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The impact of an alkasite restorative material on the ph of a streptococcus mutans biofilm and dentin remineralization: an in vitro study

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THE IMPACT OF AN ALKASITE RESTORATIVE MATERIAL ON THE PH OF A *STREPTOCOCCUS MUTANS* BIOFILM AND DENTIN REMINERALIZATION: AN *IN VITRO* STUDY

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ผลของไอออนที่ปลดปล่อยจากวัสดุอัลคาไซต์ต่อค่าความเป็นกรด-เบส ของ สเตร็พโตคอคคัส มิวแทนส์ ไบโอฟิล์ม และการคืนกลับของแร่ธาตุในเนื้อฟัน

น.ส.ภาวิณี วิริยะเสถียรกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	THE IMPACT OF AN ALKASITE RESTORATIVE MATERIAL ON THE PH OF A *STREPTOCOCCUS MUTANS* BIOFILM AND DENTIN REMINERALIZATION: AN *IN VITRO* STUDY
By	Miss Pawinee Wiriyasatiankun
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ภาวิณี วิริยะเสถียรกุล : ผลของไอออนที่ปลดปล่อยจากวัสดุอัลคาไซต์ต่อค่าความเป็นกรด-เบส ของสเตร็พโตคอคคัส มิวแทนส์ ไบโอฟิล์ม และการคืนกลับของแร่ธาตุในเนื้อฟัน. (THE IMPACT OF AN ALKASITE RESTORATIVE MATERIAL ON THE PH OF A *STREPTOCOCCUS MUTANS* BIOFILM AND DENTIN REMINERALIZATION: AN *IN VITRO* STUDY) อ.ที่ปรึกษาหลัก : รศ. ทญ. ดร.รังสิมา สกุลณะมรรคา, อ.ที่ปรึกษาร่วม : รศ. ทญ. ดร.พนิดา ธัญญศรีสังข์

วัตถุประสงค์: เพื่อศึกษาผลของไอออนที่ปลดปล่อยจากวัสดุอัลคาไซต์ต่อค่าความเป็นกรด-เบส ของสเตร็พโตคอคคัส มิวแทนส์ ไบโอฟิล์ม และความแข็งผิวของเนื้อฟัน

วัสดุและวิธีการ: วัสดุที่ใช้ในการศึกษาแบ่งเป็น 2 กลุ่ม ได้แก่ เรซินคอมโพสิต (FZ) และ อัลคาไซต์ (CN) การศึกษาแบ่งเป็น 3 การทดลอง ได้แก่ 1) การวัดค่าความเป็นกรด-เบส ของสเตร็พโตคอคคัส มิวแทนส์ ไบโอฟิล์ม ที่ยึดเกาะบนวัสดุ FZ และ CN จำนวนกลุ่มละ 31 ชิ้น เป็นเวลา 24 ชั่วโมง 2) การวัดความเข้มข้นของไฮดรอกไซด์ ฟลูออไรด์ และแคลเซียมไอออนสะสมที่ปลดปล่อยจากวัสดุ FZ และ CN จำนวนกลุ่มละ 31ชิ้น เมื่อแช่วัสดุลงในน้ำปราศจากไอออนเป็นระยะเวลา 6 ชั่วโมง, 1, 3, 7, 14 และ 28 วัน 3) การวัดค่าความแข็งผิวของเนื้อฟันกรามถาวรมนุษย์จำนวน 14 ซี่ การเตรียมฟันทำโดยแบ่งฟัน 1 ซี่ เป็น 2 ชิ้น ตัดแนวนอน ผ่านกลางตัวฟันในแนวใกล้กลาง-ไกลกลาง ฟันที่ตัด 1 ชิ้น แบ่งได้เป็น 2 ส่วน กล่าวคือฟัน 1 ซี่ แบ่งเป็น 4 จตุภาค จตุภาคที่ 1 คือเนื้อฟันปกติ จตุภาคที่ 2 ถึง 4 จะผ่านการแช่สารละลายตัวของแร่ธาตุ จากนั้นทำการบูรณะจตุภาคที่ 2 และ 4 ด้วยวัสดุ FZ และ CN ตามลำดับ ทำการวัดความแข็งผิวของเนื้อฟันในระดับความลึก 20, 40 และ 60 ไมครอน จากด้านบดเคี้ยว โดยจตุภาคที่ 2 และ 4 ทำการวัดความแข็งผิวของเนื้อฟันภายหลังบูรณะด้วยวัสดุ 30 วัน สถิติที่ใช้ในการวิเคราะห์ผล ได้แก่ Independent t-tests, Mann-Whitney-U และ repeated-measure-ANOVA

ผลการศึกษา: 1) ค่าความเป็นกรด-เบส ของสเตร็พโตคอคคัส มิวแทนส์ ไบโอฟิล์ม ที่ยึดเกาะบนวัสดุ CN (4.45) สูงกว่า FZ (4.06) อย่างมีนัยสำคัญทางสถิติ 2) ความเข้มข้นของไฮดรอกไซด์ ฟลูออไรด์ และแคลเซียมไอออนสะสมที่ปลดปล่อยจากวัสดุ CN สูงกว่า FZ อย่างมีนัยสำคัญทางสถิติทุกช่วงเวลาที่วัด 3) เนื้อฟันที่ผ่านการละลายแร่ธาตุ และบูรณะด้วยวัสดุ CN มีค่าความแข็งผิวสูงกว่าเนื้อฟันที่ผ่านการละลายแร่ธาตุทุกระดับความลึก และสูงกว่าการบูรณะด้วย FZ ที่ระดับความลึก 20 และ 40 ไมครอน อย่างมีนัยสำคัญทางสถิติ

สรุปผลการศึกษา: วัสดุ CN สามารถปลดปล่อยไฮดรอกไซด์ ฟลูออไรด์ และแคลเซียมไอออนได้ ซึ่งส่งผลเพิ่มค่าความเป็นกรด-เบส ของสเตร็พโตคอคคัส มิวแทนส์ ไบโอฟิล์ม และค่าความแข็งผิวเนื้อฟันที่ผ่านการละลายแร่ธาตุ

สาขาวิชา	ทันตกรรมหัตถการ	ลายมือชื่อนิสิต .....
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KEYWORD: Alkasite, Biofilm, Ion release, Resin composite, Streptococcus mutans, Hardness

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This study investigated the effect of an alkasite material on the pH of *Streptococcus mutans* biofilm and dentin hardness. *S. mutans* biofilms were formed on a resin composite (FZ) and an alkasite (CN) materials and their pH determined after 24 h. Hydroxide, fluoride and calcium ions released from the materials were determined at 6 h, 1, 3, 7, 14, and 28 d. Fourteen human molar teeth were cut horizontally across the middle third of the crown, bisected mesio-distally into two sections and each section divided into two, yielding four quadrants. Quadrant 1 was a sound dentin control, quadrants 2-4 were chemically demineralized, a cylinder of FZ and CN placed on the surfaces of quadrants 2 and 4, respectively. The microhardness of quadrants 1 and 3 were measured at depths of 20, 40, and 60 μm from the occlusal surface, and similarly of quadrants 2 and 4 after 30 days. Independent t-tests, Mann-Whitney-U, and repeated-measure-ANOVA tests were used for data analysis.The pH of the biofilm on CN (4.45) was significantly higher($p<0.05$) than that on FZ (4.06).The quantity of all ions released from CN was significantly higher than from FZ. The hardness of demineralized dentin under CN was significantly higher than that of demineralized dentin at all depths, and higher than that of demineralized dentin under FZ at 20 and 40 μm. In conclusion, CN released hydroxide, fluoride, and calcium ions, which was associated with raising the biofilm pH and the hardness of demineralized dentin.

Field of Study:	Operative Dentistry	Student's Signature .....
Academic Year:	2021	Advisor's Signature .....
		Co-advisor's Signature .....

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Pawinee Wiriyasatiankun

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Chapter 1

Introduction

Background and rationale

Dental caries is one of the most common oral diseases related to dental biofilm [1], and *Streptococcus mutans* (*S. mutans*) is one of the main biofilm bacteria which produce acids from fermentable sugar and cause demineralization of the tooth surface [2, 3]. The ecological plaque hypothesis is widely recognized as the most plausible explanation for caries development. More than 700 bacterial species live in the oral cavity, which in health live in symbiosis. However, frequent sugar consumption can enhance biofilm acidogenicity (lower the pH) and promote a shift in the balance towards pathogenic bacteria, including *S. mutans*, which can survive in acidic conditions (the property of aciduricity). Prolonged low pH results in tooth demineralization and eventually, a frank cavity [4]. To control dental caries, neutralization of the acid may help in the maintenance of microbial symbiosis inside the biofilm [5].

An alkasite restorative material is a new category of filling material which is classified as a subgroup of resin composite [6]. The manufacturer (Ivoclar Vivadent, Schaan, Liechtenstein) claims that the main advantage of an alkasite material is that it can release hydroxide, calcium, and fluoride ions from its alkaline (calcium fluoro-silicate glass) filler [6]. Hydroxide ions present on the surface of the material may play an important role in neutralizing acids produced by cariogenic bacteria [7]. Furthermore, the release of calcium and fluoride ions from the filler is postulated to contribute to remineralization and the prevention of dental caries [6]. Calcium is necessary for remineralization, and fluoride plays an important role in accelerating the process [8].

There has not been any previous research on the influence of hydroxide ions produced from the alkasite material on the pH of biofilm, and only one study (using polarized light microscopy) assessed the capacity of calcium and fluoride ions released by the alkasite material to inhibit demineralization. [9]. Therefore, the aims of this *in vitro* study were to assess the potential of the alkasite material to: a) raise the pH of a biofilm containing *S. mutans*; b) remineralize demineralized dentin.

Research questions

1. Do the ions released from alkasite material neutralize the cariogenic *S. mutans* biofilm?

2. Do the ions released from alkasite material remineralize the demineralized dentin?

Objectives

1. To determine pH of *S. mutans* biofilm which attach on alkasite material.

2. To determine microhardness of demineralized dentin after restored with alkasite material.

Hypothesis

Research hypothesis

1. The ions released from alkasite material can neutralize the cariogenic *S. mutans* biofilm.

2. The ions released from alkasite material can remineralize the demineralized dentin.

Statistical hypothesis:

1. H0: There is no difference between pH of the *S. mutans* biofilm which attach on alkasite material and non-ion released resin composite material.

H1: There is difference between pH of the *S. mutans* biofilm which attach on alkasite material and non-ion released resin composite material.

2. H0: There is no difference in remineralization on demineralized tooth after filling with alkasite material and non-ion released resin composite material.

H1: There is difference in remineralization on demineralized tooth after filling with alkasite material and non-ion released resin composite material

Independent variable: Alkasite material (Test), Resin composite (Control)

Dependent variable: pH of the *S. mutans* biofilm, Knoop hardness of dentin when contact with material

Outline of research

This experiment study aims to compare pH of the *S. mutans* biofilm which attach on alkasite material and resin composite material and to determine remineralization by measuring the tooth microhardness before and after contact to alkasite material and non-ion released resin composite materials. Moreover, the study will evaluate the level of hydroxide, fluoride and calcium ions release from alkasite material.

Limited of research

This experimental study cannot simulate the whole real situation such as the oral environment, oral microorganisms.

Expected benefit gain

The study will provide information on the alkasite material's properties in term of neutralization of acid condition and remineralization which may help to decrease the risk of secondary caries.

Chapter 2

Literature review

Dental caries

Dental caries is a multifactorial disease which mainly consists of host (tooth structure), the microbial biofilm formed on the tooth surface, fermentable carbohydrates (especially sucrose) and times. In a healthy condition, there is a dynamic process consisting of rapidly alternating periods of tooth demineralization and remineralization. The balance of pathological and protective factors influences the initiation and progression of dental caries. Protective factors promote remineralization and lesion arrest, while pathological factors shift the balance in the direction of dental caries and disease progression (Fig. 1) [1].

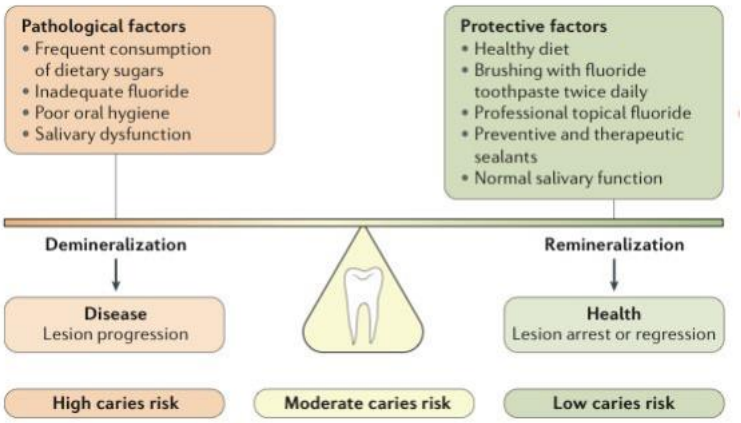


Figure 1 Balancing pathological and protected factors in dental caries [1].

