IN VITRO REMINERALIZATION OF HUMAN ENAMEL WITH BIOACTIVE GLASS CONTAINING DENTIFRICE USING CONFOCAL MICROSCOPY AND NANOINDENTATION ANALYSIS FOR EARLY CARIES DEFENSE

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2004

This work was performed in dedication to those who gave their time and love to support me and my efforts. It was the contribution of many and prayers of a select few who deserve credit for my successes. My parents and loved ones believed in me and respected my pursuits in life, even when we didn't see eye to eye. I must also thank Almighty God for giving me the strength and perseverance to overcome in times of difficulty and the humility to accept my shortcomings and still succeed as a man among many.

In memorial, I dedicate this work to my grandfather Kazi Reazuddin Ahmed, who taught me the value of sacrifice and achievement in this world. My successes are a testament to his love and guidance at critical stages in my life. I only regret that he is not here today to share this accomplishment with me.

ACKNOWLEDGMENTS

I would like to thank Ben Lee, Neel Bhatavedekar, Kevin Taylor, and Allyson Barrett in Department of Dental Biomaterials at the University of Florida for their direct involvement in making this work a success. For three months in the lab, we were almost a family. I would also like to thank Guy La Torre, Kevin McKenzie, and Donald Thibadeaux from Novamin Technologies Inc. for their help and guidance throughout the project, especially during the initial stages. Kevin contributed so much of his time helping me take this research to a new level.

From the Department of Materials Science and Engineering, I must thank Dr. Christopher Batich, my committee member, and Dr. Susan B Sinnott, without whom much of this would not have been possible. All credit to her for stimulating my interest in Materials Science and Engineering as her undergraduate student at the University of Kentucky. I shall always admire her devotion to science and academic integrity.

This project was also supported by the efforts of Major Analytical Instrumentation Center staff. Dr. Amelia Dempere, Wayne Acree, Brad Willenberg, and Jerry Bourne helped bring my hypotheses to life. Each analysis technique required their expertise and cooperation.

Confocal microscopy analysis would not have been possible without Dr. Marguerita Fontana, Dr. Carlos Gonzales-Cabezas, and Amir Haider. Dr. Fontana and colleagues at Indiana University-Purdue University at Indianapolis sacrificed their time

and convenience to advise me and help perform the analytical centerpiece of this study. They were available to answer every question and make their expertise my expertise.

Many thanks go to Dr. R.J. Hanrahan, Dr. David Reitze, Tim Vaught, and Dr. James Wefel for their time and assistance at various points thoughout the study.

I reserve special recognition for Dr. Mecholsky, Dr. Greenspan, and Dr. Anusavice for their valued advice and encouragement. They provided the resources and opportunity to this ambitious young researcher, allowing me to mature and develop my scientific curiosities. I am ever grateful for their guidance through this long and arduous journey.

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IN VITRO REMINERALIZATION OF HUMAN ENAMEL WITH BIOACTIVE GLASS CONTAINING DENTIFRICE USING CONFOCAL MICROSCOPY AND NANOINDENTATION ANALYSIS FOR EARLY CARIES DEFENSE

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December 2004

Chair: J. J. Mecholsky, Jr.
Major Department: Materials Science and Engineering

Remineralization of early caries lesions provides an oral health and economic advantage to populations suffering from this bacteria-based disease. Preventive treatment of caries in the early stages could reduce dental costs and frequency of clinical visits. This study investigated the use of bioactive glasses in dentifrice in comparison to commercial dentifrice (Colgate® Regular) to determine if additional calcium and phosphate release from the bioactive glass contributes positively to fluoride dentifrice remineralization capability. Two techniques were used *in vitro* to quantify the remineralization for dentifrice treatment of tooth enamel. Confocal laser scanning microscopy (CLSM) and nanoindentation were used to evaluate mineral changes in artificial surface lesions optically and mechanically. Eighteen human molars were sectioned into four parts : demineralized control, sound control, Novamin® (bioactive glass) and Colgate® dentifrice treatment. Upon demineralization, specified treatment sections were placed in a pH cycling regimen for 20 days. Each tooth section was further

cross-sectioned through the treatment window down the lesion depth for analysis. Novamin® tooth sections remineralized lesions more than Colgate® for two parameters, lesion area and total gray value (fluorescence), which have been directly correlated with mineral density. Single tailed T-test for treatment groups yielded significant difference ($p < 0.001$). Novamin® dentifrice treatment reduced lesion area an average of 41.9%, while Colgate® averaged 24.9%. Novamin® sections also exhibited an average 70.5% decrease in total gray value compared to 48.1% for Colgate®. Two way ANOVA testing of three tooth sections (original lesion, Novamin® treatment, Colgate® treatment) for both parameters found significant difference ($p < 0.0001$). Duncan multiple range testing found these three sections statistically different at significance level 0.01. Both parameters signify extensive remineralization *in vitro* for surface lesions. Nanoindentation did not significantly exhibit remineralization for either dentifrice treatment. Considerable hardness variability was observed throughout the cross-sectional lesion, as well as inconsistency among multiple teeth and treatment groups. Nanoindentation is not recommended for mechanical analysis of *in vitro* remineralization of human enamel. Vickers microhardness was performed on lesions as well but did not yield credible results. Indents were difficult to optically identify and measure.

CHAPTER 1
BACKGROUND AND MOTIVATION

The field of dentistry enables modern societies to effectively and preventively maintain oral health. Clinicians have been relatively successful within the realm of dental care taking into consideration the frequency of patient visits. But that dependence upon interaction between the patient and their dentist is not only costly to some, but also contrary to human nature. Our human tendency to treat ailments responsively rather than preventively forces the scientific community to develop better methods to counter the more severe stages. So it is now the motivation for dental researchers and clinicians to develop strategies and treatment methods for fighting dental diseases in their early stages. Engineers call this practice preventive maintenance while physicians refer to it as preventive medicine. Regardless of the terminology, science has been a marvelous tool for enabling individuals to care for themselves. Decades of dental science have brought society the common practice of daily tooth brushing, flossing and mouth rinse. This concept of preventive dentistry provides the foundation for this study and its long-term applications.

Dental caries remains the oldest and most prevalent oral disease in human history. It is only recently that the advent of daily dental care and clinician oversight has reduced the frequency of caries within large populations [WIN98]. Fluoridated water supplies and habitual influences, such as diet, certainly have an effect on caries management. But this study focuses primarily on daily treatment as the most effective caries defense and ways to optimize it process through materials science.

Caries pathology and its early treatment are well researched. Only the details of the caries prevention process and the best way to accomplish this task are left for dental researchers to discover. The term "caries" which originates from Latin for "rot" or "rotten" prompted original researchers of the past two centuries to develop methods to counter this process of *tooth decay* or *demineralization*. The heart of caries research and prevention lies in the opposition of these terms, thus *replacement* or *remineralization*.

Traditionally, surface or early caries treatment was centered around tooth mineral loss and gain. The progressive increase in processed sugars and acidic foods and beverages in the human diet provide oral bacteria greater opportunity to produce acids that dissolve tooth mineral. Thus, the facilitation of regaining new mineral via the natural oral process remains the ultimate goal. Tooth mineral, among other biological minerals, is composed mainly of calcium and phosphorous. Methods for providing these constituents of mineral or a new constituent to better facilitate remineralization of teeth has been the backbone for this type of research.

Dentifrices have been around for more than 2000 years [BAR04, WIN98] but only since Muhler's development of fluoride toothpastes in 1954 has such a dramatic effect been found in caries prevention. Within a few years Colgate Palmolive Company and Proctor & Gamble began marketing their own fluoride toothpastes. Decades later, the daily use of fluoride toothpaste is still accepted as the best and simplest method of caries prevention and tooth remineralization for large populations. Although a debate still exists in the dental community concerning optimization methods of tooth remineralization, it is clear that fluoride ion plays a significant role in the remineralization process. Early caries treatment is especially important with dentifrices

to reduce the risk of treating advanced stages of the disease. If individuals can effectively fight caries with daily brushing, the need for restorative treatment can be avoided.

The invention of bioactive glasses (Bioglass ®) by Dr. Larry Hench in the late 1960s provided medical researchers with a new tool to enhance bone formation that facilitates the body's own natural processes. This provided a whole new approach to biomaterials. My experience in bio-inert materials was pushed aside by the possibility of investigating new and more advanced uses for this "bio-interactive" technology. Through good fortune and the support of Novamin Technologies Inc., I found an opportunity in dental research to explore the nature of bioactive glasses in dentifrice and its potential to provide the oral environment with the essential elements (Ca and P) to remineralize teeth.

The novelty of "Bioglass® toothpaste" for this study is evident but doesn't stop here. Just as treatment methods for early caries progress, the need for newer and better technologies in assessing caries treatment continues to grow as well. Previous analysis methods for tooth remineralization do not provide the scope for detailing finer quantification of early stage caries. Transverse microradiography (TMR) and microindentation are still sound techniques for dental caries research, but do not provide the scale or quantification requirements that current and future investigators need to progress. In the case of caries, *in vitro* studies in the past used TMR for subsurface caries lesion analysis. But early caries detection and treatment requires *surface* analysis and less time. Confocal laser scanning microscopy (CLSM) and nanoindentation were viable candidates to explore early caries treatment for more current research objectives. The aims of this study are as follows:

- Compare and contrast the benefits and drawbacks of confocal laser scanning microscopy and nanoindentation for analysis of tooth remineralization *in vitro*.

- Determine the remineralization capabilities of a Novamin® (bioactive glass) containing dentifrice on human enamel *in vitro*.

CHAPTER 2
FUNDAMENTALS OF THE TOOTH

Basic tooth structure is based on layers and function. Teeth, like bone, are comprised of soft inner layers to provide nutrients and growth function, whereas outer layers are designed for structure and protection. This dual nature provides a research perspective conducive to remineralization studies. In essence, if the tooth is generally viewed as "living," the prospect of it regenerating or remineralizing becomes no different than with any other biological tissue. On a larger scale, it is advantageous to consider the oral cavity itself as a unique and independent ecosystem. The primary tissues involved in caries research are dentin and enamel. Early caries studies focus almost entirely on enamel and its surface layers.

Enamel is the visible outer layer of the tooth. It is translucent; and can vary in color from yellowish to grayish white. The different colors of enamel may be attributed to variations in thickness, translucent proprieties, the quality of the crystal structure, and surface stains. Enamel (Figure 2-1) is the calcified substance that covers the entire anatomic crown of the tooth and protects the dentin and pulp. It is the hardest tissue in the human body and consists of approximately 97% inorganic minerals, 1.5% organic materials, and 1.5% water [LEG88]. Calcium and phosphorus (as hydroxyapatite) are its main inorganic components. Enamel can endure crushing pressure of approximately 700 MPa. A layering of the dentin and periodontium below produces a cushioning effect of the tooth's different structures, enabling it to endure the pressures of mastication. Structurally, enamel is composed of millions of rods or prisms. Each rod begins at the

dentino-enamel junction (zone between the enamel and dentin) and extends to the outer surface of the crown. Enamel is formed by epithelial cells (ameloblasts) that lose their functional ability when the crown of the tooth has been completed. Therefore, enamel, after formation, has no power of further growth or repair, only mineral gain and loss.

Dentin (Figure 2-1) is the light yellow tissue beneath the enamel. It is more radiolucent than enamel, very porous, and it constitutes the largest portion of the tooth. The pulp chamber is located on the internal surface of the dentin walls. Dentin is harder than bone but softer than enamel and consists of approximately 70% inorganic matter and 20% organic matter and 10% water [LEG88]. Calcium and phosphorus are its chief inorganic components. Dentin is a living tissue and must be protected during operative or prosthetic procedures from dehydration and thermal shock. The dentin is perforated by tubules (similar to tiny straws) that run between the cemento-enamel junction (CEJ) and the pulp. Cell processes from the pulp reach part way into the tubules like fingers. These cell processes create new dentin and mineralize it. Dentin transmits pain stimuli by the way of dentinal fibers and hydrostatic pressure within the tubules. Because dentin is a living tissue, it has the ability for constant growth and repair that reacts to physiologic (functional) and pathologic (disease) stimuli.

Hydroxyapatite

Calcium phosphates are the most important inorganic constituent of biological hard tissues. In the form of carbonated hydroxyapatite, they are present in tendons, bone and teeth to give these organs stability, hardness and function. The maturation of tooth enamel and dentin involves the second major calcification process in mammals, bone being the first. The formula for tooth mineral in enamel consists primarily of calcium hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$.

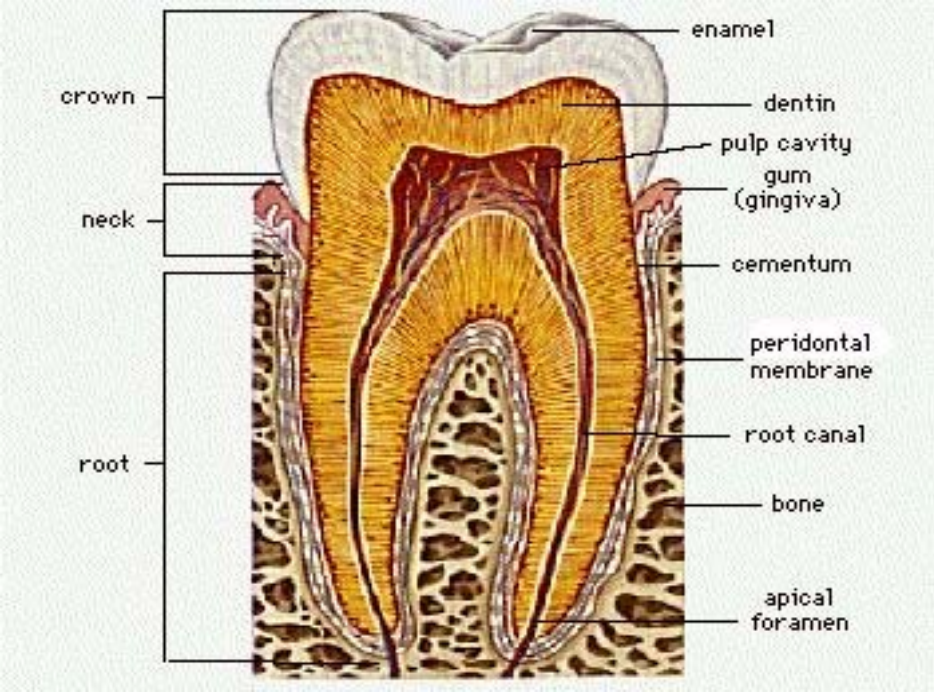

Figure 2-1 Anatomy of the human tooth. Note: Taken from Dentistry and Real Life *http://users.hol.gr/~jelian/anatomy.htm* with requested permission. [DRL03]

Human biological apatites can vary considerably based upon formation and functional conditions as well as basic Ca:P molar ratios. Enamel maintains a Ca:P ratio of nearly 1.63 as compared with the general hydroxyapatite (HA) ratio, 1.67 and bone, 1.71 [LEG88]. The similarities of biological apatite in enamel to pure HA make it possible for researchers to develop chemical relationships to study tooth minerals. Tooth enamel contains parallel crystals of biological apatite, which are much larger than those of bone and dentin. The needle-like crystal rods may grow to 100 μm in length and 50 nm wide. The enamel maturation process is vital to understand the solution dynamics involved for this study. Calcium deficient hydroxyapatite (CDHA) is the main constituent of developing enamel and considered to be quite carbonated. The subsequent mineral formation is driven by the introduction of Ca^{2+} into the apatite and simultaneous loss of carbonate. Featherstone describes newly mineralized bone and teeth as carbonated hydroxyapatite (CAP), which is essentially impure HA, represented by the

formula Ca_{10-x} $(Na)_x$ $(PO_4)_{6-y}$ $(CO_3)_z$ $(OH)_{2-u}$ $(F)_u$ [FEA00]. In enamel this "calcification" produces a mineral increase from 45 wt% to 96 – 98 wt% and a significant rise in Ca:P molar ratio. This ultimately results in the most highly mineralized and hardest skeletal tissue in the body [DOR02]. Although the biological apatite of tooth enamel varies due to crystal impurities and apatite combinations, ionic exchange at the enamel surface is based primarily upon calcium phosphate solubilities.

