# EFFECTS OF SILVER NANOPRISMS CONTAINING ACRYLIC SOFT LINING MATERIAL ON ANTIMICROBIAL ACTIVITY, PHYSICAL AND MECHANICAL PROPERTIES

A THESIS

BY

KAMONWAN KHUMSUP

Presented in Partial Fulfillment of the Requirement for the

Master of Science Degree in Prosthodontics

at Srinakharinwirot University

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AN ABSTRACT

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**Objective**: The purpose of this study was to evaluate water sorption, solubility, hardness and antimicrobial effect of silver nanoprisms-containing acrylic soft lining material against *Staphylococcus aureus* and *Candida albicans.*

Methods: In this in vitro experimental study, the specimens of Visco gel (acrylic soft lining materials) mixed with silver nanoprisms were divided into 5 groups ( 0, 25, 50, 100 and 200ppm of silver nanoprisms) and were immersed in distilled water for 1,4,7and 14 days. The antimicrobial properties were determined via turbidity test, which was measured by spectrophotometer at 530 nm. For water sorption and solubility test, disc-shaped specimens (50x0.5mm) were fabricated from stainless steel mould. For the hardness test, cylindrical specimens (28x6mm) were fabricated. The hardness was determined using a Shore A durometer. The results were analyzed by using two-way ANOVA and a tukey HSD test.

**Results**: The percent of *S.aureus* reduction ranged between 6.15-43.14%. The antibacterial effect of 50ppm group was highest followed by 25ppm, 100ppm, 200ppm and 0ppm group, respectively. The percent of *C.albicans.* reduction ranged between 12.84-58.52%. The maximum antifungal efficacy was 50 ppm. The maximum antimicrobial efficacy was 1 day.

**Conclusions**: The silver nanoprisms containing acrylic soft lining material demonstrated ability to inhibit the growth of *S. aureus* and *C. albicans*. The addition of 25 ppm of silver nanoprisms effected antimicrobial activity. An increase in silver nanoprisms concentration resulted in an increased hardness and a decrease in both water sorption and water solubility.

# ผลของการเติมสารซิลเวอร์นาโนพริซึมในวัสดุเสริมฐานฟันเทียมแบบนุ่ม ต่อการยับยั้งเชื้อจุลินทรีย์และผลต่อคุณสมบัติทางกายภาพและทางกล

บทคัดย่อ

ของ

กมลวรรณ คำทรัพย์

เสนอต่อบัณฑิตวิทยาลัย มหาวิทยาลัยศรีนครินทรวิโรฒ เพื่อเป็นส่วนหนึ่งของการศึกษา
ตามหลักสูตรปริญญาวิทยาศาสตร์มหาบัณฑิต สาขาวิชาทันตกรรมคลินิก
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**วัตถุประสงค์** เพื่อประเมินผลของการเติมสารซิลเวอร์นาโนพริซึมในวัสดุเสริมฐานฟันเทียมแบบนุ่ม ต่อการยับยั้งเชื้อจุลินทรีย์*( Staphylococcus aureus* and *Candida albicans)* และผลต่อคุณสมบัติทางกายภาพในด้านการดูดซึมน้ำ การละลายตัว และคุณสมบัติทางกลในด้านความแข็งของวัสดุ