Dental caries usually begins at the enamel surface (the initial demineralization is subsurface). Dietary fermentable carbohydrates; particularly sucrose, are metabolized by bacteria in biofilm result in organic acid production, which initiated the demineralized process of the crystalline mineral structure of the tooth [1]. Although the various types of organic acids are produced by

microorganism in dental biofilms, lactic acid which involves caries formation is the main by-product when there is an excess of sucrose [10]. When the pH is decreased until the minerals in biofilm-enamel interface becomes undersaturated, the surface layer of enamel is partially demineralized. The increased porosity allows acids diffusion into a deeper layer resulting in subsurface demineralization. Further progress of the caries process leads to the collapsed of the surface layer of the lesion and finally causes the physical cavitation [1].

The etiology of dental caries and ecological plaque hypothesis

Nowadays, the etiology of dental caries has been explained based on the ecological plaque hypothesis which proposed by Marsh in 1994 [4]. Oral-microorganisms live together with symbiosis (mutually beneficial to both host and microorganisms) in a normal circumstance. Nevertheless, the symbiosis may shift into a dysbiosis (benefit or harm to the other one) condition when the environment is changed. The shift of microbial ecology results in the initiation of the disease [4, 11].

Initial colonizers can attach on the tooth surface by producing biofilms and in the meantime, they can produce acids from sugary foods which lead to tooth mineral loss (demineralization) [11]. However, in normal circumstance, homeostatic mechanisms in the oral cavity such as the buffer capacity from saliva help to restore the mineral balance toward a net mineral gain in favor of remineralization [1]. This dynamic environment produces a stable condition of microflora, with the dominance of non-mutans streptococci and *Actinomyces* spp. (Fig.2). When sugar consumption is frequent or saliva function is impaired, the decrease of pH in the dental plaque becomes more severe and persistent. With this acidic condition, the populations of aciduric strains, which are cariogenic pathogens, in dental plaque are more increase selectively so that the demineralization/remineralization balance is disturbed over an extended period which may lead to progression of dental caries (Fig.2) [12].

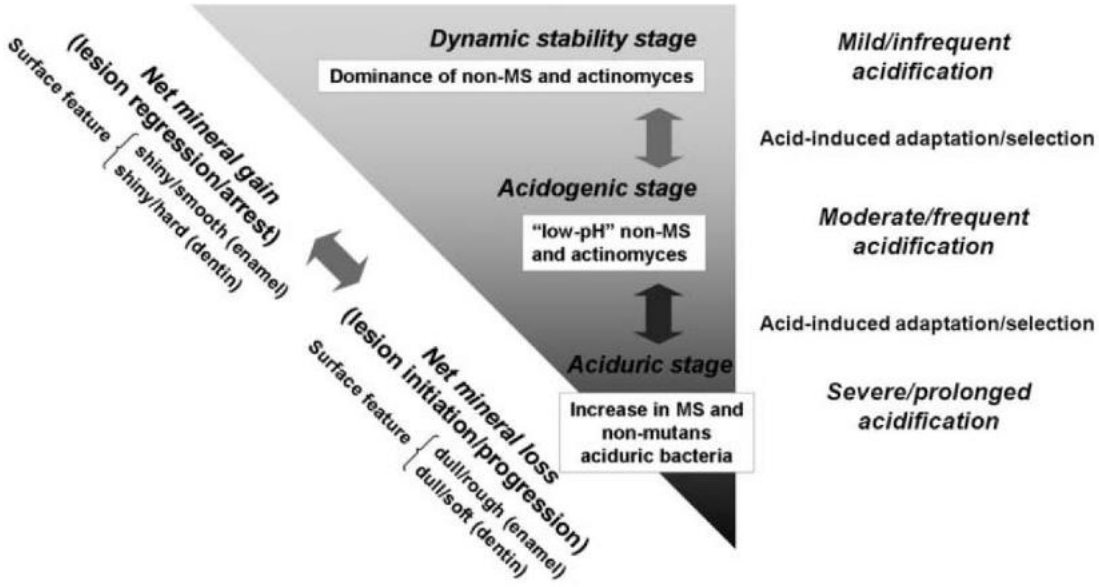


Figure 2 The caries process according to an extended caries ecological hypothesis [11].

Among oral microorganism, *S. mutans* is considered as one of the predominant pathogens causing dental caries since the bacteria can faster produce acids and better in biofilm formation than others. Moreover, it can grow well in acidic condition [13, 14].

However, based on the ecological plaque hypothesis, the disease control is not only the direct inhibition of cariogenic microorganism but also the disruption of factors that influence the microbiota shift, such as preventing the acidic condition [1].

Alkasite restorative material

Recently, the novel alkasite restorative material has been introduce in tradename of "Cention® N" (CN) (Ivoclar Vivadent; Schaan, Liechtenstein). It is a tooth-color filling material, containing alkaline filler which can release acid-neutralizing ions. CN can be categorized as a new class of resin-based filling and a subgroup of resin composite. As a dual-cured material with optional additional light-cure, bulk-filling is possibly done. Moreover, the manufacturer claimed that CN can

be used both with adhesive and without adhesive. The retentive tooth preparation is necessary in case of adhesive is not used, while the minimal invasive tooth preparation is done with bonding procedure [6].

CN comprises of liquid and powder. The liquid is composed of dimethacrylates and initiators, whilst the powder contains various glass fillers, initiators and pigment. There are 4 different dimethacrylate monomers in liquid solution including Urethane dimethacrylate (UDMA), Tricyclodecan-dimethanol dimethacrylate (DCP), Tetramethyl-xylylen-diurethane demethacrylate (aromatic aliphatic-UDMA) and Polyethylene glycol 400 demethacrylate (PEG-400 DMA). These organic monomers are responsibility for mechanical properties and stability due to crosslinks during polymerization. Each type of dimethacrylate monomers has different characteristics (Table 1). The role of the fillers in CN powder are strengthening material and enabling handling ability. The inorganic fillers comprise of a barium aluminium silicate glass filler, ytterbium trifluoride, an Isofiller (Tetric N-Ceram technology), a calcium barium aluminium fluorosilicate glass filler and a calcium fluorosilicate (alkaline) glass filler, with a particle size of between 0.1 μm and 35 μm. All filler materials in CN are showed in Table 2 and Figure 3 including their respective functions [6].

Table 1: Structural formulae and characteristics- of monomers used in Cention® N [6].

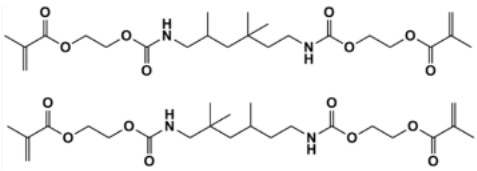
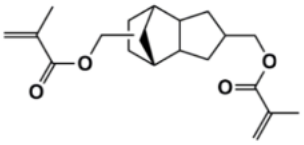
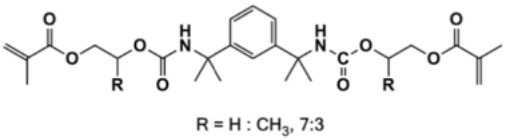
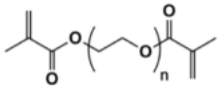
Monomer	Formula	Characteristics
UDMA		- The main component of monomer matrix - Moderate viscosity and yields strong mechanical properties - Hydrophobic and low water absorption
DCP		- Low viscosity - Enable hand-mixing - Cyclic aliphatic structure responsibility for strong mechanical properties
Aromatic aliphatic-UDMA	 R = H : CH_3, 7:3	- Hydrophobic - High-viscosity - Low tendency to discolour - Stiffness
PEG-400 DMA	 n = 9	- Enhance the flowability

Table 2: Overview of various fillers contained in Cention® N and Their respective function [6].

Filler	Function
Barium aluminium silicate glass	Strength
Ytterbium trifluoride	Radiopacity
Isofiller	Shrinkage stress relief
Calcium barium aluminium fluorosilicate glass	Strength, Fluoride release
Calcium fluorosilicate glass	F^-, OH^-, Ca^{2+} ion release

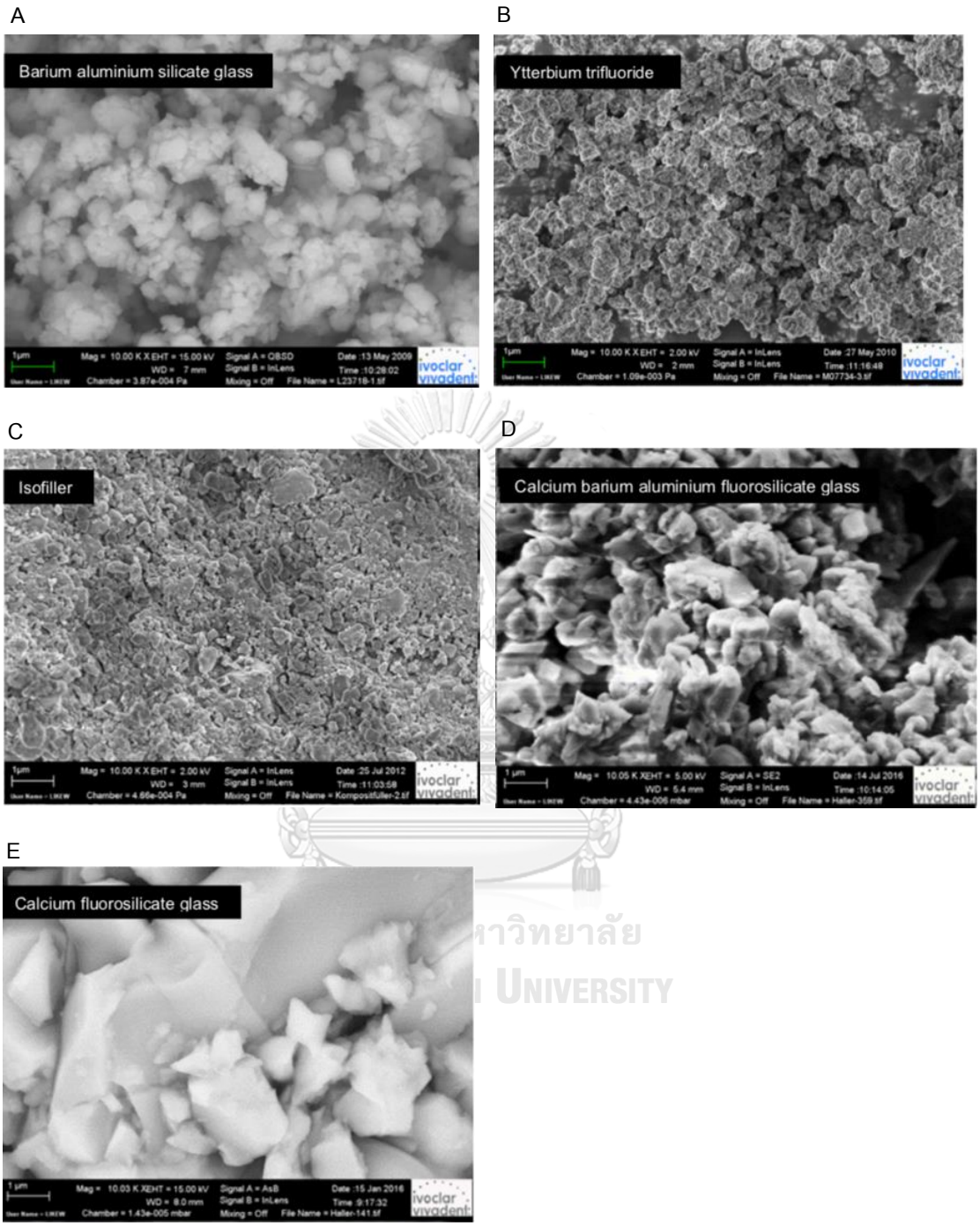


Figure 3 Electron microscopie of fillers in Cention® N [6]. **A**: Barium aluminium silicate glass, **B**: Ytterbium trifluoride, **C**: Isofiller, **D**: Calcium barium aluminium fluorosilicate glass, **E**: Calcium fluorosilicate glass

Mechanical properties

CN is an alternative material for posterior teeth restoration, since mean of compressive strength is approximately 121.395 to 321.92 MPa which is significantly higher than high viscosity glass ionomer cement and no significant different to nanofilled resin composite [15-17]. The flexural strength (107.21 ± 0.81 MPa) [16] and tensile strength (41.0 ± 12.5 MPa) [17] are significantly higher than high viscosity glass ionomer cement and not significant different to nanofilled resin composite. Moreover, there are two *in vitro* studies using CN as a core build up material for final restoration in previously endodontically treated tooth in class I [18] and class II MOD cavity [19]. The results of these studies showed that the compressive strength and the fracture resistance were similar between CN group and resin composite group.

The strength of CN is attributed to barium aluminium silicate glass (20-30 %wt) and calcium barium aluminium fluorosilicate glass filler (25-35 %wt) in powder including cross-linking of methacrylate monomer also leads to high polymer network density. In addition, CN contains both of self-cured (copper salt, peroxide and thiocarbamide) and light-cured initiator system (photoinitiator Ivocerin® and acyl phosphine oxide initiator) resulting in high degree of polymerization over the complete depth of the restoration [6, 15].

Ion releasing properties

Two types of filler in CN can release ions. Calcium fluorosilicate (alkaline) glass filler can release fluoride ions, calcium ions and hydroxide ions. Calcium barium aluminium fluorosilicate glass can release fluoride ions. The amount of releasing ions depends on pH value in oral cavity which the acidity is likely to enhance the number of ions released. [6] Aluminium ions are also detected in

acidic condition as a result of acid hydrolysis of Al-O-Si bond. Moreover, Silicon ion can be released from calcium fluorosilicate glass into Tris buffer solution (pH6.8) [20].

