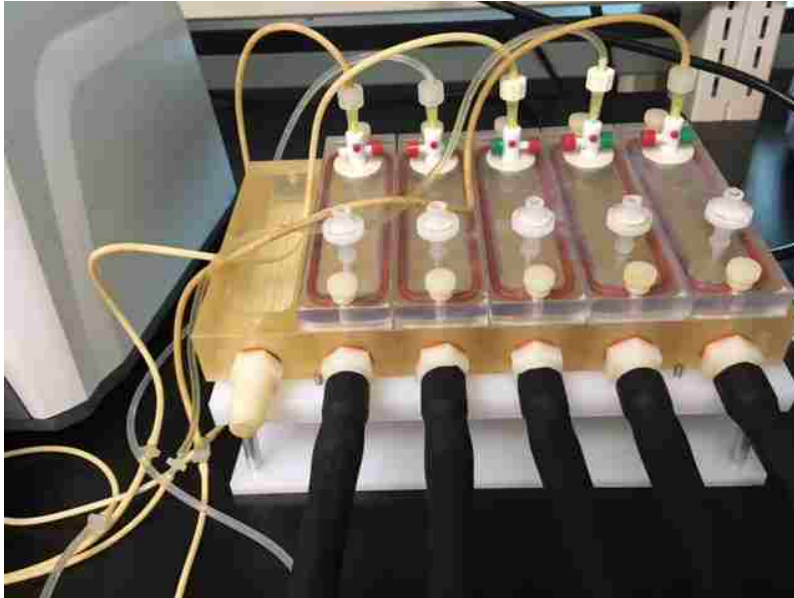


Figure 5.1: Set up of the original drip flow reactor.

SEM imaging allowed us to perform qualitative analysis of biofilm growth. Contamination was a recurrent issue with some of the earlier experimental runs. It was determined that some bacteria other than *S. mutans* were surviving the autoclaving process. Aseptic techniques were improved and experimentally determined to ensure sterility. None of the contaminated samples were analyzed or included in the final data analysis.

Variable data was collected that made it difficult to determine what was contributing to biofilm growth and what factors might be inhibiting its growth. Months of

data collection was inconclusive. Each variable of the set-up was investigated experimentally. Eventually it was determined that the nutrient broth that was running through the drip flow reactor varied in temperature because it was kept outside of the incubator. Once the nutrient broth was moved inside of the incubator, the results became more consistent and allowed for conclusive data collection and analysis (Figure 3.3).

5.3 Recommendations

Future research should focus not only on *In vitro* but also *in vivo* studies on the biofilm formation on orthodontic baseplate materials. In addition to caries-causing bacteria, those organisms involved in periodontal disease and fungal diseases such as *Candidiasis* should be investigated. Split mouth studies involving retainers consisting of varying antibacterial materials, would allow a direct comparison of the products and could demonstrate how biofilms may change with time. Another method for testing could be using fluorescence or radiolabeling to measure cell adhesion and viability. Furthermore, future studies could also determine how bacterial growth is affected by the salivary pellicle and its behavior over time as attachment sites decrease.

Studies could be performed to see how other oral bacteria would interact with the biofilm formation on PMMA with antibacterial inclusions. Specifically *Lactobacillus Acidophilus*, a secondary colonizer, as it is considered a common caries marker could be studied. For clinical relevance, LA should be investigated with SM as it uses the SM biofilm as a substrate for its own proliferation.

Different concentrations of silver should be tested *in vivo* to determine what concentration of silver of inclusion is most effective without substantial alteration of the mechanical properties. Different antibacterial substances should be investigated and compared to silver for effectiveness. Specifically, efforts should be made to find covalently-bonded antibacterial products so that its effects are not diminished upon leaching (i.e. quaternary ammonium or tethered antibiotics).

As many clinicians are now choosing Essix style retainers over the Hawley style retainers, it would be prudent to look at the possibility of incorporating antibacterial additives into the polymers that are used in Essix retainers. There are companies that are already manufacturing medical grade antibacterial plastics, but their minimum order quantities were prohibitive for this study. This may have additional application due to the increase in clear aligner therapy as a common alternative to fixed appliances.

Additional knowledge could be gained by using an improved staining method for visualizing the attachment patterns of specific bacteria and its effect on overall biofilm formation.