At neutral pH, saliva is supersaturated with calcium and phosphate. This saturation is necessary to counter the recurrent acid challenge of dietary cycles and residual sugars which are used by oral bacteria to create acid via fermentation. These ions, among others, give saliva a buffering capacity in addition to a mineralization reservoir. Below the critical pH of human enamel, 5.2 to 5.5 [LAR03], the dissolution of enamel mineral follows basic solubility laws for hydroxyapatite. At lower pH, the dissolution of apatite mineral continues until the oral pH returns to normal. Along with saliva's own buffering capacity, salivary bicarbonate in equilibrium with CO_2 from the respiratory process shifts the equilibrium to a more alkaline condition. The state of subsaturation for calcium and phosphate at lower pH prevents remineralization under these conditions. When oral pH again rises above "critical" status, the calcium/phosphate saturation of the saliva again supercedes that of the enamel and mineral deposition begins. Due to the supersaturation condition of saliva at pH 7.0, one would expect that hydroxyapatite mineral would continue to form on the enamel surface. As in many cases within the human body however, this micro-environment is equipped with its own checks and balances. The supersaturation of calcium and phosphate does not over mineralize teeth because of the protein rich film (tooth pellicle) on the enamel surface. It is thought that saliva proteins

also play a part in maintaining this balance. Still the question remains, what if the salivary pH becomes severely alkaline? This increase in pH results in dental calculus, essentially calcium phosphate precipitate in the plaque fluid. A broader look at the demineralization and remineralization process is necessary, especially when topical fluoride is introduced into the system.

Demineralization and Remineralization Phenomena

Generally, the life of dental hard tissues is well understood and research has revealed the structures and concepts involved in natural processes of the oral environment. The nature of these tissues and how they behave under certain conditions is clear, but what is not clear is the degree to which these natural processes can be influenced or even accelerated. Over the course of human life, enamel and dentin undergo unlimited cycles of demineralization and remineralization. The debate involves ways to measure and influence this process. Years of work have brought fluoride to the forefront of remineralization studies and application. But the influence of fluoride on demineralization and remineralization of enamel is yet to be agreed upon. The crux of this argument lies in the balance of these two competing phenomena. A tip in the balance one way or the other will either lead to stronger healthier teeth or greater susceptibility to dental caries and other oral complications. To promote general oral healthcare, the use of fluoride toothpastes for daily promotion of remineralization has become standard practice. Evaluating these tissues at different stages of the oral cycle and measuring the optical and mechanical properties are the key to determining a net increase or decrease in mineral flux.

The consumption of simple dietary sugars (particularly monosaccharides and disaccharides like Sucrose) provides not only nourishment for our bodies but also a food

source for oral bacteria. As bacteria making up the normal oral flora adhere to the pellicle, a bacterial mass or film called plaque is formed [MAR99]. The plaque bacteria, particularly *Streptococcus mutans* and *lactobacilli*, ingest sugars for glycolysis to produce weak organic acids (such as lactic, pyruvic, acetic acid). These acids lower the surface pH and diffuse through the plaque and into the tooth, leaching calcium and phosphate from the enamel. At this time the plaque pH may have dropped to 4.0 – 4.5 [WIN98]. This mineral loss compromises the mechanical structure of the tooth and could lead to cavitation over a long period of time. The stages of caries progression are clear and in the interest of preventive maintenance, early carious lesions appear to be the best opportunity for countering this destructive process. As alluded to earlier, saliva alone has the capability to increase plaque pH with bicarbonates although typically this process may take up to 2 hours. The susceptibility of apatite in enamel surface layers makes it critical to control the acidity of the plaque fluid and the Ca^{2+} and PO_4^{3-} ion concentrations in saliva [FEA00]. The subsequent remineralization process is nearly the reverse. When oral pH returns to near neutral, Ca^{2+} and PO_4^{3-} ions in saliva incorporate themselves into the depleted mineral layers of enamel as new apatite. The demineralized zones in the crystal lattice act as nucleation sites for new mineral deposition. In the presence of fluoride (at high concentrations), the original CAP loses its remaining carbonate and is replaced with a hybrid of hydroxyapatite (HAP) and fluorapatite (FAP) [FEA00]. This cycle is fundamentally dependent upon enamel solubility and ion gradients. Essentially, the sudden drop in pH following meals produces an undersaturation of those essential ions (Ca^{2+} and PO_4^{3-}) in the plaque fluid with respect to tooth mineral. This promotes the dissolution of the enamel. At elevated pH, the ionic supersaturation of plaque shifts the

equilibrium the other way, causing a mineral deposition in the tooth. Figure 2-2 provides an overview of these processes.

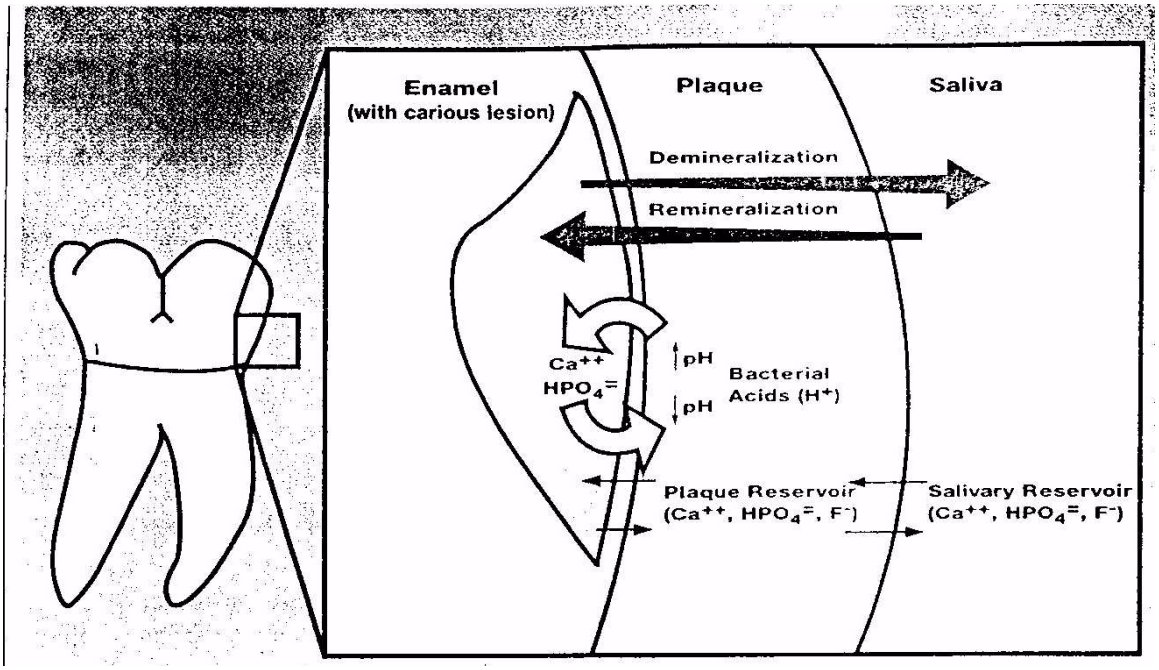

Figure 2-2 Cycle of demineralization and remineralization in enamel. Note: Taken from Winston AE, Bhaskar SN, "Caries prevention in the 21st century." *J Am Dent Assoc* 129, p 1579 - 1587, 1998 with requested permission.

Saliva

Saliva plays multiple roles in these oral processes. Aside from providing a constant rinse, the value of saliva as a reservoir for calcium, phosphate and fluoride has been well established [LAR03]. Saliva offers a myriad of other benefits, although many are not widely known but contribute significantly to enamel remineralization.

The buffering capacity of human saliva plays a major role in countering fluctuations in pH. Again, acidic beverages and/or sugary foods cause temporary pH drops during which demineralization is accelerated. Stephan's work in 1944 found patients with little to no caries activity maintained a resting salivary pH of 7.0-7.2 [STE44]. It was later understood that bicarbonates in saliva played a major role in elevating low oral pH after meals. The relationship of bicarbonate concentrations in

saliva and blood has led other investigators to study caries incidence relative to blood properties [BAC99, BIE04]. Other buffers present in saliva include urea proteins. Urease enzyme in plaque fluid metabolizes urea producing ammonia and an increase in plaque pH. Arginine rich proteins in saliva can also metabolize into alkaline substances such as arginine and ammonia. Phosphate has also been found to contribute to buffering capabilities [DOW99]. Various salivary components also demonstrate antibacterial capability. Iron binding protein lactoferrin has been shown to inhibit aerobic and facultative anaerobic bacteria (such as *Streptococcus mutans*) which require iron to metabolize. Lysozyme also exhibits direct antibacterial function. This enzyme, well known for its presence in tear and nasal secretions (discovered by Fleming in 1922), complexes with salivary ions such as bicarbonate, iodine and fluorine, which bind to bacterial cell walls and induce autolysis [AMA01, DOW99].

It is clear that a reduction in salivary flow or its constituents would negatively affect our capability to fight caries [DOW99]. Xerostomia is defined as the perception of oral dryness or hyposalivation. This is due to any number of factors including radiation, medication and diseases such as diabetes. The most widely studied disease state affecting salivary function is exocrinopathy or *Sjögren's Syndrome*. This autoimmune disease is characterized by inflammation of glands with lymphocyte infiltration. Secretory components begin to deteriorate causing decreased saliva flow. Specifically, the reduction in salivary flow has been associated with a marked increase in caries incidence [DRE76]. Although a number of remineralizing factors are affected, the near absence of calcium and phosphate from the oral cavity cannot be compensated. Remineralization becomes nearly impossible without key constituents. External constituents, such as

fluoride from dentifrice, have been proven to positively affect remineralization. The influence of saliva in the presence of topical fluoride to form greater levels of CaF_2 (a remineralization agent) was demonstrated by Larsen. This relationship also revealed a decrease in caries incidence [LAR01]. But Larsen also explains that the inter-dependence of calcium, phosphate, and fluoride enables the remineralization effect. Therefore, absence of calcium and phosphate (such as Xerostomia) would essentially deem the presence of fluoride irrelevant.

CHAPTER 3
REMINERALIZATION THEORY

While a great deal has been discovered about the remineralization and demineralization process, details regarding the mechanism of mineral deposition are not universally agreed upon. This study focuses on remineralization of early caries lesions through *dentifrice application*. This is the most common form of anti-caries treatment today and has proven quite effective in reducing caries incidence since its introduction.

Dentifrice and Fluoride

Around the turn of the century, primitive toothpastes were primarily used as abrasive cleansing agents. However, a relative explosion in dental caries forced scientific investigation to aid the affected populations. Bibby continued the functional development of toothpastes in 1942 with the first clinical trials of fluoride toothpastes. Proctor & Gamble later introduced a fluoride toothpaste in 1955 using a stannous fluoride agent. Further development led to sodium monofluorophosphate (Na_2PO_4F) and sodium fluoride (NaF) agents in dentifrice. Fluoride levels are generally regulated to 1100 ppm in modern commercial toothpastes. This topical application of fluoride is one of two ways it is believed to promote remineralization.

Systemic Benefit

Recently, scientific studies have shown the systemic action of fluoride is secondary to a topical application. It is nevertheless a contributor to the overall beneficial effect for prevention of tooth decay. Over time, fluoride ions ingested from fluoridated water, fluoridated milk, fluoride supplements and foods processed with fluoridated water,

can become incorporated into the structure of developing teeth. It has also been suggested that teeth which develop under the effect of systemic fluorides have shallower pits and grooves, allowing less trapping of oral bacteria and food particles, which contribute to tooth decay [BRU90].

Topical Benefit

On the surface of the tooth, there is a constant exchange of mineral ions between crystals of the enamel surface and plaque fluid bathing the tooth surfaces. When topical fluorides are regularly applied to the teeth (through toothpastes, mouth rinses, fluoridated water, and professional applications) it is possible that even a poorly mineralized enamel surface can be progressively improved by the natural fluoride exchange equilibrium described above. In the case of early caries lesions, studies have shown the availability of fluoride in relatively low concentrations to result in the arrest and even remineralization of targeted enamel. From this information, we see that adults may benefit from fluorides as well as children via the topical benefit of fluorides [BRU90, ISM95].

Extensive studies on the use of dentifrice to introduce fluoride have proven its anti-caries efficacy. Fluoride has three principal topical mechanisms: 1) Free fluoride ion combines with H^+ to produce hydrogen fluoride, which migrates throughout acidified plaque. This ionized form is lipophilic and can readily penetrate bacterial membranes. Bacterial cytoplasm is relatively alkaline, which forces the dissociation of H^+ and F^-. Fluoride ion inhibits various cellular enzymes key to sugar metabolism. Hydrogen ions simultaneously acidify the cytoplasm, thus slowing cellular activities and inhibiting bacterial function. [MAR99] 2) Fluoride integrated in the enamel surface (as fluorapatite, FAP) makes enamel more resistant to demineralization than HAP during acid challenge. 3) Fluoridated saliva not only decreases critical pH, but also further inhibits

demineralization of the deposited CaF2 at the tooth surface. Fluoride at the enamel surface has been found to attract and "bind" to calcium ions. This enhances nucleation of new mineral at specific demineralized zones. Computer simulation has advanced this assertion and supported previous studies involving fluoride applications on early caries or surface lesions [FEA00, PEP04].

The exact mechanism of fluoride in the surface lesions is still not well understood but a few details are clear. The generalized reaction of HAP (enamel) dissolution below critical pH:

$$Ca_{10}(PO_4)_6(OH)_2 + 8H^+ \longrightarrow 10Ca^{2+} + 6HPO_4^{2-} + 2H_2O \qquad (3\text{-}1)$$

But fluoride application allows for a substitution reaction to produce FAP

$$Ca_{10}(PO_4)_6(OH)_2 + 2F^- \longrightarrow Ca_{10}(PO_4)_6F_2 + 2OH^- \qquad (3\text{-}2)$$

FAP is more resistant to acid challenge due to lower solubility (**HAP** $Ksp = 2.34x10^{-59}$ and **FAP** $Ksp = 3.16x10^{-60}$) [LAR03]. In fact, even before FAP formation, ten Cate, Nelson, Featherstone and colleagues found that topical fluoride levels in solution around synthetic CAP (3% carbonate similar to enamel) reduced solubility significantly. The same studies revealed that systemic fluoride incorporation did not significantly improve apatitic acid resistance, thus arguing in favor of topical applications throughout lifetime as the best defense against early caries [FEA00, TEN99].

The argument for topical fluoride gained strength with a number of *in vitro* and in situ studies involving dentifrice sources and inherent fluoridated enamel. Ten Cate and Duijsters found that enamel dissolution was primarily a function of two external properties, pH and fluoride concentration of surrounding solution. Low pH environments were especially sensitive. Transverse microradiography (TMR) confirmed that surface

lesion depth and mineral loss were a direct function of fluoride concentrations. Figure 3-1 summarizes their findings graphically [TEN83, TEN99].

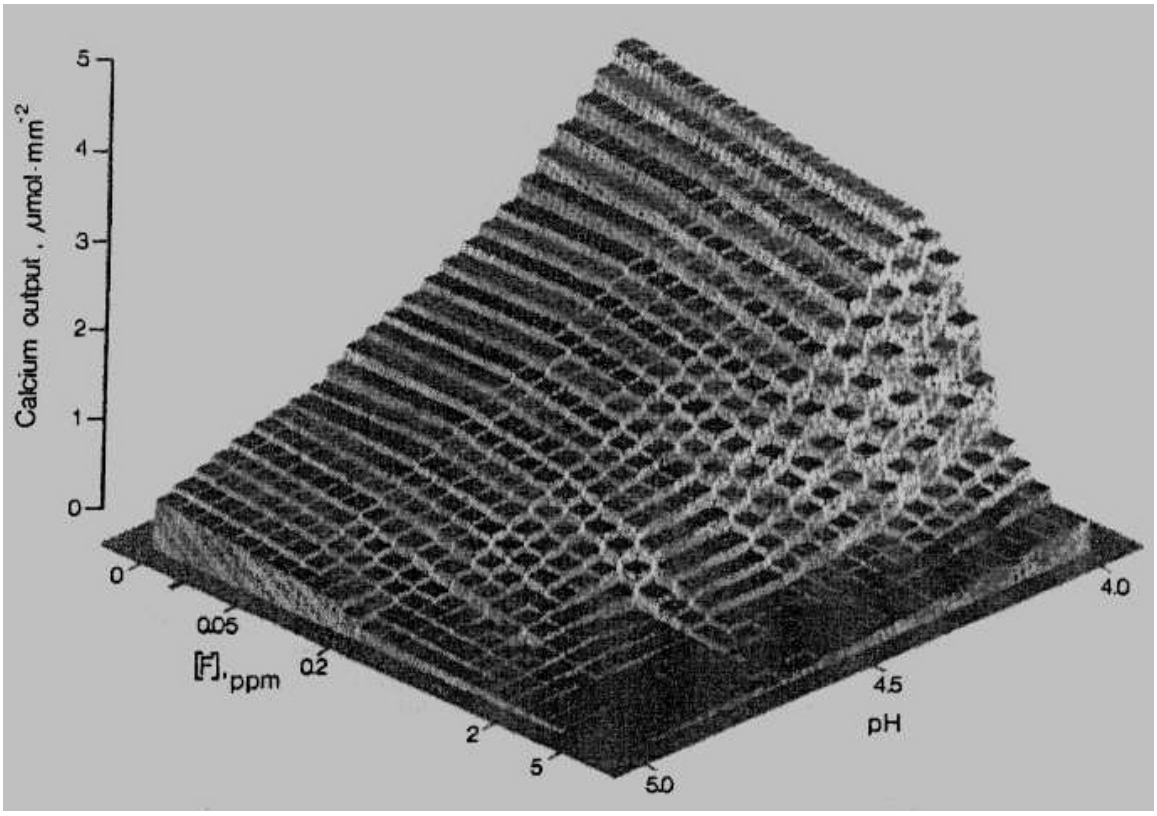

Figure 3-1 Mineral Loss (expressed as Ca output) as a function of pH and [F^-]. Note: From ten Cate JM, Duijsters PPE, "The influence of fluoride in solution on tooth enamel demineralization I. Chemical data." Caries Res 17, p 193 - 199, 1983, courtesy of Karger AG, Basel with permission.