Hydroxide ion released

The manufacturer claimed that hydroxide ions can be released from alkaline glass filler. [6] There are two studies measured pH value in various solutions for hydroxide ion detection. Tiskaya and colleague found a slight increase of pH about 0.2 and 0.35 when immersed CN discs in artificial saliva (pH4) and Tris buffer (pH 6.8) for 6 weeks, respectively [20]. However, the other showed the results that the significant increase in pH was found only in acidic medium [21].

Fluoride ion released

Recently, several studies showed ability of CN in constantly released fluoride ions throughout the experiment without fluoride burst effect as conventional glass ionomer cement (GIC) and resin-modified glass ionomer cement (RMGIC) [20, 22-25]. Kelić's study reported the longest fluoride released was up to 168 d [24]. Gupta *et al*. compared fluoride ion release from CN (self-curing and light-curing mode) with GIC in neutral (pH 6.8) and acidic (pH 4) conditions [21]. The result showed that the highest fluoride ions released material was CN immersed in acidic solution with self-curing mode. In acid condition, the deterioration rate of CN was faster than GIC because the resin matrix of CN was exposed and fluoride ions were subsequently released. However, in the neutral condition, GIC released more fluoride ions because of the fillers of CN were surface modified to resisting the degradation. Besides, they found the tightly bound matrix and hydrophobic matrix of light-polymerized CN may affect the lesser extent of fluoride ions released when compared to self-curing mode [21]. Another study found the amount of fluoride ions released from CN (light-curing mode) in acidic condition (artificial saliva-pH4) was similar to those in neutral (Tris buffer-pH 7.3) [20].

Calcium ion released

Tiskaya and colleague studied the level of calcium ions released from CN compared with Bioactive composite (ACTIVA™ BioACTIVE-RESTORATIVE™, Pulpdent, USA) when immersed in artificial saliva pH 4 and 7 and Tris buffer solution pH 7.3 for 42 days. CN significantly released amounts of calcium ion up to 60 ppm when immersed in Tris buffer pH 7.3 and the release had linear relationship to square root time. Nevertheless, the higher amount of calcium ions from CN was found in artificial saliva (pH 4) which the cumulative calcium ions released at six weeks was approximately 240 ppm, while Bioactive composite released only 111 ppm. The authors suggested that the high calcium ions released from CN into artificial saliva (pH 4) might be due to the degradation of the glass fillers that was influenced by the exchange of hydrogen ions in artificial saliva pH 4 with calcium ions [20].

Effect of hydroxide ions to pH in biofilm

The pH stabilizing properties of an ions-releasing composite resin (Ariston pHc, Ivoclar Vivadent, Liechtenstein) was evaluated *in vivo,* which can release same ions with CN (fluoride, calcium and hydroxide ions). They measured pH change of saliva over the occlusal surface of the restoration (Class I cavities) of fifty patients between the ages 18-20 years who had active carious lesion in the posterior area. The result showed the mean pH significantly increased only in the first day of post-operative. However, the pH at each time point remained higher than pre-operative measurement [26]. Another study evaluated the effect of the hydroxide ion released form old ions-releasing composite resin (Ariston pHc) on plaque acidogenicity, *in vivo*. After 1 min rinsing of 10% sucrose solution, the final plaque pH (at 60 min) on 34 months old Ariston pHc was higher than resin composite restoration [7].

Effect of calcium and fluoride ions on tooth remineralization

Dental caries is a dynamic and reversible process. Remineralization can occur following demineralization. When acid is formed at the tooth-biofilm interface in sufficient amount to reduce the local pH below the critical pH, the local environment at the interface becomes under-saturated in calcium and phosphate. Calcium and phosphate from the tooth enamel are dissolved into the surrounding local environment, resulting in demineralization. As acid is removed, saliva becomes saturated in calcium and phosphate and these calcium and phosphate ions are driven back into the demineralized tooth tissue resulting in net mineral gain and repair of enamel's hydroxyapatite structure. Thus, adequate calcium and phosphate levels are crucial for inhibiting mineral loss during periods of low pH and promoting mineral gain when pH return to neutral [27, 28].

When fluoride is present, the critical pH for solubilization of calcium and phosphate ions is lowered. The solubility is reduced due to fluoride ions can be incorporated into tooth minerals by replaced hydroxide in hydroxyapatite to from fluorapatite or fluoride-enriched hydroxyapatite. Thus, fluoride-releasing materials could help inhibit demineralization and facilitate remineralization [29, 30].

There is a study on enamel and dentin demineralization inhibition at three different restoration margins after artificial caries challenge. At two weeks, the mean area of demineralization at enamel and dentin adjacent to CN were significantly less than Z100 and higher than Vitremer group [9].

Evaluation of ion release

Measurement of the released hydroxide ions

The Molar (M) concentration of hydroxide ions can be calculated from pOH which obtains from measuring pH of a solution. The calculation is based on this equation [31] :

$$pH + pOH = 14$$

$$pOH = - \log [OH^-]$$

$$[OH^-] = 10^{- pOH}$$

Measurement of the released fluoride ions

Ion-selective-electrode (ISE) is a method used for measuring fluoride ion released from a testing material into an aqueous solution [32]. The principal of ISE technique is a direct measurement of the electrode potential difference (mV) of electrochemical cells. Two electrodes consisting of sensing half-cell and reference half-cell are immersed in a sample solution. When an electric current is flown, ions will exchange between two electrodes [33]. Thus, the electrode potential difference can be measured. Total ionic strength adjuster buffer (TISAB) is usually added to the solution in order to control pH and prevent the binding of fluoride ion with hydrogen that becomes the complex fluoride. Thus, free fluoride ion levels can be determined more accurately [34].

The important component of ISE meter consists of (Fig. 4) [33]

1. A voltmeter which is an electrical circuit that connects to electrical cells for measuring electrode potential difference.
2. An electronic amplifier in millivolt which can increase electric signal.
3. A sensing electrode or ion-selective electrode; ISE which is an important component. It is classified by type of membrane. For measuring fluoride ion, solid-state membrane electrode is used.
4. A reference electrode which is an electrochemical half-cell which known the electrode potential. It can be used as a fixed reference for measuring electrode potential.

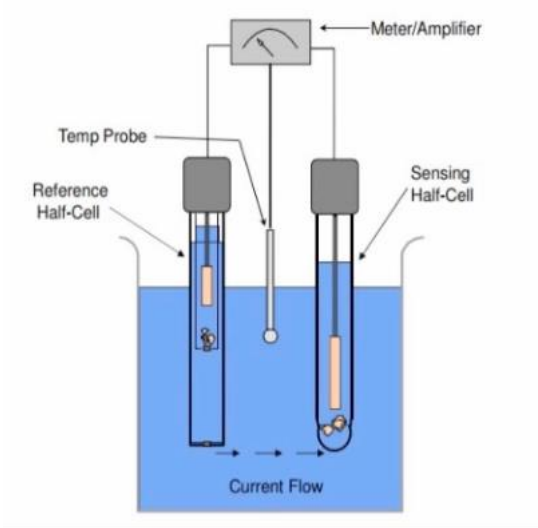


Figure 4 The important component of fluoride ion analyzer [33].

Measurement of the released calcium ions

For the determination of calcium ion release, the sample solution can be analyzed by inductively coupled plasma-optical emission spectroscopy (ICP-OES). ICP-OES is a quantitative method used for determining the trace elements in liquid samples. The advantages of ICP-OES are high sensitivity, high reliability, wide dynamic range and relatively less affected by coexisting elements. However, this technique cannot be used for a sample that contained high-purity materials or unknown elements. The schematic of typical ICP-OES apparatus is showed in Figure 5 This tool is consisted of a sample introduction section, a plasma source, a spectrometer, a detector, and a data processing system. The process of ICP-OES works by a sample solution is sprayed into a high-temperature plasma generated by subjecting argon gas to a high-frequency field. Then the heat stimulates an electron goes from ground state to excited state and releases energy in form of luminous intensity. The spectrometer can detect the wavelengths and intensities of the emissions from excited atoms and ions [35]. When ICP-OES is used for quantitative analysis, a calibration curve

is made by adjusting a calibration solution to a known concentration of the analyte element. Then, the concentration of the analyte element is determined by comparing the intensity of emissions of the sample solution from the calibration curve [36].

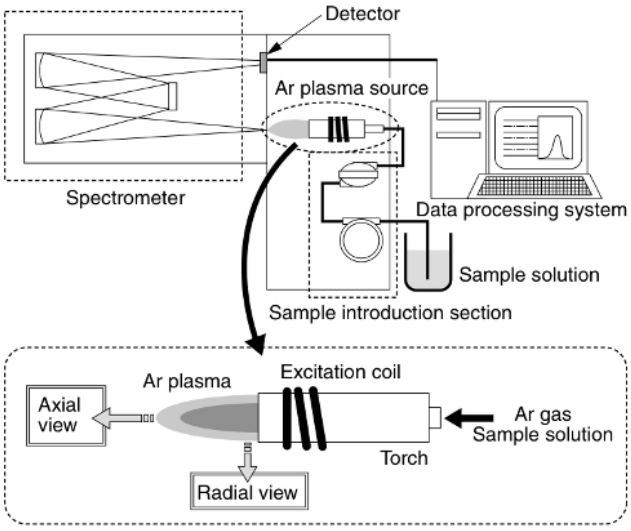


Figure 5 Schematic of ICP-OES [35]

Determination of tooth demineralization and remineralization

Microhardness measurement

Different techniques can be used to assess demineralization and remineralization including microhardness cross-sectional surface, polarized light microscope, microradiography and microCT scan [37, 38]. Polarized light microscope has been widely used in hard tissue research but is usually used as a qualitative method. For the sectioned analysis , transverse microradiography is preferred to polarized light microscopy, mainly because it is more directly related to mineral content [38]. Microradiography is the technique of mineral quantification by means of x-ray absorption. Two parameters: the lesion depth and the mineral loss value are also determined by microradiography. However, both methods require prepared sections as thin slices (thickness about 90 or 200 μm for

enamel or dentin, respectively) before investigated at cross-sectional surface. Thus, these methods are difficult and time consuming [38, 39]. MicroCT frequently used in experiments exploring mineral density or structure of bones or other mineral tissues such as, teeth. MicroCT scan can evaluate mineralized tissues non-destructively in three dimensions and measure mineral changes on hard tissues. Nonetheless the main drawback of microCT is the huge amount of data processing will make the whole process difficult and time consuming [40].

The longitudinal microhardness technique can be used for the quantitative analysis of mineral density profiles within both demineralized and remineralized enamel and dentin [41]. It can be used for quantitative evaluation and comparison of different treatment group effects on the overall progression or reversal of the caries process [42]. Hardness value is a measure of the mechanical resilience of the enamel, dentin or cementum due to the penetration of the indenter. Inorganic and organic components of tooth structure render the hardness value for its mineralized tissues. The operator error for hardness measurements was found to be less than 5% result in the accuracy and reproducibility of the test which is very reliable for sound or demineralized hard dental tissue [43]. Featherstone [44] demonstrated a linear relationship between microhardness on the cross-sectional surface and mineral content assessed by microradiography.

Several hardness testing instruments have been used to measure the hardness of mineralized tooth tissue, including Knoop microhardness. The Knoop indentation hardness, developed by Knoop *et al*, is the accurate measurement for the hardness of various materials, including dental materials and mineralized tooth structure. Knoop indenter has an elongated diamond pyramid-shaped point in which the ratio of the long and short diagonal is 7:1. The indenter presses on test sample resulting in surface area deformation at the mineralized tissue under analysis which the length of long diagonal of indenter is used for calculating the Knoop hardness value by this

equation: KHN (kg/mm^2) = 14230K/L^2 (K = applied force in grams, L = the indentation length) [41]. The elastic recovery of the Knoop indentation occurs only in the short diagonal, so that is not affecting the Knoop hardness value [45].

Chapter 3

Research methodology

The experimental design

This study was divided into 3 experiments as showed below:

1. Determination of biofilm pH
2. Determination of ion release from materials
    2.1. Hydroxide ions
    2.2. Fluoride ions
    2.3. Calcium ions
3. Determination of remineralization: Microhardness measurement

Restorative materials were used in the first and the second experiments (Table 3), whereas human teeth were used in the third experiment. The instruments and the materials used in experiments were showed in Table 4.

Table 3 Restorative materials.

Materials (Manufacturer)	Compositions
Alkasite: Cention® N (CN) (Ivoclar Vivadent, Schaan, Liechtenstein)	**Powder:** Barium aluminium silicate glass, Ytterbium trifluoride, Isofiller, Calcium barium aluminium fluorosilicate glass, Calcium fluoro silicate glass **Liquid:** UDMA, DCP, aromatic aliphatic-UDMA, PEG-400 DMA, initiators and pigment
Resin composite: Filtek™ Z350XT (FZ) (3M ESPE, St. Paul, MN)	Bis-GMA, BIS-EMA, UDMA, TEGDMA, Particles of silica and zirconia/silane, BHT, Photoinitiator and Pigments

Table 4 The instruments and the materials used in the experiments.