New studies could investigate biofilm growth on the other surfaces of orthodontic retainers. The traditionally rougher intaglio surface of a Hawley retainer which rests on the palate or gingival floor may differ in adhesion properties from those found elsewhere. These projects could investigate how the additional physical attributes of a Hawley retainer, such as stainless steel clasps, or the surface contours that reflect the actual physical morphology of the palate, might contribute to bacterial adhesion. Testing could also be extended to examine polished versus unpolished surfaces to more accurately explain the contribution of surface energy to biofilm formation.

5.4 Conclusion

Results from this study demonstrated significant reduction in biofilm formation in silver-infused PMMA. Outcomes provided a compelling argument that the drip flow system and materials could be investigated further to determine if antibacterial PMMA can be used safely in the oral environment and explore their effectiveness in reducing biofilm formation. Future work with *in vivo* testing would be particularly beneficial.



**UNLV Biomedical IRB - Administrative Review
Notice of Excluded Activity**

DATE: March 4, 2016
TO: Katherine Howard, PhD
FROM: UNLV Biomedical IRB
PROTOCOL TITLE: [875905-1] Exempt protocol: Antibacterial activity of orthodontic appliance base plate material PMMA infused with Ag and other novel antibacterial compounds
SUBMISSION TYPE: New Project
ACTION: EXCLUDED - NOT HUMAN SUBJECTS RESEARCH
REVIEW DATE: March 4, 2016
REVIEW TYPE: Administrative Review

Thank you for your submission of New Project materials for this protocol. This memorandum is notification that the protocol referenced above has been reviewed as indicated in Federal regulatory statutes 45CFR46.

The UNLV Biomedical IRB has determined this protocol does not meet the definition of human subjects research under the purview of the IRB according to federal regulations. It is not in need of further review or approval by the IRB.

We will retain a copy of this correspondence with our records.

Any changes to the excluded activity may cause this protocol to require a different level of IRB review. Should any changes need to be made, please submit a Modification Form.

If you have questions, please contact the Office of Research Integrity - Human Subjects at IRBi@unlv.edu or call 702-895-2794. Please include your protocol title and IRBNet ID in all correspondence.

Office of Research Integrity - Human Subjects
4505 Maryland Parkway, Box 451047, Las Vegas, Nevada 89154-1047
(702) 895-2794, FAX: (702) 895-0805, IRBi@unlv.edu

BIBLIOGRAPHY

- Addy, M., Shaw, W. C., Hansford, P., & Hopkins, M. (1982). The effect of orthodontic appliances on the distribution of candida and plaque in adolescents. *Br J Orthod*, 9(3), 158-163.
- Ahn, S. J., Lim, B. S., Lee, Y. K., & Nahm, D. S. (2006). Quantitative determination of adhesion patterns of cariogenic streptococci to various orthodontic adhesives. *Angle Orthod.*, 76(5), 869-875.
- Ahrari, F., Eslami, N., Rajabi, O., Ghazvini, K., & Sahar, B. (2015). The antimicrobial sensitivity of *streptococcus mutans* and *streptococcus sangius* to colloidal solutions of different nanoparticles applied as mouthwashes. *Dental Research Journal*, 12(1), 44-49.
- Alexander, S. A. (1991). Effects of orthodontic attachments on the gingival health of permanent second molars. *American Journal of Orthod. Dentofac. Orthop.*, 100(4), 337-340.
- Alves, P. V., Alviano, W. S., Bolongnese, A. M., & Nojima, L. I. (2008). Treatment protocol to control streptococcus mutans level in an orthodontic patient with high caries risk. *American Journal of Orthod. Dentofac. Orthop.*, 133(1), 91-94.

- Ammons, M., Ward, L., & James, G. (2011). Anti-biofilm efficacy of lactoferrin/xylitol wound hydrogel used in combination with silver wound dressings. *Int Wound Journal*, 8, 268-273.
- Appelbaum, B., Golub, E., Holt, S. C., & Rosan, B. (1979). In vitro studies of dental plaque formation: Adsorption of oral streptococci to hydroxyapatite. *Infect Immun*, 25(2), 717-728.
- Arendorf, T., & Addy, M. (1985). Candidal carriage and plaque distribution before, during and after removable orthodontic appliance therapy. *Journal of Clinical Periodontology*, 12(5), 360-368.
- Atack, N. E., Sandy, J. R., & Addy, M. (1996). Periodontal and microbiological changes associated with the placement of orthodontic appliances. A review. *Journal of Periodontology*, 67(2), 78-85.
- Balenseifen, J. W., & Madonia, J. V. (1970). *Journal of Dental Research*, 49(2), 320-324.
- Batoni, G., Pardini, M., Giannotti, A., Giuca, M. R., Gabriele, M., & Senesi, S. (2001). Effects of orthodontic attachments on the gingival health of permanent second molars. *European Journal of Oral Sciences*, 109(6), 388-392.
- Birdsall, J., & Robinson, S. (2008). A case of severe caries and demineralisation in a patient wearing an essix-type retainer. *Primary Dental Care : Journal of the Faculty of General Dental Practitioners*, 15(2), 59-61.

