2008

Permeability Alterations of Bio-Silicified Treated Human Dentin: an In Vitro Study

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Permeability Alterations of Bio-silicified Treated Human Dentin

-An In Vitro Study-

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University of Connecticut,

School of Dental Medicine

A Thesis

Submitted in Partial Fulfillment of the Requirements

For the Degree of Master of Dental Science

At the University of Connecticut

2008

Approval Page

Master of Dental Science Thesis

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-An In Vitro Study-

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2008

Acknowledgements

I am grateful to Almighty God for giving me strength to complete this project and for giving me the opportunity to work with such tremendous people who supported me all the way.

To Doctor A. Jon Goldberg, I thank you for inspiring and challenging me to accomplish this task. Your insight and encouragement throughout the entire research process provided me with a memorable experience that I'll carry with me throughout life. I am deeply indebted to you for your guidance, statistical expertise and countless hours of editing. Thank you.

To Doctor Reza B. Kazemi, thank you for teaching me the understanding principles of dentin permeability. I sincerely appreciate your help with technical skills in dentin disc preparation and hydraulic conductance apparatus. Further, I would like to thank to the unhindered use of your laboratory as well as your help in acquiring materials. Without these, my project would not have been possible.

To Doctor Maria Athena C. Advincula, thank you very much for your enthusiastic support for my project. You showed me the procedure for conducting the bio-silicification reaction. I deeply appreciate your guidance, support and wisdom throughout the entire research process.

To my program director, Doctor Kamram Safavi, thank you for believing in me and giving me the time and support I needed to make this project a reality.

I am grateful to Doctor Larz Spangberg for the tremendous support. Especially your advice on particle size and shape of Mineral trioxide aggregate (MTA) and Portland

cement projects were unforgettable. It was highly appreciated that you introduced me many research opportunities.

To Ms Sandra Pelletier and Ms. Deborah J. Osborne, thank you very much for your administrative help for three years.

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1.0 Introduction

1.1 Background

The Clinical Problem in Restorative Dentistry and Endodontic (Dentin-material interface)

Dentin is a structurally anisotropic biological composite, and exhibits regional differences in mineral concentration, tubule density and diameter, and collagen orientation [1, 2]. Restorative dentistry procedures create an interface, usually with dentin, that can become susceptible to the common clinical problems of microleakage, sensitivity and secondary caries. Endodontic procedures create an interface, always with dentin, that also can become susceptible to microleakage. Many current clinical problems are associated with the interface between the restorative/endodontic material and tooth structure. Accordingly, many dental materials and technologies have been developed to stabilize the interface and particularly seal dentin tubules. This includes dentin bonding agents and approaches that could also occlude exposed dentin.

The question of how to seal a dentin-material interface involves questions of how to prevent leakage from the oral environment fluids at different hierarchical levels ranging in scale from the nanometer to the micrometer. The breakdown of marginal seal results in dentin sensitivity, marginal staining, and hydrolytic attack of the adhesive resins at the interface that eventually cause recurrent caries. Historically in the field of dentin bonding agents, for example, efforts were made to reduce polymerization shrinkage [3, 4] and achieve a closer match of modulus and coefficient of thermal expansion to dentin [5]. As a result of property improvement, dentin bonding agents are widely used in clinical setting.

However, dentin bonding agents still have unsolved clinical question of long term success. Potential causes of deterioration of the dentin/adhesive interface in regards to longevity of a stable seal are focused on long term hydrolytic attack to both material and dentin [6-11]. Separation of phases at or near dentin/adhesive interface leads the leakage of micro or nanometer level when using dentin bonding agent for many years. The mechanism of separation is widely examined by various scientists but final conclusion has not yet obtained. The possible causes of dentin aspects are, for example, dentin permeability in relation to dentin structure, dentin tubule orientation/ size/ density, dentin related enzymes released by bacteria, and pulpal pressure. The possible causes of material aspects are, for instance, hydrolysis of material, and stress caused to dentin due to polymerization shrinkage, and inconsistent inter/ intra operator technical errors. ,

Epidemiology and Mechanism of Dentin Hypersensitivity

Dentin hypersensitivity (DH) is defined as transient pain arising from exposed dentin, typically in response to chemical, thermal, tactile, or osmotic stimuli, which cannot be explained by any other dental defect or pathology [12]. The reported prevalence of DH is 8-35%, depending on the population studied [13, 14], and peak prevalence of DH patients is in the third and fourth decades [15-19]. The percentage of elderly people in the United States and many industrialized countries is increasing. The number of teeth retained will subsequently rise. Further, gingival recessions among elderly people are frequently observed in the clinical setting. Therefore, the treatment needs for dentin hypersensitivity and primary and secondary carious lesions are increasing. Epidemiological surveys indicate that the majority of sensitive surfaces are at

the buccal-cervical margins [15, 16, 19], although other surfaces may also be affected [20, 21]. Any site of exposed dentin may exhibit sensitivity, but not all exposed dentin is sensitive. The condition may affect any tooth [15, 16, 19, 21].

Although DH is common, its etiology is poorly understood [22], though the exposure of dentin due to gingival recession is of primary importance. Current evidence [23-25] favors the “Hydrodynamic Theory”, originally postulated in the twentieth century by Gysi and later developed by Brannstrom [26]. Stimulus induces a pressure change across the dentin layer, and associated fluid changes within the tubule cause excitation of the sensory nerves. This theory suggests that dentin tubules act as capillary tubes and that the fluid within them obeys the laws of fluid movement. The rapid movement of fluid in dentin tubules, in response to certain stimuli, may cause distortion of intradental nerves and generate a pain response. Scanning Electron Microscopy (SEM) studies have demonstrated patency of the tubules on the outer dentin surface in sensitive teeth [27-31]. Current treatments tend to concentrate on two approaches, to occlude the tubules and to block neural transmission. Although the mechanisms of pain transmission across dentin are not fully understood, DH is reduced when the dentin tubules are occluded [32, 33]. Most treatments, which range from toothpastes (home-use) to dentin bonding agents (in-office), are therefore based on the hydrodynamic mechanism of stimulus transmission and attempt to inhibit sensitivity either by sealing the tubules, by altering their contents, or by creating insoluble calcium complexes, thus forming mechanical or chemical plugs.

The mechanism of toothpaste desensitizing action is to occlude tubules by agents such as calcium carbonate, alumina, dicalcium phosphate, silica or other mineral particles [34, 35]. However, a toothbrush mechanically removes occluded plugs of protection at

the cervical portion of teeth surfaces when brushing. The occlusion by toothpaste and the mechanical removal by toothbushing is a confounding action each other. Accordingly, although toothpastes work as a short-lived treatment of DH, in-office treatment is necessary for a long-lived treatment of DH.

Various agents are used as in-office treatments of DH. Five percent neutral sodium fluoride varnish, for example, “Cavity Shield” (Omnii oral pharmaceuticals, West Palm Beach, FL) and “Duraflor” (Pharmascience, Inc. Montreal, Quebec, Canada), is used in clinics at the University of Connecticut School of Dental Medicine. Other approaches for DH treatment include light-cured bonding agents or resin composite restorations.

Cervical area of the teeth frequently lacks enamel, particularly in DH-affected areas of teeth. Gingival recession and decreased saliva flow are common among elderly people [36]. Since dentin is more easily dissolved by acids in the mouth than enamel, the risk of developing cervical and root surface caries should be considered. If the dentin is infected by root caries, caries removal will result in the decreased distance between sound dentin and pulp. This leads the increased density and diameter of tubules, making dentin tubule occlusion more challenging in relation to DH treatment.

Open dentin tubules in teeth with vital pulp are known to cause DH [26]. Endodontic treatment is needed for persisting DH with significant symptoms and for irreversible pulpitis or a necrotic pulp due to caries. This is because bacteria intra- and extra-cellular components and certain chemical substances in contact with the dentin surface can act through the dentin tubules and generate pulp inflammation reactions [37, 38]. Treatment modality for persisting DH and irreversible pulpitis is pulpectomy,

whereas for necrotic pulp is root canal treatment. Both treatments require access through the pulp chamber and complete removal of pulp tissue by mechanical and chemical methods. Most DH and root caries treatments are focused on dentin tubule occlusion from the outer surface of dentin. In contrast, endodontic treatment is focused on dentin tubule occlusion from the inner surface of dentin, which is located adjacent to the pulp where the density and diameter of dentin tubules shows maximum number and size.

The Effect of Microleakage and Clinical Outcome

As mentioned at the beginning of this thesis, restorative and endodontic procedures create an interface, usually with dentin, that can become susceptible to the common clinical problems of microleakage. Many current clinical problems are associated with the interface between the restorative/endodontic material and tooth structure. The effect of microleakage on restorative and endodontic treatment outcomes has been studied for decades. In 1956, Strindberg considered that a major cause of endodontic failure was the apical tissue fluid leakage due to an inadequate root filling [39]. In 1961, Marshall and Massler utilized a radioactive tracer and showed the apical microleakage of the teeth with coronal restoration [40]. Madison et al. in 1988 reported that there was no statistically significant difference between experimental and control groups utilized in the monkey study and that controlling microleakage was a major concern in endodontic treatment and outcome [41]. Ray and Trope indicated in a cross-sectional study in 1995 that the technical quality of coronal restorations was significantly more important than the technical quality of root fillings [42]. However, the limitations of this study include multiple providers, unknown restorative materials used, and evaluation

of overhanging/inadequate/defected restorations. Riccuci et al. reported in 2000 in his cohort study, which was evaluated by one provider, that coronal leakage may not be of such a great clinical importance as implicated by numerous studies *in vitro* [43].

Despite the large number of publications on microleakage, it is hard to draw firm conclusions from them because of the disparity that exists in the findings of most studies.

The Principle of Sealing Tubules with Minerals

Many researchers are working for sealing dentin tubules with resin. However, there are many reports that resins deteriorate with time [6-11]. As a result of deterioration, common clinical problems occurs. Accordingly, it will be favorable to look at minerals instead of resins.

The principle of sealing tubules with mineral is not a new concept. The mechanisms could be classified into the following three general categories: (1) use of existing particles, (2) precipitation of particles from saturated or non-saturated solutions, (3) particles catalyzed from saturated or non-saturated solutions.

The use of existing particles is the oldest method. A typical example is use of inorganic restorative cements such as silicate cement or glass ionomer cement. Existing particles are expected to block the entry of dentin tubules mechanically. If the size of particles is smaller, it is possible that particles will flow into tubules. The disadvantages of this method include lack of interfacial bonding/adhesion and dislodgement/separation of particles, leaving dentin tubules open. The transport of intact particles such as oxalates is reported by Pashley and Galloway in 1985 and Yiu et al. in 2005 [44, 45].

The use of particles precipitated from saturated or non-saturated solutions was introduced by Imai and Akimoto in 1990 as a new method of treatment for dentin hypersensitivity by precipitation of insoluble calcium phosphate *in situ* [46]. In this method, serial application of 5% disodium phosphate solution and 10% calcium chloride solutions using cotton pellets immediately occluded dentin tubules with calcium phosphate crystals precipitates as a result of an ionic reaction. Later in 1993, Imai and Ikemura reported that the phosphate solution was modified by addition of fluoride ion to improve the acid-resistance of the precipitate [47, 48].

In 1995, Suge et al. studied the calcium phosphate precipitation method using somewhat clinically dangerous acidic and basic solutions for patient safety. In this method, an application of an acidic solution (1 mol/L $CaHPO_4.2H_2O$ dissolved in 2 mol/L H_3PO_4) and basic post-treatment solution (1 mol/L NaOH, from 0 to 0.1 mol/L NaF) were studied [49]. Later in 2008, Suge et al. was modified for the safety by changing to the topical application of ammonium hexafluorosilicate solution on dentin [50].

The use of particles precipitated from super- or mildly- saturated solutions was studied by Cherng et al. In 2004, he reported a reduction in dentin permeability by using a slurry containing a supersaturated calcium phosphate solution in 2004 [34] by using a mildly saturated calcium phosphate solutions in 2006 [51]. In 2007, Tay and Pashley reported occlusion of tubules by dicalcium phosphate using mineral trioxide aggregate (MTA) [52].

The method of sealing tubules with mineral particles catalyzed from saturated or non-saturated solutions is a new approach that is introduced in this study. The conceptual

advantage of using “synthetic biocatalyzed mineralization” is the potential to position the catalyst at locations not accessible to particles with subsequent application of a liquid precursor to form mineral *in situ,* obviating the need to transport particles to desirable locations such as within the tubules or at an interface to seal, reinforce, or otherwise stabilize these regions [53, 54].

Synthetic Catalyzed Biomineralization

Biomineralization means the process by which living organisms produce minerals. Bone morphogenic protein, dentin sialoprotein, amelogenin, and bone sialoprotein mediate the mineralization of skeletal bone, dentin, enamel, and cartilage, respectively [55-57].

Recent discoveries about the biomineralization process in primitive marine species may hold promise for developing an analogous synthetic technology appropriate for dentistry [53, 54]. In particular, it is an important discovery that silica-forming proteins such as silaffins or silicateins play a catalytic role in protective silica formation in primitive species such as the diatom *Cylindrotheca fusiformis* [58] or the sponge *Tethya aurantia* [58, 59]. These proteins remain active in vitro and within a polymer host [60].

In 1999, Cha and his colleagues isolated proteins which allow silica formation. In short, biosilicification means the formation of silica by protein catalysis together with precursor solution [61]. The advantage of this discovery is a silica formation at physiologic conditions, such as temperature and pH in the mouth, whereas conventional silica formation requires high temperature and pressure.

Simplified cationic macromolecules can be synthesized to be are structurally similar to the functional regions of the natural proteins. A separation process from natural proteins is not required for simplified synthetic cationic macromolecules, which are usually commercially available in the form of chemical agents. Simplified synthetic cationic macromolecules have been shown to promote silicification [62-64]. The mechanisms and reaction parameters that influence biomimetic mineralization have been reviewed [65, 66].