The experiments	The instruments and the materials
Materials preparation	- Resin composite Filtek™ Z350XT shade A2 (3M ESPE, St. Paul, MN) - Cention® N (Ivoclar Vivadent, Schaan, Liechtenstein) - Acrylic mold size 10 mm x 2 mm - Glass cover - Light curing unit (Elipar™ light; 3M, St. Paul, MN)
Bacterial preparation	- *Streptococcus mutans* UA159 - Brain heart infusion (BHI) agar and broth - 5% (w/v) sucrose - Deionized water - Pipette and pipette tip - Test tube
Determination of pH biofilm	- Polyvinylsiloxane Zhermack elite HD+ putty soft (Zhermack, RO, Italy) - 24 well cell culture plate (Costar®; Washington, DC, USA) - pH meter (LAQUA pH-22; HORIBA, Kyoto Japan)
Determination of ion release from materials - Hydroxide ions	- Advanced electrochemistry meter (Thermo Scientific™ Orion™ Versa Star™; Thermo Fisher Scientific, Waltham, MA) - Plastic container 10 ml - Pipettes and pipette tip - Deionized water

- Fluoride ions	- Fluoride standard 100 PPM (Orion Calibration Standard 940907) - TISAB III solution (Orion Calibration Standard 940911) - Advanced electrochemistry meter (Thermo Scientific™ Orion™ Versa Star™; Thermo Fisher Scientific, Waltham, MA) - Plastic container 5 ml - Pipettes and pipette tip - Deionized water
- Calcium ions	- Calcium pure standard 1000 PPM (CAS Number 7440-70-2, Perkin Elmer®, Waltham, MA) - ICP-OES spectrometer (Optima™ 7300 DV, Perkin Elmer®, USA) - Filter paper No.1 - Centrifuge tube 15 ml - 10% HNO_3 solution - Pipettes and pipette tip - Deionized water
Tooth preparation	- Human molar teeth with no any caries and pathology - 0.1% thymol solution (M-dent, Bangkok, Thailand) - Ultrasonic scaler - Pumice without fluoride - Silicon carbide sand paper No.400, 800, 1000 - Automatic polishing machine (NANO 2000;

	Pace Technologies, AZ, USA) - Epoxy resin - Silicone mold - Nail vanish - Low speed cutting machine (Isomet® 1000; Buehler, Lake Bluff, IL) - Demineralizing solution - Deionized water - Resin composite Filtek™ Z350XT shade A2 (3M ESPE, St. Paul, MN) - Cention® N (Ivoclar Vivadent, Schaan, Liechtenstein) - Clearfil™ SE Bond dentin bonding agent (Kuraray, Osaka, Japan) - Microbrush (Kerr, Orange, CA, USA) - Light curing unit (Elipar™ TriLight, 3M ESPE, USA) - Glass cover - Metal mold ¼ circle (Dental art lab, Bangkok, Thailand)
Determination of remineralization - Microhardness measurement	- Microhardness tester (FM-810; FUTURE-TECH, Kanagawa, Japan)

Methods

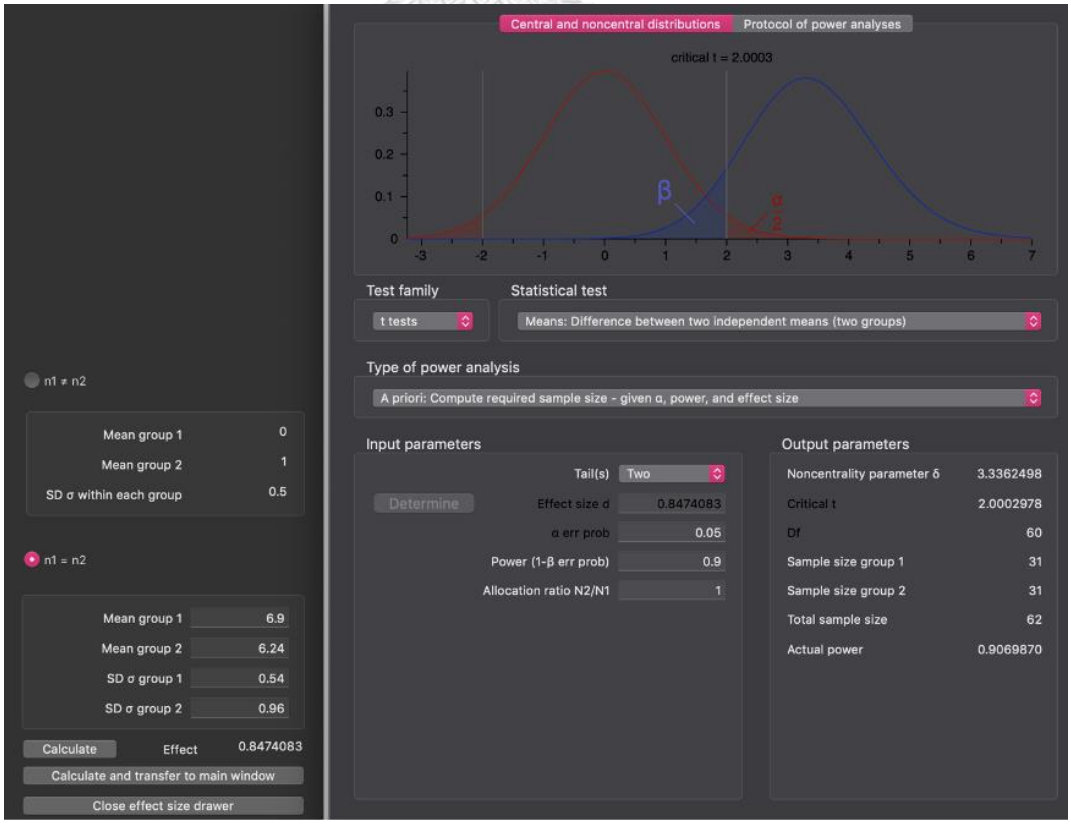
Sample sizes calculation

For determination of biofilm pH and ion release, the sample size was estimated using two independent means and the power is set at 90% and significance level at 5% (G*Power 3.1 Program). The data of similar study comparing final plaque pH (at 60 minutes) of 34-months age of

restoration between an ion-releasing composite resin (IRCR) and a universal composite resin (CR) was used to calculate. The means of pH ± SD of IRCR and CR were 6.90 ± 0.54 and 6.24 ± 0.96, respectively. [7] The total sample sizes was 62 (CN = 31, FZ = 31) (Fig. 6A).

For determination of remineralization (microhardness measurement), the sample size was estimated using ANOVA: repeated measures, within-between interaction and the power is set at 80% and significance level at 5% (G*Power 3.1 Program). The similar study compared dentin microhardness after restoring with various fluoride-containing restorative materials and resin composite, but the data did not show the variance, so the default values were used [46]. The medium effect size f of 0.25 is used to calculation. The number of groups was two (CN and FZ) and the number of measurements is three (before demineralization, after demineralization and after restoration). The total sample sizes are 28 (CN = 14, FZ = 14) (Fig. 6B).

A



B

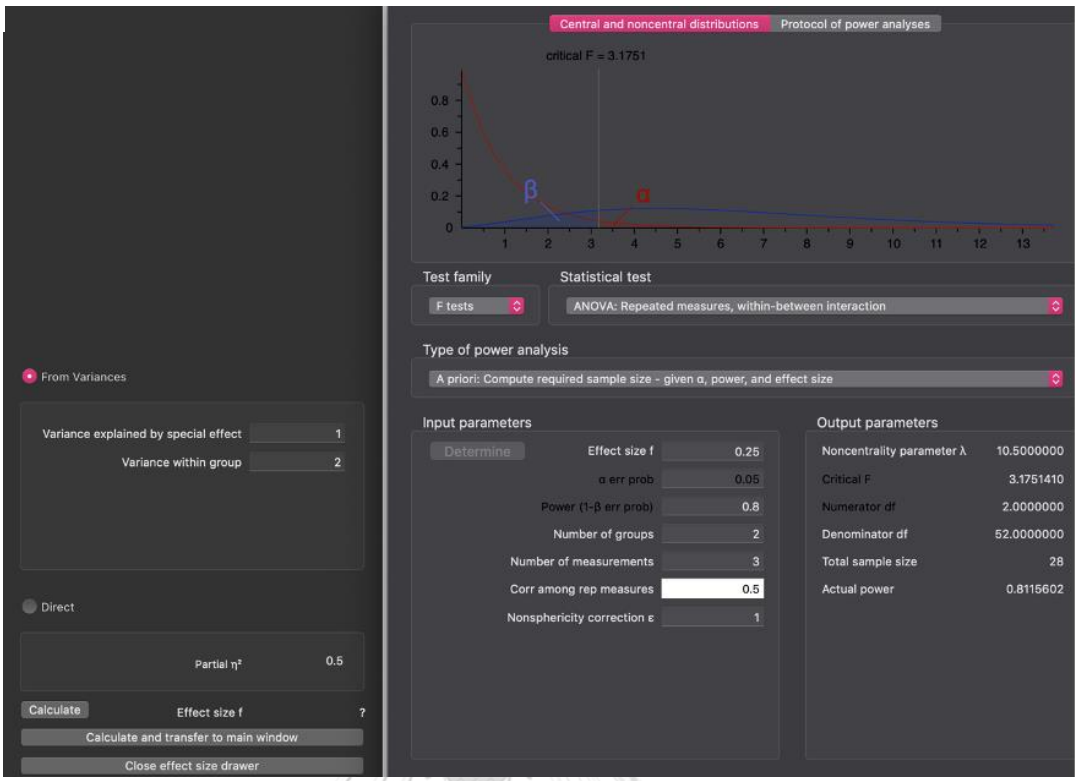


Figure 6 The sample size calculation using G*Power 3.1 Program. (**A**; The sample size calculation in for determination of biofilm pH and ion release, **B**; The sample size calculation in for determination of remineralization)

The study was approved by the Ethics Committee (HREC-DCU 2020-040) and Institutional Biosafety Committee (DENT CU-IBC 022/2020) of Faculty of Dentistry, Chulalongkorn University.

Determination of biofilm pH and ion release

Material preparation

Thirty-one specimens of each of two restorative materials: 1) alkasite (Cention® N; Ivoclar Vivadent, Schaan, Liechtenstein (CN)) and 2) resin composite (Filtek™ Z350XT; 3M ESPE, St. Paul, MN(FZ) (Table 3) were prepared. For CN, the powder bottle was shaken well,

the scoop provided overfilled with powder and the excess powder scraped off. The liquid bottle was held vertically and one drop of liquid extruded, ensuring that it was free of bubbles. One scoop of powder and one drop of liquid were mixed for 45-60 sec. The mixed CN and the FZ were placed in acrylic molds, 10 mm diameter x 2 mm deep [20], the molds placed on a glass slide and covered with a second glass slide to extrude excess material. The discs of FZ were cured for 40 sec from the upper surface using a curing light (Elipar^{TM}; 3M ESPE, St. Paul, MN) with a light intensity $\geq$ 500 mW/cm^2.

Determination of biofilm pH

Bacterial preparation

Streptococcus (*S.*) *mutans* UA159 from frozen stocks (-80°C) was prepared as described by Wongpraparatana *et al*. with some modifications [47], cultured in a brain heart infusion (BHI) on an agar plate and incubated at 37°C with 5% CO_2 for 24 h. The isolated colonies were inoculated in BHI broth and incubated at 37°C with 5% CO_2 overnight (16 h). The culture was adjusted to OD600nm of 0.1 as measured by a spectrophotometer (Pharmacia LKB Biotechnology Inc, Uppsala, Sweden) and was further incubated until reach the log phase (OD600nm = 0.5-0.6, approximately 3 h). The medium was changed from BHI broth to BHI broth supplemented with 5% sucrose for biofilm formation.

Biofilm formation and pH measurement

The 31 FZ and CN specimens were embedded in a polyvinyl siloxane putty impression material in 48-well plates, with only the upper surface exposed. The specimens and plate were sterilized by plasma sterilization (STERRAD®100NX; ASP^{TM}, Irvine, CA). 500 µl of the prepared log-phase culture was added to each well and the plate incubated at 37°C with 5% CO_2 for 24 h to form biofilm [47]. The medium was removed after 24 h, biofilms scraped off the specimens

using a cell scraper and transferred to a pH meter (LAQUA pH-22; HORIBA, Kyoto, Japan), pre-calibrated using standard buffer pH values of 4.01, 7.00, and 10.01 [48], to measure the pH (Fig. 7).