- Bjerklin, K., Garskog, B., & Ronnerman, A. (1983). Proximal caries increment in connection with orthodontic treatment with removable appliances. *Br J Orthod*, *10*, 21-24.
- Boersma, J. G., Van der Veen, M. H., Lagerweij, M. D., Bokhout, B., & PrahI-Andersen, B. (2005). Caries prevalence measured with QLF after treatment with fixed orthodontic appliances: Influencing factors. *Caries Research*, *39*(1), 41-47.
- Bollen, C. M., Lambrechts, P., & Quirynen, M. (1997). Comparison of surface roughness of oral hard materials to the threshold surface roughness for bacterial plaque retention: A review of the literature. *Dental Materials : Official Publication of the Academy of Dental Materials*, *13*(4), 258-269.
- Botha, S. J. (1993). Oral lactobacilli isolated from teenage orthodontic patients. *The Journal of the Dental Association of South Africa = Die Tydskrif Van Die Tandheelkundige Vereniging Van Suid-Afrika*, *48*(4), 177-181.
- Bowden, G. H. W., & Li, Y. H. (1997). Nutritional influences on biofilm development. *Advances in Dental Research*, *11*(1), 81-99.
- Busscher, H. J., Weerkamp, A. H., van der Mei, H. C., van Pelt, A. W., de Jong, H. P., & Arends, J. (1984). Measurement of the surface free energy of bacterial cell surfaces and its relevance for adhesion. *Applied and Environmental Microbiology*, *48*(5), 980-983.

- Cephas, K. D., Kim, J., Mathai, R. A., Barry, K. A., Dowd, S. E., Meline, B., et al. (2011). Comparative analysis of salivary bacterial microbiome diversity in edentulous infants and their mothers or primary care givers using pyrosequencing. *PLoS One*, 6(8)
- Cerny, R. (2001). Permanent fixed lingual retention. *J. Clin Orthod*, 35(12), 728-732.
- Cerny, R., & Lloyd, D. (2008). Dentists' opinions on orthodontic retention appliances. *J. Clin Orthod*, 42(7), 415-419.
- Chang, H. S., Walsh, L. J., & Freer, T. J. (1999). The effect of orthodontic treatment on salivary flow, pH, buffer capacity, and levels of mutans streptococci and lactobacilli. *Australian Orthodontic Journal*, 15(4), 229-234.
- Choi, D. S., Cha, B. K., & Jost-Brinkman, P. G. (2009). Microbiologic changes in subgingival plaque after removal of fixed orthodontic appliances. *The Angle Orthodontist*, 79(6), 1149-1155.
- Cohen, B. (1980). Research into dental caries. *British Dental Journal*, 149(1), 21-24.
- Dasanayake, A., Caufield, P., Cutter, G., & Roseman, J. (1995). Differences in the detection and enumeration of mutans streptococci due to differences in methods. *Arch Oral Biology*, 40, 345-351.
- Demir, A., Babacan, H., & Nalcaci, R. (2012). Comparison of retention characteristics of essix and hawley retainers. *Korean Journal of Orthodontics*, 42(5), 255-262.

- Doyle, R. J., & Rosenberg, M. (1995). Measurement of microbial adhesion to hydrophobic substrata. *Methods Enzymol*, 253, 542-550.
- Espinosa-Cristobal, L., & Martinez-Castanon, G. (2009). Antibacterial effect of silver nanoparticles against streptococcus mutans. *Materials Letters*, 63, 2603-2606.
- Fan, C., Lianrui, C., Rawls, H., Norling, B., Cardenas, H., & Whang, K. (2010). Development of an antimicrobial resin--A pilot study. *Academy of Dental Materials*, 11, 8.
- Fang, M., Chen, J., & Xu, X. (2006). Antibacterial activities of inorganic agents on six bacteria associated with oral infections by two susceptibility tests. *International Journal of Antimicrobial Agents*, 27, 513-517.
- Forssten, S. D., Björklund, M., & Ouwehand, A. C. (2010). *Streptococcus mutans*, caries and simulation models. *Nutrients*, 2(3), 290-298.
- Furno, F., Morley, K., & Wong, B. (2004). Silver nanoparticles and polymeric medical devices; a new approach to prevention of infection? *Journal of Antimicrobial Chemotherapy*, 54, 1019-1024.
- García-Godoy, F., & Hicks, M. J. (2008). Maintaining the integrity of the enamel surface: The role of dental biofilm, saliva and preventive agents in enamel demineralization and remineralization. *Journal of American Dental Association*, 139(Supplemental), 25S-34S.