Later studies found that low concentrations of fluoride, ambient 0.06 ppm and dentifrice 1100 ppm, significantly decreased enamel mineral loss by 5% and 9% respectively under simple pH cycling regimen [TEN95]. Histomorphometric analysis of lesions after remineralization with fluoride found the new enamel crystallites to be dimensionally larger than the original sound enamel. They were also found to be randomly oriented (lacking organization) which rendered this enamel slightly less dense. Still the ability of fluoride to bind to free calcium and phosphate, including ions leaving the tooth during demineralization to form FAP reduces the probability for enamel

dissolution in *subsequent* acid challenges. Because FAP is much less soluble and has significantly less buffering capacity, an even lower pH will be required to force enamel dissolution [SHE94].

Bioglass®

The discovery of bioactive glasses by Hench in 1969 pushed the boundaries of biomaterials capability and function. In an era of bio-inert materials and implantation, Hench determined the critical steps for bioactive glass ceramic interaction with the human body in order to bond. Bioglass® is a multi-component inorganic compound made of elements (silicon, calcium, sodium and phosphorous) naturally found in the body [HEN96]. The development and success of bioactive glasses is due to their highly biocompatible nature. Previous implant materials regardless of initial success, failed over long periods of time. Numerous metals and polymers eventually succumbed to the aggressive defense mechanisms and corrosive nature of body fluids. Hence, the advantage of bioactive glass is not only acceptance within the body, but also its ability to chemically *bond*. More than three decades of study has revealed the series of reaction steps involved in Bioglass® bonding mechanisms within the body. This study involves the use of bioactive glasses in powder particulate form. This form provides easy dispersion in this dentifrice application and exploits the fact that fine glass powder particulates resorb much faster than bulk implants. Upon implantation, Bioglass® in aqueous environment immediately begins surface reaction in three phases, leaching and exchange of cations, network dissolution of SiO_2, and precipitation of calcium and phosphate to form an apatite layer. The 5 critical stages for glass surface reactions are detailed below [HEN93].

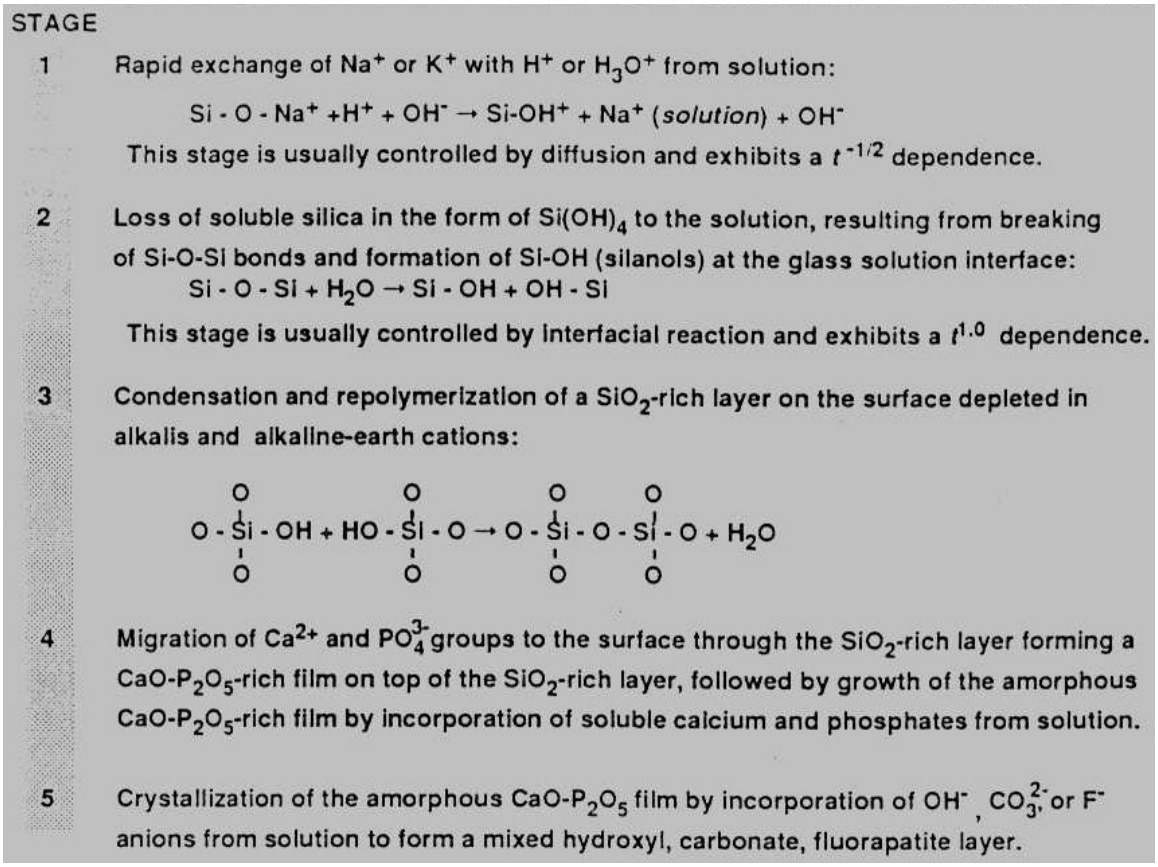

Figure 3-2 Stages of bioactive glass surface reactions. Note: Taken from Hench LL, Wilson J, An Introduction to Bioceramics Singapore, World Scientific Publishing, 1993 with requested permission.

The initial Na^+ and H^+/H_3O^+ ion exchange and de-alkinization of the glass surface layer is quite rapid, within minutes of implantation and exposure to body fluids. The net negative charge on the surface and loss of sodium causes localized breakdown of the silica network with the resultant formation of (silanol) Si(OH) groups, which then repolymerize into a silica rich surface layer [GRE99]. This stage involves the base catalyzed hydrolysis of Si-O-Si bonds of the glass structure. This mechanism is based on previously well documented corrosion studies of alkali silicate glasses as well as infrared spectroscopy studies that appear to show the formation of nonbridging oxygen species following Si-O-Si bond breakage [HIL96]. The subsequent stages (4 and 5) involve the development of silica rich and amorphous calcium phosphate layers respectively. These

stages incorporate the noticeable presence of biological moieties such as blood proteins, growth factors and collagen. Within 3-6 h *in vitro*, the calcium phosphate layer will crystallize into the carbonated hydroxyapatite (CAP) layer which is essentially the bonding layer. Chemically and structurally, this apatite is nearly identical to bone and tooth mineral, thus allowing the body to attach directly to it. These Bioglass® surface reactions from implantation to 100-150 μm CAP layer formation takes 12 to 24 h [HEN93, KON02]. Hench further investigated 6 more stages primarily focused around bone and tissue bonding. These stages, although not completely understood, depend significantly on the specific formulation of Bioglass® involved.

The standard for Bioglass® formulation is commonly known as 45S5 which has been used extensively in research studies. It contains 45 wt% SiO_2, 24.5 wt% Na_2O and CaO, and 6 wt% P_2O_5. Bioactive glasses have traditionally kept the P_2O_5 fraction constant while varying the SiO_2 content. In fact, the network breakdown of silica by OH^- was found to be time dependant upon the concentration of SiO_2. It is now understood that keeping the silica below 60 wt% and maintaining a high CaO/P_2O_5 ratio guarantees a highly reactive surface.

Novamin®, a trade name for bioactive glass, is manufactured by Novamin Technologies Inc. (Alachua, FL). The material is reported to have a long record of safety and efficacy as an implant material used to regenerate new bone in defects [GRE99]. When bioactive glass is incorporated into toothpaste formulations, the ions released from the amorphous calcium phosphate layer are believed to contribute to the remineralization process of the tooth surface. Bioactive glasses have been successfully used clinically as bone grafting material for over 15 years and has been cleared by the FDA for use in oral

and orthopedic bone grafting for nearly 10 years [LOW96, WIL87]. Bioglass® is also marketed worldwide under the trade names PerioGlas® and NovaBone®. Recently, it has been demonstrated that fine particulate bioactive glasses (<90 μm) incorporated into an aqueous dentifrice have the ability to clinically reduce the tooth hypersensitivity through the occlusion of dentinal tubules by the formation of the CAP layer [LIT97]. Investigators using bioactive glass compositions have demonstrated a significant anti-microbial effect towards caries pathogens (*S. mutans, S. sanguis*) upon exposure to bioactive glass powders as well as solutions and extracts [ALL01, STO96, STO98]. Despite advances in oral care over the last 40 years – in large part because of the incorporation of fluoride into a large number of products – there are still greater than 150 million cavities filled in the US every year [ADA90] at an estimated cost of $12 – 20 billion. A substantial number of these cavities result from inadequate saliva, without which fluoride is of limited value [LEO01, SPA94]. This study could benefit many individuals who experience reduced calcium, phosphate and fluoride ions caused by hyposalivation resulting from old age, prescription drug use, Sjögren's Syndrome, diabetes and radiation therapy. In addition, women are at increased caries risk due to inadequate salivary calcium levels at different points in their lives including ovulation, pregnancy and post-menopause, resulting in the same net effect as reduced saliva fluoride efficacy. Approximately 9.7% of the general population is estimated to have insufficient salivation or chronic xerostomia [PUJ98], representing over 20 million Americans who consume $100-160 million annually in consumer toothpaste. Currently there are several additional strategies for preventing, reversing or arresting the caries process including application of fluorides, sealants, anti-microbials, salivary enhancers as well as patient

education [NHI01]. But these approaches represent a time and financial investment for the patient. A reformulation of fluoride dentifrice containing Novamin® can 1) enhance remineralization, 2) counteract demineralization, 3) control anti-caries activity more effectively than current fluoride toothpastes and may be a significant oral health contribution to the general population.

The Novamin® dentifrice used in this study resembles commercial toothpaste formulations, including the Colgate® Regular product also used in this study. Novamin® 4505 was added at 5 wt% in addition to the 1100 ppm fluoride (from sodium monofluorophosphate) which is used in current Colgate® products. The Novamin® 4505 used in this study is similar to 45S5 glass compositionally, but is designed to be a 5 μm diameter particle. Particle size distribution of this particulate ranges from 15 μm to less than 1 μm and serves as a more highly reactive bioactive glass because of its greater surface to volume ratio.

CHAPTER 4
MINERAL QUANTIFICATION

The quantification of apatite mineral in dental studies is an evolving process. The past 50 years have shown us that a greater understanding of dental caries also brought about the explosion of consumer products to treat the disease. The United States Food and Drug Administration (FDA) is the regulatory agency for potential consumer products to be marketed to the masses. Although widely understood that early dentifrices cleaned the teeth well, the degree of efficacy in fighting dental caries was yet to be defined. Traditionally, scientific study requires researchers to provide proofs and logic to support their hypotheses. Qualification of these scientific arguments only provides proof of their existence but quantification provides the *magnitude* to which those arguments can be made and compared. Dental researchers understood the role of tooth mineral as the exchange commodity in cariology study. To justify research efforts, the FDA and numerous researchers have developed a number of analytical techniques to quantify the remineralization of teeth and caries resistance.

Microradiography

Transverse microradiography (TMR) has been developed over time to become the standard bearer for tooth remineralization studies. Theoretically it draws similarity to other photo-X ray techniques. The ability to quantify mineral density changes in addition to obvious visual qualification makes TMR the most powerful and trusted technique among researchers.

Sample preparation for TMR requires thin (typically <100 μm) sections that are polished and glued to glass slides. A cathode tube source emits polychromatic X-rays that are filtered (Ni) and directed toward the reference system and specimen. Figure 4-1 depicts the TMR setup in detail [MJO86].

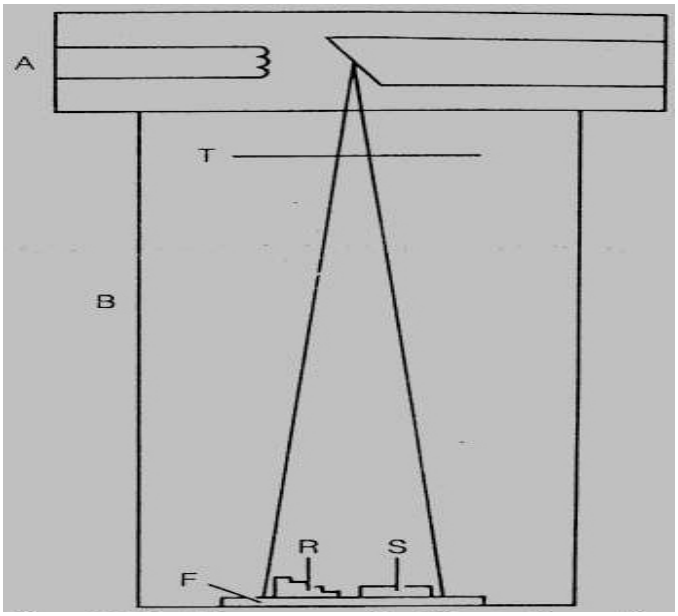

Figure 4-1 Microradiography setup (A) Cathode Tube, (B) light-proof casing, (T) filter, (R) reference step wedge, (S) specimen, and (F) radiographic film

Filtered monochromatic X rays reach the specimen and reference system, usually an aluminum step wedge. Typically these steps range from 10 – 150 μm. Gelhard and Arends further developed this method running at 20 kV and 15-20 amp to produce a wavelength of $\lambda = 0.15$ nm, which in turn guaranteed 95% absorption inorganic (mineral) [GEL84, MJO86]. Sample thickness was estimated to 1 μm accuracy on the radiographic film. Quantification of enamel tooth mineral is made with microdensitometric tracing of the film using specially designed software. Angmar's formula is used to calculate relative mineral volumes with respect to cross-sectional depth with transverse microradiography [DIJ86].

$$V = 50.48\ (t_a / t_s) \bullet 100\% \qquad (4\text{-}1)$$

where t_a is the step wedge thickness and t_s is the specimen thickness

The volume percent mineral is then plotted against cross sectional depth from the surface to graphically produce the Z parameter. Z is defined as the integrated area under the densitometric tracing for each specimen in units of vol% mineral • μm. Plotting Z for both sound (original) and demineralized enamel provides a direct quantified measurement of mineral density flux, thus the parameter ΔZ [GEL84]. A decrease in ΔZ for a specimen therefore signifies mineral deposition de novo. This is represented in Figure 4-2 [DIJ86].