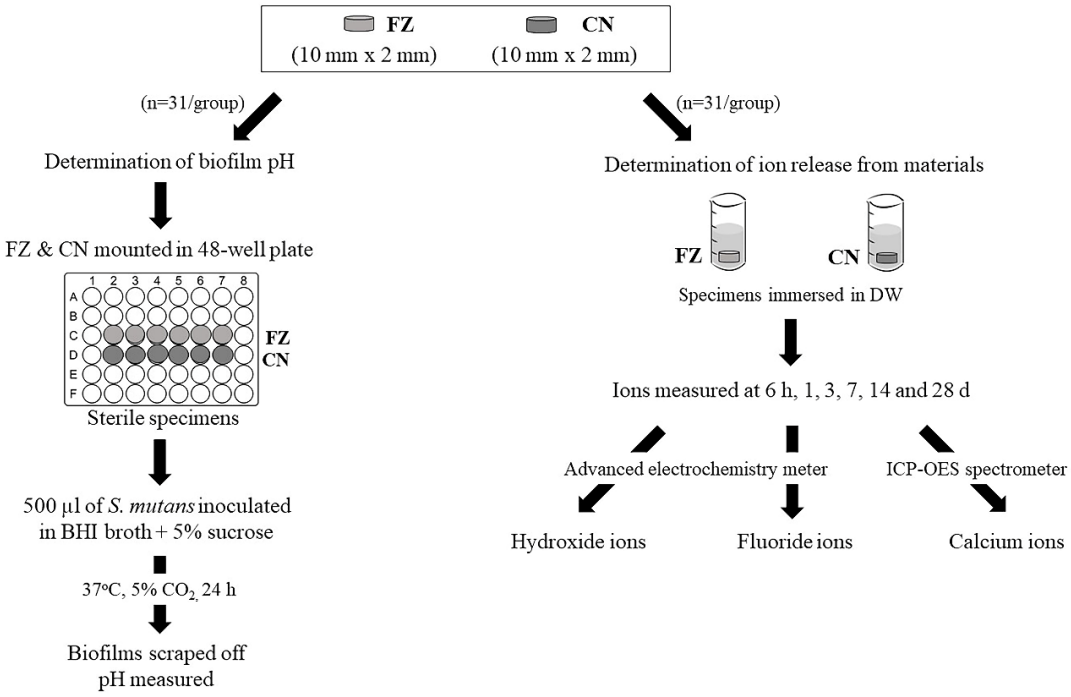


Figure 7 Flow diagram illustrating the determination of biofilm pH and ion release

Determination of ion release

Thirty-one of each of FZ and CN specimens prepared as described above were individually immersed in 10 ml deionized-water (DW) and incubated at 37°C (= specimen immersed DW) [49]. After 6 h of incubation, the specimen immersed DW was collected for ion measurement. Then, the specimens were re-immersed into 10 ml of fresh DW [49]. This procedure was repeated at 1, 3, 7, 14, and 28 d [20] (Fig. 7). The means and standard deviations (SD) of the amount of ions released were calculated at each time point.

Hydroxide ions

The pH of the testing solutions and DW were measured using the advanced electrochemistry meter in pH mode (Thermo Scientific™ Orion™ Versa Star™, Thermo Fisher Scientific, Waltham, MA). Before the measurement, the standard buffer pH 4.01, 7.00 and 10.00 were used for calibration. The pH of 2 ml of DW was measured as a baseline. Six ml of specimen immersed DW were collected at the assigned time points and divided equally into three plastic containers (2 ml for each) for pH measurement. Then, the molar (M) concentration of hydroxide ions ($[OH^-]$) was calculated based on the equations [31]:

$$pH + pOH = 14$$

$$pOH = - \log[OH]$$

$$[OH^-] = 10^{- pOH}$$

The concentration of hydroxide ion released by the materials was calculated using the formula:

$[OH^-]$ released from materials = $[OH^-]$ of specimen immersed DW - $[OH^-]$ of DW

The mean (and standard deviation) of hydroxide ion concentration was calculated for each time point.

Fluoride ions [32, 50]

Fluoride ion release was determined using an advanced electrochemistry meter in ion-selective electrode (ISE) mode (Thermo Scientific™ Orion™ Versa Star™). Fluoride standard 100 ppm (Orion Calibration Standard 940907) was diluted to three concentrations (0.1, 1.0, and 10 ppm) for ISE calibration. Three ml of specimen immersed DW were collected at the assigned time points and divided equally into three plastic containers (1 ml for each) for measurement. To determine fluoride released from the specimen, TISAB III solution (Orion Calibration Standard

940911) was mixed with specimen immersed DW in a 1:10 by volume ratio (0.1 ml: 1 ml) and the probe was then placed into the mixture to measure fluoride ion in ppm unit. The mean (and standard deviation) of fluoride ion concentration was calculated for each time point.

Calcium ions [51]

To prepare the calibration curve (CPS/ppm), a 1000 ppm calcium pure standard solution (CAS Number 7440-70-2, Perkin Elmer®, Waltham, MA) was diluted to 0.5, 1, 10, 50, and 100 ppm solutions and measured by ICP-OES spectrometer (Optimax 7300 DV, Perkin Elmer®). Centrifuge tubes were immersed in 10% nitric acid solution for 24 h at room temperature and rinsed with DW. Ten ml of specimen immersed DW was filtered through Grade 1 filter paper and transferred to the centrifuge tube for measurement. The specimen-immersed DW was measured 3 times by an ICP-OES spectrometer, which automatically calculated the average calcium ion concentration of the specimen-immersed DW in the counts per second (CPS) unit. The CPS units were then converted to ppm using the calibration curve.

Determination of remineralization

Tooth specimen preparation

Fourteen sound human extracted molars were collected from dental clinics, cleaned using pumice-water slurry with rubber cup and an ultrasonic scaler, immersed in 0.1% thymol solution at 37°C for 2 wk and used within 2 mth after extraction [52]. The teeth were sectioned horizontally approximately 3 mm above the cemento-enamel junction using a slow speed cutting machine (Isomet® 1000, Buehler, Lake Bluff, IL) with water coolant (Fig. 8). The occlusal section was discarded, and the radicular section embedded in epoxy resin in a 15 x 15 mm silicone mold, exposing only the cut surface (Fig 8). The cut surface was divided into four quadrants,

quadrants 1and 3 coated with nail varnish, and the specimen sectioned in half mesiodistally along the border of the nail varnish. Quadrants 2, 3 and 4 were immersed in demineralization solution (50 mM acetate, 2. 2 mM $CaCl_2 \cdot 2H_2O$ and 2. 2 mM KH_2PO_2; pH=4.6) for 96 h at 37°C [53] and washed in DW for 30 sec [54].

The FZ material was bonded to quadrant 2 following the manufacturer's instructions and using Clearfil™ SE Bond dentin bonding agent (Kuraray, Osaka, Japan). Briefly, the primer was applied to the cut surface for 20 sec and dried with a mild air flow. The bond was applied, spread with a mild airflow and light-cured for 10 sec (Elipar™ light; 3M, St Paul, MN). FZ was placed on the center of the cut dentin surface in a quarter circular metal mold, radius 7 mm, height 1 mm, and light-cured for 40 sec. The CN material was mixed as described in the material preparation subtopic, then bonded to quadrant 4 using the adhesive and mold as described for FZ, and light-cured for 40 sec. Specimens were immersed in DW at 37°C for 30 d [55].

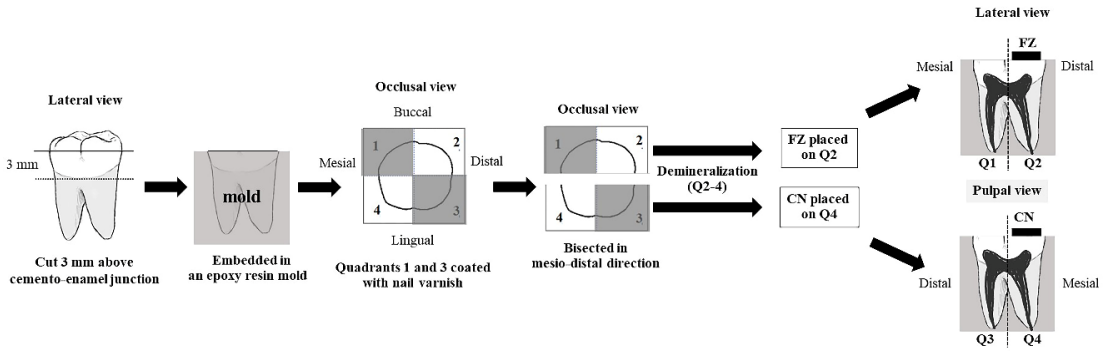


Figure 8 Specimen preparation. *Q1 = sound dentin; Q3 = demineralized dentin control; Q 2, and Q4 = chemically demineralized dentin with FZ and CN placed on their respective surfaces.*

Microhardness measurement

Microhardness was evaluated using Knoop indenter with a load of 25 g for 15 sec (FM-810, FUTURE-TECH, Kanagawa, Japan) [56]. Quadrants 1 and 3 were measured before and after demineralization respectively, and quadrants 2 and 4 measured 30 d after bonding the materials. Indentations were made at 20, 40 and 60 μm belowthe occlusal surface, and at the same depths, three spots 100 μm apart below the center of the materials (Fig. 9). The results were presented as the mean Knoop hardness number values (KHN) in the same row.

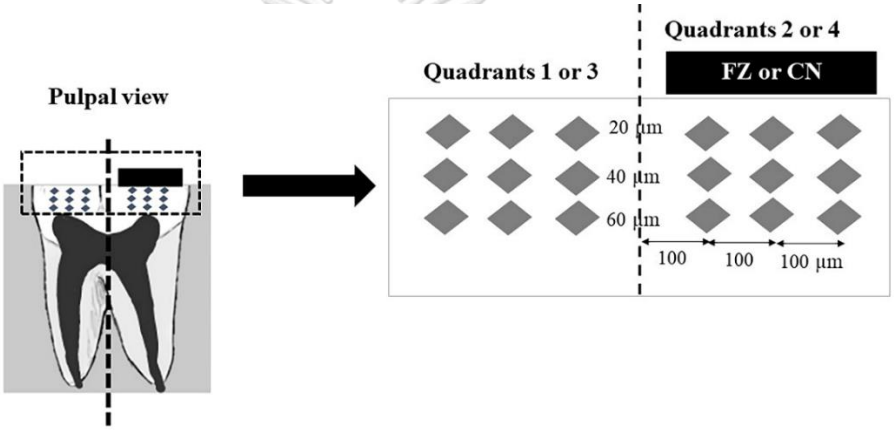


Figure 9 Microhardness measurement

Statistical Analysis

The statistical analysis was done by SPSS version 28.0 software (SPSS Inc, Chicago, IL), using an independent t-test to compare the pH of biofilms between the FZ and CN groups. Ion concentrations released from FZ and CN specimens at each time point were compared using Mann-Whitney U test, and ion concentrations released from the same material at different time points were compared using repeated measure ANOVA. The repeated measures ANOVA with Bonferroni correction for multiple comparisons were used to compare the microhardness of dentin across groups at 20, 40, and 60 μm depth. Statistical significance level was set at α = 0.05.

Chapter 4

Results

pH of biofilm

The pH of the *S. mutans* biofilm on the CN restoration was 4.45, which was significantly higher than the pH of the biofilm on the FZ restoration (4.06; $p<0.001$) (Fig. 10).

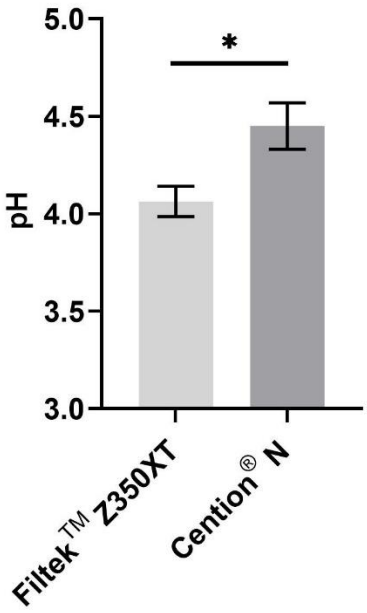


Figure 10 The pH of S. mutans biofilm formed on Filtek™ Z350XT (FZ) and Cention® N (CN) specimens. Each dot represents the pH of each specimen.

Hydroxide ion release

The hydroxide ions released from FZ and CN at each time point are shown in Figure 11. The hydroxide ions of FZ 6.4 ± 1.3, 2.4 ± 0.4, 13.3 ± 1.1, 11.4 ± 1.1, 12.4 ± 1.2, 28.7 ± 2.0 nM at 6 h, 1, 3, 7, 14 and 28 d, respectively, whereas those of CN were 176.4 ± 12.8, 261.9 ± 22.7, 443.4 ± 25.6, 618.6 ± 33.9, 1,033.5 ± 170.1, 5,621.0 ± 956.9 nM. Over the measured time

intervals, the hydroxide ions produced by FZ increased by approximately 70 nM, whereas the hydroxide ions produced by CN increased by approximately 8,000 nM. CN was significantly higher than FZ at each time point ($p<0.001$). When comparing the number of ions at different time intervals within the CN group, a statistically significant difference ($p<0.05$) was found at all periods except between 7 d and 14 d.