Mechanisms behind Bio-silicification (sol-gel reaction)

Biosilicification is a two-step reaction. The first is hydrolysis, and the second is condensation. Hydrolysis involves hydrolysis of a silicon alkoxide precursor (Si-OR such as a part of TMOS) to produce silanol (Si-OH) and liberate alcohol molecules. Silanol (Si-OH) is not stable. Thus, the condensation reaction involves the condensation of two silanol substrates to form siloxane bonds (Si-O-Si) which will eventually form the silica network ($Si(OH)_4$, silicic acid) and the release of water molecules. These water molecules can then hydrolyze another alkoxide precursor (Si-OR). This is a solution-gelation reaction [67, 68] (Figure 1).

Conventional silica formation requires high temperature and pressure. However, in biosilicification, proteins act as catalysts for solution-gelation reactions. Therefore, silica formation occurs at low temperature and pressure and neutral pH, under normal physiologic conditions [61, 62, 69]. Several scientists reported that the catalyst in a form of protein promotes the hydrolysis and condensation reaction of silica precursors to form

silica in vitro such as wafers, but not on human dentin surface of extracted teeth or dentin [64, 70, 71].

Before trying biosilicification on human dentin disc, the research group at the University of Connecticut Health Center studied biosilicified coating on inorganic wafers. [72, 73]. In this preliminary study, angular silica particles were observed as dendritic structures on wafers and demonstrated the process parameters which can control the morphology of film coating. The results of these studies were used as useful preliminary data when determining the Materials and Methods in this study on human dentin in vitro.

Reasons for Considering Silica Mineralization

As mentioned earlier, there are various mineral components available to seal dentin tubules with. Since the major components of human dentin are calcium and phosphate, these have been studied in the form of precipitate. Likewise, it is logical to use hydroxyapatite (HA) for mineralization. However, there are several reasons for considering silica mineralization.

Chemistry of biosilicification is sol-gel reaction, whereas HA mineralization is a traditional precipitation from super-saturated solutions such as dissolution reactions of carbonate or phosphate phases. Although HA could be driven by a catalyst as all mammals form bone and teeth, most biocatalyzed mineralization has been conducted on the silica system because of aforementioned discovery of the silicatein and sillafin proteins. The silica system has been studied in solutions and on surfaces [64, 71, 72, 74]. Both the silica and HA biomineralized systems bear strikingly similar nano-particulate morphologies [58]. So far, the rate of silicification and the size and morphology of the

formed silica are better understood than HA mineralization. Further, it is reported that biocatalyzed silica is capable of entrapping enzymes and their inhibitors, such as, for example, chlorhexidine [75, 76]. Accordingly, it is speculated that simultaneous delivery of therapeutic agents may be achieved using a silica system.

Comparison of Leakage Study Techniques

It is important to study the effect of a silicified layer on human dentin surfaces and degree of dental tubules obturation on dentin permeability *in vitro*. There are various techniques to evaluate dentin-material interface leakage that we considered for use in this study including dye penetration, radioactive isotopes penetration, microorganism penetration, and fluid filtration systems. Traditionally, the most popular technique in restorative and endodontic research has been the linear dye penetration technique. The theory of liner penetration technique expects that the nano- or micro-scale tracer would indicate the gap between the restorative/root filling/sealer and dentin surface. The dye penetration is measured by sectioning the root perpendicular to the long axis of the crown/root or decalcifying the entire sample [77]. However, considerable variation in results has been reported within the same authors [78, 79]. Among different authors using a similar experimental protocol, for instance, dye penetration distances were minimum of 0.12 mm and maximum of 9.25 mm have been reported [78, 80]. These variations are partially explained by, for example, entrapped air has been shown to affect the capillary action of the dye and prevents complete dye penetration [81, 82]. Factors such as ionic charge /size /pH of dye and might be important in penetration technique and a cause of considerable variation [77]. As problems increase with dye penetration techniques, the

use of fluid filtration systems with positive pressure has become a current standard of dentin-material interface leakage evaluation techniques.

Dentin Permeability Test System (Hydraulic Conductance System)

In 1974, Pashley and co-investigators published the first experimental work utilizing a laboratory method to measure dentin permeability, which can be determined by a number of variables, including the pressure of moving fluid across the dentin, the length of the dentinal tubules, the viscosity of the fluid, and the radius of the tubule [23, 24]. In this work, a split-chamber device was described wherein thin slices (approximately 1 mm) of coronal dentin from extracted human molars were placed between two O-rings and tightened within the plexiglass reservoirs. One end of the chamber would be connected to a source of hydrostatic pressure or treatment solution and the other end to a means of measuring flow rate or collecting diffusal fluid. Movement of the injected air-bubble in the system controlled and measured by a micropipette was found to be an accurate flow meter [83, 84]. By using this device, investigators determined the fundamental properties of dentin permeability [32, 85].

The fluid filtration system was developed by Derkson and Pashley in 1986 to quantify microleakage around coronal restorations [86]. This system has been modified and applied to many other experimental situations and has been found to be reliable and reproducible [87-90].

Standardizing variables such as dentin thickness, surface area, and applied pressure in this technique significantly advanced the understanding of dentin permeability [84, 91]. The fluid filtration system is considered to be a non-destructive reproducible

system, allowing the same dentin discs to be evaluated after multi-treatment episodes [92]. The fluid filtration system is simple but contains much uniqueness and thus the system is well received by researchers worldwide.

Advancements of understanding of dentin permeability are reported by various researchers. Sena suggested that alteration of permeability by therapeutic agents could be a useful treatment for DH patients [84]. Ciucchi et al. reported that it was possible to measure dentin fluid flow and estimate tissue pressure *in vivo* [93]. Levinkind et al. suggested that it is possible to measure permeability from the ion movement within an electrolyte conductance system using impedance [94]. Other investigators have also attempted to measure fluid flow by confocal optical microscopy [95] and scanning electro-chemical microscopy (SECM) [96, 97].

The clinical question of outcome success in relation to fluid tight seal by using fluid filtration system was widely studied by many researchers including Endodontics. However, Wu et al. noted that in 1990, one of every 4.3 articles published in the *Journal of Endodontics* and the *International Endodontic Journal* dealt with microleakage. In particular, the results are often non-reproducible, and standard deviations are relatively large [77]. The assessment of sealability in endodontics has a long and controversial history with unclear clinical implications [98, 99].

In December 2007, the editorial board of the *Journal of Endodontics* made a decision that it would not publish permeability papers effective on July 1, 2008 because of question of reliability. The editorial board encourages investigators to study the validity of the assessment of sealability methods themselves in endodontics [100]. The editorial board of the *Journal of Endodontics* used reasons for questioning the reliability

based on the report of Wu and Wesselink in 1993 [77]. However, as already mentioned, the fluid filtration system has the advantage of non-destructive assessment, allowing use of multi-treatment scenarios on the same specimen as well as in combination with other evaluation protocols such as, for example, SEM analysis [86].

Scanning Electron Microscopy and Image Analysis

Most attention to the treatment of DH has been directed at blocking the hydrodynamic mechanism of dental tubules [12, 101, 102]. However, how the numerous compounds actually achieve their effects has received limited attention [103, 104]. Scanning election microscopy (SEM) can be used to measure uptake onto the dentin surface [105-112] or assess the characteristics of the smear layer [113-115] and hybrid layers following etching the dentin and application of resin bonding [116, 117]. The X-ray microanalysis can also be used to characterize the nature of the deposits on the surface [118]. Other investigators have used SEM and/or transmission electron microscopy (TEM) to evaluate both dentin morphology in sensitive and nonsensitive dentin and/or the surface characteristics following application of desensitizing agents [30, 31, 119-122], as well as the ultrastructure of the resin-dentin interdiffusion zone [123], or x-ray tomographic microscopy and atomic force microscopy to evaluate mineral distribution and dimensional changes in human dentin during mineralization [124]. Ide et al. used confocal optical microscopy to determine the surface characteristics induced by selected desensitizing toothpastes [125]. Confocal optical microscopy has also been used to assess resin composite systems [126], the influence of handling techniques on the hybrid layer with bonding agents [127], and the sealing ability of selected dentin bonding

agents [128]. Fourier transform infrared photoacoustic spectroscopy has been used to evaluate dentin morphology in abrasion/erosion cavities [129]. SEM and image analysis have been utilized together to quantify dentin tubule morphology and/or following application of desensitizing agents and restorative materials, or the effect of smear layers on dentin permeability [91, 94, 130-133].

1.2 Rationale/Purpose of Present Study

Restorative and endodontic procedures create an interface with dentin that can become susceptible to the common clinical problems of microleakage, sensitivity, and secondary caries. Many current clinical problems are associated with the surface of an exposed dentin and interface between the restorative/endodontic material and dentin structure.

The question of how to protect the exposed dentin surface and permanently seal a dentin-material interface involves different strategies to prevent leakage from the oral environment fluids at different hierarchical levels ranging in scale from the nanometer to the micrometer into the dentin tubules, causing dentin hypersensitivity, dentin-material interface/marginal deterioration, and eventually, primary or recurrent/secondary caries and pulpal irritation.

A new strategy may be available based on the isolation and identification of the proteins responsible for biosilicification in primitive marine species [58-61]. The effects of the various process parameters such as catalyst, precursor, reaction rate, particle size/morphology, and environment have been studied [62-64].

Biosilicification could lead to a solution to the dental clinical problems if studies demonstrate that PLL can catalyze silica particles onto a dentin surface from TMOS precursor. Biosilicification could potentially be used to occlude dentin tubules or otherwise seal dentin-restorative interfaces [134].

The purposes of this study were, therefore, to (a) determine if the biomineralization reaction consistently forms silica particles *in vitro* onto human dentin discs surfaces and within the dental tubules, (b) determine if hydraulic conductance (permeability) is reduced; and (c) identify clinical factors that affect these outcomes.

2.0 Materials and Methods

Selection of Extracted Human Molar Teeth and Dentin Disc Preparation

Fully erupted and non-carious permanent molar teeth extracted due to periodontal disease were used in this study. These human teeth were donated from the University of Connecticut School of Dental Medicine clinics and private practices to our research team. The research team would like to express sincere appreciation to the Oral and Maxillofacial Surgery Department and Dr. Joel Ziegler regarding teeth from school and Dr. Kazemi regarding teeth donation from local private practices. Immediately after donation, the teeth were cleaned and stored in 0.3 % sodium azide solution in polyethylene screw-cap jars for purpose of infection control until used. Teeth donated from the school clinic were usually processed within 24 hours, whereas teeth donated from local private practice were processed case-by-case but in a timely manner. This entire procedure was approved by the University of Connecticut Health Center Internal Review Board.

Teeth were examined by using a digital radiograph system (Schick CDR, Schick Technologies, Inc., Long Island City, NY), at the University of Connecticut Health Center Endodontic Clinic. Digital radiographs of extracted teeth were taken, and image analysis was conducted. The following methods were used to ascertain and eliminate teeth inappropriate for this study. First, teeth with any restoration were excluded (Figure 2A). Second, teeth with caries or defects were excluded (Figure 2B). Third, teeth with open apex were also excluded (Figure 2C). Finally, measurements were made to verify that thickness of occlusal dentin was adequate. The adequate dentin thickness was defined as more than 1.1 mm distance between the dentin-enamel boarder/dentino-

enamel junction (X) and the pulp-dentin boarder/pulp horn (Y) and was confirmed by using the measurement feature of the digital radiograph system (Figure 2D). The rationale of the 1.1-mm-thickness requirement is due to the consideration of the thickness of the diamond blade used and followed the polishing procedure of the sectioned/prepared dentin discs. After washing under running tap water for 30 minutes, the teeth were mounted in a cutting machine (Isomet 1000, Buehler, Ltd. Lake Bluff, IL) and sectioned perpendicular to the long axis of the tooth just inside the DEJ with a slow-speed running diamond saw. To prepare the diamond-saw cutting machine, the bath of cutting machine was filled with distilled water to cover ¼ of the diameter of the diamond blade disc. The blade was initiated at 100 rpm to create an indent to the dentin, and the speed was gradually increased to 300 rpm. At the final stages of cutting, the weight load was adjusted by hand to minimize any unnecessary enamel lips, which makes the polishing procedure difficult due to the uneven surface of the tooth (Figure 3). The disc was polished at 240-grit through 320-grit metallurgical paper to obtain discs nominally 0.5 ± 0.05 mm thick. The discs were examined with a stereomicroscope at 10X magnification (Zeiss, Germany), and any dentin discs with remaining enamel island or pulp horn exposure were discarded. Digital calipers (Mitutoyo, Tokyo, Japan) were used to measure the thickness of the dentin discs. Although individual sample thickness was not recorded, thickness within the range of 0.45 to 0.55 mm was confirmed using the digital caliper for all dentin discs. After polishing, the dentin disc was placed in 30 ml of distilled water, and an ultrasonic machine (B300 tabletop ultrasonic cleaner, Branson Ultrasonics Corporation, Danbury, CT) was used at 40 kHz for 5 seconds to remove debris.

To remove the smear layer, the discs were immersed in 17% EDTA for 5 minutes, rinsed in water for 30 minutes, immersed in 5.25% NaOCl for 5 minutes, and rinsed again for 30 minutes. The EDTA pH was unknown for samples 1-11, pH 12.5 for samples 12-14, pH 8.7 for samples 15-17 (Accumet AB15E University pH/mV/T Meter, Fisher scientific, Pittsburgh, PA).