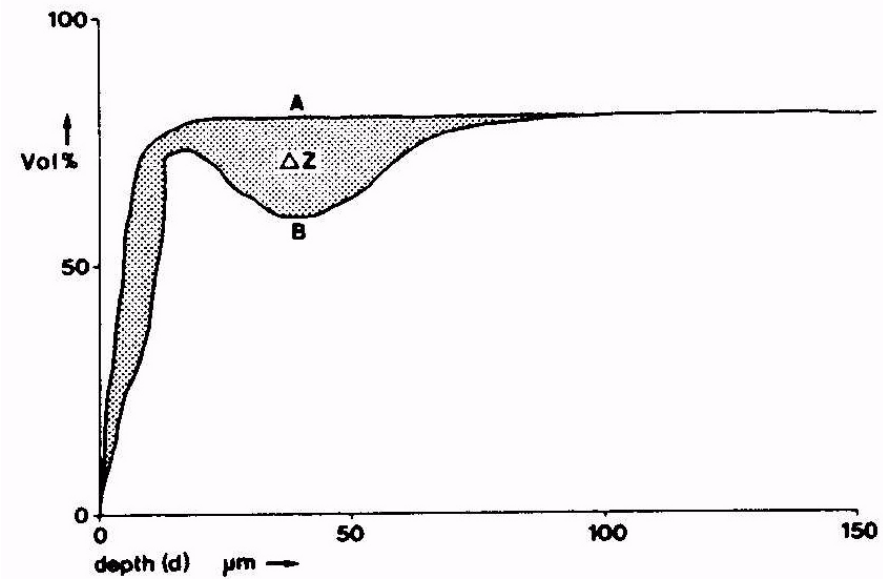

Figure 4-2 Mineral density quantified as ΔZ in TMR.

Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy (CLSM) is a powerful imaging technique based upon optical behavior of light within specimens. Generally, confocal analysis features excitation (such as fluorescence) although emission detection is also possible. The fluorescence phenomenon involves the absorption of light of a given wavelength by

a fluorescent molecule followed by the emission of light at longer wavelengths. Fluorescence detection has three major advantages over other light-based investigation methods: high sensitivity, high speed, and safety, not only for the operator but also for the sample because they are not affected or destroyed in this process [WEB99]. This provides an extremely fine scale view of desired specimen planes. Although planar imaging is common among many techniques, the ability of CLSM to analyze thick (3D) specimens makes it so valuable in the scientific community. Conceptually, real time imaging of a single specimen for various depths or "sections" provides researchers with a practical advantage, especially considering biological specimens and time dependant reactions. For this study, it is imperative that fluorescence detection and imaging be understood. A typical confocal microscope is shown in Figure 4-3.

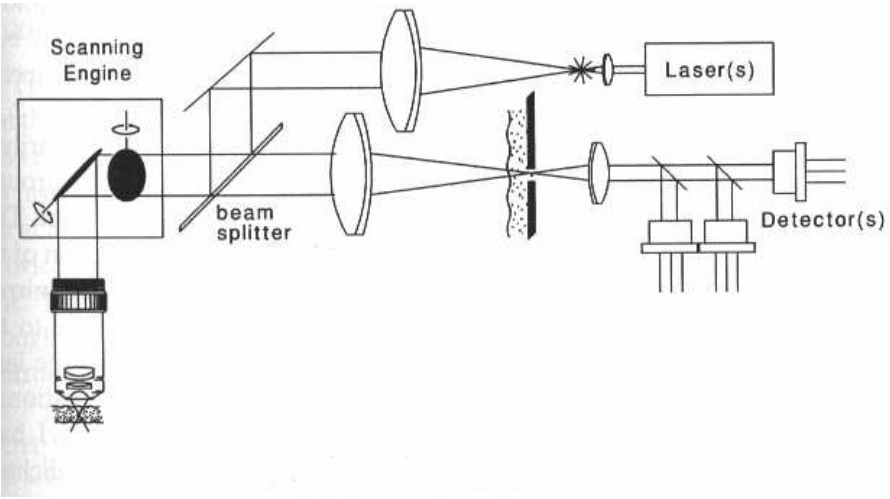

Figure 4-3 Schematic of a generic confocal microscope.

The theory of CLSM originates with the laser light source being focused upon the specimen and reflected back through a dichroic mirror to the detector. For fluorescence, the light returning from the sample through the objective lens reaches the beam splitting mirror and passes as excitation light. The detector processes the image based upon the

focal intensities it receives through this path [WEB99]. Figure 4-4 depicts the imaging of a thick sample in greater detail.

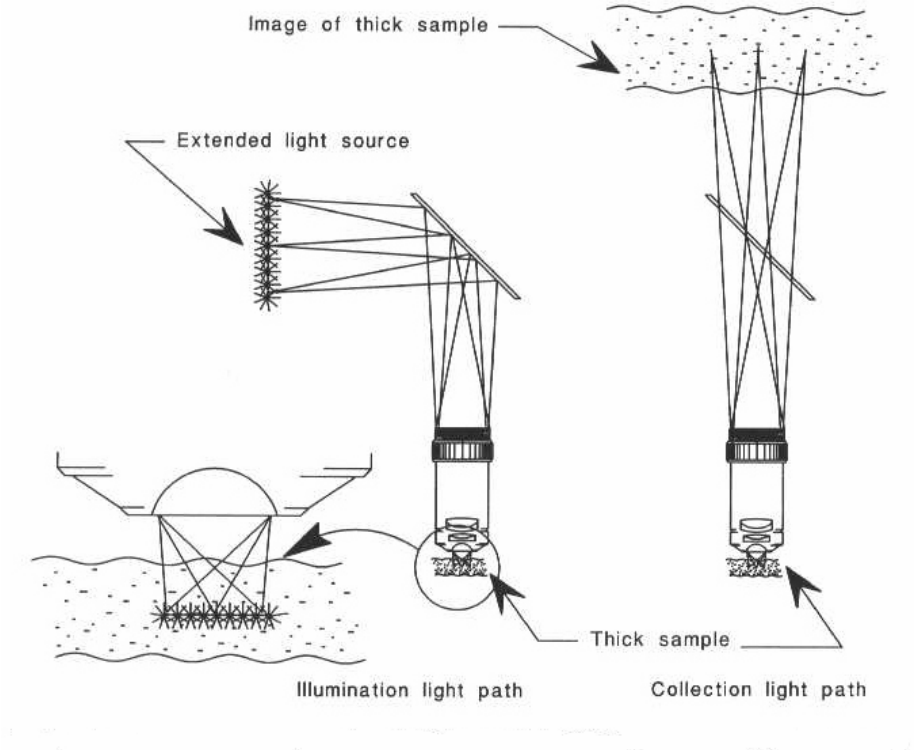

Figure 4-4 Conventional microscope for fluorescence in epitaxial configuration.

The florescence of teeth was observed by both dye-assisted imaging and auto-fluorescence. Preliminary confocal work performed at the McKnight Brain Institute (University of Florida) showed a significant degree of autofluorescence in demineralized enamel through both fluorescein isothiocyanate (FITC) and Texas Red filters. Autofluorescence of enamel has been shown to significantly correlate with TMR and dye-assisted CLSM results [BEN89, GON99]. Similar work on nearly identical specimens was performed at the Oral Health Research Institute at Indiana University-Purdue University at Indianapolis (IUPUI). Both sets of imaging revealed again a high fluorescing lesion compared to near zero for sound enamel. Fontana and Gonzales-Cabezas [FON96][GON99] have shown a significant correlation between cross-sectional

confocal analysis in fluorescence and mineral flux. Their fluoride toothpaste studies revealed a significant match for two parameters (lesion area and total fluorescence) between confocal microscopy and microradiography of tooth enamel and dentin. *In vitro* demineralization and remineralization of surface enamel was easily imaged and quantified. Significant correlations were also found for half tooth (thick sections) remineralization comparing TMR and confocal microscopy. For remineralization studies, ΔZ pre and post remineralization were combined as a single parameter representing their mathematical difference, ΔM. Fontana and Gonzales found Pearson correlation coefficients (perfect correlation = 1) of 0.71 for ΔM vs. total lesion area and 0.70 for ΔM vs. total fluorescence. A previous study involving only demineralized samples were found to correlate with TMR to the same degree. A subsequent study also revealed nearly identical correlation in mineral changes with TMR when comparing autofluorescence (no dye) of enamel vs. the dye. The fluorescent dye (0.1 mM Rhodamin B) was used to enhance fluorescence imaging of lesion areas in these studies [BEN89].

Nanoindentation

Nanoindentation is a relatively new method for characterization of material mechanical properties on a very fine scale. Features less than 100 nm across and thin films less than 5 nm thick can be evaluated. Test methods include indentation for comparative and quantitative hardness measurements, and scratching for evaluation of wear resistance and thin film adhesion. Nanoindentation is often performed in conjunction with atomic force microscopy (AFM). The area for testing is located by AFM imaging and indentations are imaged by AFM after testing. A three-sided, pyramid-

shaped diamond probe tip (Berkovich) is commonly used for sample indenting and scratching. For indenting, the probe is forced into the surface at a selected rate and maximum force. For scratching, the probe is dragged across the sample surface while controlling the force, rate, length, and angles. Imaging is performed using the same probe for intermittent contact via (tapping mode) AFM. A force-displacement curve based on piezoresistive loading is generated during indentation and provides further indication of mechanical properties [HAN03, HYS03].

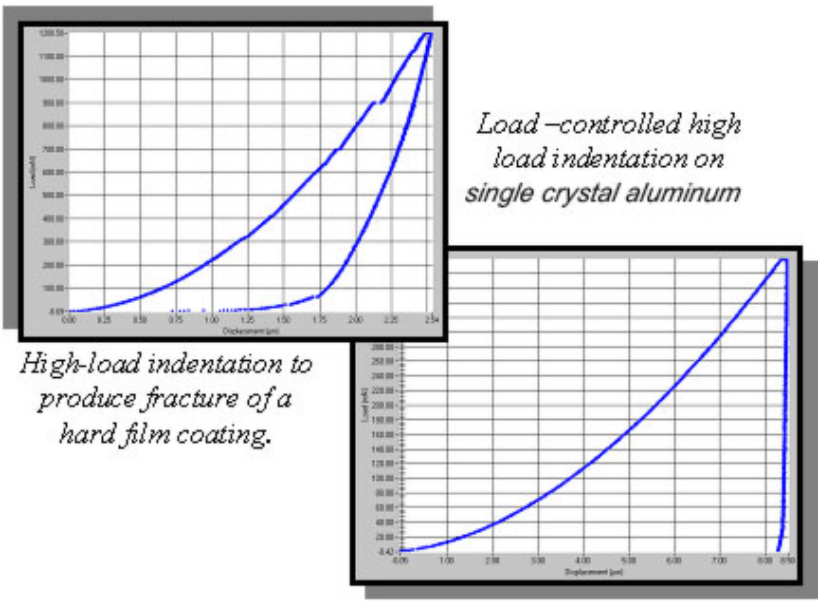

Figure 4-5 Force-displacement (loading) curves on various materials.

This study considers two main mechanical properties obtained through the Triboindentor® (Hysitron Inc., Minneapolis, MN): hardness and reduced modulus. The load-displacement data from the unloading curve is fit to a power law relation to determine mechanical properties.

$$P=A(h_0-h_f)^m \qquad (4\text{-}2)$$

where m = 2 (conical), P is the load , h_o is the initial depth, h_f is final depth, and A is the area.

The derivative of this power law with respect to the depth gives the material stiffness (S) at the maximum load, P_{max}

$$S = dP/dh \quad at \ P_{max} \tag{4-3}$$

The contact depth, h_c, can be calculated from:

$$h_c = h_{max} - 0.75(P_{max}/S) \tag{4-4}$$

The hardness, H, and reduced modulus, E_r are then found by:

$$H = P_{max}/Ah_c \tag{4-5}$$

$$E_r = [S\sqrt{\pi}] / [2\sqrt{(Ah_c)}] \tag{4-6}$$

A typical loading curve for sound enamel is shown in Figure 4-6.

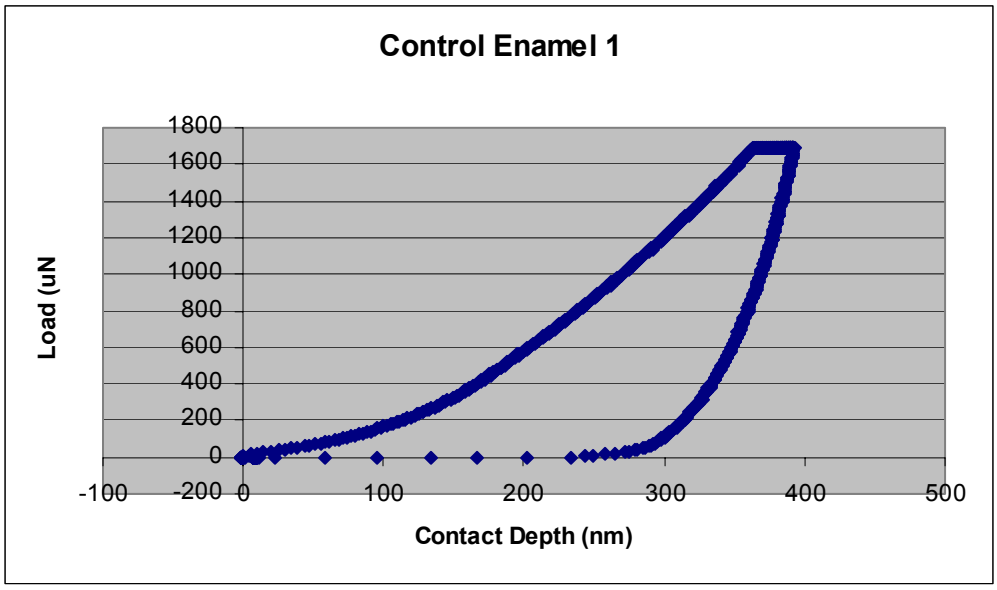

Figure 4-6 Loading curve for nanoindentation on sound enamel.

Microindentation

Microindentation, on the other hand, is a more established technique for measuring the hardness of materials. For ceramics in particular, hardness is a critical mechanical property. For engineering and characterization applications, approximately 60% of worldwide published ceramic hardness values are obtained using Vickers diamond

indentation, with loads typically in the range of a few Newtons to 9.8 N (1 kg-f) and occasional data for high-toughness ceramics as high as 98 N (10 kg-f). At small indentation loads, problems arise from the load dependence of hardness and from measurement uncertainty due to the small indentation size. At higher loads, cracking and fracture become problems in some cases, making measurement impossible. Typical hard ceramics have Vickers hardness in the 10-30 GPa range [QUI98]. Teeth particularly are considered ceramic composites. Typical hardness values for enamel range from 2.9-3.9 GPa and dentin 0.6 GPa [FOR91, WIL92].

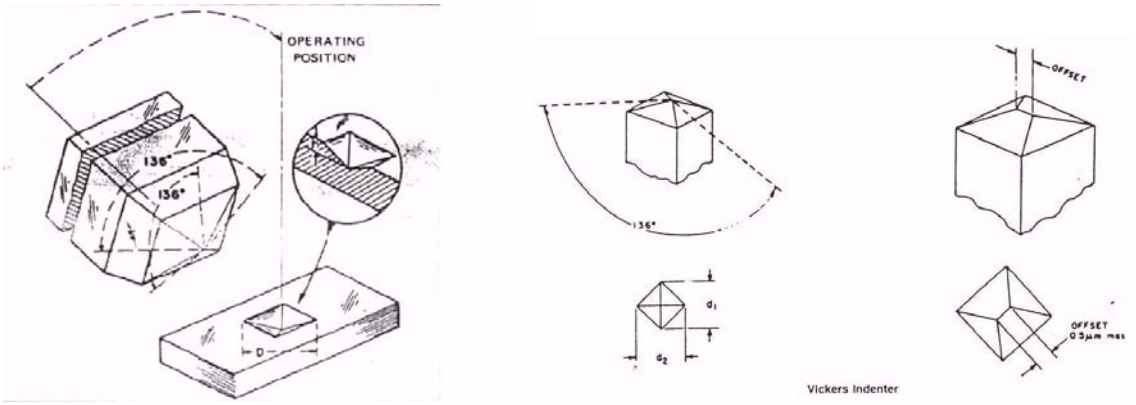

Figure 4-7 Vickers microhardness testing.

The square pyramidal shape of the Vickers indenter creates a smaller deeper impression, although more likely to crack than Knoop indentations. ASTM standard E 384, Microhardness of Materials, covers Vickers hardness; C 1327 is the new standard for Vickers hardness of advanced ceramics and recommends a load of 9.8 N. The universal standard [AST00] for this calculation is described in equation 4-7.

$$\textit{Vickers Hardness (HV)} = 1854.4 \cdot P/d^2 \qquad (4\text{-}7)$$

where P is the load in grams force
d is the mean diagonal length of indentation in μm,
assuming standard face angle of indenter tip is 136°

To obtain GPa from these units (kgf/mm^2), multiply by 0.0098. ASTM standard E 384 section 7.1 clearly states that optimum accuracy of measurement requires that the specimen be flat with a polished or otherwise suitably prepared surface [AST00]. Although it was developed in the 1920s (by engineers at Vickers Ltd. in the U.K.), this hardness testing method continues to be a significant standard, especially for hard ceramics.