- Gedik, H., & Ozkan, Y. K. (2009). The effect of surface roughness of silicone-based resilient liner materials on the adherence of candida albicans and inhibition of candida albicans with different disinfectants. *Oral Health Prev Dent*, 7(4), 347-353.
- Ghorbanzadeh, R., Pourakbari, B., & Bahador, A. (2015). Effects of baseplates of orthodontic appliances with in situ generated silver nanoparticles on cariogenic bacteria: A randomized, double-blind cross-over clinical trial. *The Journal of Contemporary Dental Practice*, 16(4), 291-298.
- Gibbons, R. J., & van Houte, J. (1973). On the formation of dental plaques. *Journal of Periodontology*, 44(6), 347-360.
- Gizani, S., Papaioannou, W., Haffajee, A. D., Kavvadia, K., Quirynen, M., & Papagiannoulis, L. (2009). Distribution of selected cariogenic bacteria in five different intra-oral habitats in young children. *International Journal of Paediatric Dentistry / the British Paedodontic Society [and] the International Association of Dentistry for Children*, 19(3), 193-200.
- Glans, R., Larsson, E., & Ogaard, B. (2003). Longitudinal changes in gingival condition in crowded and noncrowded dentitions subjected to fixed orthodontic treatment. *American Journal of Orthod. Dentofac. Orthop.*, 124(6), 679-682.
- Gorelick, L., Geiger, A. M., & Gwinett, A. J. (1982). Incidence of white spot formation after bonding and banding. *American Journal of Orthodontics*, 81(2), 93-98.

- Gracco, A., Mazzoli, A., Favoni, O., Conti, C., Ferraris, P., Tosi, G., et al. (2009). Short-term chemical and physical changes in invisalign appliances. *Australian Orthodontic Journal*, (25), 34-40.
- Gudkina, J., & Brinkmane, A. (2008). Caries experience in relation to oral hygiene, salivary cariogenic microflora, buffer capacity and secretion rate in 6-year olds and 12 year olds in riga. *Stomatologija*, 10(2), 76-80.
- Hahnel, S., Rosentrit, M., Bürgers, R., & Handel, G. (2008). Surface properties and in vitro streptococcus mutans adhesion to dental resin polymers. *J Mater Sci Mater Med*, 19(7), 2619-2627.
- Haruki, T., & Little, R. M. (1998). Early versus late treatment of crowded first premolar extraction cases: Postretention evaluation of stability and relapse. *Angle Orthod.*, 68(1), 61-68.
- Haussener, T., Paul, S., Haripradasa, K., & Williams, D. (2016). A practical synthesis of N-alkyl- and N,N'-dialkyl-polyamines. *Tetrahedron Letters*, 57, 2845-2848.
- Hichens, L., Rowland, H., Williams, A., Hollinghurst, S., Ewings, P., Clark, S., et al. (2007). Cost-effectiveness and patient satisfaction: Hawley and vacuum-formed retainers. *European Journal of Orthodontics*, 29(4), 372-378.
- Holla, G. Yeluri, R. Munshi, AK. (2012). Evaluation of minimum inhibitory and minimum bactericidal concentration of nano-silver base inorganic anti-microbial agent (Novaron[®]) against *streptococcus mutans*. *Contemp Clin Dent*, 3, 288-293.