Dentin Permeability Measurement before Biosilicification

The dentin permeability of smear layer free condition of dentin disc was quantitatively measured prior to biosilicification. A dentin disc was attached to the modified fluid filtration apparatus, shown in Figure 4A, as originally described by Derksen et al. in 1986 [86]. External or applied hydrostatic pressure was provided by a 37.5-cm column of 0.02 % sodium azide solution at room temperature. This apparatus is connected by clear-color tubes and it is filled with 0.02 % sodium azide solution. A three-way faucet controls direction of the solution. The apparatus was flushed to ensure that there were no air bubbles trapped in the tubing.

Determining the occlusal side of the dentin disc is critical. A letter on the plastic ruler was magnified on the occlusal side, whereas a letter on the plastic ruler appeared smaller on the pulp side in wet condition of the disc and ruler (Figure 5). The occlusal side of the dentin disc was placed upward, with the pulp side of the dentin disc placed downward on the high pressure side (Figure 4B). Using two inert rubber O-rings whose internal diameter was 6 mm, dentin disc was securely tightened within the screw chamber by fingers to obtain a fluid-tight seal as described in Figures 4B and 4C. Careful attention was paid so as not to apply excessive tightening force of the chamber to prevent cracks

on dentin discs. The area inside the O-ring was equivalent to the area of dentin exposed to the fluid.

The assembled chamber together with a dentin disc was connected to the tubing of the fluid-filtration apparatus. An air bubble approximately 3 mm in length was introduced into the system for the purpose of visible measurement marker, and its movement was controlled with a microsyringe (Figure 6).

The surface of dentin discs usually appears dry immediately after being placed in the chamber. As time progresses, the dentin disc became wet. Comparisons between a smeared dentin disc (i.e. no EDTA/NaOCl treatment disc as a control) and a smear-layer-free disc were made. Smeared dentin discs do not show fluid flow, where as smear-layer-free discs shows fluid flow, whose phenomena looks similar to “sweating.” Therefore, the first 5-minute elapsed time was considered as the fluid system equilibration period, and no actual measurement was conducted. After the equilibration period by confirming the “sweating” of the disc, permeability of the disc was measured three times, and microsyringe readings were recorded in millimeters. The relationship between the air bubble movement on the ruler and microsyringe readings is as follows. At the time of start opening the three-way faucet, the air bubble should be located at the position zero on the ruler. Concurrently, it is necessary to record the reading of the microsyringe. After five minutes, the location of the air bubble has moved. By using the microsyringe, the air bubble should move back to the positing when the measurement started. At the same time, it is necessary to record the reading of the microsyringe. Microsyringe reading informs the operator the fluid flow in 5 minutes.

Between measurements, a 5-minute break was taken for a system equilibration and the bubble was backed to the original baseline position using microsyringe. The disc was not moved until all three sets of measurements were completed. This ensured that the exposed dentin area in a disc was in the same location and dentin tubule configuration, size and density for all three measurements. Medium-sized endodontic absorbent paper points (Mynol, Ada products, Milwaukee, WI) were used to remove fluid that had penetrated on the dentin disc surface after each measurement. The methods for this fluid absorbency process are summarized in Figure 7.

The movement of the air bubble per unit time was measured and converted to microliters per five minutes using the known inner diameter of the microtubing (Figure 6).

Hydraulic conductance (permeability) was calculated from the volume of water that was passed through the disc, adjusting for the pressure, length of time, and cross-sectional area of the disc, as summarized below.

Equation; $Lp=Jv/ ((Po-Pi) X A X t)$

		Values	Unit
Jv	fluid flow	Microsyringe	μL
Po	external or applied hydrostatic pressure	37.5	cmH_2O
Pi	internal or pulpal tissue pressure	0	cmH_2O
A	the area of dentin	0.2826	cm^2
T	time	5	minute

Silicification and Analysis of Dentin Discs; Material used for Biosilicification

The precursor solution was 100mM tetramethyl orthosilicate (TMOS) in anhydrous ethanol prepared from 1M TMOS (Sigma-Aldrich, St. Louis, MO) pre-

hydrolyzed with 0.001 M HCl. The polypeptide catalyst solution was 0.001M 30-70 kDa polylysine-HBr (PLL, 085K5100, Sigma-Aldrich, St. Louis, MO) in water. For TMOS preparation, 0.152 gm of TMOS was measured inside the safety hood. A disposable glass pipette was used for this procedure. One mL of 0.001M HCl was added to 0.152 gm of TMOS using a micro pipette (P1000) together with disposable plastic chip. For the purpose of pre-hydrolyzation, a mixture of TMOS and HCl was stored in a small container inside the safety hood for ten minutes. The pH of the mixture was not checked for samples 1-11, whereas it was confirmed for samples 12-17.

PLL was prepared as following procedures. PLL was originally stored in a freezer. After defrosting in the refrigerator, 0.0025 g of PLL was measured. Ten ml of filtered distilled water was added to PLL. After mixing, the mixture was stored in the refrigerator until use. Expected shelf life was up to three months. The PLL pH was not checked for samples 1-11, whereas it was confirmed for samples 12-17.

TMOS/PLL/TMOS Application Methods used for Samples 1-11

TMOS/ PLL/TMOS application methods for Samples 1-11 are summarized below.

Sample	Application	Technique	Storage*
1-11	TMOS/PLL/TMOS	Immersion	0 day

*; Dentin permeability was measured between 10 and 30 minutes after completed application of the TMOS and PLL.

The biosilicification reaction was conducted on each dentin disc by sequential immersions in (1) TMOS for 5 minutes, (2) PLL for 30 minutes followed by a water (pH 11.0) rinse, and (3) TMOS for 5 minutes for a second time. After each TMOS treatment

in (1) and (3) for 5 minutes, the discs were rinsed in ethanol and then water (pH 7.0) for 1 minute to hydrolyze the alkaloid. Dentin discs were gently dried with an office-use portable dust cleaner and then stored in a clean container. Dentin permeability was measured between 10 and 30 minutes after completed application of the TMOS and PLL.

Reasons Why We Decided to Study Samples 12 through 17

The result of samples 1-11, which will be described in a later section of this thesis, indicated inconsistent permeability. Therefore, we decided to review the experiential protocol to eliminate possible process parameters contributing to the variability. As a result, (1) dentin drying method, (2) discontinuation of dip method of TMOS/PLL application, and (3) use of premixed TMOS/PLL were adapted for following experiments for samples 12 -17.

TMOS/PLL Application Methods used for Samples 12-14

TMOS/ PLL application methods for samples 12-14 are summarized below.

Sample	Application	Technique	Storage
12, 13	TMOS/PLL/TMOS	Cotton pellet coating	7 days
14	Pre-Mixed TMOS/PLL	Immerse 24 hours	6 days

For the procedure for samples 12 and 13, 100 mM TMOS pre-hydrolyzed with 0.001M HCl, PLL, and H_2O (pH 7.0), H_2O (NaOH) (pH 11.0) and ethanol (200 proof) were prepared in advance. Also, size #2 cotton pellets (5.5 mm in diameter) were prepared. (Richmond Dental, Charlotte, NC) First, 3 ml of 100 mM TMOS pre-hydrolyzed with 0.001M HCl was poured into a disposable plastic container. Second, the occlusal side of the dentin disc was placed upward on a clean glass plate, and the excess

water was removed. A cotton pellet dipped in fresh TMOS gently scrubbed the occlusal side of dentin disc for 30 seconds. This 30-second application was defined as one set. Ten sets of applications were continuously repeated using fresh TMOS on the same cotton pellet. Thus, TMOS was on the disc for a total of 5 minutes.

The dentin disc was dipped into 30 ml of ethanol for a few seconds, and the excess ethanol was gently removed by using 2 X 2-inch gauze without touching the occlusal side of the disc.

The dentin disc was dipped into 30 ml of H_2O (pH 7.0) for 1 minute. Metal cotton pliers were used to keep the disc standing inside the container so that it did not touch the container walls. After removing the disc, excess water was removed by using 2 X 2-inch gauze.

The dentin disc was immersed into 10 ml of 0.001M PLL for 30 minutes and then placed on the bottom of the container, occlusal side up, allowing complete coverage of the disc, as the bottom of the container was not flat. After 30 minutes, the disc was removed from the container, and excess PLL was removed by using 2 X 2-inch gauze.

The dentin disc was then placed into 30 ml of H_2O (NaOH) (pH 11.0) for 1 minute, using the procedure described above. The disc was then placed into 30 ml of H_2O (pH 7.0) for 1 minute, following the same process.

Fresh TMOS was then applied using a cotton pellet, gently scrubbing the occlusal side of the dentin disc for 30 seconds using metal cotton pliers (one set). Ten sets of applications were continuously repeated using fresh TMOS on the same cotton pellet. Thus, TMOS was on the disc for a total of 5 minutes.

The dentin disc was then dipped into 30 ml of ethanol for 10 seconds, and excess ethanol was removed, using care not to touch or scrub the occlusal side of the disc. This was followed by again soaking in 30 ml of H_2O (pH 7.0) for 1 minute.

Visible water was removed using air from the central facility in a gentle manner, mimicking a clinical wet-bonding procedure to avoid collapsing the collagen network in the dentin. The drying process was performed once. The disc was then placed into a plastic tissue culture container, which was placed into 37°C and 100% humidity in a dark environment (tissue culture incubator) for 7-days for the purpose to equilibrate (or aging) of dentin disc.

A dark environment of 37°C and 100% humidity is similar to the human oral environment. Further, no evaporation of pre-mixed solution was observed as a result of the 100% humidity environment. The concentration of CO_2 was 95% at the incubator, leading to a slight acidic environment in long term storage. Actually, in a pilot study of the pH change of DI water, the pH of DI water changed from 7.0 to 6.0 in one week.

Permeability of the dentin disc was measured 1 week after biosilicification.

In sample 14, pre-mixed TMOS/PLL was used. Details of the preparation, application, and storage technique used are as follows.

TMOS pre-hydrolyzed with 0.001M HCl, PLL, and distilled water was prepared in advance. TMOS (200 µL), PLL (400 µL), and distilled water (1400 µL) were placed into a cell of a plastic container for tissue culture research (cell diameter = 2.5 cm). The total volume of pre-mixed TMOS/PLL/distilled water solution was thus 2000 µL (2 ml).

The dentin disc was immersed in a tissue culture container cell with the occlusal side up, and stored at 37°C and 100% humidity for 24 hours, when the disc was removed

from the solution and its appearance was observed. Gentle air from the central facility was applied to remove excess solution. The disc was then placed into a new tissue culture cell with no solution, occlusal side up. This was then stored placed at 37°C and 100% humidity for 6 days to equilibrate, at which time permeability was measured. The pH of the HCl used for pre-hydrolization of TMOS in samples 12-14 was unknown.

Since the research day was limited on Tuesdays, the experimental protocol was prepared with consideration of this schedule. Thus, the samples immersed for 24 hours became 6-day storage samples, and the cotton pellet coating samples became 7-day storage samples.

TMOS/PLL Application Methods used for Samples 15-17

TMOS/PLL application methods used for samples 15-17 are summarized below.

Sample	Application	Technique	Storage
15, 16	Pre-Mixed TMOS/PLL	Immerse 24 hours	6 days
17		30 second brush coating	7 days

Samples 15-17 were prepared in the same manner as sample 14, described above. However, since the pH of the HCl used in sample 14 was uncertain, the HCl used in samples 15, 16, and 17 was confirmed using a pH meter. Storage methods and measurement of permeability for samples 15 and 16 were as for sample 14, described above.

For sample 17, a disposable brush for operative dentistry (3M ESPE Disposable Applicator Brush Tips, #59723, St. Paul, MN) and a clear glass plate were prepared in advance. The occlusal side of the dentin disc was placed upward on a clean glass plate, and pre-mixed TMOS/PLL was applied to the dentin-disc surface using a disposable

brush. Side-by-side brushing motion was performed for 30 seconds. The disc was then placed into a new tissue culture container cell with no solution. With the occlusal side up, the disc was aged at 37°C and 100% humidity for 7 days, after which permeability was measured.

Special Caution paid in regards to Dentin Permeability Measurement after Biosilicification

One of the advantages of the fluid filtration system is a reproducible dentin disc use for dentin permeability measurement. As mentioned previously, dentin permeability was measured before biosilicification and its value was considered as a baseline. Exposed dentin area was 6 mm in diameter, which corresponds to the size of inert rubber O-rings. Although biosilicification treatment was completed on the entire occlusal side of dentin, the location of exposed dentin area should accurately be duplicated as same as dentin permeability measurement before biosilicification. If the location duplicated is not accurate, dentin tubule configuration, size and density is different from between before and after biosilicification.

An O-ring mark on a dentin disc was inevitable at the end of permeability measurement at smear layer free condition. Our research team examined O-ring mark on a dentin. This examination was exercised in cooperation with material experts at the University of Connecticut at Storrs campus. As a result of examination, O-ring marks are inert with regard to material point of view. Accordingly, the O-ring mark emerged at the time of dentin permeability measurement before biosilicification was found to be utilized as a useful positioning landmark for the placement of dentin discs after biosilicification.

As a useful technique for accurate positioning, a clinical surgical loupe (2.5X magnification) was used by carefully looking at previously marked O-ring marks for the poison duplication.

Further, the figure tighten power within the screw chamber should be duplicated. If there is a discrepancy of figure tightens power between two measurements, the comparison will be less accurate due to the excessive or insufficient force against collagen fibers on dentin, which may lead to obtaining a fluid-tight seal. There is currently no relevant objective method available to measure the power. Accordingly, the power duplication was dependent on the dexterity of single operator.

In summary, the exposed dentin area in a disc was ensured to be duplicated in the same location using the same finger tighten power used when the permeability measurement before biosilicification. The permeability was repeatedly measured three time and hydraulic conductance (permeability) was calculated.

SEM Observation of Selected Dentin Discs

In SEM observation, only the occlusal side of the dentin disc surface was observed. “To examine for the presence of the anticipated silica reaction product and its morphology, two of the TMOS/PLL/TMOS treatment dentin discs and one of each type of control were sputter coated (Hummer X, Analtech Ltd, Newark, DE) with approximately 12 nm of gold/palladium and examined with SEM (JEOL JSM-6320F, Tokyo, Japan) for selected samples 1-11” [134].