The analysis theory is quite different for these two techniques. As mentioned earlier, nanoindentation is based upon a loading/unloading curve calculation whereas Vickers is solely dependent upon the indent surface area. Oliver and Pharr demonstrated (Table 4-1 and 4-2) that results from the two techniques are comparable [OLI92].

Table 4-1 Comparison of hardness and Young's modulus data obtained with Vickers and Berkovich indenters.

MATERIAL	BERKOVICH INDENTER		VICKERS INDENTER	
	Elastic Modulus [GPa]	Hardness [GPa]	Elastic Modulus [GPa]	Hardness [GPa]
Sapphire	483	26.92	496	27.04
Soda Lime Glass	78	5.24	82	6.18
Fused Silica	72	8.44	71	8.87
Silicon	169	11.13	169	12.32
Window Glass	77	5.96	78	7.14
Alumina	449	26.10	439	25.89
Nickel	218	5.87	218	6.73
Barium Titanate	228	11.54	219	11.92

Table 4-2 Comparison of indentation fracture toughness obtained by different indenters.

Instrument	Indenter Tip	Material	K_c MPa $m^{1/2}$(stdev)
Nanoindenter	Cube Corner	Silicon Carbide	3.60 (0.50)
Wilson/Tukon	Vickers	Silicon Carbide	2.18 (0.35)
Nanoindenter	Vickers	Barium Titanate	0.66 (0.15)
Wilson/Tukon	Vickers	Barium Titanate	0.62 (0.21)

Preliminary evaluation of the nanoindentation technique was performed and imaged for bovine enamel with AFM in conjunction with nanoindentation, see Figures 4-8 and 4-9.

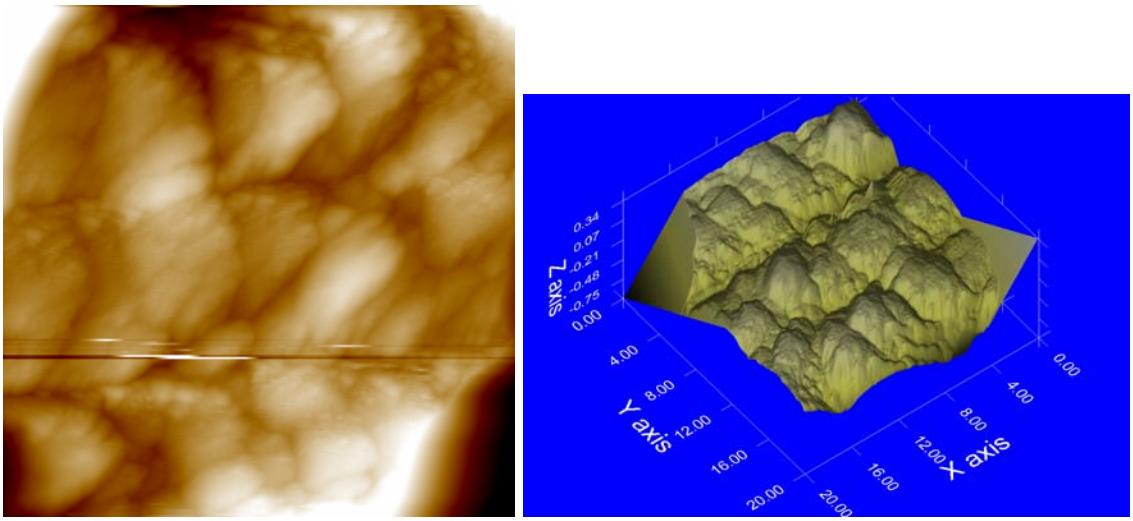

Figure 4-8 Demineralized Enamel. Axis units in μm.

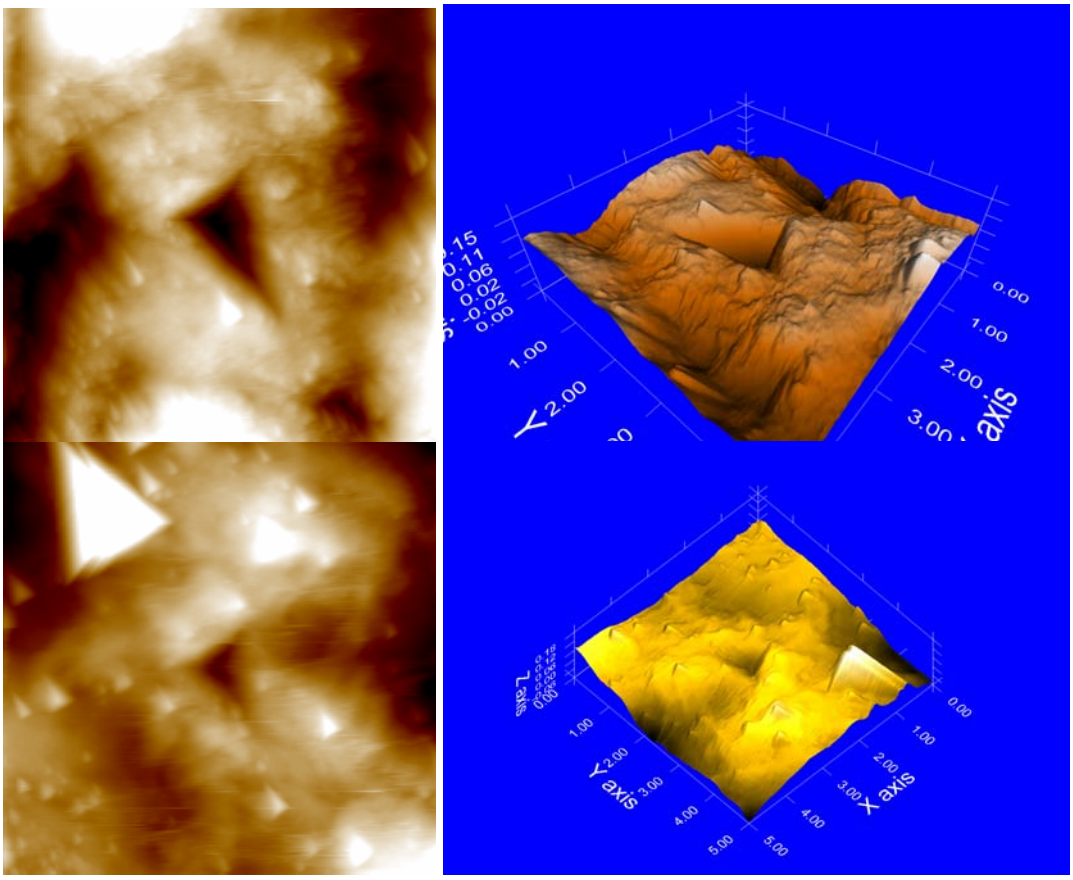

Figure 4-9 Indents in Novamin® treated enamel. Axis units in µm.

Imaging for preliminary Vickers indents was performed with SEM for bovine enamel also. All Vickers testing for this group was performed at 300g load with 15s dwell time.

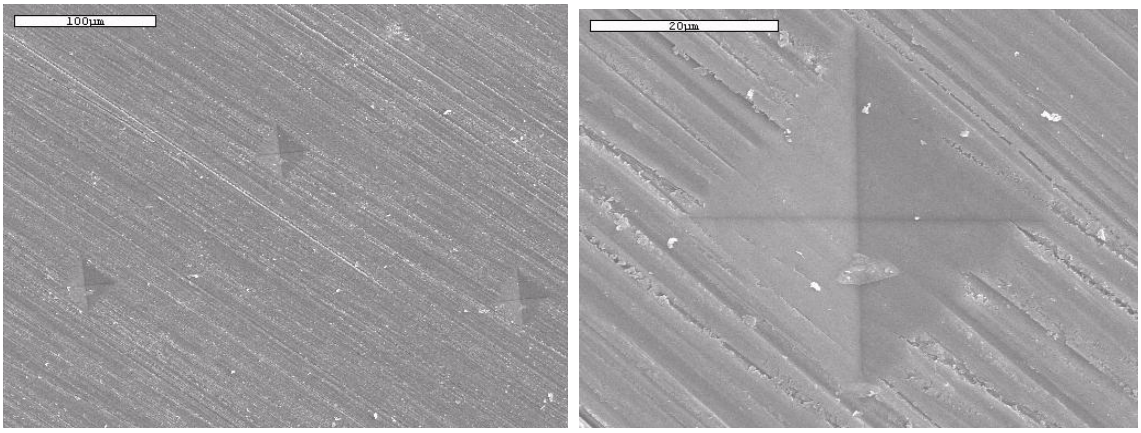

Figure 4-10 Vickers indents in control enamel @ 300X and 2000X.

CHAPTER 5
EXPERIMENTAL METHODS AND MATERIALS

Collection and Storage of Teeth

A total of 48 extracted human molars were collected from dental surgical clinics at the University of Florida. Teeth were required to have intact surfaces, no carious lesions, and no restorations. In keeping with the Health Insurance Portability and Accountability Act (HIPAA) regulations, we were not aware of the cause for tooth extraction, age, name, or gender of the patients. The use of human teeth in this study was also approved by the Institutional Review Board (IRB) at the University of Florida. A total of 18 (out of 48) of the collected teeth were randomly segregated for use in this toothpaste study. All teeth were stored in 0.1% Thymol (w/v) solution until sterilization [BIS03]. Teeth were cleaned by removal of soft tissue debris and later sterilized using Co^{60} source for gamma irradiation. They were subjected to a dose of 500 krad, well above the required 173 krad. This process does not alter the tooth structure or caries susceptibility [HAN62, WHI94].

Sectioning and Mounting

Each tooth (n = 18) was sectioned into quadrants along both mesiodistal and buccolingual planes (Figure 5-1) using a diamond tipped circular saw (Buehler Isomet® 300 Low Speed). A strict labeling method was assigned to each tooth section designating tooth number and treatment protocol (see Table 5-1). Sections were mounted individually in epoxy mounting resin and labeled. A 3-mm diameter treatment window was opened on each tooth section by grinding off 200 μm of surface enamel. Removal of the outer enamel layer was essential for standardizing the experiment.

Table 5-1 Tooth labeling scheme. Example *36BX*

Tooth Section	Tooth #	Label	Treatment
Mesiolingual	31 thru 48	LC	None (Control)
Distolingual	31 thru 48	LD	Demineralized Control
Mesiobuccal	31 thru 48	BX	Colgate® Regular
Distobuccal	31 thru 48	BY	Novamin® Dentifrice (5 wt%)

Fluoridation and cyclic remineralization/demineralization of the surface layers makes each tooth surface inherently inhomogenous. In order to reduce variability, these layers were removed to expose the virgin enamel deeper in the tissue [RIC89]. Mechanical polishing was performed with successive 120, 400, 600 and 1200 grit paper and confirmed with a digital caliper.

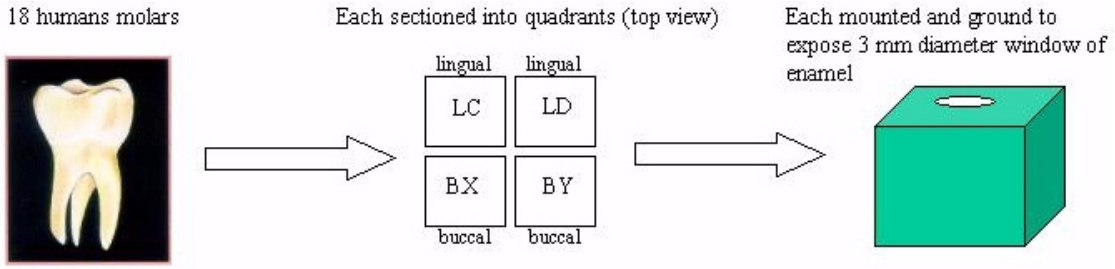

Figure 5-1 Experimental flow diagram of specimen sectioning and mounting.

Lesion Formation

Tooth sections LD, BX and BY (Figure 5-2) were demineralized in stirred solution containing 2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4, 0.05 M Lactic Acid, and 0.5 ppm F^- adjusted to pH 4.5 with 50% NaOH. Surface lesion formation was maintained at 37°C in order to produce a uniform lesion depth of 100-150 μm [IVA03].

Treatment Regimen

Section BX and BY were treated in a pH cycling regimen including 3:1 toothpaste solutions of Colgate® Regular and Novamin® dentifrice respectively. Both dentifrices, nearly identical in composition, contain 1100 ppm fluoride although the Novamin® paste contains 5 wt% bioactive glass particles in place of silica abrasive. Tooth sections were

immersed cyclically in stirred treatment solutions, demineralization solution (detailed above) and Fusayama's synthetic saliva [LEU97] for 20 days. Complete pH cycling was performed at 37°C with exception to dentifrice solution treatment, which was done at 25°C. The daily treatment regimen (pH cycling) included the following sequence:

T_0	Demineralization solution 30 minutes
T_0 + 0.5 hrs	Dentifrice treatment 3 minutes / distilled H_2O wash / synthetic saliva
T_0 + 7.5 hrs	Demineralization solution 30 minutes
T_0 + 8.0 hrs	Dentifrice treatment 3 minutes / distilled H_2O wash / synthetic saliva for 16 h

Cross-sectioning

All four sections of each tooth (LC, LD, BX and BY) were cross-sectioned through the treatment window to expose the lesion depth along the cross-sectional surface. Two halves of each tooth section were then available for optical and mechanical analysis. Throughout the study, tooth sections were intermittently kept in refrigeration for storage (~6°C). Teeth were individually wrapped in distilled water soaked Kimwipes® tissue.

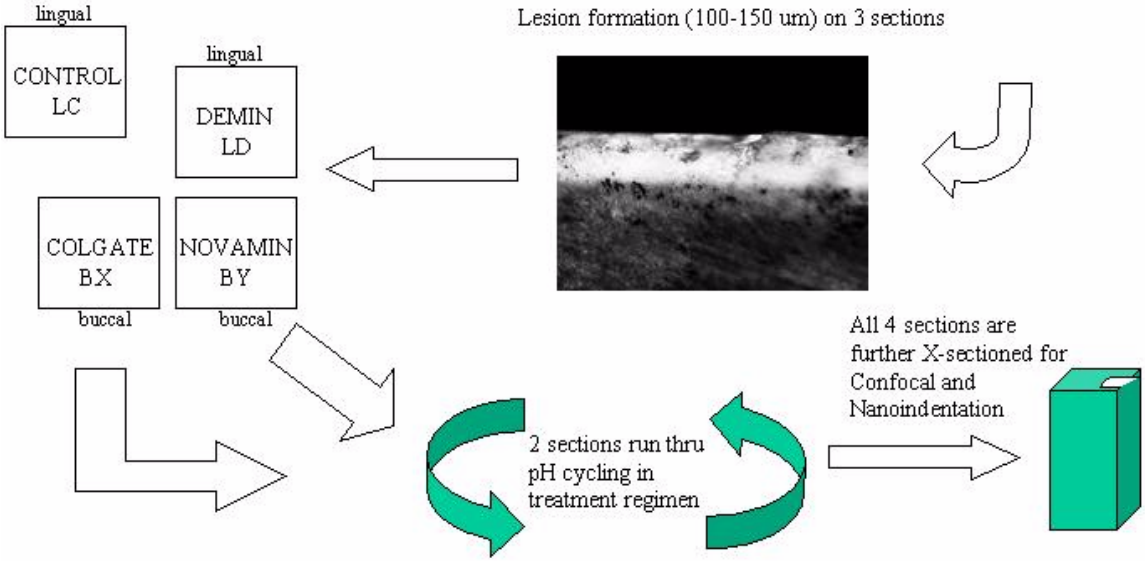

Figure 5-2 Flow diagram of lesion formation, pH cycling and cross sectioning.

Analysis

CLSM was performed on 72 cross-sections (4 sections of 18 teeth) at the Oral Health Research Institute at Indiana University-Purdue University at Indianapolis (IUPUI) under the supervision of Dr. Marguerita Fontana. Randomly selected cross-sections of the remaining halves were chosen for nanoindentation at the Major Analytical Instrumentation Center (MAIC) at the University of Florida. Microindentation was then performed (Buehler Ltd. Micromet 3 Microhardness Tester, Lake Bluff, Illinois) on the same sections for hardness comparison of both techniques.