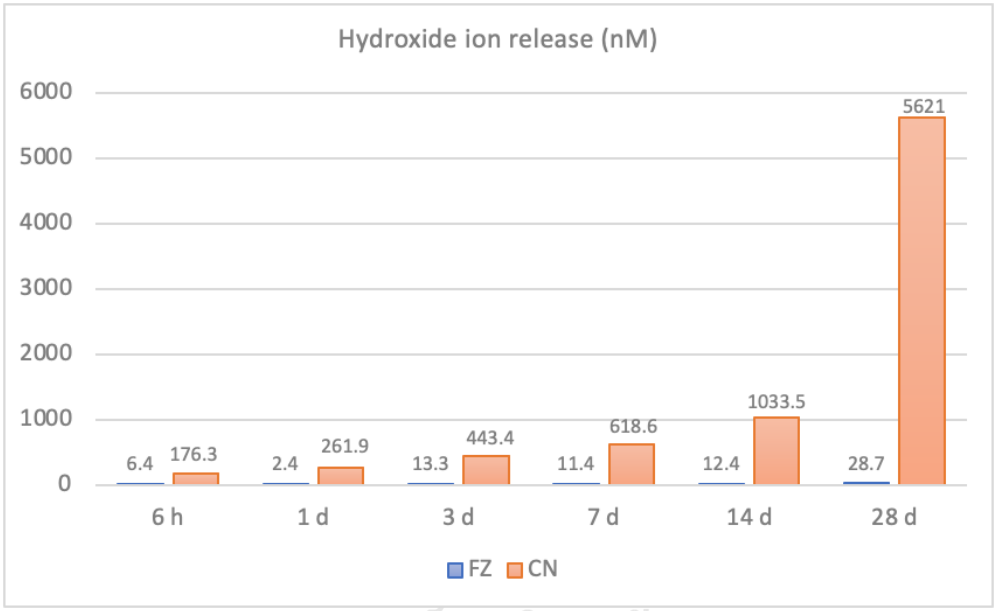


Figure 11 The mean hydroxide ions released from Filtek™ Z350XT (FZ; ■) and Cention® N (CN; ■) at six time points (6 hours, 1, 3, 7, 14 and 28 days).

Fluoride ion release

The fluoride ions released from FZ and CN at each time point are shown in Figure 12. The fluoride ions from FZ were 0.008 ± 0.001, 0.003 ± 0.000, 0.004 ± 0.000, 0.003 ± 0.000, 0.005 ± 0.000 and 0.004 ± 0.000 ppm at 6 h, 1, 3, 7, 14 and 28 d, respectively, whereas those from CN were 1.137 ± 0.059, 1.947 ± 0.075, 5.245 ± 0.355, 7.433 ± 0.530, 8.107 ± 0.852 and 10.457 ± 1.263 ppm, which were significantly higher than FZ at each time point ($p<0.001$).

Throughout the test period, CN continually released fluoride, accumulating a total of 34.3 ppm on day 28th. A statistically significant difference ($p<0.05$) was detected in both groups when comparing the amount of fluoride ions at different time periods within the CN group except between 7 d and 14 d.

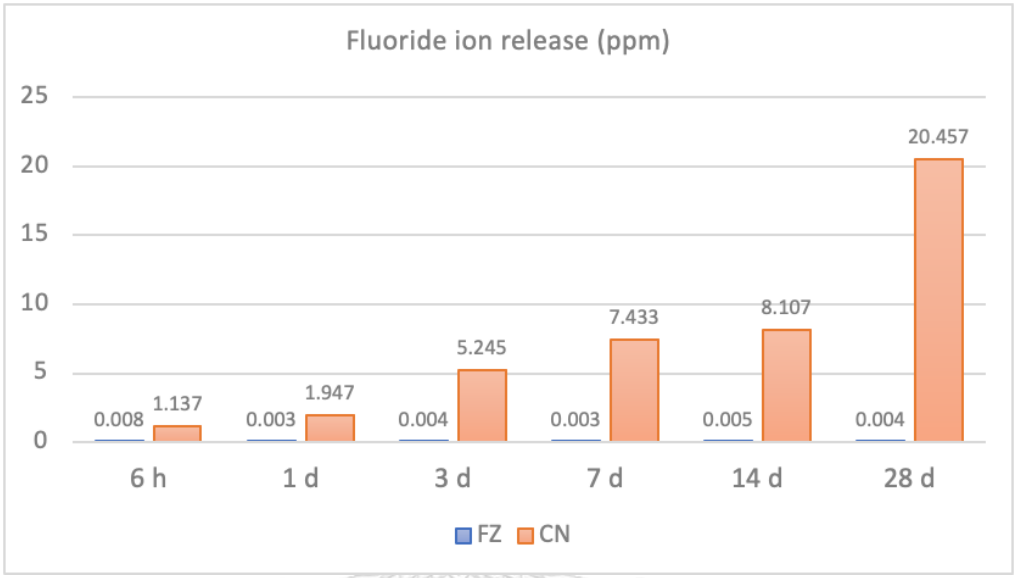


Figure 12 The means fluoride ions released from Filtek™ Z350XT (FZ; ■) and Cention® N (CN; ■) at six time points (6 hours, 1, 3, 7, 14 and 28 days).

Calcium ion release

The calcium ions released from FZ and CN at each time point was shown in Figure 13. The calcium ions of FZ were 0.140 ±0.023, 0.015 ± 0.002, 0.015 ± 0.004, 0.013 ± 0.002, 0.011 ± 0.001 and 0.023 ± 0.002 ppm at 6 h, 1, 3, 7, 14 and 28 d, respectively whereas those of CN were 3.570 ± 0.201, 6.824 ± 0.295, 14.725 ± 0.901, 18.823 ± 1.150, 20.510 ± 1.835 and 23.431 ± 2.496 ppm. At each time point, the calcium ions released from CN were significantly higher than those released by FZ ($p<0.001$); the total calcium ions from CN were 87.9 ppm after 28 days. A

statistically significant difference ($p<0.05$) was detected when comparing the amount of calcium ions at different time periods within the CN group except between 7 d and 14 d.

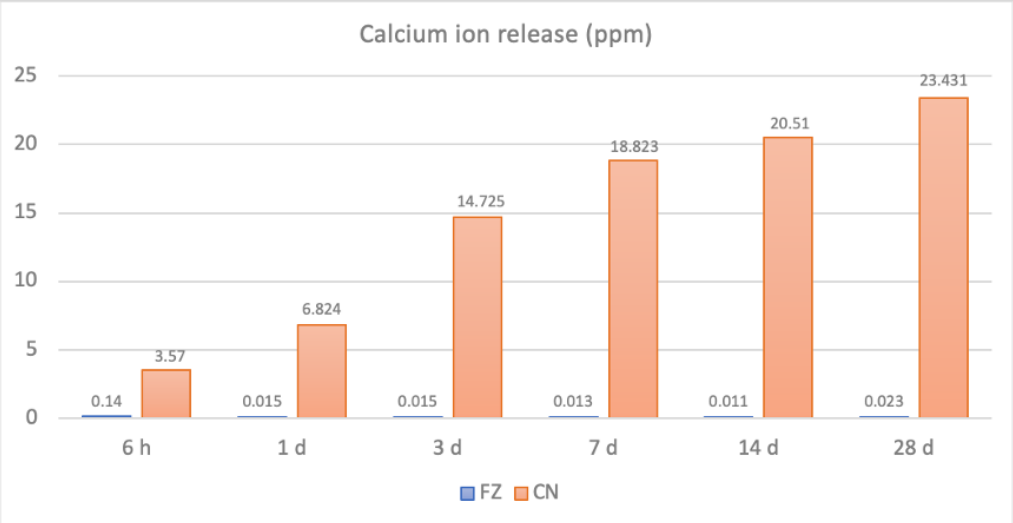


Figure 13 The mean calcium ions released from Filtek™ Z350XT (FZ;) and Cention® N (CN;) at six time points (6 hours, 1, 3, 7, 14 and 28 days).

Knoop microhardness

When the Knoop hardness number (KHN) was examined between depths of 20, 40, and 60 μm within each group,no difference was detected in all groups ($p>0.05$) except that the KHN of sound dentin at 60 μm was significantly higher than at 20 and 40 μm ($p=0.03$ and $p=0.02$, respectively). However, when the groups were compared at the same depth, only specimens in the CN group had a significantly higher KHN than that in the demineralization group at all distances ($p<0.05$), while the FN group specimens exhibited no difference (Fig. 14). Moreover, the KHN of the CN group specimens was significantly greater than that of the FZ group at 20 and 40 μm ($p<0.05$, Fig 14).

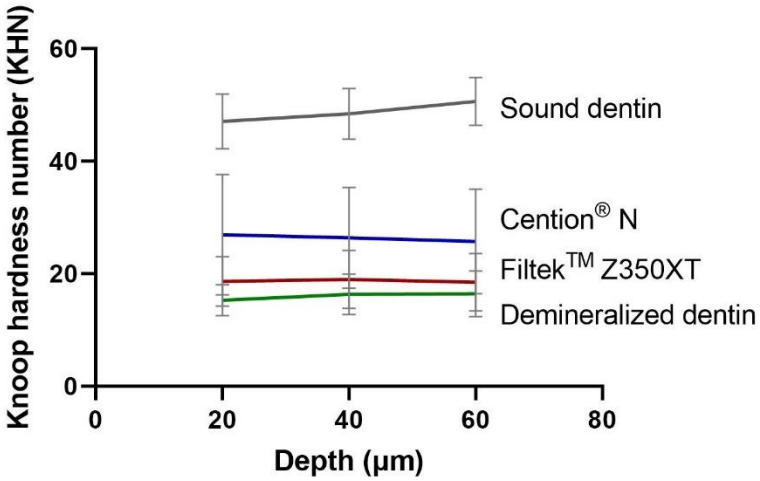


Figure 14 Mean value of Knoop hardness number (KHN) of dentin specimens at different depths.

Chapter 5

Discussion

This laboratory study revealed that the CN had the capacity to increase the pH of a biofilm and the KHN of demineralized dentin more than did FZ (negative control). These findings were supported by the determination of ion release. The release of hydroxide, fluoride, and calcium ions was significantly higher from CN than from FZ at all time intervals (6 h, 1, 3, 7, 14 and 28 d), suggesting that the alkaline glass filler (calcium fluoro-silicate glass) is responsible forthe substantial level of ions release from CN [6]. The releasing mechanism of ions is due to water absorption and degradation of the filler which may affect mechanical properties of CN [20, 57]. The detection of minute concentration of ions from the FZ groups may be due to some ionic impurities from the DW. DW used in this study was also measured the hydroxide, fluoride and calcium ions concentration, which were 8.9 nM, 0.006 ppm and -0.029 ppm, respectively. However, the measured concentration was lower than the limit of quantitation (LoQ), which implies the unreliable value of measurement [58].

Over the period of 28 d, we found that CN continuously released hydroxide ions with a final concentration that was approximately 5621 nM. The release of hydroxide ion contributes to the regulation of the pH of the biofilm. This pH-regulatory property is useful in current caries management, which focuses on environmental control to accommodate commensal microorganisms, rather than eliminating pathogens [5]. As a result, hydroxide ion release may help preventing demineralization by increasing the pH of the acidic biofilm. Although, this study found hydroxide ion released from CN can increase the pH level to 4.45, it did not reach a critical pH (5.5).

However, CN also released fluoride and calcium ions along with hydroxide ions which may promote caries prevention.

The results from present study showed that demineralized dentin subjacent to the CN restoration was higher in hardness compared to the control over a period of 30 d. Similarly, two previous studies using a nanoindenter and polarized light microscopy to detect the nanohardness and the demineralization inhibition effect respectively of CN at an enamel restoration margin, reported that the nanohardness of enamel adjacent to CN margin was not different from that when conventional glass-ionomer cement (GIC) was used [9, 59]. Furthermore, another study showed that the mean area of demineralization at the enamel and dentin adjacent to CN was significantly less than that adjacent to resin composite at 2 wk [9]. In our study, we demonstrated that the release of calcium and fluoride ions from the CN material may result in the higher microhardness of CN at every depth compared to the control.

CN continuously released not only hydroxide ions, but also fluoride and calcium ions over a 28-day period. These findings were consistent with previous fluoride release studies, which showed that CN consistently released fluoride ions throughout the experimental periods of 21, 42, and 168 d, with a cumulative concentration of around 4, 7 and 12 ppm, respectively [20, 21, 24]. In the same way, Tiskaya *et al.* [20] demonstrated that CN can continue to release calcium ions up to 6 wk, which corresponded with our findings.

Although the CN ion release profile found in our study was consistent with previous reports, the amount of released ions was not. The number of ions released from CN in the current study differed from the others because of various factors influencing the release such as specimen' s surface area, polymerization methods of materials (self-cured or light-cured),

and storage media, including immersion time. Williams *et al.* [60] reported that the surface area of conventional GIC specimens affected fluoride ion release, with the greater the surface area, the greater the amount of fluoride ions released. Moreover, Tiskaya's study [20] used 10 mm diameter and 1.2 mm thickness of CN specimen (surface area = 1.6 cm^2) which had smaller surface area than ours (surface area = 2.2 cm^2) and produced less fluoride and calcium ions. The number of ions released tends to increase in self-cured CN during immersion in an acidic media [20, 21]. It could be because light-cured polymerization results in a more tightly bound or less hydrophilic matrix, and a low pH solution induces more rapid degradation of glass filler and ion release when compared to a neutral solution [20, 21]. Several studies have shown that CN can release fluoride ions over long periods of time resulting in high fluoride accumulation [20, 21, 24, 25]. As a result, the amount of fluoride released is likely to be affected by the immersion time. CN has the ability to release fluoride ions, similar to conventional GIC and resin-modified glass-ionomer cement (RMGIC), nevertheless a difference in amount and pattern of fluoride ion release were found in a previous study [25]. Singh's study [25] showed that conventional GIC and RMGIC released the highest fluoride ions in day 1 due to the burst effect, and the fluoride release gradually decreased over time. However, CN released the least fluoride ions in day 1 and significantly more fluoride than conventional GIC and RMGIC at all other time periods. From the literature, the polymerized resin matrix in RMGIC can limit water diffusion into the cement and impede the release of fluoride ions, which may be the same for CN [57, 61]. However, several studies, including ours, found that CN continuously released ions over the total experimental time, which may be due to the presence of 24.6 % by weight alkaline filler in CN which is as much as one-third of all inorganic filler contained in CN [6]. Fluoride recharge is an important property of fluoride-releasing materials, and CN has been

found to have this property as well as conventional GIC [62]. The number of porosities in a set material probably affects the quantity fluoride released both before and after recharge, resulting in GIC and RMGIC having more fluoride recharge ability than resin-based materials such as CN [63].