For samples 12-17, a SEM (Hitachi TM-1000, Tokyo, Japan) was used for observation. No desiccations or sputter-coating were required. SEM images were digitally stored and analyzed.

Statistical Analysis

As mentioned preciously, 17 samples were included in this study. The dentin permeability of both before and after biosilicification was measured for each sample disc. Measurements were repeated three times and averaged. Raw data of fluid flow movement values were calculated into hydraulic conductance (permeability). Permeability of before and after biosilicification was compared in percentage of increase or decrease.

First 11 samples were included for the statistical analysis. Differences of permeability before and after biosilicification were compared with a matched-pairs *t* test. Statistical analysis was performed with Prism 4 (Graphpad Software, Inc.). The confidence level used throughout the experiment was 95% ($P < 0.05$).

3.0 Results

3.1 SEM Evaluation of Dentin Surfaces

3.1.1 Controls

The SEM image of sample 1 confirmed that the EDTA/NaOCl treatment produced the intended smear layer free dentin surface with patent tubules (Figure 8A). Only sample 1 was evaluated for the purpose of smear layer free confirmation. As the experiment progress, the question was raised as to whether the smear layer had been appropriately removed since the dentin permeability results were inconsistent, which will describe later in this thesis. Selected silicification completed discs (perhaps samples 8-11) were preliminary evaluated to confirm smear layer free. As a result incomplete smear-layer removal was observed using SEM. After careful review of the protocol, the EDTA pH was focused as a possible factor of incomplete smear-layer removal.

EDTA used for sample 1 was approximately pH 7-9, which was prepared from EDTA powder. After completion of the first several samples, there was no more EDTA powder in the research laboratory. The ready-to-use EDTA solution was purchased without knowing the fact of approximately pH 13 of that solution. No EDTA pH information was recorded until sample 14 was studied. Immediately after notify this problem in our research group meeting, the appropriate EDTA powder was purchased. The EDTA pH for samples 15-17 was thus 8.7.

EDTA/NaOCl treated discs were observed using new Hitachi table top SEM to confirm smear-layer free. These discs were treated by (1) 17% EDTA pH 13.0 /5.25% NaOCl and (3) 17% EDTA pH 7.7 /5.25% NaOCl. The application time of EDTA was 5

minutes, followed by a tap water rinse for 30 minutes. The application time of NaOCl was 5 minutes, followed by a tap water rinse for 30 minutes. SEM images of these discs were shown in Figures 9A and 9B, respectively. As a result of comparative SEM observations, it was confirmed that high pH EDTA did not remove the smear layer (Figure 9A). This corresponds to the report by Serper and Calt in 2002 [135].

3.1.2 First 11 samples

In sample 1, the baseline permeability at smear-layer-free condition was measured, and then the SEM image (Figure 8A), was taken as the smear-layer-free condition disc. After biosilicification and the second permeability measurement, the surface was examined with the SEM (Figure 8B). In the SEM observation, critical drying was required with sputter coating. There are differences between the two SEM images. Most importantly, a 98% permeability decrease was observed after biosilicification. Since sample 1 was successful, the same experiment was repeated to obtain more data.

Figure 8B shows a uniform coating of silica. The dentin discs treated with TMOS precursor and PLL catalyst solutions were covered with a layer of material clearly visible with SEM (Figure 8B). As determined by XPS system (VG Escalab MK II and Phi Multiprobe System with 15-255GAR Analyzer and 04-548 Dual Anode X-ray Source), hydrated silica was probably formed on the dentin disk [134]. The tubules are partially occluded, but the volcano-like appearance is observed at the openings. The volcano appearance is probably due to fluid flow during the permeability test. Higher magnification SEM images suggested that the silica coating is an agglomeration of nanoscale-size particles. The coating consisted of uniform agglomerated spherical

particles approximately 0.2-0.6 µm in diameter [134]. In most cases, the particles formed a continuous bridge over the tubules and covered the patent tubules, although the coating was not uniform over the entire surface of the dentin discs. Tubule observation may suggest silica formation inside the tubules. Approximately one-quarter of the surfaces remained uncoated. SEM examination, in addition to the schematic of 4 quadrants (Figure 10), shows open tubules in 1 quadrant, partially-covered tubules in 2 quadrants, and what is likely a smear layer in the 4th quadrant. It appears that the smear layer was not removed uniformly from the discs. Insufficient removal of smear layer may have a relation to uncertain EDTA pH.

Although not all SEM images were included in this thesis, selected samples exhibited successful deposition of at least a partial coating onto the dentin surface. The extent to which the dentin discs were covered with the coating varied from 25% to 100%.

Discs receiving the TMOS treatment, but no PLL catalyst (Figure 11A), showed little evidence of any deposited reaction product. Discs receiving only PLL (Figure 11B) showed no deposited material and appeared similar to the untreated surface shown in Figure 8A. These TMOS-only and PLL-only discs are considered control discs for comparison.

3.1.3 Description of Fragmented Coating

Interpretations of SEM images of samples 12-14 will be explained in a later section with the explanation of permeability.

Figures 12 (magnification; 1000X) and 13 (magnification; 2000X, 5000X) are SEM images of sample 15, which showed a 62 % decrease of dentin permeability. Most

dentin tubules were open. However, fragment shape deposits (more than 10 μm in length) partially cover the dentin tubules. The deposit size of sample 15 was larger than that of sample 14. The deposit shape of sample 15 was more rectangular than that of sample 14. The "rectangular" pieces in Figure 12 are fragments of a larger coating that fragmented as an artifact of the sample preparation. The deposit may have cracked during high suction vacuum within the SEM chamber, and it is possible the fragmentation was caused by this drying process.

3.2 Permeability

Dentin permeability is expressed in Lp, which is $\mu L\ cm^{-2}\ min^{-1} cm\ H_2O$. Table 1 (below) shows the dentin permeability of samples 1-11.

Table 1 Summary of 11 samples (Lp)

Sample	Permeability (Lp) before biosilicification	Permeability (Lp) after biosilicification
1	0.15	0.00
2	0.23	0.02
3	0.23	0.21
4	2.23	3.00
5	3.89	2.72
6	0.51	1.00
7	0.51	0.47
8	0.17	0.23
9	0.70	0.43
10	1.09	0.68
11	0.19	0.42

Cherng et al. reported in 2004 that permeability of smear layer free condition at 0.5mm thickness third molar dentin disc after 6% citric acid etching for three minutes ranged from 0.44 to 4.03 [51]. This seems reasonably close to the data of the permeability

(Lp) before silicification shown in Table 1. Therefore, the validity of the permeability test method which was introduced by Pashley is appropriate in our study.

After silicification, samples 1, 2, 3, 5, 7, 9, and 10 showed decreased permeability, whereas samples 4, 6, 8, and 11 showed increased permeability.

Differences before and after bio-silicification were compared with a matched-pair statistical test and coefficients of variation were calculated. The mean hydraulic conductance (permeability) of the four TMOS/PLL/TMOS treated dentin discs was reduced from 0.90 (1.17) $\mu L\ cm^{-2}\ min^{-1} cm\ H_2O$ in the smear layer free condition to 0.83 (1.04) following silicification (mean (standard deviation)) and the difference was not statitistically significant. ($p = 0.675$). The coefficient of variation (CV) was 1.30 before and 1.26 after biosilicification. Permeability results had a large coefficient of variation, contributing to the lack of statistically-significant differences. Figure 14 and Figure 15 show paired t-test data and visualized statistical interpretation.

The decreased permeability suggests that the silica had sufficient integrity to block the flow of water during the test. However, the increased permeability is also reported here. Interestingly, the uniformity or percentage of the dentin disc covered did not correspond to the change in dentin permeability. In order to correct the inconsistent permeability of samples 1-11, the experimental control was modified, as described in the Materials and Methods section and applied to samples 12-17.

Table 2 (below) summarizes the dentin permeability of samples 12, 13, and 14.

Table 2 Summary of samples 12, 13, and 14

Sample	Permeability (Lp) before biosilicification	Permeability (Lp) after biosilicification	
12	1.01	1.47	46% Increase
13	0.77	0.85	10% Increase
14	0.82	0.09	89% Decrease

Sample 14 showed an 89% permeability decrease. In the interpretation of this researcher, using sequential application of TMOS/PLL/TMOS is highly possible that process parameters contributed to the variability which had been observed in 11 samples. Sample 14 used the application of premixed TMOS/PLL. The premixed TMOS/PLL seemed to work better than did sequential application of TMOS/PLL/TMOS. The sample 14 protocol was therefore repeated for the subsequent experiments.

Table 3 (below) summarizes the dentin permeability of samples, 15, 16, and 17.

Table 3 Summary of samples 15, 16, and 17

Sample	Permeability (Lp) before biosilicification	Permeability (Lp) after biosilicification	
15	0.62	0.24	62% Decrease
16	1.76	1.86	6% Increase
17	2.94	3.08	5% Increase

Sample 15 showed permeability decrease, however, sample 16 showed permeability increase. Samples 15 and 16 are the same experimental condition and protocol, but the dentin permeability result was quite different. Accordingly, SEM observation was recommended to explain the difference.

In sample 14, a “sandy appearance” was observed on the dentin disc, whereas in samples 15 and 16, “smooth-continuous-shinny appearances” (Figure 16) were observed

on dentin discs. Nothing was observed in sample 17, meaning that it was very similar to a smear layer free condition disc surface.

In sample 14, the EDTA used for dentin disc to remove smear layer was pH 12.5 for previously mentioned reasons and, thus, it was speculated that unopened tubules existing in the smear layer free condition. In samples 15 and 16, the EDTA used for dentin disc was pH 8.7 and, thus, the problem of unopened tubules should resolve, expecting a good smear layer free condition.

Figures 17 (magnification of 1000x) and 18 (magnification of 2000x and 5000x) are SEM images of sample 14, which showed an 89% decrease of dentin permeability. Interestingly, most of the dentin tubules are open. No relation was observed between the number of open tubules and the decrease of dentin permeability. The change in permeability with silicification was inconsistent in the extent of coating coverage. The higher magnification SEM observation (Figure 18) showed that the deposit size was a few micrometers and the shape was round. This size is much larger than in samples 1-11.

Figures 19 (magnification of 1000x) and 20 (magnification of 2000x and 5000x) are SEM images of sample 16, which showed a 6% increase of dentin permeability. Samples 15 and 16 are the same experimental protocol, but the dentin permeability result was quite different. No deposit was observed on dentin and thus all dentin tubules were open, as shown in the left of sample 16 (Figure 19). However, deposit was observed on enamel, as shown in the right of sample 16 (Figure 19). The deposit size and shape were very similar to those of sample 15. In summary, pre-mixing of the TMOS and PLL did not eliminate the inconsistent permeability result.

In all 17 samples, samples 1, 2, 3, 5, 7, 9, 10, 14, and 15 showed decreased permeability, whereas samples 4, 6, 8, 11, 12, 13, 16, and 17 showed increased dentin permeability. No statistical comparison was made for all 17 samples in regard to permeability changes because the experimental protocol had changed significantly from samples 12 through 17.

3.3 Possible Explanations for Inconsistent Results

All previous studies have evaluated the catalyzed silicification reaction under well-controlled experimental conditions. Therefore, it seems likely that the inconsistency observed here is related to the dentin surface, which might appear consistent by normal SEM standards of evaluation, but may be sufficiently heterogeneous (within the disc and/or between discs) to have a significant effect on the biosilicification reaction. Accordingly, factors such as the catalyst, its molecular weight, and the type and concentration of the alkoxide precursor may need evaluation. In addition, the techniques used to apply the precursor and catalyst might influence the outcomes. Finally, tooth selection, storage, and method of smear layer removal may have affected the results.

3.3.1 Tooth Selection, Storage, Smear Layer Removal

Tooth storage methods and the quality of dentin disks were carefully reviewed. As a result of this review, smear layer removal by EDTA and NaOCl, tooth selection, and tooth storage methods were improved. As mentioned previously, high pH EDTA left unopened tubules due to residual smear layer. Unfortunately, the extracted teeth used in this study had no data on patient age, sex, radiograph, and reason for extraction. Tooth

storage conditions were standardized; however, the duration of storage varied. More precise and stringent documentation of individual teeth will be helpful in future research. In summary, careful dentin disc selection and preparation did not eliminate the inconsistent permeability result.

3.3.2 Attempts at Identifying Technique Variables That Affected Results

a. Pre-mixing TMOS and PLL (Samples 14-17)

In samples 1-13, the dentin discs were sequentially immersed into the TMOS, PLL, and TMOS. For samples 14-17, pre-mixed TMOS/PLL was used. However, inconsistency of permeability was not eliminated.

The change in technique from the sequential to the pre-mixed method is due to the results of samples 12, 13, and 14. Sequential immersion method was used for samples 12 and 13, whereas pre-mixed TMOS/PLL method was used for sample 14. In respect to application method, a cotton pellet was used for samples 12 and 13, whereas immersion into pre-mixed TMOS and PLL for 24 hours was done for sample 14. Among samples 12-14, sample 14 showed an 89% decrease in permeability. The SEM image of sample 14 had a sandy appearance. This is why it was thought that pre-mixed TMOS and PLL worked better than the sequential method. Accordingly, the protocol for samples 15-17 was planned, using pre-mixed TMOS and PLL.

As a side note, the pH of the HCl used for pre-hydrolization of TMOS in samples 12-14 was unknown. The HCl used in samples 15, 16, and 17 was checked, using a pH meter, because a pH check was critical in the sol-gel chemistry used in this study.