CHAPTER 6
RESULTS AND DISCUSSION

Cross sectional analysis of enamel lesions with CLSM were based upon digital images taken at specified controlled conditions. Sound enamel registers near zero fluorescence (grayscale value ~ 0) and appears pitch black. Lesions slightly autofluoresce but imbibition of the Rhodamine B dye (0.1 mM) allows the porous demineralized layer to fill and appear with considerable contrast. Analysis of all samples was conducted with a specially modified Nikon microscope fitted with Odyssey confocal capability (Odyssey, Noran Instruments, Inc., Middleton, WI). The accompanied software (Metamorph® version 4.1.6, Universal Images Corp., West Chester, PA) calculates image-based parameters of selected lesion zones. Using a 10X Nikon objective, the specimens were illuminated with an argon laser at 50% intensity using a 488 nm excitation wavelength. Confocal slits were set at 25 μm with a 515 nm long-pass filter. A 350-μm characteristic length was randomly chosen within a representative part of the lesion for each sample. Figure 6-1 shows a typical cross-sectional image.

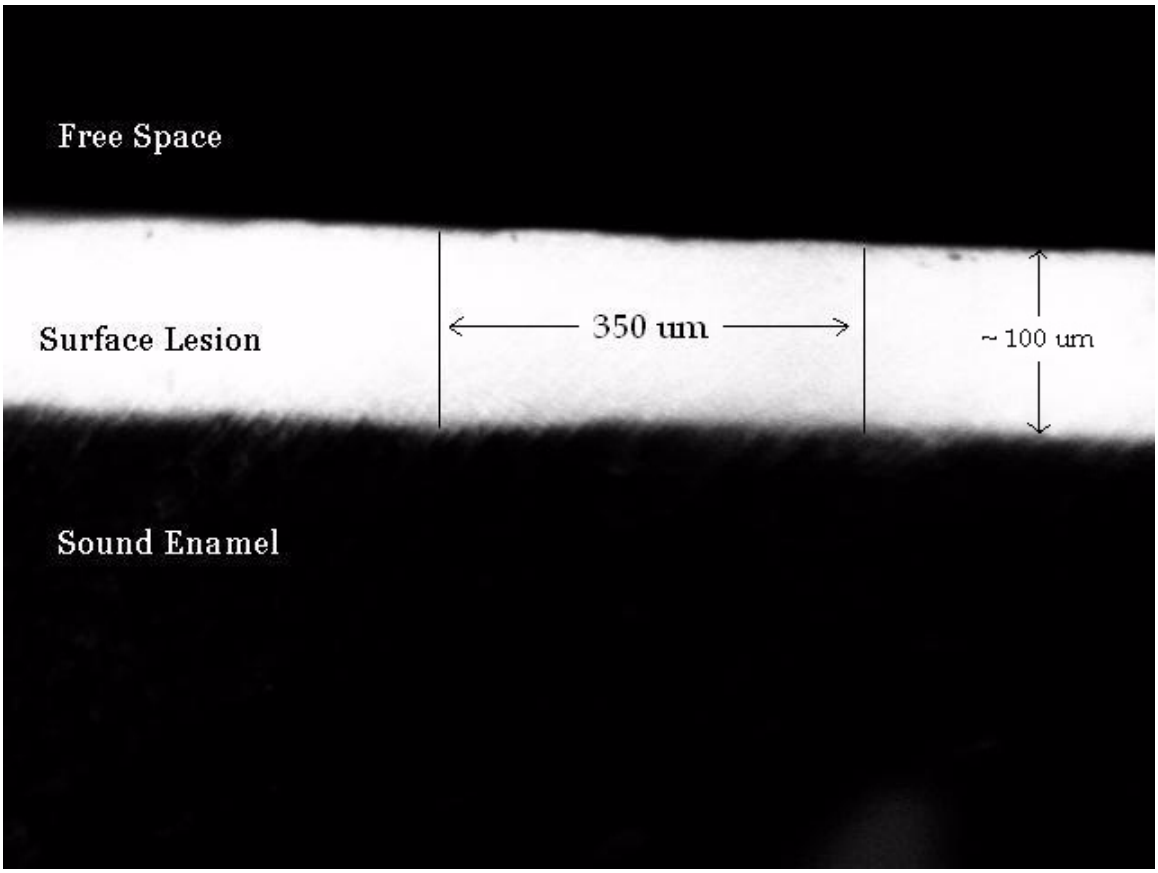

Figure 6-1 Confocal image of enamel lesion, tooth section *38LD*

The complete analysis (all 4 tooth sections of 18 teeth) was performed at contrast level 1500 and minimum threshold value 50 (grayscale). The two parameters that correlate well with TMR are lesion area and total gray value (or total fluorescence). Data for these two parameters was collected and tabulated to compare sections within each tooth to determine changes in lesion properties after 20 days of dentifrice treatment. Data could not be collected for LC (sound enamel control) naturally because of non-fluorescence. Parameters are specifically defined as:

- *Lesion Area – direct summation of fluoresced (above threshold) pixels in characteristic length of lesion.*
- *Total Gray Value – direct summation of gray values (0 – 255) for pixels within characteristic length of lesion.*

Lesion areas for 3 sections of all 18 teeth were plotted to compare the effect of Novamin® and Colgate® dentifrices in Figure 6-2.

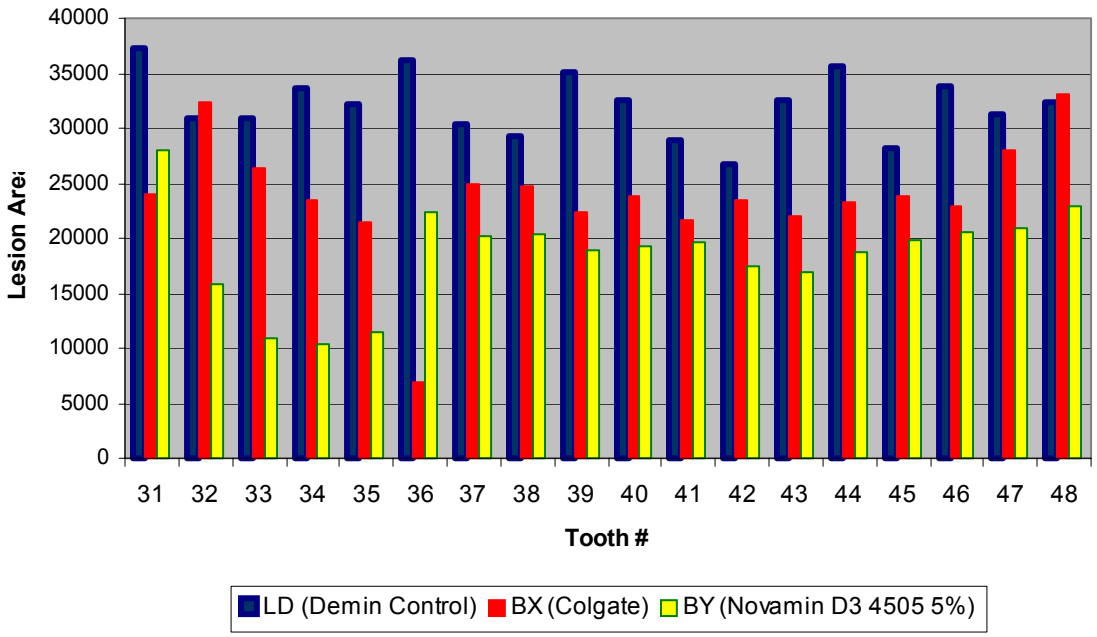

Figure 6-2 Lesion area for each tooth for the demineralized control, Colgate and Novamin® treatment condition.

The Novamin® dentifrice reduced the lesion area more than Colgate® for 16 out of 18 teeth. Single tailed T-testing (assuming unequal variances) found these two groups to be significantly different ($p < 0.001$). For nearly all samples, the 20 day dentifrice treatment under pH cycling halted and reversed the caries process as evident in the reduction of the lesion area. Colgate® reduced the lesion area by an average of 24.9% from the original lesion LD. Novamin® decreased the lesion area by 41.9%.

Total gray value was also plotted (Figure 6-3) to determine the fluorescence levels within the lesions. This parameter is commonly reported as total fluorescence because greater fluorescence corresponds to higher gray values. Thus, smaller values are indicative of less porosity and dye penetration, or more mineral.

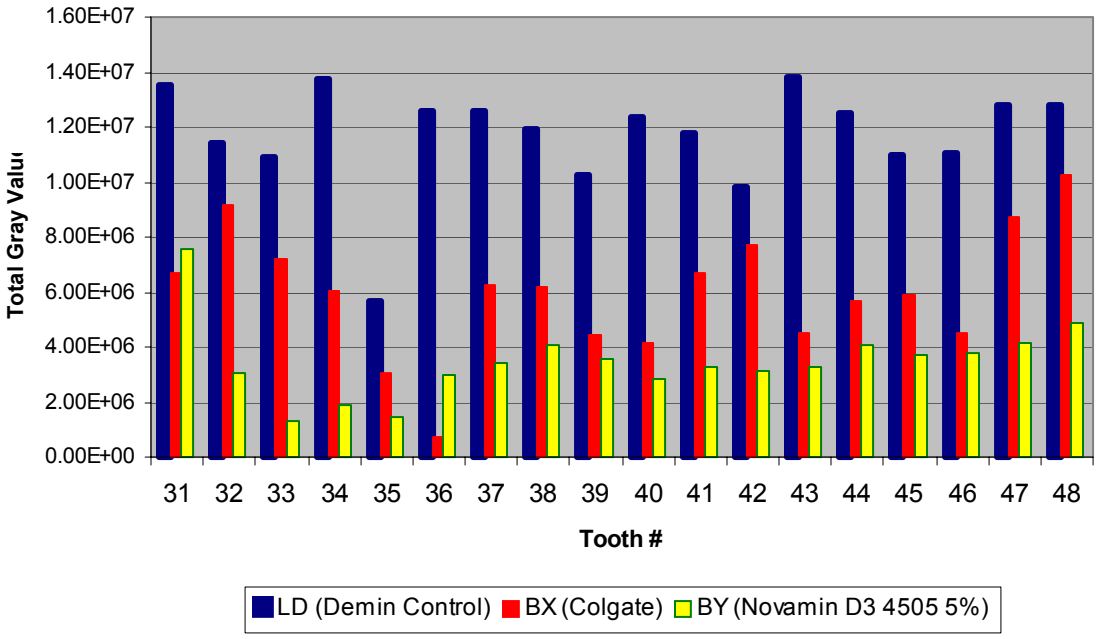

Figure 6-3 Total Gray Value for each tooth for the demineralized control, Colgate and Novamin® treatment condition.

The Novamin® dentifrice reduced the total gray value more than Colgate® for the same 16 out of 18 teeth. Treatment groups were again found to be significantly different ($p < 0.001$). Colgate® reduced the fluorescence (total gray value) by an average of 48.1%, Novamin® 70.5%.

Statistical review (SAS System software) using two-way analysis of variance (ANOVA) of all three groups (LD, BX and BY) yielded statistical significance ($p < 0.0001$) for both confocal parameters, lesion area and total fluorescence. Duncan multiple range testing for both parameters found all three groups to be statistically different in means at a significance level of $\alpha = 0.01$.

Cross sectional images for these teeth sections show remineralization "bands" revealing the depth at which most remineralization occurred.