In a clinical context, when acids from cariogenic biofilm demineralize enamel, calcium and phosphate ions are released and accumulate in saliva. When the pH rises, minerals from highly saturated saliva return to the demineralized enamel, resulting in net mineral gain and hydroxyapatite structure repair. For this reason, adequate calcium and phosphate levels are critical for preventing mineral loss during low pH periods and encouraging mineral gain when the pH returns to normal [27]. When fluoride is present, the critical pH for calcium and phosphate ion solubilization decreases. Fluoride ions can integrate into a tooth by replacing hydroxide ions in hydroxyapatite to form fluorapatite or fluoride-enriched hydroxyapatite, which has a lower solubility than hydroxyapatite and calcium-deficient hydroxyapatite [64]. Because of this, the release of fluoride and calcium from CN may improve the inhibition of demineralization and the facilitation of remineralization. Several laboratory studies have demonstrated a higher potential of GIC and RMGIC than non-releasing fluoride materials to inhibit demineralization at a restoration margin [65-67]. Clinically, however the results are conflicting [68]. As mentioned above, CN can inhibit demineralization at the enamel and dentin adjacent to restorations in a similar way to conventional GIC, based on two laboratory studies [9, 59]. Until now, the evidence that CN can inhibit secondary caries or remineralize demineralized enamel and dentin is still weak without clinical support, thus it is necessary for further laboratory and *in vivo* studies to confirm the results.

Chapter 6

Conclusions

With the limitations of this laboratory study, we concluded that Cention® N, an alkasite restorative material, released hydroxide, fluoride, and calcium ions, which may result in an elevation of the pH of the *S. mutans* biofilm and an increase in the hardness of demineralized dentin.

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Appendix A

Study protocol and consent form approval certificated of exemption

No. 033/2020

Study Protocol and Consent Form Approval
Certificate of Exemption

The Human Research Ethics Committee of the Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand has approved the following study to be carried out according to the protocol and patient/participant information sheet dated and/or amended as follows in compliance with the **ICH/GCP**

Study Title	: The effects of ions releasing from the alkasite material to pH of *Streptococcus mutans* biofilm and dentin remineralization: An *in vitro* study
Study Code	: HREC-DCU **2020-040**
Study Center	: Chulalongkorn University
Principle Investigator	: Ms. Pawinee Wiriyasatiankun
Protocol Date	: April 28, 2020
Date of Approval	: May 7, 2020
Date of Expiration	: May 6, 2022

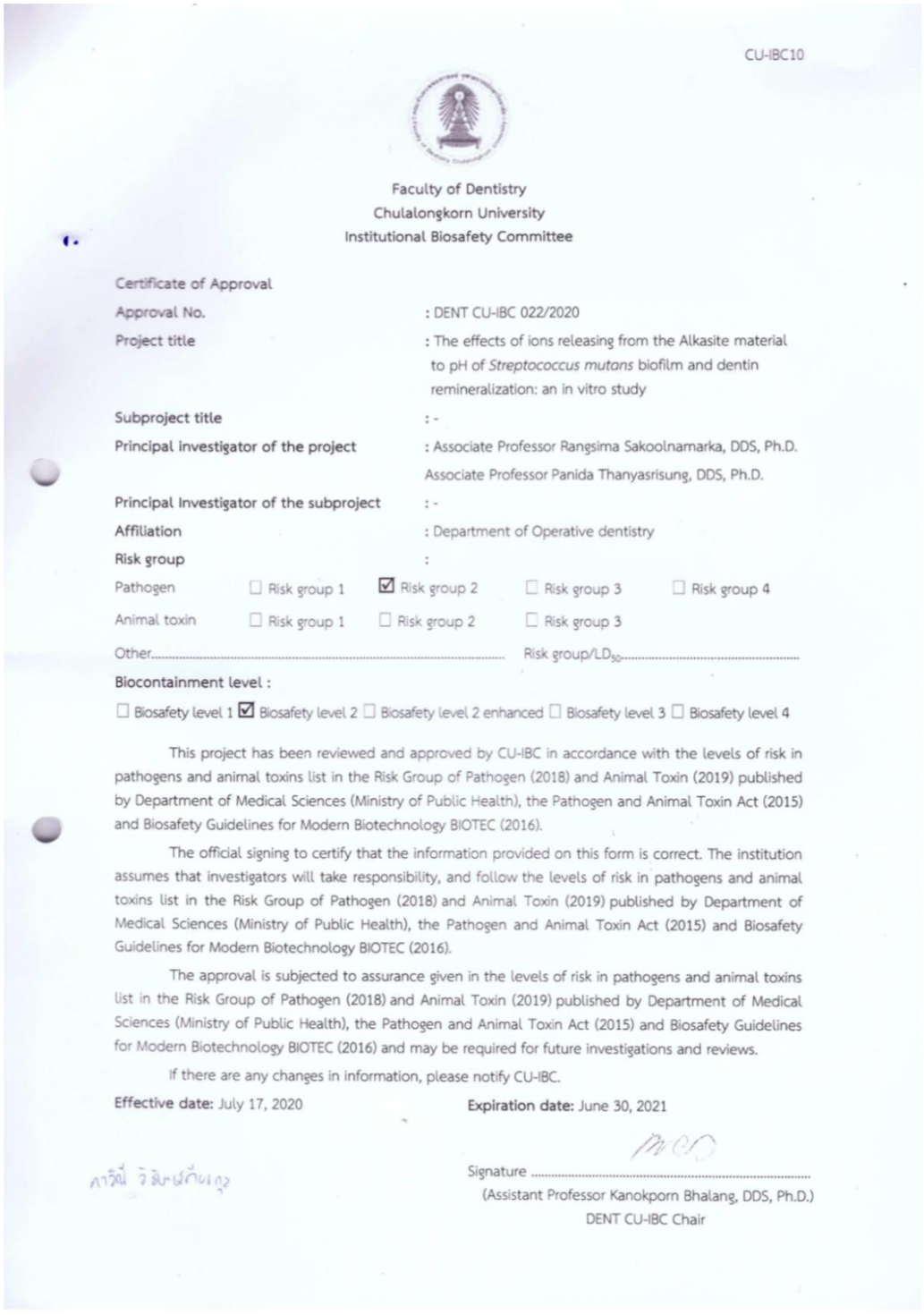
.....
(Associate Professor Dr. Kanokporn Bhalang)
Chairman of Ethics Committee
Associate Dean for Research

*A list of the Ethics Committee members (names and positions) present at the Ethics Committee meeting on the date of approval of this study has been attached (upon requested). This Study Protocol Approval Form will be forwarded to the Principal Investigator.

Approval is granted subject to the following conditions: (see back of the approval)

Appendix B

The certificate from the Institutional Biosafety Committee of Chulalongkorn University



CU-IBC10

Faculty of Dentistry
Chulalongkorn University
Institutional Biosafety Committee

Certificate of Approval

Approval No. : DENT CU-IBC 022/2020

Project title : The effects of ions releasing from the Alkasite material to pH of *Streptococcus mutans* biofilm and dentin remineralization: an in vitro study

Subproject title : -

Principal investigator of the project : Associate Professor Rangsima Sakoolnamarka, DDS, Ph.D.
Associate Professor Panida Thanyasrisung, DDS, Ph.D.

Principal Investigator of the subproject : -

Affiliation : Department of Operative dentistry

Risk group :

Pathogen ☐ Risk group 1 ☑ Risk group 2 ☐ Risk group 3 ☐ Risk group 4

Animal toxin ☐ Risk group 1 ☐ Risk group 2 ☐ Risk group 3

Other..... Risk group/LD_{50}.....

Biocontainment level :

☐ Biosafety level 1 ☑ Biosafety level 2 ☐ Biosafety level 2 enhanced ☐ Biosafety level 3 ☐ Biosafety level 4

This project has been reviewed and approved by CU-IBC in accordance with the levels of risk in pathogens and animal toxins list in the Risk Group of Pathogen (2018) and Animal Toxin (2019) published by Department of Medical Sciences (Ministry of Public Health), the Pathogen and Animal Toxin Act (2015) and Biosafety Guidelines for Modern Biotechnology BIOTEC (2016).

The official signing to certify that the information provided on this form is correct. The institution assumes that investigators will take responsibility, and follow the levels of risk in pathogens and animal toxins list in the Risk Group of Pathogen (2018) and Animal Toxin (2019) published by Department of Medical Sciences (Ministry of Public Health), the Pathogen and Animal Toxin Act (2015) and Biosafety Guidelines for Modern Biotechnology BIOTEC (2016).

The approval is subjected to assurance given in the levels of risk in pathogens and animal toxins list in the Risk Group of Pathogen (2018) and Animal Toxin (2019) published by Department of Medical Sciences (Ministry of Public Health), the Pathogen and Animal Toxin Act (2015) and Biosafety Guidelines for Modern Biotechnology BIOTEC (2016) and may be required for future investigations and reviews.

If there are any changes in information, please notify CU-IBC.

Effective date: July 17, 2020 **Expiration date:** June 30, 2021

Signature .....
(Assistant Professor Kanokporn Bhalang, DDS, Ph.D.)
DENT CU-IBC Chair

Appendix C

The result of determination of biofilm pH

N	pH (FZ)	pH (CN)
1	4.15	4.55
2	4.03	4.35
3	4.16	4.66
4	4.12	4.37
5	4.13	4.73
6	4.12	4.39
7	4.16	4.42
8	4.15	4.37
9	4.11	4.19
10	4.11	4.42
11	4.15	4.54
12	4.19	4.51
13	4.15	4.35
14	4.08	4.45
15	3.99	4.49
16	4.05	4.48
17	4	4.58
18	4.02	4.56
19	4.07	4.4
20	4.01	4.28
21	3.99	4.43
22	3.92	4.46
23	3.94	4.54
24	4.08	4.61
25	4.11	4.29
26	3.97	4.3
27	3.97	4.56
28	3.97	4.45
29	3.99	4.36
30	3.97	4.5
31	4.1	4.38

Appendix D

The result of determination of hydroxide ion release (FZ group)

N (FZ)	Hydroxide 6 h (nM)	Hydroxide 24 h (nM)	Hydroxide 3 d (nM)	Hydroxide 7 d (nM)	Hydroxide 14 d (nM)	Hydroxide 28 d (nM)
1	29.3	1.1	7.1	7.2	10.8	17.2
2	21.9	1.2	10.8	8.5	10.8	18.1
3	9.5	1.1	10.8	6.2	8.9	12.9
4	14.7	1.1	13.0	7.9	19.3	13.8
5	14.0	4.2	3.0	9.1	18.9	19.6
6	12.6	4.4	8.0	8.0	7.0	15.9
7	6.3	4.6	9.9	6.2	13.9	28.0
8	9.6	5.1	22.7	6.2	12.0	24.9
9	8.1	4.8	23.0	9.2	1.9	28.4
10	17.0	10.5	24.1	8.0	7.4	29.3
11	9.0	4.9	18.0	10.3	12.9	3.6
12	6.9	2.6	17.1	11.4	12.6	31.7
13	5.5	2.5	22.6	7.9	8.8	29.0
14	3.9	1.8	17.4	3.6	9.3	26.5
15	5.7	3.0	12.6	9.7	26.2	13.8
16	1.8	1.6	20.8	8.5	13.2	27.0
17	1.8	1.0	8.3	10.9	13.3	25.4
18	0.9	0.6	13.7	8.5	7.9	25.3
19	1.1	0.5	13.1	5.9	9.9	35.6
20	1.5	1.2	18.1	8.4	27.4	24.1
21	0.4	0.7	11.2	11.9	21.2	41.7
22	1.9	2.6	6.2	12.2	16.2	41.1
23	3.7	1.7	4.4	11.8	9.9	44.4
24	1.8	1.9	5.7	12.8	30.6	35.3
25	1.3	2.3	6.5	10.0	11.9	32.5
26	2.6	0.6	18.1	19.6	9.6	31.0
27	0.2	1.3	17.8	22.5	3.1	39.7
28	1.4	0.9	18.0	18.2	10.0	55.0
29	1.8	1.1	17.5	20.9	7.4	45.6
30	0.9	1.4	6.8	27.4	5.3	43.7
31	1.1	0.6	6.9	24.9	7.3	30.3

Appendix E

The result of determination of hydroxide ion release (CN group)