Pre-mixed TMOS and PLL were used for samples 15-17. Samples 15 and 16 were the duplicate protocols of sample 14. In sample 17, a 30-second brush coating technique was used. Although samples 15 and 16 were the same experimental condition and protocol using premixed TMOS/PLL, the permeability result was the opposite. SEM observation of sample 15 and 16 showed a smooth-continuous shiny appearance, whereas sample 14 had a sandy appearance. It is still unknown why the permeability results of samples 15 and 16 were so different, as both the SEM images of samples 15 and 16 showed smooth-continuous shiny appearances. In samples 15 and 16, fragments were observed, which were more than 10 µm in length but not consistent. In terms of the size and shape of fragment, it is possible that this fragment was a group of granulated silica. When samples 15 with 16 were compared, the deposit was partially observed on enamel in sample 16. The size and shape of deposit in sample 16 on enamel was very similar to that of sample 15 on dentin. (See *3.1.3 Description of Fragmented Coating*)

b. Storage Following Application of Silicification Chemistry

In samples 1-11, no dentin disc storage was considered after silicification. Dentin permeability was measured between 10 to 30 minutes after completion of silicification.

Later in this study, it became obvious that dentin disc storage after application of the TMOS and PLL was critical. For example, the storage period is more important than the immersion period, which is known as “aging” in sol-gel chemistry. Six-day or seven-day storage of the dentin disc after application of the TMOS and PLL was completed in samples 14-17, but the permeability result was inconsistent. It is uncertain whether storage following application of silicification chemistry made a difference.

Six-day storage at 37°C /100 % humidity conditions following application of silicification chemistry was done for sample 14. Since sample 14 was very successful, it seemed prudent to retain that methodology. This is why samples 15 and 16 were kept the same protocol as sample 14, except for the pH check.

In regard to SEM, it is difficult to evaluate whether storage affects the appearance of the surface. This is because more than one factor in the experimental protocol changed for samples 12-17.

4.0 Discussion

4.1 Storage Following Application of Silicification Chemistry

Two methods were used for the application of silicification chemistry, immersion and cotton pellet/brush coating. There are also two lengths for application time; one is short, approximately 30 seconds, and the other is long, such as 24 hours. The following three patterns are considered: (1) short immersion, (2) long immersion, and (3) cotton pellet/brush coating, which is always for a short period.

Among the above three patterns, (1) short time (approximately 30 seconds) immersion of dentin disc to the silicification chemistry was conducted to the first 11 samples, such as samples 1-11. However, permeability was inconsistent in these samples. Thus, (2) immersions for a long period and (3) cotton pellet/brush coatings were conducted for samples 12-17. In this, better results were obtained by 24-hour immersion than by those using a short-time, cotton pellet/brush coating. Although permeability results were still inconsistent among samples 12-17, the impression from the results of samples 14 and 15 was that the longer the application time of silicification chemistry, the better the chance for decreased permeability.

Sealing tubules with minerals is not a new concept, as described in the Introduction section. For example, Imai and his research team worked with calcium/phosphate mineralization for precipitation on dentin discs using ionic reaction, which is a fast reaction but which covers the dentin tubule surface only, without much penetration inside the dentin tubules. In our study, the time required for mineral formation in biosilicification was longer than in the precipitate method. In sol-gel

reaction in our study, the amount and rate of silica formation is slower than ionic reaction since pre-hydrolyzed TMOS was reacted with PLL for a length of time. However, the appropriate time required for biosilicification remains unknown because biosilicification is a new technology in material science. No one tried to apply biosilicification technology to human dentin so ever. In this respect, this research is challenging, but the exciting translational research from the latest material science into clinical dentistry.

The advantage of using a catalyst to form the mineral may include reaction rate control by the catalyst. If this control is achieved, it will also be possible to control the location of particle formation and the morphology of the particles. This philosophy applies to the whole thesis. However, in first eleven sample results showed inconsistent permeability results. Accordingly, in study of sample 12-17, attention was paid to resolve the inconsistency. There were many possible candidates of process parameters which may resolve the inconsistency. Among them, a storage method of discs was considered as one of the highest possibility due to the following reasons.

Storage methods of discs was considered critical based on the fact in adhesive dental material use in operative dentistry. The surface of the dentin where the adhesive dental material applies is recommended to keep certain moistures. Currently, there is no clinical method to measure the moisture content of dentin surface in quantity such as percentage. In dental school setting, faculty members teach students moisture keeping as technique sensitive. For example, the term “no puddle” accounts for preventing visible over wetting. The term “no bone dry” accounts for preventing excessive dryness. Clinical wet-bonding procedure is critical to avoid collapsing the collagen network in the dentin [136]. Wet-bonding is a key for success in adhesive bonding. Likewise in this study of

biosilicification, avoiding collapsing the collagen network in the dentin could be important for consistent dentin permeability. Further, in terms of clinical applications, such as dentin hypersensitivity treatment, the oral environment is always wet. Accordingly, two options were considered. One is immersion of the dentin disc in water; the other is placement in a 100% humidity chamber. With the former, the leaching out of silicification chemistry is a concern. Thus, the latter was adopted.

As seen by the necessity for pre-hydrolyzation of TMOS with 0.001M HCl, the acidic environment will be beneficial for the bio-silicification process because the cationic nature of PLL can alter the formed silica from spheres several hundred nm in size to planar structures up to 2 µm in scale [64]. A pH check is critical in hydrolysis and condensation reaction, especially for pre-hydrolyzation of TMOS with 0.001M HCl. A pH check is also critical in regards to monitor between cationic and anionic.

The permeability and SEM result of samples 15 and 16 were opposite and confusing, even though the protocol was the same. To make sense of the confusion, the following factors are considered. First, the dentin discs used were different, although the immersion time of pre-mixed TMOS/PLL was 24 hours for both samples 15 and 16. It is possible that 24-hour immersion was sufficient to occlude dentin tubules for sample 15, but not for sample 16. An appropriate deposit of silica particles must occur during immersion, because the storage stage does not increase the silica deposit. Therefore, the storage stage is important only for stabilization of the silica. Upon visual observation after 6-day or 7-day storage periods, deposit was observed on the dentin discs. However, interestingly, the amount of deposit did not correlate to a decrease in permeability. This result was confusing and provided the research question if the storage time might be

shortened to less than 6 or 7 days. In many cases, research protocol for dentin in an incubator requires 72 hours for equilibration, with progress determined by observing the recovery of the collagen network within dentin [136]. The general view of “aging” in silicification chemistry is that the longer the storage time, the better silicification is expected [137]. However, a set standard for this time has not been established, and the chemistry research regarding this point is scarce since biosilicification is a new technology. It may be that weeks or months of storage are needed, considering the slow reaction of biosilicification.

4.2 Attachment or Adhesion Strength of Silicified Coating

Sample 1 SEM was observed after permeability testing, and high permeability decrease was observed. This observation indicated the silica did not remove during the repeated fluid flow and contributed to high permeability decrease. The interfacial relationship between silica coating and dentin could be helpful to understand by utilizing the general concept of the interfacial relationship between adhesive bonding and dentin. In this, satisfactory adhesive strength between adhesive bonding and dentin leads to improved seal. The higher the adhesive strength obtained, the better the interfacial seal is achieved. The restorative dental materials such as resin composites or cements were historically followed this philosophy and the significantly improved clinical dentistry. However, the primary purpose of this thesis study is to occlude dentin tubules and no tensile strength is expected. Therefore, the interfacial relationship between silica coating and dentin should have a minimum strength to bond each other so as the deposited silica

could tolerate the physical power of fluid flow. It is speculated that biosilicification is not necessary to achieve high bond strength which is usually requires for operative dental materials. Sample 1 SEM of after biosicilification may suggest that interfacial attachment strength between silica coating and dentin was high enough to resist the water flow during the test. If attachment strength is low, water flowing inside dentin tubules may flush out the formed silica.

Further, mechanical integrity within the formed silica is probably important, but specifics are unknown. In particular, the silica particle is very small compared to other inorganic dental cements, such as silicate and glass-ionomer cements.

In the future research, the study of interfacial attachment strength between silica coating and dentin, together with mechanical integrity within the formed silica, will provide us one of useful hints to interpret the dentin permeability inconsistency in this study.

4.3 Possible Future Work

4.3.1 Viewing cross sections of tubules

The permeability results were inconsistent and did not correspond to the silica coverage area on the dentin discs observed by SEM images. If I have a future opportunity to continue studying or the other researcher will have a chance to carry over this study, it is highly recommended to consider sectioning the silicified dentin disc parallel to the long axis of the tubules. SEM images taken in this study are limited to the observation of cross sectional view. Although SEM is a useful equipment for three dimension observation, it is critical how to prepare the sample and focus the specific area of interest. As mentioned previously in relation to the interface between silica and dentin or within

silica, cross sectional view has limitations and it shows the surface area in only two dimensions. SEM images of sectioned silicified dentin disc parallel to the long axis of the tubules could evaluate the formed mineral morphology and its relation to dentin tubules walls.

It is speculated that silica particles flow into dentin tubules and occlude inside. In order to evaluate this phenomenon, it is necessary to evaluate to the dentin and silica in three dimensions. Viewing cross sections of tubules by SEM is traditionally used, although achieving a vertical split of discs along with the tubules is a sensitive technique to prepare samples. Perhaps a cone focal microscope or a high-grade cone beam CT system could make three-dimensional observation possible. Seeing inside dentin tubules by these methods would be helpful for a future research.

4.3.2 Consideration of Hydrophilicity

As mentioned previously, the moisture of dentin in biosilicification could be important as well as in resin dentin bonding. In this, it could be helpful for biosilicification to utilize the general concept exercised in dentin bonding. Enamel consists of highly mineralized structures, whereas dentin contains both inorganic and organic structures. Because of the organic component, dentin has a hydrophilic nature. In particular, the collagen network is a key factor to consider in dentin bonding. A wet bonding technique is recommended for eliminating water but keeping the surface moist so as not collapsing collagen network. In general, dentin bonding material maintains a hydrophobic nature. To achieve affinity between a hydrophilic substrate and a

hydrophobic adherent, a primer is used in dentin bonding. I would like to consider this mechanisum in dentin bonding into biosilificiation due to the following reason.

Biosilicification to dentin is a very new approach and it is considered as a clinical translational research from a fundamental biosilicification chemistry, which was mostly studied by the University of California-San Diego research team. The nature of silica formed by biosilicification is hydrophilic but is still unclear in certain respects. In this study, since both the substrate (dentin) and the adherent (silica) are hydrophilic, the original assumption was that dentin tubule occlusion would be easy. However, it was not.

For samples 1-11, some attention was paid to maintaining dentin disc dryness; however, there were some flaws. One example was that, two operators were involved in drying. Though the two researchers tried to use similar methods, there were no objective methods to assure similarity. The inconsistent permeability result was once thought to be due to the technical variations between two operators; however, this assumption was refuted by confirming experimental notes and results. The other flaw was that the office dust cleaner was used for dentin disc drying, which is not the standard for dentin or dentin bonding research.

For samples 12-17, only one researcher performed the tasks, and compressed oil-free air from a central facility was used as a gentle air for the dentin disc. This air application technique is more appropriate to imitate wet bonding for patient care setting.

Though there was careful control, as shown above, the permeability results were still inconsistent throughout this study. The current assumption is that pH, temperature, humidity, and residual water inside dentin tubules may be factors. The experiments are also affected by processing history, concentration, ionic strength, and other parameters.

Another possibility may be that an acidic environment can cause dissolution of occluded dentin tubules. It will be helpful if pH and humidity both inside and outside the dentin tubules can be tracked when the biosilicification is in progress. Further, using Tetraethyl orthosilicate (TEOS) instead of TMOS will be a consideration. In future research to achieve permeability consistency, attention should be paid to precursor/catalyst combination, speed of biosilicification, use of accelerator, timing for biosilicification chemistry application, biosilicification chemistry concentration, high/low PLL molecular weight, and temperature for biosilicification chemistry and dentin. However, high pH or high temperature is not suitable for applications in the mouth when the clinical application is considered.

4.3.3 Consideration of Particle Size and Shape

The reported particle sizes of the silica formed from biosilicification range from 0.04 to 0.1 µm according to Roth et al. in 2005 [138]. As an evaluation of our first three sample SEM images, a coating of 0.2-0.6 µm agglomerated particles was formed on all dentin discs [134]. When comparing dentin tubule diameter (2 to 5 µm) to silica particle size, theoretically, biosilicification on the dentin surface could potentially penetrate deep inside dentin tubules to form and deposit silica. This is very beneficial in respect to many dental treatments, such as hypersensitivity treatment, restorative dentistry, and endodontics. In dentin hypersensitivity treatment, the cold water pain will be relieved by dentin tubule occlusion. In endodontics, apical and coronal seals of the root canal system are essential for success in root canal treatment. The particle size of currently available commercial products in dentisty are generally a few micrometers and some of them

penetrate into dentin tubules and others not. Dentin permeability is historically tested there commercial products in range of micrometer. Dentin permeability is not tested for products in range of submicron, which is silica formed. In this respect, this thesis study could be a first achievement in this size. Accordingly, it is speculated that this small particle size of silica may be one of the factor of dentin permeability variability.

In many commercially available dental materials, especially dentin bonding materials, deterioration of the dentin-material interface will ultimately take place. The reason for this deterioration is still debated, however nanoleakage and dentin tubule orientation and occlusion are considered as a part of possible reasons. Theologically, to evaluate nano-scale silica particles in size could be achieved by using high magnification of SEM or other current technology, although the current technology limitation is not certain. In our SEM observation at the maximum magnification of up to several thousand, it was unknown whether the tubule occlusion was complete or incomplete, and permanent or non-permanent. In this thesis study, we did not use methods that might have given the information. In future research, very high magnification of SEM, specially designed sample preparations, the use of state-of-the-art technology are expected to have the information in regards to silica particle size, precise dentin anatomy, and relation between submicron particle and current permeability testing system. Further, comparison among silica, hydroxyapatite, and calcium precipitation will yield more information. The long-term success of many methods in biosilicification has not been demonstrated, presenting a future research possibility.