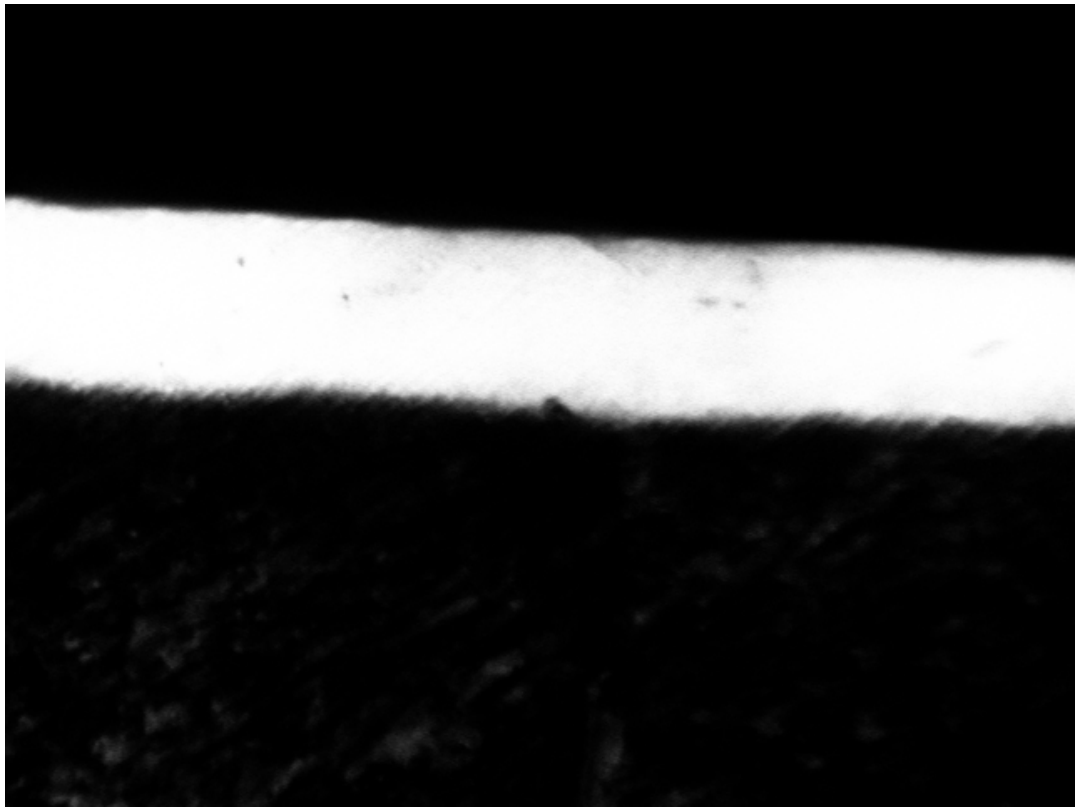

Figure 6-4 Confocal image of tooth section *41LD (Demineralized Control)*

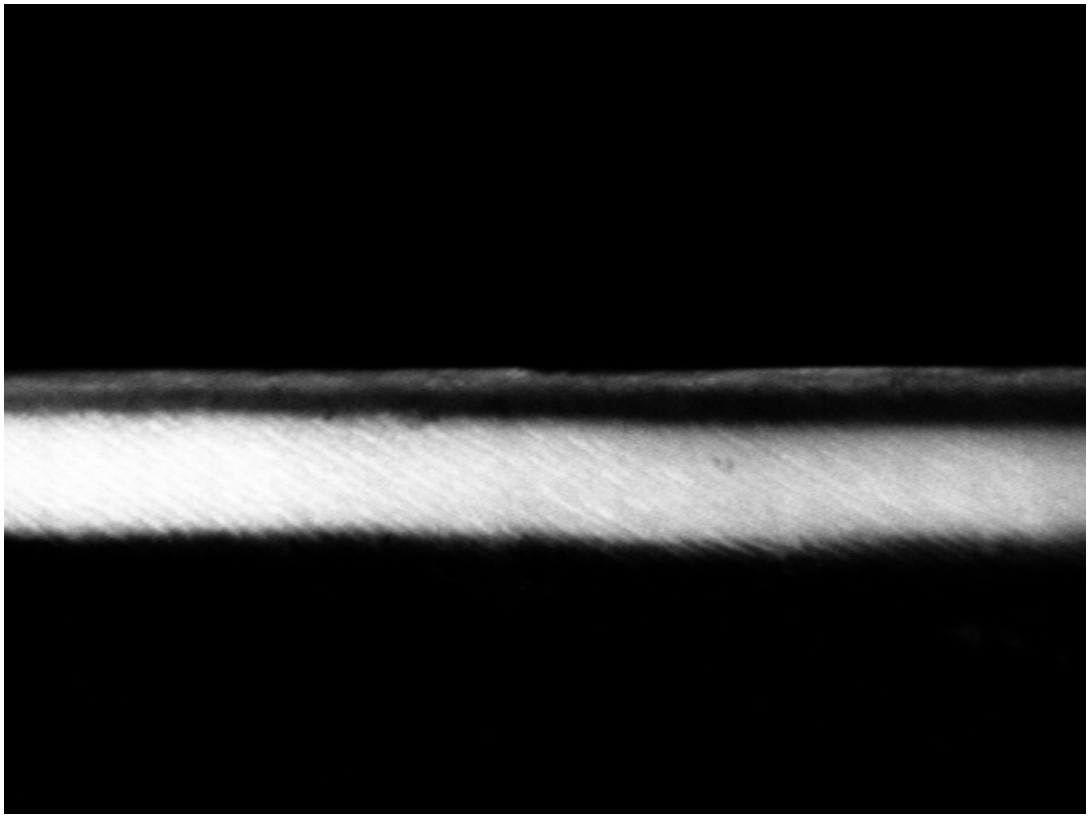

Figure 6-5 Confocal image of tooth section *41BX (Colgate® treated)*

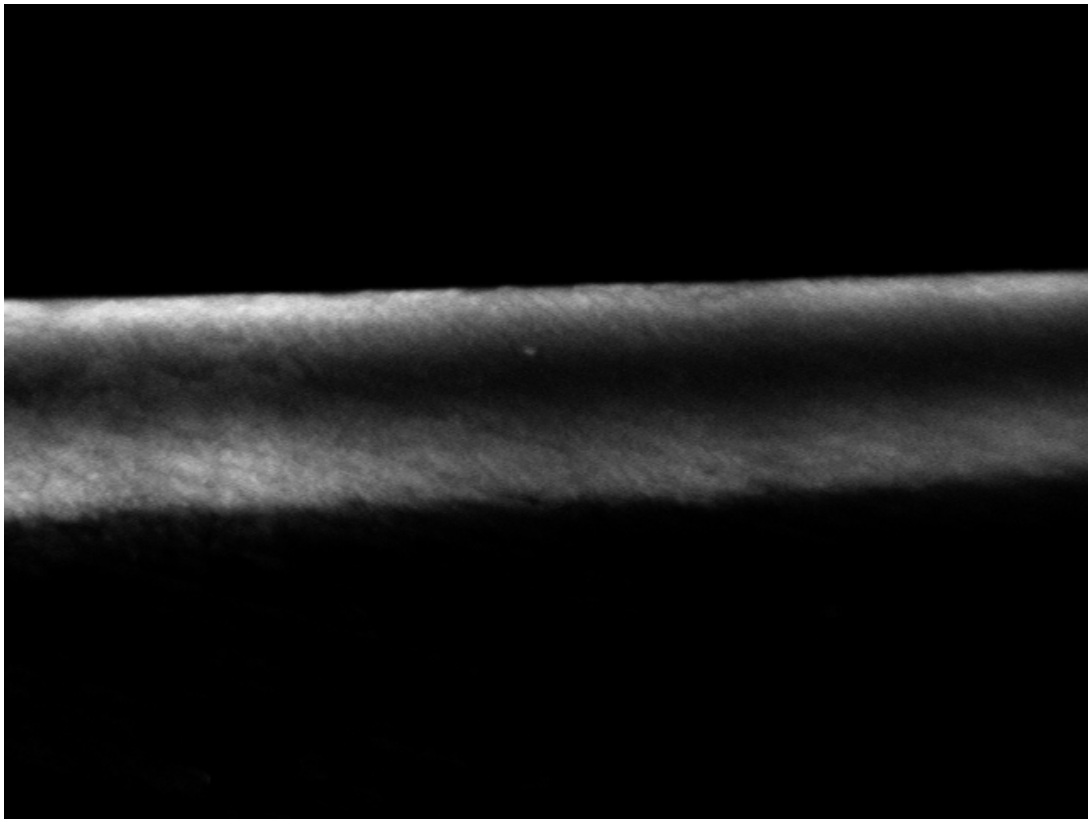

Figure 6-6 Confocal image of tooth section *41BY (Novamin® treated)*

The remineralization "bands" are clearly visible signifying mineral deposition, similar to those reported in previous toothpaste studies. The depth of these bands could be due to several variables. A combination of diffusion rates for fluoride, calcium, and phosphates into the enamel surface in addition to twice daily acid challenge may influence band depth. Fractional variations in composition from one tooth to another probably play a role as well. Slight differences in impurity element levels in the tooth structure cannot be ignored. Although the natural surface enamel was removed, the maturation history of these specimens could vary considerably. It is likely that fluoridated water sources and dietary habits of the original patients play some role in the demineralization and remineralization capability of these teeth. Ultimately, these variables also determine the size and shape of these bands. The most influential factor,

however, is the daily acid challenges and acid diffusion capability. The noticeable (bright) demineralization layer above the remineralization band is unusual considering this layer always receives the first and heaviest dose during remineralization treatment. Logically, the remineralization band should begin at the surface and fade with depth. But this would be true if only remineralization was taking place within the lesion. The fact that pH cycling includes daily acid challenges keeps the outer surface demineralized and in a state of constant flux. The depth of that demineralized band is most likely controlled by acid diffusion, possibly an Arrhenius relationship. Novamin® samples clearly show a larger remineralization band for nearly all samples and a noticeably darker lesion.