- Horiuchi, M., Washio, J., Mayanagi, H., & Takahashi, N. (2009). Transient acid-impairment of growth ability of oral streptococcus, actinomyces, and lactobacillus: A possible ecological determinant in dental plaque. *Oral Microbiology and Immunology*, 24(4), 319-324.
- Hotta, M., Nakajima, H., Yamamoto, K., & Aono, M. (1998). Antibacterial temporary filling materials: The effect of adding various ratios of ag-zn-zeolite. *Journal of Oral Rehabilitation*, 25(7), 285-289.
- Hwang-Sog, R., In-Ho, B., & Kyung-Gu, L. (2012). Antibacterial effect of silver-platinum coating for orthodontic appliances. *Angle Orthod.*, 82(151), 157.
- Kassaei, M., Akhavan, A., Sheikh, N., & Sodagar, A. (2008). Antibacterial effects of new dental acrylic resin containing silver nanoparticles. *J. Appl Polym Sci*, 110(3), 1699-1703.
- Kawahara, K., Tsuruda, K., Morishita, M., & Uchida, M. (2000). Antibacterial effect of silver-zeolite on oral bacteria under anaerobic conditions. *Dental Materials*, 16, 452-455.
- Kawashita, W., Tsuneyama, S., Miyaji, F., Kokubo, T., Kozuka, H., & Yamamoto, K. (2000). Antibacterial silver-containing silica glass prepared by sol-gel method. *Biomaterials*, 21, 393-398.
- Kim, J., Kuk, E., & Yu, K. (2007). Antimicrobial effects of silver nanoparticles. *Nanomedicine; Nanotechnology, Biology, and Medicine*, 3, 95-101.

- Kitada, K., de Toledo, A., & Oho, T. (2009). Increase in detectable opportunistic bacteria in the oral cavity of orthodontic patients. *International Journal of Dental Hygiene*, 7(2), 121-125.
- Kolenbrander, P. E. (2000). Oral microbial communities: Biofilms, interactions, and genetic systems. *Annual Review of Microbiology*, 34, 413-437.
- Kolenbrander, P. E., Andersen, R. N., Blehert, D. S., Eglund, P. G., Foster, J. S., & Palmer, R. J., Jr. (2002). Communication among oral bacteria. *Microbiology and Molecular Biology Reviews : MMBR*, 66(3), 486-505.
- Lansdown, A. (2006). Silver in health care: Antimicrobial effects and safety in use. *Current Problems in Dermatology*, , 17-34.
- Lara-Carrillo, E., Montiel-Bastida, N. M., Sanchez-Perez, L., & Alanis-Tavira, J. (2010). Effect of orthodontic treatment on saliva, plaque and the levels of streptococcus mutans and lactobacillus. *Medicina Oral, Patologia Oral y Cirugia Bucal*, 15(6), e924-e929.
- Lessa, F., Enoki, C., Ito, I., Faria, G., Matsumoto, M., & Nelson-Filho, P. (2007). In-vivo evaluation of the bacterial contamination and disinfection of acrylic baseplates of removable orthodontic appliances. *American Journal of Orthod. Dentofac. Orthop.*, 131(705), e11-e17.
- Leung, N. M., Chen, R., & Rudney, J. D. (2006). *Oral Bacteria in Plaque and Invading Buccal Cells of Young Orthodontic Patients. American Journal of Orthodontics and*

Dentofacial Orthopedics : Official Publication of the American Association of Orthodontists, its Constituent Societies, and the American B, 130(6), 698.e11-698.e18.

Levrini, L., Mangano, A., Montanari, P., & Margherini, S. (2015).

Periodontal health status in patients treated with the invisalign® system and fixed orthodontic appliances: A 3 months clinical and microbiological evaluation. *European Journal of Dentistry*, (9), 404-410.

Lewis, E. A., Ogle, R. E., Sorensen, S. E., & Zysik, D. A. (1988). Clinical and laboratory evaluation of visible light-cured denture base resins and their application to orthodontics. *American Journal of Orthod. Dentofac. Orthop.*, 94(3), 207-215.

Lipp, C., Kirker, K., Agostinho, A., & James, G. (2010). Testing wound dressings using an in vitro wound model. *J Wound Care*, 19(6), 220-226.

Little, R. M., Riedel, R. A., & Artun, J. (1988).

An evaluation of changes in mandibular anterior alignment from 10 to 20 years postretention. *American Journal of Orthod. Dentofac. Orthop.*, 93(5), 223-228.

Little, R. M., Riedel, R. A., & Stein, A. (1990). Mandibular arch length increase during the mixed dentition: Postretention evaluation of stability and relapse. *American Journal of Orthod. Dentofac. Orthop.*, 97(5), 393-404.