This researcher is interested in the shape of the minerals formed in this study. Particle size of the mineral may correspond to the size of the aforementioned dentin

tubules. Particle shape of the mineral may relate to the depth of penetration into the dentin tubules. Penetration depth may affect the shape since direction and orientation of the particle may determine the depth of penetration. For example, with a thin, elongated particle, the length may prevent deep penetration. Thus, it may be profitable to study not only particle size but also shape. Morphology will be addressed, for example, as a surface area and aspect ratio. In regard to surface area information, this will help understand the reaction rates of biosilicification. Aspect ratio will help in understanding a flow of catalyst/precursor solutions into the dentin tubules. The minerals formed in this study are irregular in shape and exist as aggregates. In this, inter- and intra-particle relationships will become a key factor. This relationship is probably related to the shape of the particles.

Extracted teeth used in this study had no data regarding patient age, sex, radiograph, or reason for extraction. To compensate this disadvantage, the dentin permeability value (Lp) was compared with other reports, which was described in result section. By looking at these reports, it was found that the relationship between the thickness and Lp were reported as follows. Reeder et al. reported in 1978 that the thinner the dentin disc, the more the Lp value increased; third molar Lp after 50% citric and etching for two minutes was 0.121, 0.185, 0.390, at dentin disc thickness 0.9, 0.7, 0.6 mm, respectively [85]. Tagami et al. in 1989 and Schmalz et al. in 2001 reported that human dentin Lp showed an inverse linear relationship with dentin thickness [139, 140]. These reports speculated that Lp has a lot of to do with dentin thickness and micro anatomy of dentin such as size and shape of the dentin tubules.

For the future study, using old versus young dentin and root versus coronal dentin is interesting, because the size and shape of the dentin tubules are different. As a substrate changes, the silica application will be changed, too. Dentin permeability in the crown is related to the numerical density of dentin tubules [24]. Studies of the numerical density of dentin tubules have focused primarily on coronal dentin [141-148]. Mean numerical density in coronal dentin in previous studies varied markedly: 15,000 to 24,500 /mm^2 for outer dentin; 35,000 to 40,400 /mm^2 for middle dentin; and 43,000 to 65,000 /mm^2 for inner dentin [1]. Careful dentin disc selection and preparation did not eliminate the inconsistent permeability result. However, in future research, if a way is found to restrict or target tooth selection much more meticulously, it is possible that dentin can be made permeability consistent.

5.0 Conclusions

Within the constraints of this experimental design, the following conclusions can be drawn:

1. A coating of 0.2-0.6 µm agglomerated particles was formed on many dentin discs. The coating covered 25% to 100% of the dentin discs. The coating was most likely hydrated silica.

2. The coating was able to bridge and cover the dentin tubules. Tubule observation may suggest silica formation inside the tubules.

3. Permeability results had a large coefficient of variation, contributing to the lack of statistically significance differences.

4. The change in permeability with silicification was inconsistent with the extent of coating coverage.

5. Careful dentin disc selection and preparation did not eliminate the inconsistent permeability result.

6. Pre-mixing of the TMOS and PLL did not eliminate the inconsistent permeability result.

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Figure 1
Mechanisms behind biosilicification

1. Hydrolysis

   $Si\text{-}OR + H_2O \Rightarrow Si\text{-}OH + R\text{-}OH$

2. Condensation reaction

   $Si\text{-}OH + HO\text{-}Si \Rightarrow \text{-}(Si\text{-}O\text{-}Si)n\text{-} + H_2O$

   $Si\text{-}OR + HO\text{-}Si \Rightarrow \text{-}(Si\text{-}O\text{-}Si)n\text{-} + R\text{-}OH$

(R. K. Iler, The Chemistry of Silica,1979)
(Silicon & Siliceous Structures in Biological Systems, 1981)

Figure 2A
An example of tooth with restoration

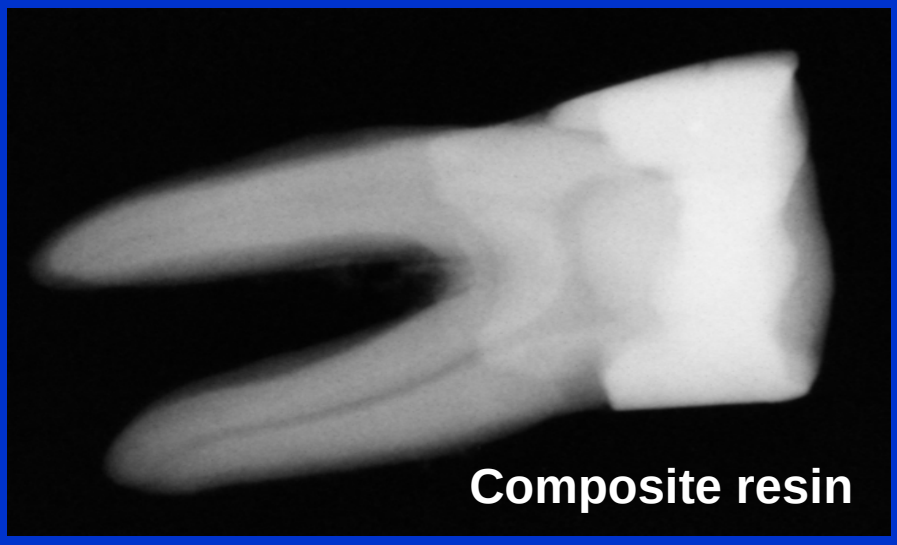


Figure 2B
An example of tooth with caries or defects

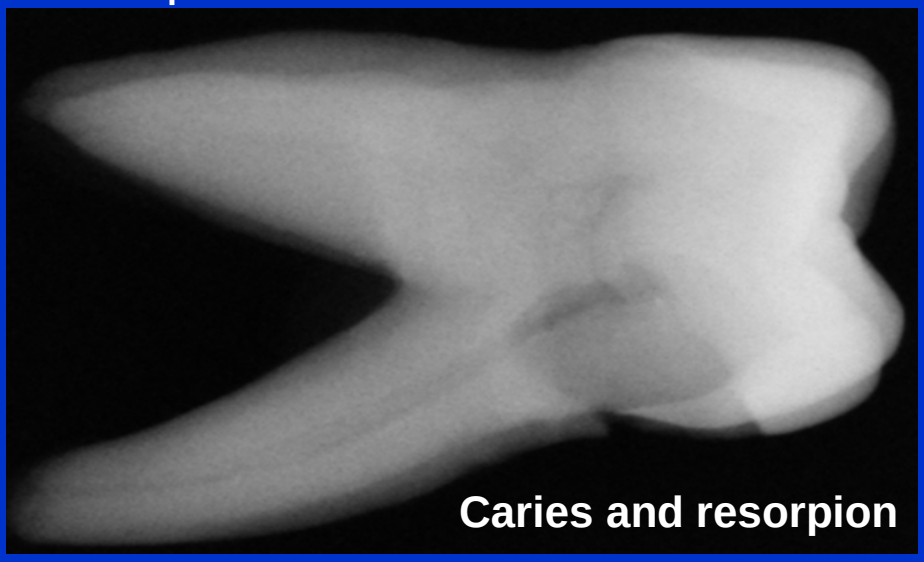


Figure 2C
An example of tooth with open apex

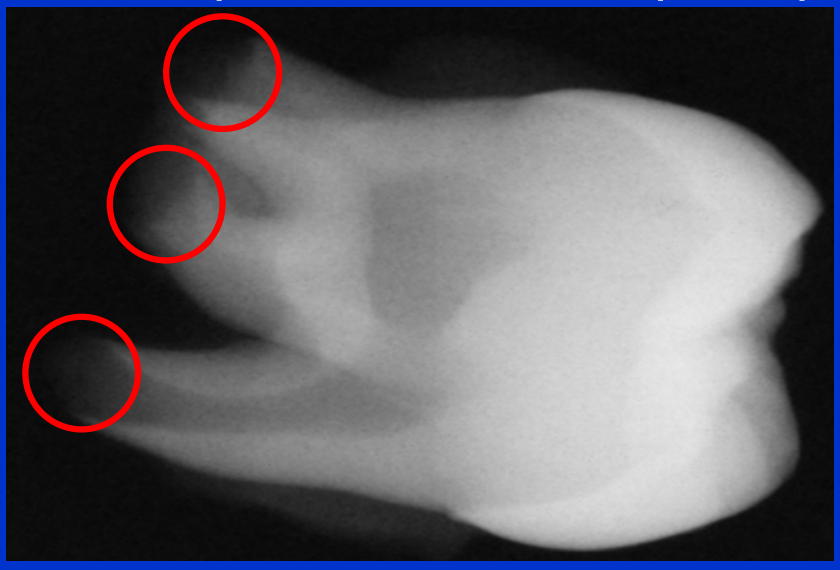
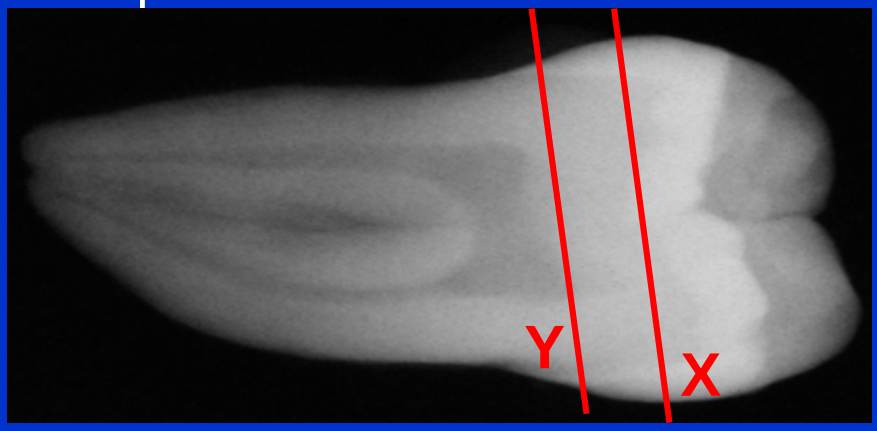
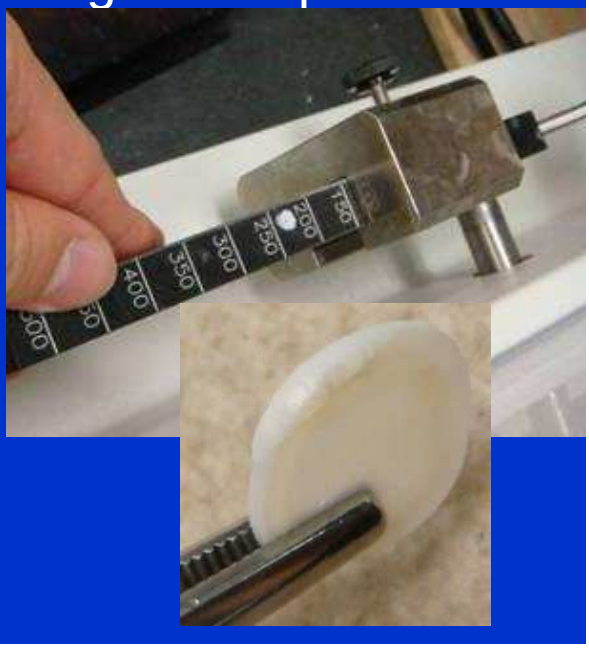


Figure 2D
The adequate dentin thickness confirmation



X; Dentin-enamel boarder/dentino-enamel junction
Y; Pulp-dentin boarder/pulp horn