Figure 6-7 Confocal image of tooth section *47LD (Demineralized Control)*

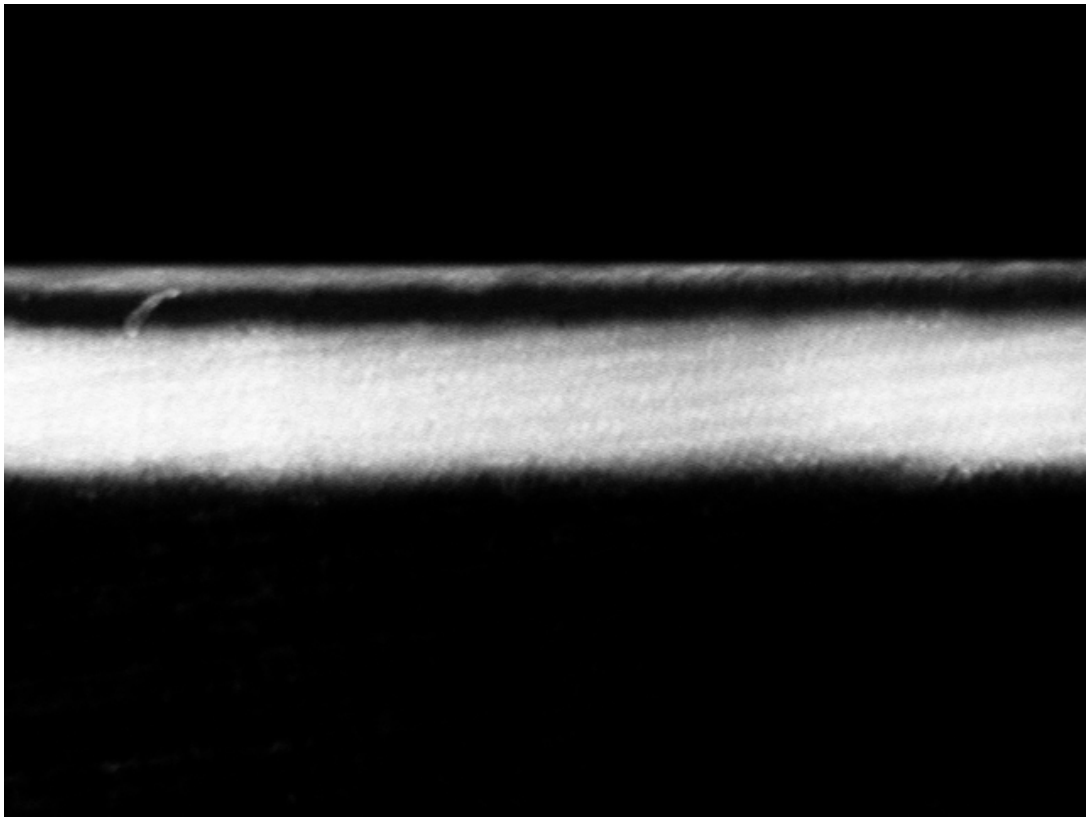

Figure 6-8 Confocal image of tooth section *47BX (Colgate® treated)*

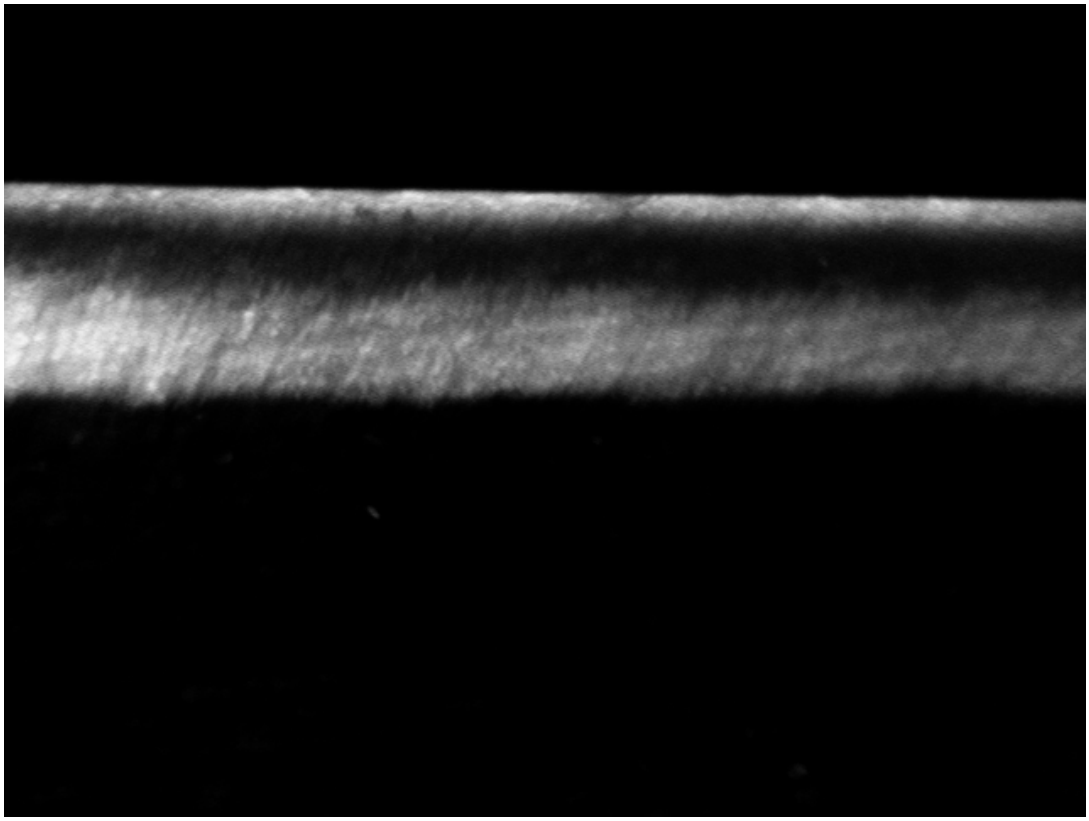

Figure 6-9 Confocal image of tooth section *47BY (Novamin® treated)*

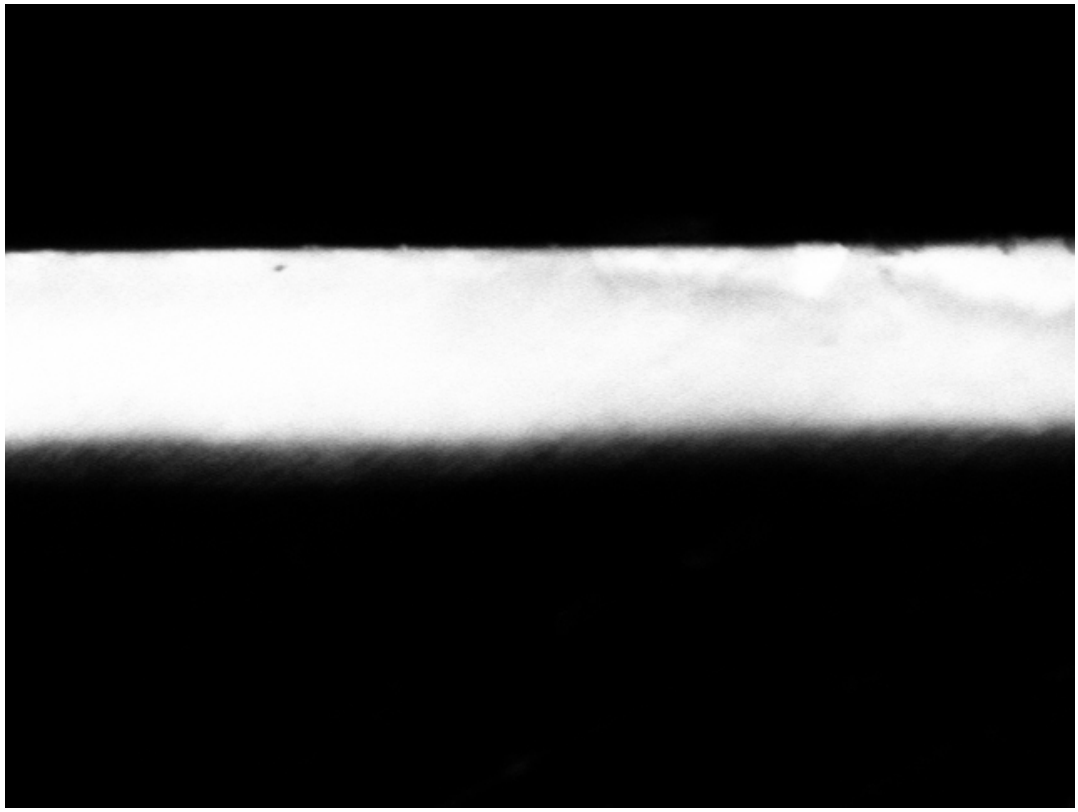

Figure 6-10 Confocal image of tooth section *48LD (Demineralized Control)*

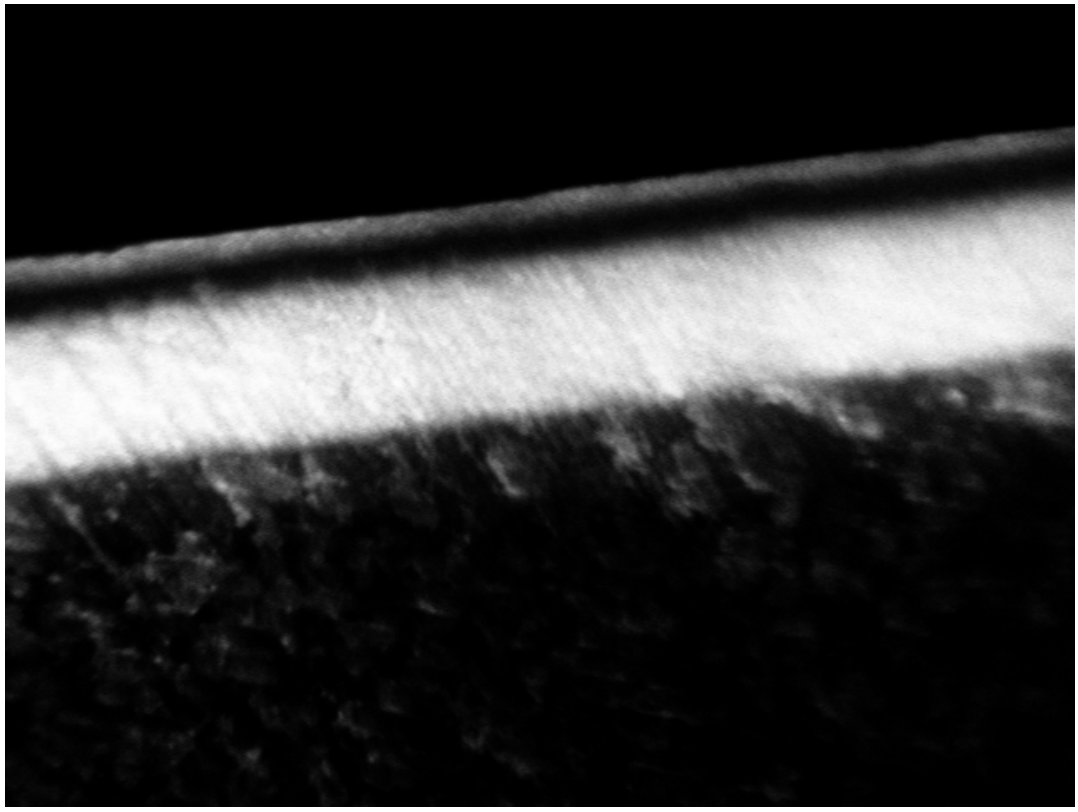

Figure 6-11 Confocal image of tooth section *48BX (Colgate® treated)*

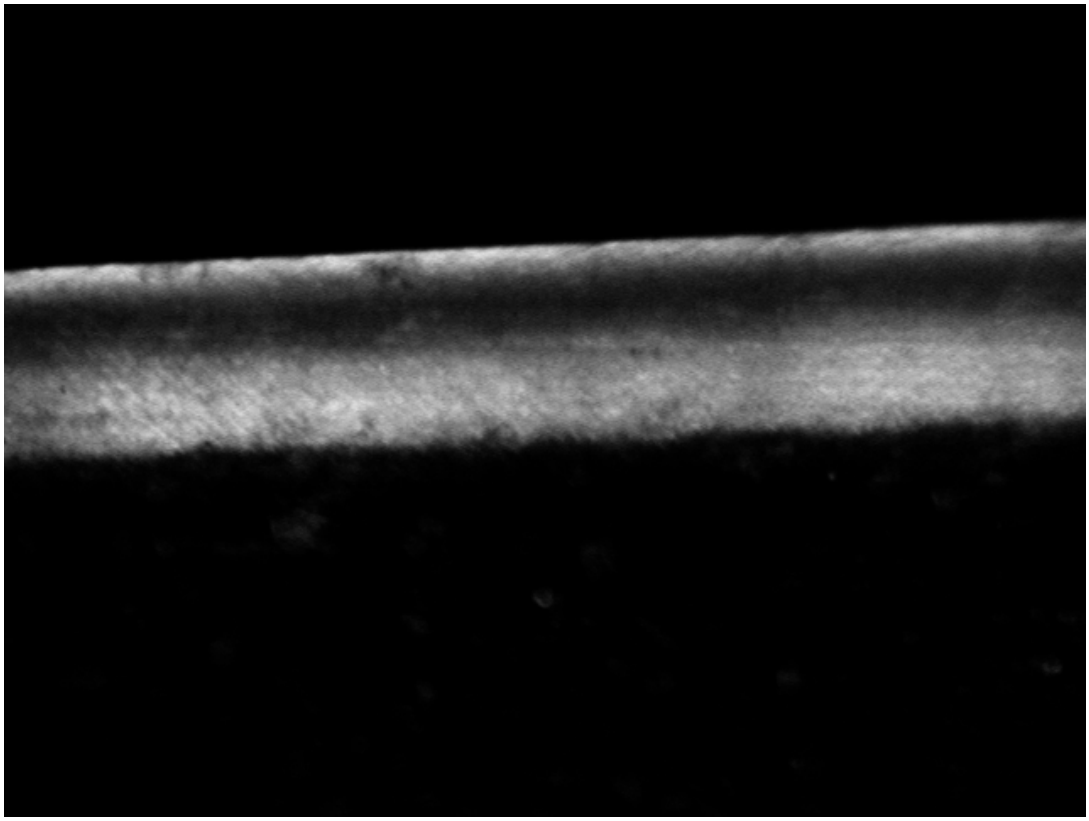

Figure 6-12 Confocal image of tooth section *48BY (Novamin® treated)*

The nature and degree of interaction between fluoride and Novamin® is clearer. It appears that *in vitro*, these two agents positively interact and do not inhibit the remineralization function of the other. Although the techniques used in this study cannot confirm or deny this hypothesis, ultimately the lack of oral bacteria and plaque leave room for question. An in vivo study with plaque is necessary to better determine the degree to which these two agents contribute to the remineralization process at the tooth interface.

CLSM has proven to be not only a powerful technique to assess remineralization, but also an efficient one. The samples, considered large by past experimental methods, required no special post-treatment preparation for analysis other than cross-sectioning and 24 h dye soaking. This technique reduces time and cost as well as improved operator safety compared to with TMR. The most valuable asset may be yet to come however. If

autofluorescence of surface lesions could be correlated with TMR to the degree of dye-assisted fluorescence analysis, timed studies could be performed at intervals during the treatment of *same* tooth sections rather than different sections of the same tooth. This provides a more accurate account of the remineralization process because it eliminates the slight variability among different sections of the same tooth.

Cross-sectional CLSM analysis proved to be the optimal approach to surface remineralization study *in vitro*. From cross-sectional imaging it was clear that most remineralization took place somewhat below the surface during exposure to fluoride containing toothpaste. This is consistent with earlier studies [GON99]. Therefore data from the treatment surface alone would be inaccurate, misleading, and incomplete.

Reducing variability is extremely important for remineralization studies because teeth are inherently inhomogeneous, especially surface layers. To obtain meaningful data, *in vitro* analysis requires the investigator to eliminate as much experimental variability as possible. Relative success of this study should be credited to meticulous standardization in each procedural step. Sample preparations, solutions, and processes were all subject to strict uniformity control for all tooth sections. This leaves only inherent specimen variability unaccounted.

It was also confirmed that *in vitro* remineralization/demineralization studies are predominantly controlled by basic chemistry fundamentals such as pH, ion concentrations and (enamel) solubility. This study did not include organic components such as oral bacteria or plaque. Therefore anti-microbial effects were not active. The key factors for remineralization here were ions (Ca^{2+} and PO_4^{3-}) and the pH at the tooth surface. The release of these ions from Novamin® provides a solubility gradient in favor

of mineral deposition. This explains why the samples treated with Novamin® dentifrice experienced greater remineralization, the environment was more conducive to this process. This presents a stronger case for Novamin® dentifrice in Xerostomic conditions. Surface pH appeared to be influential because Novamin® dentifrice solution maintain a significantly higher pH than Colgate® solution. Dentifrice treatment solutions were changed daily and pH differences within that period were recorded for each solution. Average pH for freshly made Novamin® solution was measured at 9.8. After 24 h, the same solutions averaged pH 10.4. Fresh Colgate® solution pH averaged 7.2 while 24 h aged solution averaged 7.3. It is likely the Novamin® dentifrice solution neutralized the preceding acid challenge significantly better than its counterpart. While half of the cross-sectioned sample was sent to IUPUI for CLSM, the remaining half was tested with nanoindentation for mechanical property changes.

Nanoindentation analysis of enamel cross-sections was performed throughout the depth of the lesion and into the sound enamel for two selected teeth. A graphical plot of hardness versus the cross sectional depth from the surface confirmed the demineralized nature of the lesion within the first 100 μm of these teeth. The hardness within the lesion was consistently low for all sections with the exception of those areas previously identified as remineralization bands. These bands, typically half way through the lesion depth, exhibited a noticeable hardness spike for both dentifrice treated sample groups. Figures 6-13 and 6-14 illustrate this point.

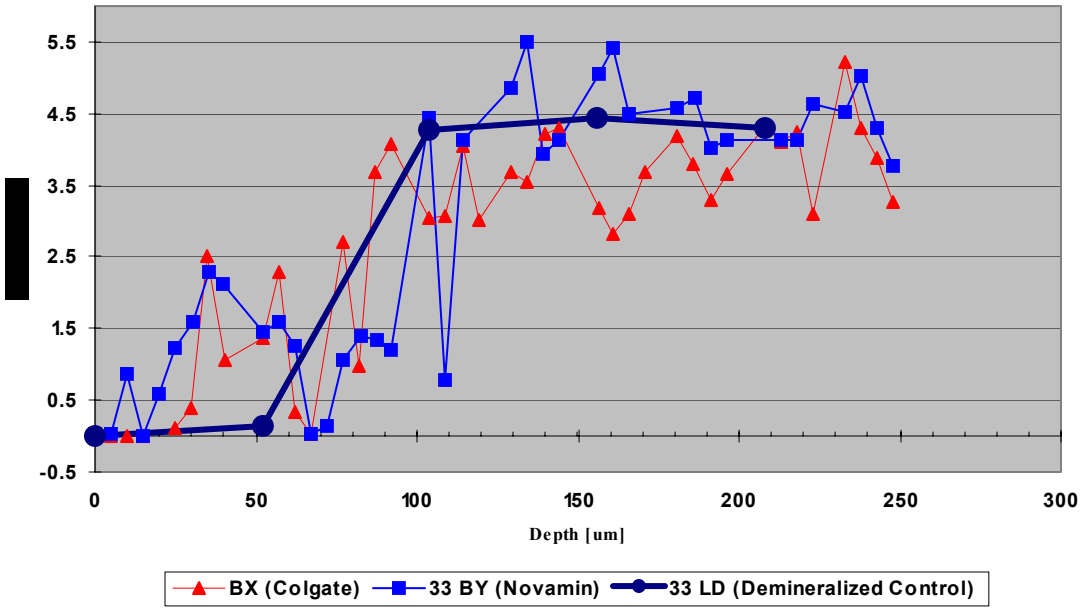

Figure 6-13 Cross sectional nanoindentation of tooth 33.

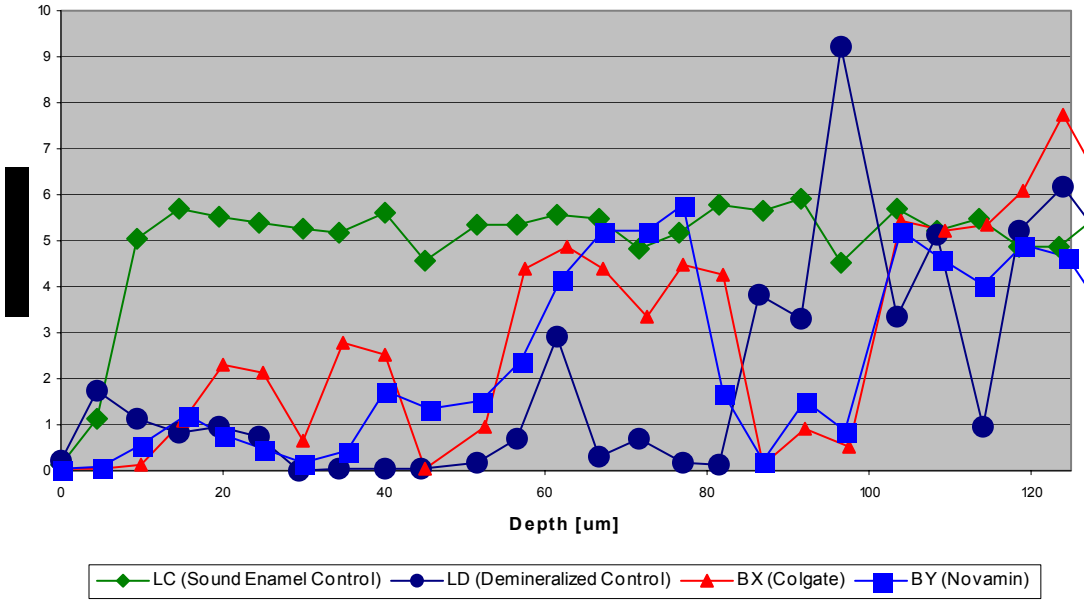

Figure 6-14 Cross sectional nanoindentation of tooth 44.

At greater depths, the hardness increases to the expected range of sound enamel. For tooth 33, the hardness spike appears at 40-50 μm depth while tooth 44 shows a similar spike between 60-80 μm. Both dentifrices (BX and BY) exhibited hardness fluctuations throughout the lesion depth without statistically significant difference ($p >$

0.05). Both groups had markedly larger hardness values than the original lesion section (LD) within the lesion (below 100 μm). However, such variability within sample groups is not advantageous to quantitative analysis. Five more teeth were plotted similarly and showed no significant difference between either test group. In fact, a number of tooth sections exhibited highly irregular hardness behavior. Some displayed greater hardness values for the demineralized section compared to sound and treated counterparts. Multiple tooth sections also exhibited gross hardness fluctuations of 10 GPa or more.

Nanoindentation did not graphically or statistically provide significant evidence for remineralization of lesions for the treatment groups. Select teeth exhibited positive trends but quantitative analysis of enamel remineralization with nanoindentation is not recommended. Cross-sectional indentation does however have some qualitative value although inconsistency is clearly an issue. Any number of factors could influence these results.

Primarily, nanoindentation is heavily dependent upon sample preparation. Variation in polishing or sample tilt could overshadow any differences in mineral content. These samples were particularly re-mounted in epoxy and again fine polished (with 0.05 μm gamma alumina) in order to reduce surface roughness for indentation. Multiple polishing effects or residual epoxy resin deposition could possibly influence mechanical properties of the porous lesion. A degree of operator skill is also relevant. Initial placement of the indents is key to determining the hardness relationship to depth. A number of samples appeared as though initial indents were started in the bordering epoxy region, thus introducing an inherent offset for subsequent indents that are related to the former by position.

Similar to microindentation, soft porous surfaces are extremely difficult to measure for hardness. The lesion zones may have presented pore sizes similar to that of the indenter tip. Features such as inherent crystal growth inhomogeneity and mineral deposition also introduce influential variables on a scale within range of the indenter tip. Crystal diameters within lesions of 0.1 μm [EKS88] could affect hardness measurements for this study considering the indentation depths of 0.5 – 2.0 μm at 5000 μN load. In sound enamel, crystal orientation (growth angles) and enamel mineral anisotropy have proven to affect hardness significantly [HAB01], thus demineralization and subsequent mineral deposition could introduce additional variation. Rather than representative hardness, nanoindentation may be too sensitive a technique to determine mineral changes over large lesion areas. It appears that ultra-fine features tend to dominate hardness data in enamel, preventing broad range determination of remineralization effect within enamel lesions.

Vickers microhardness was performed on the same samples analyzed with nanoindentation (seven total). A 100 g load at 15 s dwell time was used with a standard Vickers diamond tip. It was impossible to visually identify indents in the porous lesion for most samples. Hardness for the few samples in which indents were optically visible was still difficult to quantify. Indent edges were blurred and only marginally in focus, thus measuring diagonals was reduced to a subjective judgment. Generally, microhardness data collection was unsuccessful. If these indents were visible, it is still unlikely that microhardness data could indicate a mineralization difference. The size of the indents nearly matched that of the lesion, thus providing only representative hardness on much a much larger scale than nanoindentation. Microhardness presents the reverse

problem relative to nanoindentation. It appears to be too insensitive a technique to distinguish differences in remineralization of enamel surface lesions.

CHAPTER 7
CONCLUSIONS

A number of conclusions can be made from the results we observed. First, *in vitro* study using confocal laser scanning microscopy (CLSM) has shown that Novamin® dentifrice exhibits a greater degree of remineralization than Colgate® dentifrice of early caries lesions in human enamel. Statistical analysis (T-test) found significance ($p < 0.001$) among treatment groups. For both CLSM parameters, ANOVA ($p < 0.0001$) and Duncan multiple range testing ($\alpha = 0.01$) also yielded significant difference among groups. CLSM has also proven to be an efficient analysis technique for cross-sectional study. Post-treatment tooth preparation was minimal and non-destructive.

It appears that remineralization of surface lesions was successful independent of any anti-microbial effect. There were no organic components, such as plaque or bacteria, involved in this study. Therefore, surface variables including pH and Ca^{2+}/PO_4^{3-} most likely influenced the conditions at which remineralization of the porous lesions took place.

Reducing variability throughout the study notably enabled relevant data collection. Inherent tooth inhomogeneity was beyond experimental control, therefore strict uniformity in laboratory technique enhanced the statistical significance of the two treatment groups. Statistical analysis appears to confirm this.

Hardness testing for *in vitro* remineralization of enamel surface lesions was statistically insignificant and provided minimal quantitative value. Nanoindentation hardness testing was dependant on too many variables. Sample preparation and porosity

(relative to the tip size) makes it apparent that nanoindentation could be too sensitive a technique to determine remineralization effect of dentifrice on demineralized enamel. Conversely, Vickers microhardness is too insensitive a technique to distinguish differences in the remineralization effect on the desired scale. Thus, neither microhardness nor nanonindentation techniques are recommended for remineralization studies. However, confocal laser scanning microscopy is potentially a very good technique for studying remineralization.

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BIOGRAPHICAL SKETCH

Sammel Alauddin was born in Dhaka, Bangladesh, and immigrated to the United States at the age of two. Raised in Frankfort, Kentucky, he graduated high school at the age of 16 to pursue higher study at the University of Kentucky in Lexington. In 2002, he completed both a B.A. in Organic Chemistry and a B.S. in Materials Science Engineering. A chance meeting with a former professor at a conference led to her suggestion that he pursue graduate studies and consider her new institution, the University of Florida. At UF, he expanded his interest in biomaterials research and improving the health of future generations. With these goals in mind, he strives to make his contribution to science.