- Littlewood, S. J., Millett, D. T., Doubleday, B., Bearn, D. R., & Worthington, H. V. (2006). Retention procedures for stabilising tooth position after treatment with orthodontic braces. *Cochrane Database Syst Rev.* 2016, 25(1), CD002283.
- Liu, J., Yali, G., & Lianli, X. (2012). Study of antibacterial effect of polymethyl methacrylate resin base containing ag-TiO₂ against streptococcus mutans and saccharomyces albicans in vitro. *West China Journal of Stomatology*, 30(2), 201-205.
- Loesche, W. J. (1979). Clinical and microbiological aspects of chemotherapeutic agents used according to the specific plaque hypothesis. *Journal of Dental Research*, 58(12), 2404-2412.
- Loesche, W. J. (1986). Role of streptococcus mutans in human dental decay. *Microbiological Reviews*, 50(4), 353-380.
- Marcotte, H. (1998). Oral microbial ecology and the role of salivary immunoglobulin A. *microbiology and molecular biology reviews . Mmbr*, 62(1), 71-109.
- Marsh, P. D. (1994). Microbial ecology of dental plaque and its significance in health and disease. *Advances in Dental Research*, 8(2), 263.
- Marsh, P. D. (2005). Dental plaque: Biological significance of a biofilm and community life-style. *Journal of Clinical Periodontology*, 32(Suppl 6), 7-15.

- Matsuura, T., Abe, Y., Sato, Y., Okamoto, K., Ueshige, M., & Akagawa, Y. (1997). Prolonged antimicrobial effect of tissue conditioners containing silver-zeolite. *J.Dent.*, 25(5), 373-377.
- Meithke, R., & Vogt, S. (2005). A comparison of the periodontal health of patients during treatment with the invisalign® system and with fixed orthodontic appliances. *Journal of Orofacial Orthopedics*, (66), 219-229.
- Melrose, C., & Millett D.T. (1998). Toward a perspective on orthodontic retention? *American Journal of Orthod. Dentofac. Orthop.*, 113(5), 507-514.
- Monteiro, D., Gorup, L., Takamiya, A., deCamargo, E., & Barbosa, D. (2012). Silver distribution and release from an antimicrobial denture base resin containing silver colloidal nanoparticles. *J Prosthodontics*, 21(1), 7-15.
- Nasidze, I., Li, J., Quinque, D., Tang, K., & Stoneking, M. (2009). Global diversity in the human salivary microbiome. *Genome Res*, 19(4), 636-643.
- Nikawa, H., Egusa, H., Yamashiro, H., Nishimura, M., Makihira, S., Jin, C., et al. (2006). The effect of saliva or serum on bacterial and candida albicans colonization on type I collagen. *Journal of Oral Rehabilitation*, 33(10), 767-774.
- Nyvad, B. (1993). Microbial colonization of human tooth surfaces. *APMIS.Supplementum*, 32, 1-45.

- Oei, J., Zhao, W., Lianrui, C., DeSilva, M., Ghimire, A., Rawls, H., et al. (2012).
Antimicrobial acrylic materials in situ generated silver nanoparticles. *J Biomed Mater Res, 100B*(Part B), 409-415.
- Ogaard, B., Alm, A. A., Larsson, E., & Adolfsson, U. (2006). A prospective, randomized clinical study on the effects of an amine fluoride/stannous fluoride toothpaste/mouthrinse on plaque, gingivitis and initial caries lesion development in orthodontic patients. *European Journal of Orthodontics, 28*(1), 8-12.
- Ogaard, B., Larsson, E., Henricksson, T., & Bishara, S. (2001).
Effects of combined application of antimicrobial and fluoride varnishes in orthodontic patients *Ajo-do, 120*(1), 28-35.
- Ohashi, S., Saku, S., & Yamamoto, K. (2004). Antibacterial activity of silver inorganic agent YDA filler. *Journal of Oral Rehabilitation, 31*, 364-367.
- Ollila, P. S., & Larmas, M. A. (2008). Long-term predictive value of salivary microbial diagnostic tests in children. *European Archives of Paediatric Dentistry : Official Journal of the European Academy of Paediatric Dentistry, 9*(1), 25-30.
- Padois, K., & Bertholle, V. (2012).
Chlorhexidine salt-loaded polyurethane orthodontic chains: In vitro release and antibacterial activity studies *American Association of Pharmaceutical Scientists, 13*(4), 1446-1450.