Figure 3
The tooth cutting technique



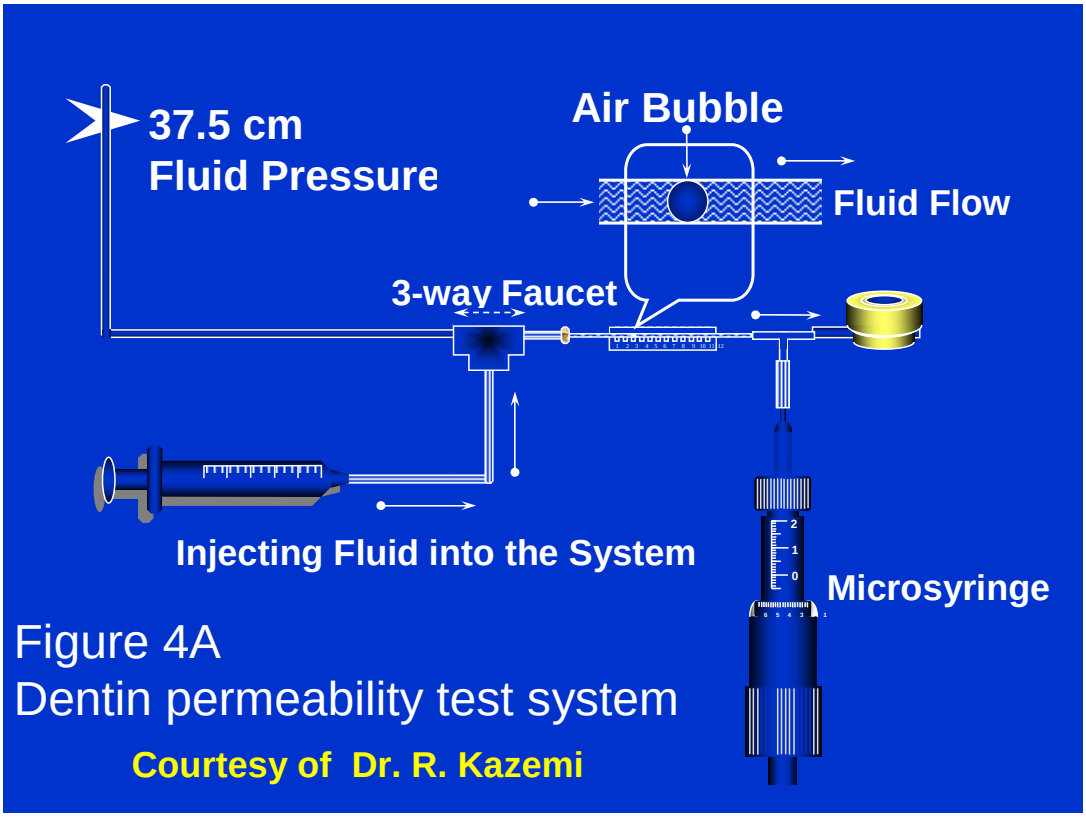


Figure 4A
Dentin permeability test system

Courtesy of Dr. R. Kazemi

Figure 4B
The dentin disc placement using O-rings

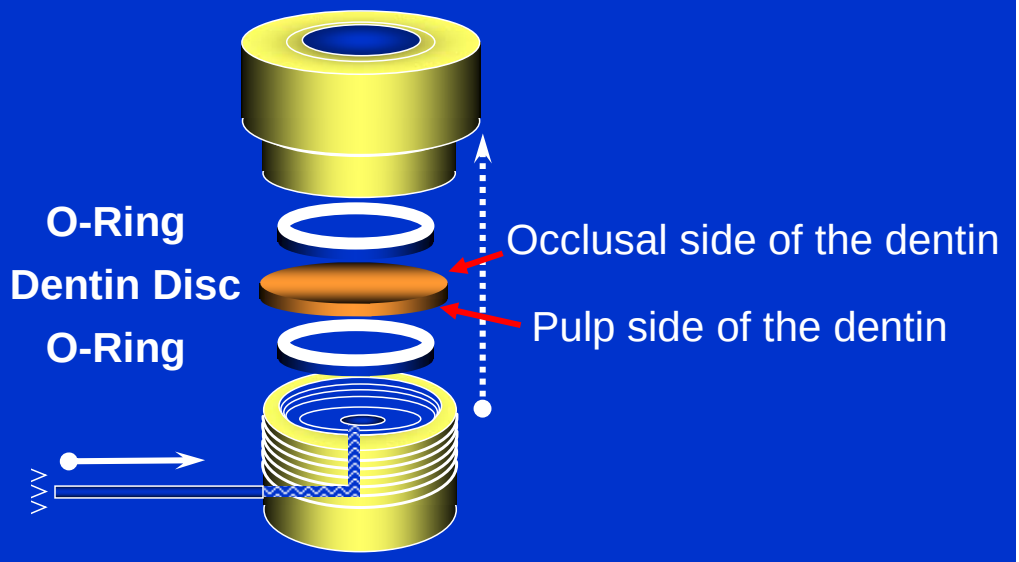


Figure 4C
The dentin disc placement using O-rings (detail)

Figure 5
Detecting method of occusal side of dentin disc

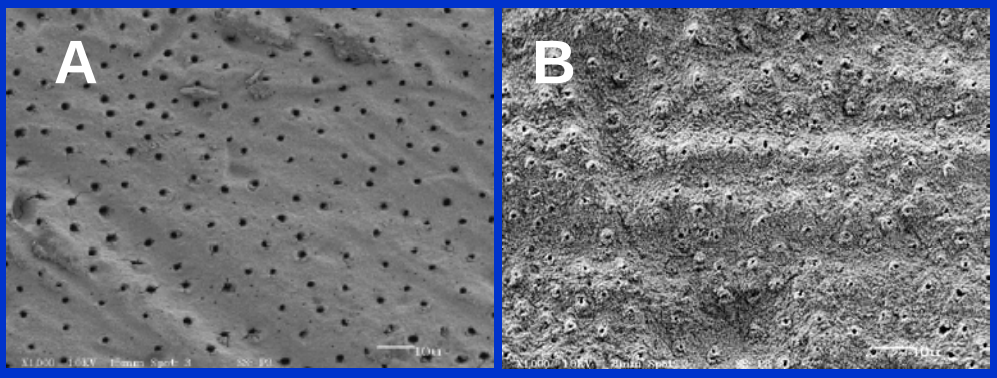
The letter magnifies on occusal side of dentin disc

Figure 6
Microsyringe and air bubble

Figure 7
Fluid absorbency process

A; Fluid is visible when the cover was screwed by fingers
B; Excess fluid removal using absorbent paper points
C; The condition which is ready for permeability measurement

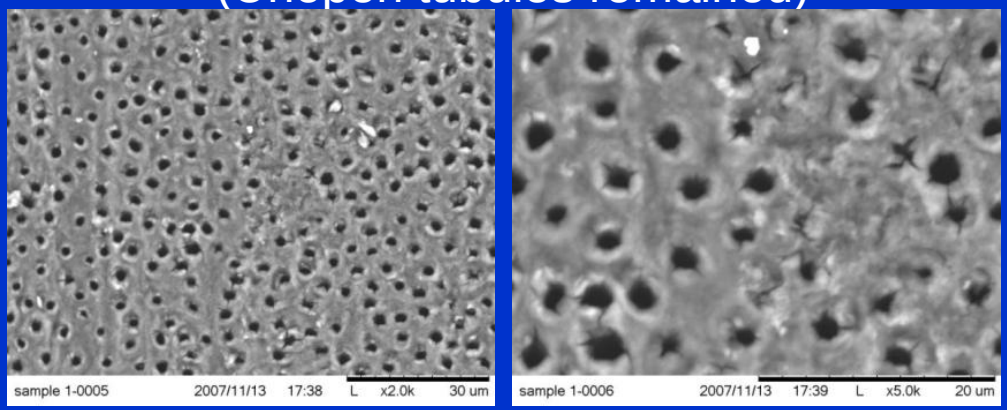
Figures 8A, 8B
SEM of silicification of dentin surface



A; Smear layer free surface with open, exposed tubules after treatment with EDTA and NaOCl

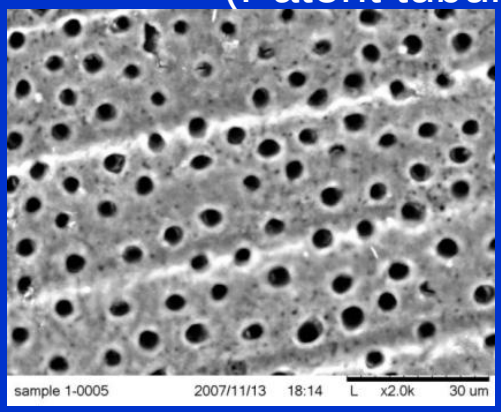
B; Silicified surface following permeability test (1000X)

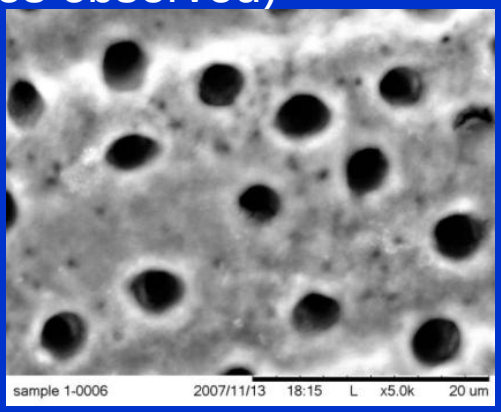
Figure 9A
Smear layer removal using EDTA pH 13.0 /NaOCl
(Unopen tubules remained)



2000X **5000X**

Figure 9B
Smear layer removal using
EDTA pH 7.7 /NaOCl
(Patent tubules observed)

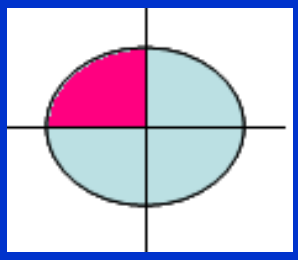


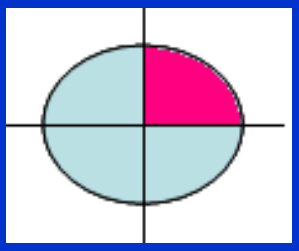


2000X **5000X**

Figure 10
The schematic of 4 quadrants of the dentin
disc surfaces for SEM observation

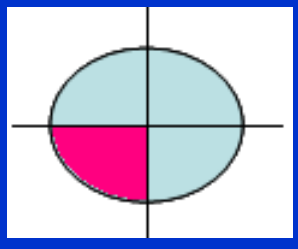
1st

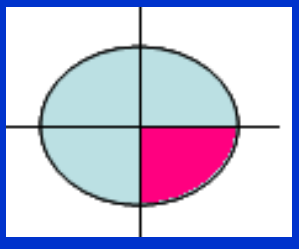




2nd

4th





3rd

Figure 11A
SEM images of TMOS only

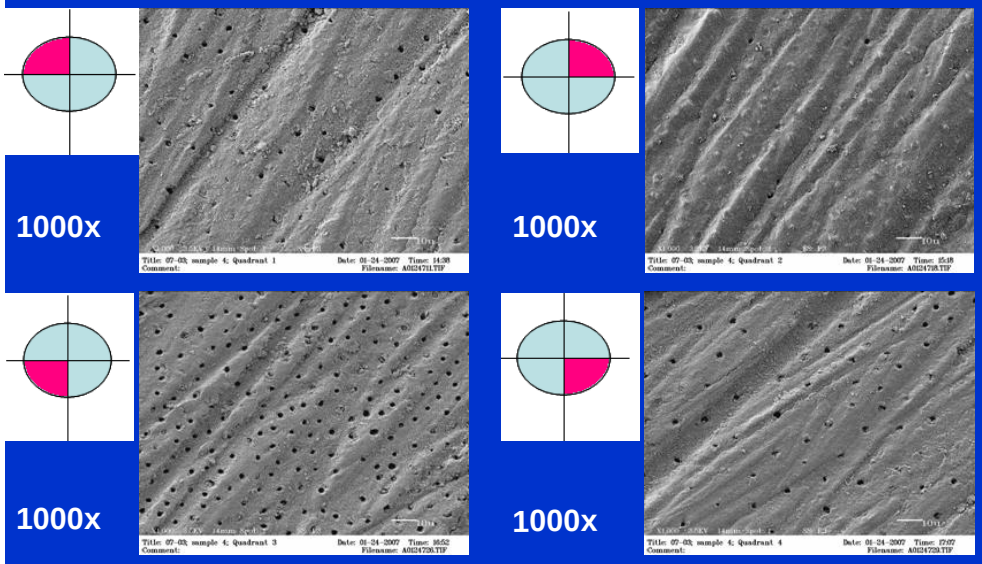


Figure 11B
SEM images of PLL only

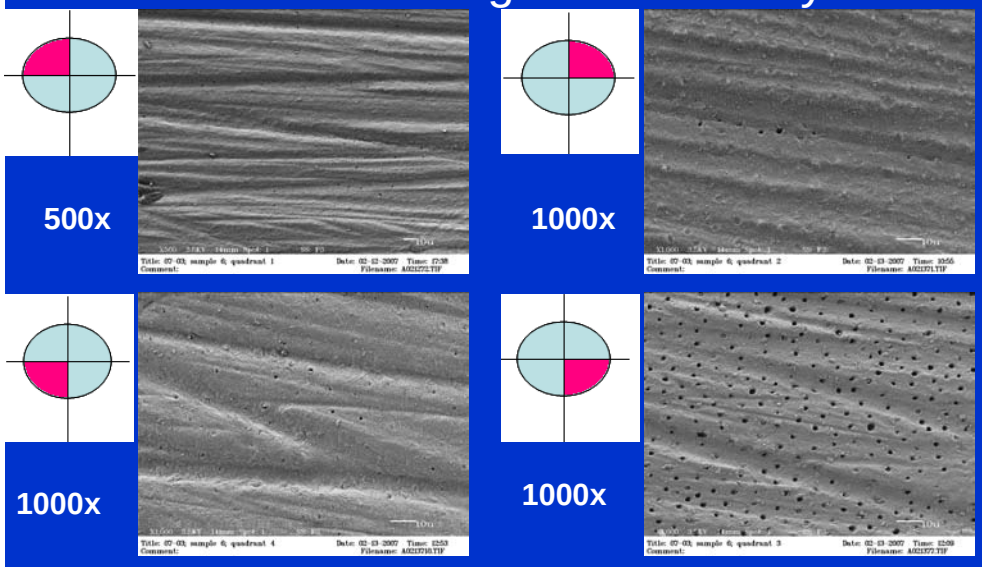


Figure 12
SEM of silicification of dentin surface
Sample 15 (1000x)