N (CN)	Hydroxide 6 h (nM)	Hydroxide 24 h (nM)	Hydroxide 3 d (nM)	Hydroxide 7 d (nM)	Hydroxide 14 d (nM)	Hydroxide 28 d (nM)
1	233.4	245.5	339.6	479.7	677.9	865.8
2	150.4	288.6	663.7	787.2	3190.7	7744.7
3	252.7	401.7	678.8	969.9	3358.1	9459.6
4	183.9	313.7	545.1	613.2	1861.4	7934.4
5	143.8	400.3	599.0	744.2	999.0	9632.4
6	451.9	513.2	677.9	721.9	1425.8	13094.6
7	163.6	446.7	598.5	939.7	2702.4	10876.5
8	139.1	366.6	608.0	903.7	2720.1	9399.4
9	178.0	431.5	683.3	952.7	946.6	8319.1
10	307.5	529.9	750.1	1071.3	3107.6	10236.6
11	220.5	454.5	558.0	823.0	962.5	996.1
12	307.4	306.8	318.0	473.5	398.7	307.5
13	101.3	277.8	359.9	470.8	456.3	563.2
14	136.8	312.3	411.2	540.7	622.2	8248.5
15	140.3	312.0	354.6	504.3	377.6	451.9
16	144.8	131.4	383.2	485.4	540.9	481.0
17	127.4	140.6	354.2	473.6	365.8	589.7
18	184.7	195.4	338.6	540.9	644.6	18786.2
19	161.4	133.1	418.2	492.4	544.9	431.3
20	179.0	146.2	380.2	551.5	710.3	896.5
21	133.8	162.3	411.2	699.9	549.6	9618.6
22	131.4	130.2	346.1	524.6	340.6	606.5
23	136.4	167.6	365.6	566.9	795.3	11048.8
24	132.4	141.3	302.6	545.1	575.5	2745.8
25	167.4	148.5	351.5	477.2	566.9	3539.8
26	154.6	148.4	317.5	480.9	477.2	829.9
27	163.7	224.4	332.9	549.4	365.6	15848.5
28	144.8	166.2	300.3	380.3	365.6	7582.2
29	150.6	157.1	305.2	411.3	359.9	1931.9
30	125.0	166.2	322.5	571.0	613.1	688.5
31	118.9	160.9	371.3	431.0	414.9	496.3

Appendix F

The result of determination of fluoride ion release (FZ group)

N (FZ)	Fluoride 6 h (ppm)	Fluoride 24 h (ppm)	Fluoride 3 d (ppm)	Fluoride 7 d (ppm)	Fluoride 14 d (ppm)	Fluoride 28 d (ppm)
1	0.018	0.008	0.005	0.007	0.008	0.009
2	0.014	0.008	0.004	0.005	0.006	0.008
3	0.011	0.006	0.004	0.005	0.005	0.006
4	0.011	0.005	0.004	0.005	0.005	0.006
5	0.010	0.004	0.004	0.004	0.004	0.005
6	0.008	0.004	0.003	0.004	0.004	0.005
7	0.008	0.003	0.003	0.004	0.004	0.004
8	0.003	0.003	0.003	0.004	0.004	0.004
9	0.010	0.003	0.003	0.003	0.004	0.004
10	0.008	0.003	0.003	0.003	0.003	0.004
11	0.007	0.003	0.003	0.003	0.003	0.004
12	0.006	0.002	0.003	0.007	0.003	0.004
13	0.006	0.002	0.003	0.006	0.004	0.004
14	0.006	0.002	0.003	0.005	0.003	0.004
15	0.005	0.002	0.003	0.002	0.003	0.004
16	0.005	0.002	0.003	0.003	0.013	0.004
17	0.005	0.005	0.003	0.003	0.008	0.004
18	0.005	0.001	0.003	0.003	0.007	0.004
19	0.018	0.004	0.003	0.003	0.006	0.004
20	0.012	0.004	0.003	0.003	0.005	0.003
21	0.009	0.001	0.007	0.003	0.005	0.003
22	0.007	0.002	0.005	0.003	0.005	0.003
23	0.006	0.001	0.004	0.003	0.004	0.003
24	0.006	0.004	0.004	0.003	0.004	0.003
25	0.006	0.002	0.003	0.003	0.003	0.003
26	0.005	0.004	0.003	0.002	0.003	0.003
27	0.005	0.002	0.004	0.002	0.003	0.003
28	0.004	0.001	0.003	0.002	0.003	0.003
29	0.004	0.003	0.003	0.002	0.003	0.003
30	0.004	0.001	0.003	0.002	0.003	0.003
31	0.004	0.001	0.008	0.002	0.003	0.003

Appendix G

The result of determination of fluoride ion release (CN group)

N (CN)	Fluoride 6 h (ppm)	Fluoride 24 h (ppm)	Fluoride 3 d (ppm)	Fluoride 7 d (ppm)	Fluoride 14 d (ppm)	Fluoride 28 d (ppm)
1	0.929	1.874	6.239	7.546	9.491	9.074
2	1.628	2.506	9.036	12.983	17.670	20.077
3	1.950	2.837	8.851	13.097	16.033	25.667
4	1.667	2.423	8.175	10.983	13.020	21.703
5	1.255	2.439	6.838	10.041	13.610	15.317
6	1.634	2.744	8.729	10.580	16.003	23.030
7	1.422	2.487	7.470	12.493	14.187	20.677
8	1.409	2.366	7.225	10.333	14.373	17.773
9	1.568	2.515	7.813	11.973	14.337	18.220
10	1.673	2.422	8.418	11.203	15.153	23.953
11	1.236	2.187	5.863	7.813	9.287	10.893
12	1.180	1.620	3.500	4.422	3.612	3.362
13	1.064	1.531	4.260	6.984	6.343	5.778
14	1.186	1.701	4.426	5.586	5.782	7.210
15	0.995	1.552	3.410	4.518	3.540	3.970
16	1.032	1.834	3.770	4.710	4.279	4.947
17	0.839	1.477	3.998	5.388	3.875	4.272
18	1.033	1.943	4.370	5.723	5.282	7.262
19	0.986	1.530	4.436	6.804	7.156	5.037
20	1.087	1.884	4.536	5.920	5.709	6.764
21	0.675	1.883	4.478	9.235	6.882	6.548
22	0.962	1.642	3.489	4.404	2.981	3.820
23	0.930	1.850	4.216	7.419	5.883	7.423
24	0.877	1.700	3.910	5.231	4.257	6.158
25	0.924	1.731	3.968	5.165	4.875	7.565
26	0.989	1.775	3.727	5.718	5.329	6.256
27	0.821	1.678	3.503	5.053	4.013	6.971
28	0.822	1.495	3.297	4.415	4.274	6.064
29	0.804	1.535	3.412	4.553	3.774	5.955
30	0.869	1.743	4.025	6.181	6.882	7.279
31	0.794	1.448	3.212	3.948	3.431	5.129

Appendix H

The result of determination of calcium ion release (FZ group)

N (FZ)	Calcium 6 h (ppm)	Calcium 24 h (ppm)	Calcium 3 d (ppm)	Calcium 7 d (ppm)	Calcium 14 d (ppm)	Calcium 28 d (ppm)
1	0.715	0.017	0.034	0.041	0.008	0.025
2	0.192	0.056	0.028	0.016	0.005	0.045
3	0.203	0.024	0.023	0.009	0.007	0.025
4	0.070	0.016	0.011	0.011	0.012	0.040
5	0.244	0.012	0.003	0.010	0.015	0.036
6	0.117	0.015	0.006	0.006	0.009	0.023
7	0.113	0.017	0.001	0.009	0.011	0.034
8	0.166	0.018	0.020	0.008	0.016	0.030
9	0.085	0.018	0.001	0.013	0.008	0.043
10	0.119	0.020	0.001	0.074	0.005	0.019
11	0.170	0.013	0.009	0.015	0.017	0.046
12	0.063	0.035	0.006	0.012	0.006	0.008
13	0.054	0.003	0.007	0.008	0.004	0.018
14	0.279	0.017	0.116	0.005	0.010	0.020
15	0.058	0.006	0.010	0.010	0.003	0.006
16	0.088	0.007	0.004	0.009	0.013	0.003
17	0.134	0.018	0.004	0.009	0.014	0.033
18	0.123	0.009	0.006	0.004	0.005	0.011
19	0.229	0.016	0.019	0.010	0.001	0.004
20	0.062	0.022	0.012	0.007	0.018	0.022
21	0.045	0.013	0.062	0.009	0.024	0.030
22	0.093	0.011	0.009	0.006	0.008	0.006
23	0.326	0.007	0.016	0.009	0.009	0.018
24	0.047	0.005	0.004	0.013	0.017	0.037
25	0.105	0.008	0.000	0.012	0.004	0.008
26	0.043	0.004	0.000	0.015	0.007	0.009
27	0.053	0.012	0.012	0.009	0.032	0.008
28	0.063	0.003	0.010	0.003	0.021	0.021
29	0.085	0.029	0.005	0.011	0.005	0.030
30	0.105	0.007	0.005	0.011	0.009	0.020
31	0.095	0.001	0.010	0.007	0.016	0.034

Appendix I

The result of determination of calcium ion release (CN group)

N (CN)	Calcium 6 h (ppm)	Calcium 24 h (ppm)	Calcium 3 d (ppm)	Calcium 7 d (ppm)	Calcium 14 d (ppm)	Calcium 28 d (ppm)
1	2.7	9.299	26.609	46.429	68.929	93.819
2	5.602	15.117	38.897	69.827	107.717	150.377
3	5.85	15.698	38.968	71.678	109.688	162.728
4	6.474	16.26	37.33	63.69	93.81	133.16
5	3.716	12.286	30.916	55.296	89.116	126.506
6	4.882	14.644	36.994	62.344	100.214	146.844
7	4.372	13.254	34.354	64.074	97.414	142.224
8	4.259	12.751	33.801	60.441	94.421	134.241
9	4.982	13.824	35.194	63.234	97.574	134.574
10	5.293	14.116	37.696	59.326	95.006	145.066
11	3.849	11.584	27.254	45.124	69.304	95.584
12	3.804	9.19	19.64	30.02	40.15	48.134
13	3.161	8.302	21.182	34.052	50.832	64.202
14	3.627	9.54	21.28	36.59	53.6	71.34
15	2.996	8.333	19.253	33.023	42.563	52.317
16	3.061	9.371	21.481	35.501	47.711	59.291
17	2.542	7.604	19.084	34.784	45.554	55.187
18	3.122	9.883	22.853	39.063	54.393	72.903
19	3.108	8.52	22.38	40.43	59.85	72.32
20	3.434	9.745	21.825	37.735	54.095	71.145
21	3.09	9.61	24.42	48.59	67.15	81.98
22	2.989	8.408	18.282	30.062	38.12	46.416
23	2.839	8.976	22.216	42.566	58.616	76.776
24	2.64	8.344	17.981	32.571	44.901	59.871
25	2.828	8.738	19.578	34.058	48.058	67.388
26	2.99	8.649	18.819	34.089	49.249	64.339
27	2.403	8.247	18.257	32.367	43.597	60.657
28	2.471	7.618	18.328	30.938	42.838	57.628
29	2.485	7.992	19.162	31.982	42.462	56.622
30	2.69	8.826	18.976	35.686	54.996	72.996
31	2.419	7.482	15.69	26.65	36.009	47.669

Appendix J

The result of determination of remineralization

N	Sound dentin (depth 20 μm) (KHN)	Sound dentin (depth 40 μm) (KHN)	Sound dentin (depth 60 μm) (KHN)	Demineralized dentin (depth 20 μm) (KHN)	Demineralized dentin (depth 40 μm) (KHN)	Demineralized dentin (depth 60 μm) (KHN)	CN (depth 20 μm) (KHN)	CN (depth 40 μm) (KHN)	CN (depth 60 μm) (KHN)	FZ (depth 20 μm) (KHN)	FZ (depth 40 μm) (KHN)	FZ (depth 60 μm) (KHN)
1	46.213	50.440	52.613	15.537	19.037	20.203	23.910	24.323	21.753	19.337	15.937	15.120
2	52.603	52.257	57.257	17.503	18.347	17.420	33.037	29.527	24.510	17.783	19.167	18.960
3	46.757	48.217	50.263	15.827	16.013	15.407	56.873	47.180	42.683	13.287	12.650	16.040
4	45.167	43.103	43.517	19.507	23.567	25.840	32.063	32.797	31.543	23.350	23.703	24.433
5	50.980	53.317	56.293	15.680	15.637	16.033	36.757	35.187	35.410	29.690	32.220	30.657
6	41.000	48.580	53.793	12.283	14.573	15.223	17.710	16.253	16.303	16.923	18.183	16.800
7	56.887	55.857	54.000	17.120	18.037	17.457	31.910	35.643	41.553	19.617	21.203	21.417
8	41.993	42.947	45.810	18.640	20.647	20.247	21.500	24.717	33.070	16.110	15.957	14.553
9	46.380	45.963	47.260	15.480	12.273	11.927	20.553	18.457	20.127	11.170	10.803	9.403
10	43.707	42.017	49.743	12.380	12.780	12.367	19.040	15.543	17.773	15.517	16.173	14.693
11	47.070	50.977	51.067	13.790	14.080	15.143	15.943	19.780	17.600	19.767	21.607	19.997
12	49.640	54.010	54.230	16.257	16.753	16.587	24.443	27.880	23.337	18.217	17.527	16.290
13	39.210	45.163	45.837	14.873	17.387	17.373	23.407	19.907	16.647	19.530	20.430	18.933
14	51.183	45.190	47.107	8.973	9.567	8.983	19.910	21.963	18.067	20.147	20.193	21.120

VITA

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