- Papaoiannou, W., Gizani, S., Nassika, M., Kontou, E., & Nakou, M. (2007). Adhesion of streptococcus mutans to different types of brackets. *The Angle Orthodontist*, 77(6), 1090-1095.
- Parisay, I., Boskabady, M., Abdollahi, M., & Sufiani, M. (2014). Treatment of severe rotations of maxillary central incisors with whip appliance: Report of three cases. *Dental Research Journal*, 11(1), 133-139.
- Parker, W. S. (1989). Retention-- retainers may be forever. *American Journal of Orthod. Dentofac. Orthop.*, 95(6), 505-513.
- Pereira-Cenci, T., Deng, D. M., Kraneveld, E. A., Manders, E. M., Del Bel Cury, A. A., Ten Cate, J. M., et al. (2008). The effect of streptococcus mutans and candida glabrata on candida albicans biofilms formed on different surfaces. *Arch Oral Biology*, 53(8), 755-764.
- Petti, S., Barbato, E., & Simonetti D'Arca, A. (1997). Effect of orthodontic therapy with fixed and removable appliances on oral microbiota. *The New Microbiologica*, 20(1), 55-62.
- Phan, T., Buckner, T., Sheng, J., & Baldeck, J. (2004). Physiologic actions of zinc related to inhibition of acid and alkali production by oral streptococci in suspensions and biofilms. *Oral Microbiology and Immunology*, 19, 31-38.
- Proffit, W., Fields, H., & Sarver, D. (Eds.). (2012). *Contemporary orthodontics* (5th ed.) Mosby.

- Pusateri, C. R., Monaco, E. A., & Edgerton, M. (2009). Sensitivity of candida albicans biofilm cells grown on denture acrylic to antifungal proteins and chlorhexidine. *Arch Oral Biology*, 54(6), 588-594.
- Quirynen, M. (1994). The clinical meaning of the surface roughness and the surface free energy of intra-oral hard substrata on the microbiology of the supra- and subgingival plaque: Results of in vitro and in vivo experiments. *J.Dent.*, 22(Suppl 1), S13.
- Quirynen, M., & Bollen, C. M. (1995). The influence of surface roughness and surface-free energy on supra- and subgingival plaque formation in man. *Journal of Clinical Periodontology*, 22(1), 1-14.
- Radford, D. R., Sweet, S. P., Challacombe, S. J., & Walter, J. D. (1998). Adherence of candida albicans to denture-base materials with different surface finishes. *J.Dent.*, 26(7), 577-583.
- Rai, M., & Yadav, A. (2009). Silver nanoparticles as a new generation of antimicrobials. *Biotechnology Advances*, 27, 76-83.
- Ramazansadeh, B., Jahanbin, A., & Yaghoubi, M. (2015). Comparison of antibacterial effects of ZnO and CuO nanoparticles coated brackets against streptococcus mutans. *J.Dent.*, 16(3), 200-205.

- Ristic, M., Vlahovic Svabic, M., Sasic, M., & Zelic, O. (2008). Effects of fixed orthodontic appliances on subgingival microflora. *International Journal of Dental Hygiene*, 6(2), 129-136.
- Rosenbloom, R. G., & Tinanoff, N. (1991). Salivary streptococcus mutans levels in patients before, during, and after orthodontic treatment. *American Journal of Orthodontics and Dentofacial Orthopedics : Official Publication of the American Association of Orthodontists, its Constituent Societies, and the American Board of Orthodontics*, 100(1), 35-37.
- Rossini, G., Parrini, S., Castroflorio, T., Deregibus, A., & Debernardi, C. (2014). Periodontal health during clear aligners treatment; a systematic review. *The European Journal of Orthodontics Advance Access*, December, 1-5.
- Ryu, H., Bae, I., Lee, K., Hwang, H., Lee, K., Koh, J., et al. (2012). Antibacterial effect of silver-platinum coating for orthodontic appliances. *The Angle Orthodontist*, 82(1), 151-157. doi:10.2319/021411-111.1
- Samaranayake, L. P., McCourtie, J., & MacFarlane, T. W. (1980). Factors affecting the in-vitro adherence of candida albicans to acrylic surfaces. *Arch Oral Biology*, 25(8-9), 611.
- Sari, E., & Birinci, I. (2007). Microbiological evaluation of 0.2% chlorhexidine gluconate mouth rinse in orthodontic patients. *The Angle Orthodontist*, 77(5), 881-884.