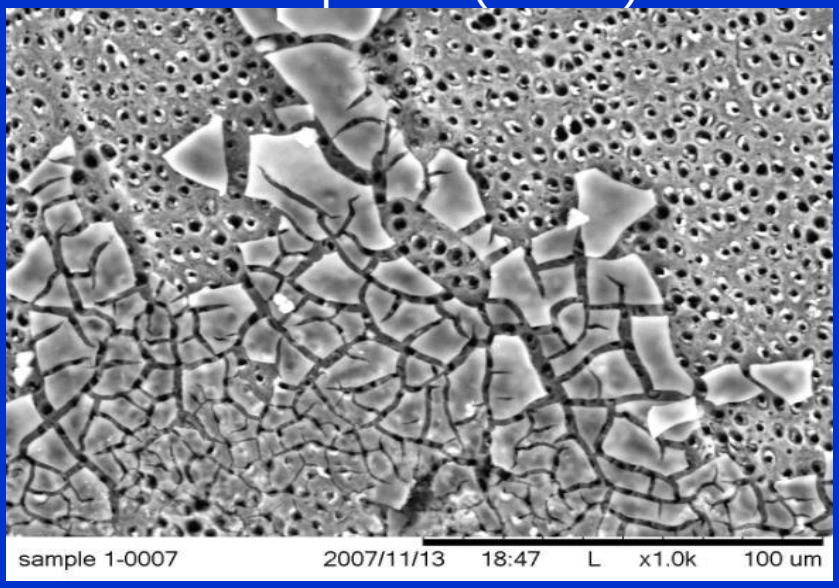
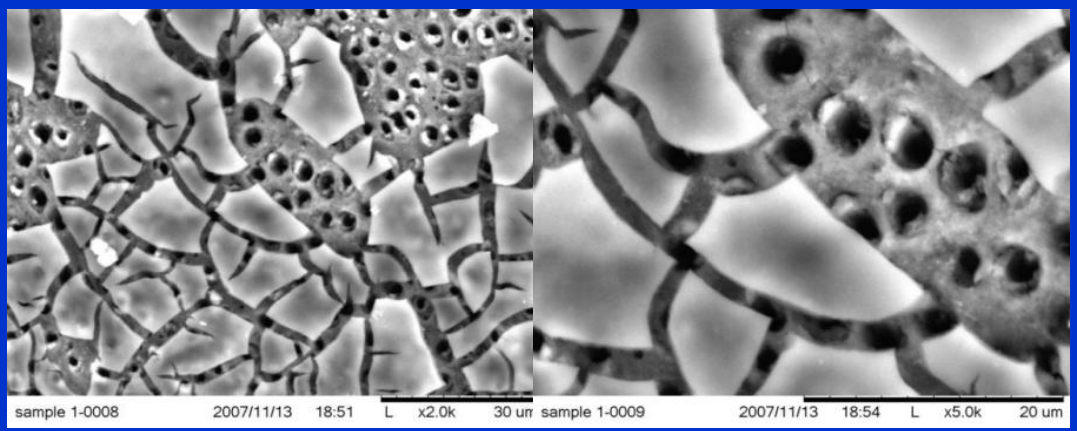


Figure 13
SEM of silicification of dentin surface
Sample 15 (2000X, 5000X)



2000x **5000x**

Figure 14
Permeability comparison
before and after biosilicification

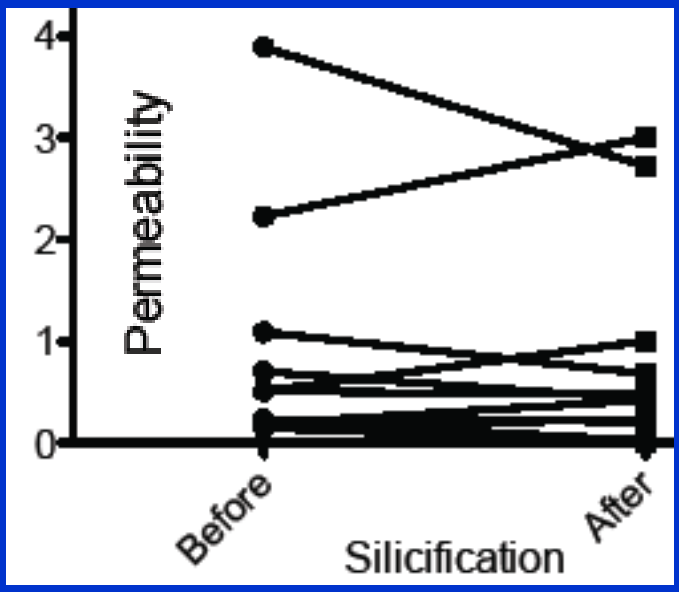


Figure 15
Visualized statistical interpretation
before and after biosilicification

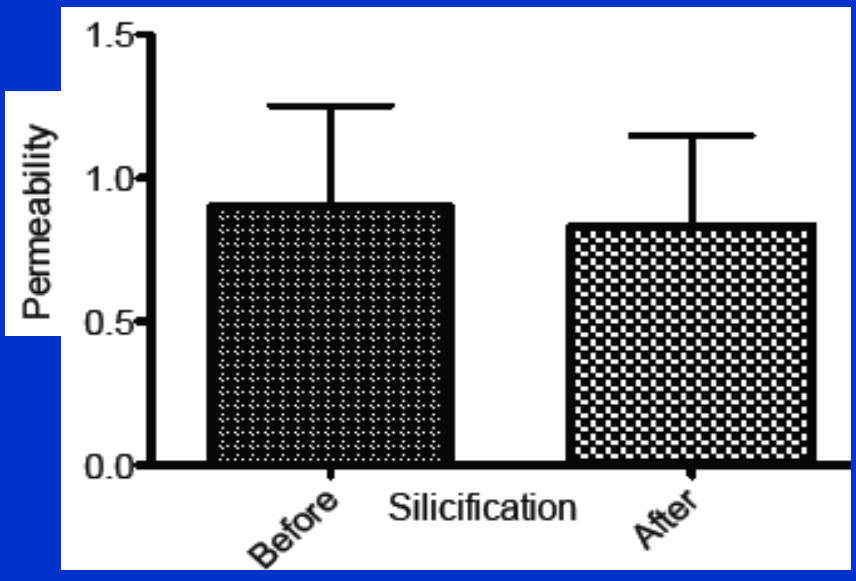


Figure 16
Sandy appearance (A) and
smooth-continuous-shiny appearances (B)

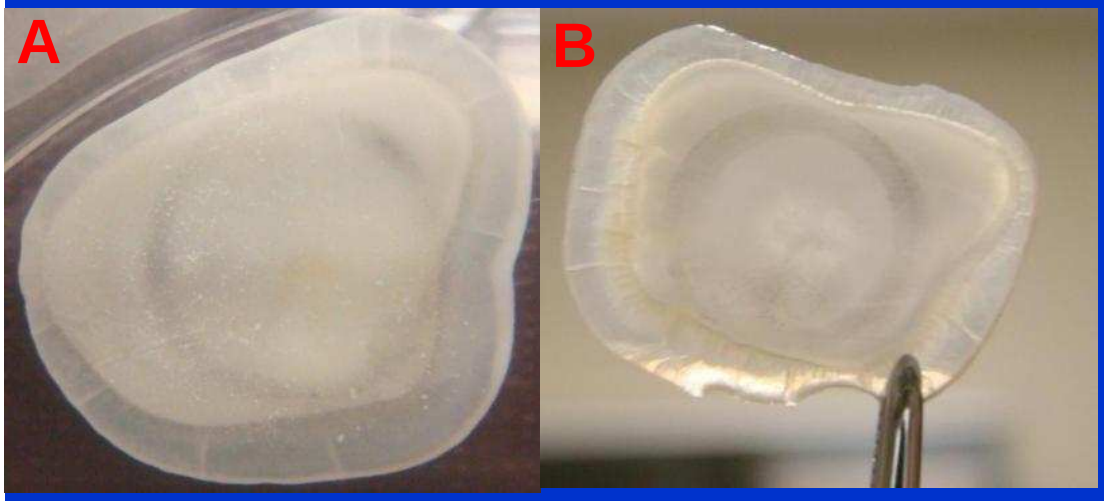


Figure 17
SEM of silicification of dentin surface
Sample 14 (1000x)

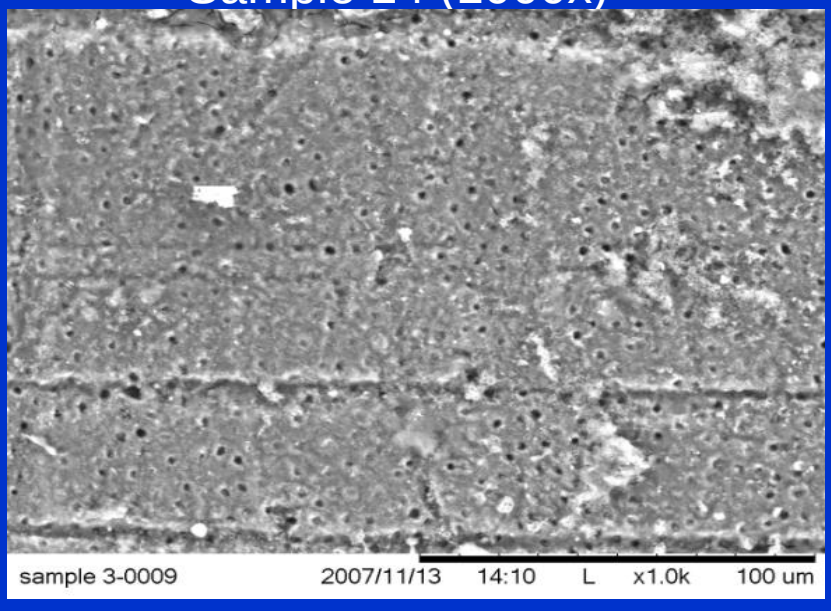
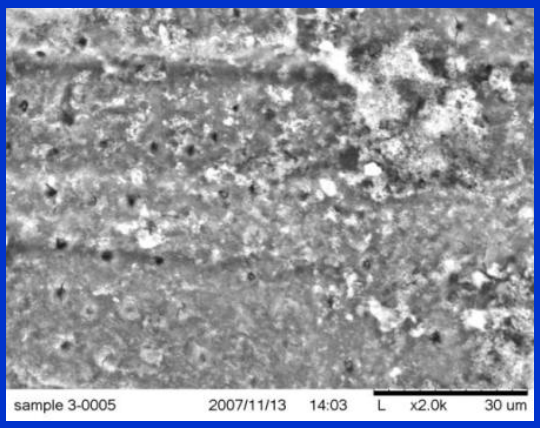
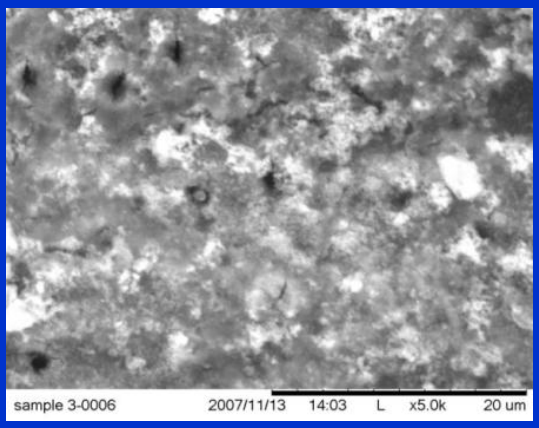


Figure 18
SEM of silicification of dentin surface
Sample 14 (2000x, 5000x)

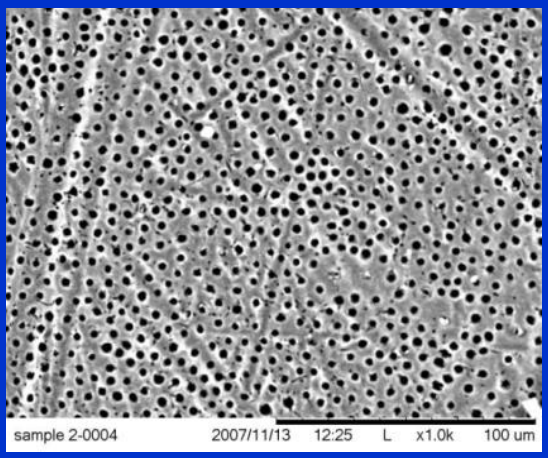




2000X **5000X**

Figure 19
SEM of silicification of dentin/enamel surface
Sample 16 (1000x)

Dentin tubules were open



Deposit on enamel observed

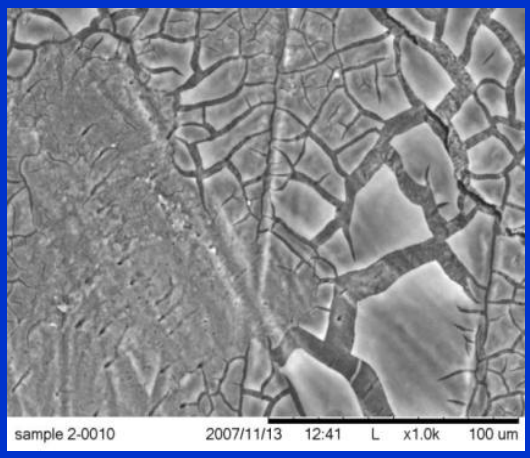
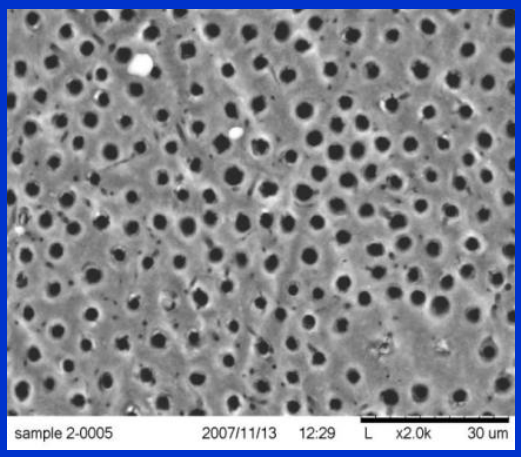
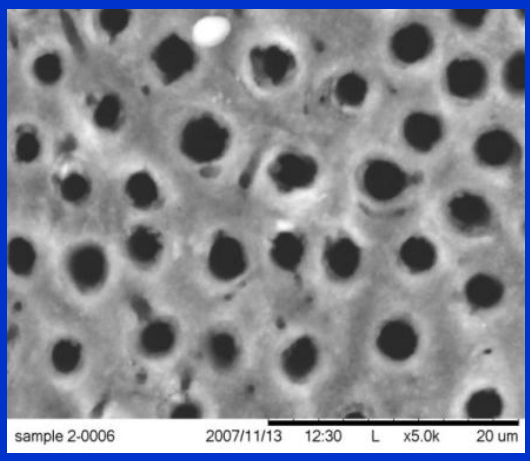


Figure 20
SEM of silicification of dentin surface
Sample 16 (2000x, 5000x)



2000x



5000x