- Sheridan, J. J., LeDoux, W., & McMinn, R. (1993). Essix retainers: Fabrication and supervision for permanent retention. *J. Clin Orthod*, *27*(1), 37-45.
- Shpack, N., Greenstein, R. B., Gazit, D., Sarig, R., & Vardimon, A. D. (2014). Efficacy of three hygienic protocols in reducing biofilm adherence to removable thermoplastic appliance. *The Angle Orthodontist*, *84*(1), 161-170. doi:10.2319/012413-75.1
- Smiech-Slomkowska, G., & Jablonska-Zrobek, J. (2007). The effect of oral health education on dental plaque development and the level of caries-related streptococcus mutans and lactobacillus spp. *European Journal of Orthodontics*, *29*(2), 157-160.
- Socransky, S. S., & Manganiello, S. D. (1971). The oral microbiota of man from birth to senility. *Journal of Periodontology*, *42*(8), 485-496.
- Struzycka, I. (2014).
The oral microbiome in dental caries. *The Polish Journal of Microbiology*, *63*(2), 127-135.
- Svec, P., Sedlacek, I., Zackova, L., Novakova, D., & Kukletova, M. (Folia Microbiologica). Lactobacillus spp. associated with early childhood caries. *2009*, *54*(1), 53-58.
- Syafiuddin, T., Hisamitsu, H., Toko, T., Igarashi, T., Goto, N., & Fujishima, A. (1997). In vitro inhibition of caries around resin composite restoration containing antibacterial filler. *Biomaterials*, *18*, 1051-1057.

- Tanner, J., Carlen, E., Soderling, E., & Valittu, P. K. (2003). Adsorption of parotid saliva proteins and adhesion of streptococcus mutans ATCC 21752 to dental fiber-reinforced composites. *Journal of Biomedical Materials Research. Part B, Applied Biomaterials*, 66(1), 391-398.
- Teanpaisan, R., Thitasomakul, S., Piwat, S., Thearmontree, A., Pithpornchaiyakul, W., & Chankanka, O. (2007). Longitudinal study of the presence of mutans streptococci and lactobacilli in relation to dental caries development in 3-24 month old thai children. *Internantional Dental Journal*, 57(6), 445-451.
- Ten Cate, J. M. (2009). The need for antibacterial approaches to improve caries control. *Advances in Dental Research*, 21(1), 8-12.
- Thomas, R. Z., van der Mei, H. C., van der Veen, M. H., de Soet, J. J., & Huysmans, M. C. (2008). Bacterial composition and red fluorescence of plaque in relation to primary and secondary caries next to composite: An in situ study. *Oral Microbiology and Immunology*, 23(1), 7-13.
- Turkoz, C., Bavbek, N. C., Varlik, S. K., & Akca, G. (2012). Influence of thermoplastic retainers on streptococcus mutans and lactobacillus adhesion. *Ajo-do*, (141), 598-603.
- van Houte, J. (1994). Role of micro-organisms in caries etiology. *Journal of Dental Research*, 73(3), 672-681.

- Volker, A., Thorsten, B., & Steinrucke, P. (2004). An in vitro assessment of the antibacterial properties and cytotoxicity of nanoparticulate silver bone cement. *Biomaterials*, 25, 4383-4391.
- Wang, Y., Chatzistavrou, X., & Faulk, D. (2015). Biological and bactericidal properties of Ag-doped bioactive glass in a natural extracellular matrix hydrogel with potential application in dentistry. *European Cells and Materials*, 29, 342-355.
- Wilson, M., & Harvey, W. (1989). Prevention of bacterial adhesion to denture acrylic. *Journal of Dentistry*, 17(4), 166-170.
- Yamamoto, K., Ohashi, S., Kokubo, T., Yamada, I., & Yamauchi, J. (1996). Antibacterial activity of silver ions implanted in SiO₂ filler on oral streptococci. *Dental Materials*, 12, 227-229.
- Yoshida, K., Tanagawa, M., & Atsuta, M. (1999). Characterization and inhibitory effect of antibacterial dental resin composites incorporating silver-supported materials. *Journal of Biomedical Materials Research. Part B, Applied Biomaterials*, 47, 516-522.
- Zachrisson, B. U., & Zachrisson, S. (1971). Caries incidence and oral hygiene using orthodontic treatment. *Scandinavian Journal of Dental Research*, 79(6), 394-401.
- Zhang, N., Chen, C., & Weir, M. (2015). Antibacterial and protein-repellent orthodontic cement to combat biofilms and white spot lesions. *Journal of Dentistry*,

Curriculum Vitae

Graduate College
University of Nevada, Las Vegas

Dr. Ryan Jolley

Degrees:

Bachelor of Science, Business, 2000
Brigham Young University

Doctor of Medical Dentistry, 2014
University of Nevada Las Vegas

Thesis Title: Antibacterial activity of orthodontic appliance base plate material, PMMA, infused with silver and a novel antibacterial compound

Thesis Examination Committee:

Chairperson, Dr. Katherine Howard
Committee Member, Dr. Karl Kingsley
Committee Member, Dr. James Mah
Graduate College Representative, Dr. Jennifer Phar