**วัสดุอุปกรณ์และวิธีการ** ชิ้นงานในการทดลองเตรียมจากวัสดุเสริมฐานฟันเทียมแบบนุ่มยี่ห้อวิสโคเจลผสมกับสารซิลเวอร์นาโนพริซึมที่ความเข้มข้นต่างๆ(0,25,50,100,200 ppm) และแบ่งกลุ่มแช่ในน้ำกลั่นที่เวลาต่างๆ(1,4,7,14วัน) การยับยั้งเชื้อจุลินทรีย์ทดสอบโดยการวัดความขุ่น (turbidity test) โดยเครื่องสเปกโตรโฟโตมิเตอร์ที่ความยาวคลื่น530นาโนเมตร การวัดคุณสมบัติการดูดซึมน้ำ การละลายตัวนั้น ใช้แม่แบบสแตนเลสเตรียมชิ้นงานที่มีลักษณะเป็นแผ่นกลมขนาดเส้นผ่านศูนย์กลาง 50 มม. หนา 0.5 มม. ส่วนการวัดความแข็งของวัสดุนั้นใช้แม่แบบพีวีซีเตรียมชิ้นงานที่มีลักษณะเป็นแท่งกลมขนาดเส้นผ่านศูนย์กลาง 28 มม. หนา 6 มม. และวัดค่าความแข็งโดยใช้ Shore A durometer วิเคราะห์ข้อมูลทางสถิติโดยใช้การวิเคราะห์ความแปรปรวนสองทางและการทดสอบทูคีย์ที่ระดับนัยสำคัญ 0.05

**ผลการศึกษา** ร้อยละการลดลงของเชื้อแบคทีเรียอยู่ในช่วง 6.15-43.14 พบว่าในกลุ่ม 50ppm ยับยั้งเชื้อแบคทีเรียสูงสุด ตามด้วยกลุ่ม 25ppm, 100ppm และ 200ppm ร้อยละการลดลงของเชื้อราอยู่ในช่วง 12.84-58.52 และพบว่าในกลุ่ม 50ppm ยับยั้งเชื้อราสูงสุด ร้อยละการลดลงของเชื้อแบคทีเรีย การยับยั้งเชื้อจุลินทรีย์เกิดขึ้นมากสุดในวันที่หนึ่ง

**บทสรุป** การเติมสารซิลเวอร์นาโนพริซึมในวัสดุเสริมฐานฟันเทียมแบบนุ่มมีผลต่อการยับยั้งเชื้อจุลินทรีย์ ในกลุ่มที่เติมสารซิลเวอร์นาโนพริซึม 25ppm มีผลต่อการยับยั้งเชื้อแบคทีเรีย เมื่อเพิ่มปริมาณสารซิลเวอร์นาโนพริซึมในวัสดุทำให้วัสดุมีคุณสมบัติในด้านการดูดซึมน้ำ การละลายตัวลดลงและวัสดุมีความแข็งเพิ่มขึ้น

The thesis titled

"Effect of silver nanoprisms-containing acrylic soft lining material on antimicrobial activity, physical and mechanical properties "

by

Kamonwan Khumsup

has been approved by the Graduate school as partial fulfillment of the requirement for the Master of Science degree in Prosthodontics of Srinakharinwirot University.

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From

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# CHAPTER 1

# INTRODUCTION

## Background and rational

Fixed or removable completed denture is an alternative treatment for replacement of the missing teeth, and resin acrylic is the most common material for a denture base, which is a conventional hard material. Although this, hard denture base has been used widely, it has been notified that it cannot tolerate to a cushion functional force of thin edentulous ridge, non-resilient mucosa and severe alveolar resorption or extensive bony prominence. In addition, gradual bone resorption, which occurs after wearing the completed denture, may cause denture instability and denture stomatitis. Accordingly, soft lining materials are applied to improve fit of the denture after tissue abused. They have been extensively used for relining denture to enhance the recovery of denture bearing area from trauma, damage or destruction and get accurate adaptation to the edentulous ridge.

Soft lining material can be classified into two main categories, temporary soft lining materials and permanent soft lining materials. Temporary soft lining materials will remain soft texture for a limited period (days to weeks) therefore it can be temporarily used as a rebasing or relining material of completed denture. In some cases, the temporary material was used for functional impression of immediate denture, immediate surgical splint and cleft palate speech aids.[1] However, the long term application of this soft lining material has shown the evidence of adverse effects in clinic.

The main problems are related to the pathogens and the deterioration of soft lining materials. Particularly, the microorganisms may adhere to the surface of the soft lining material prior penetrating into the material.[2] *Candida albicans* has been reported to colonize on the soft lining material .[2-4] and some studies have found that yeast infection can occur after wearing soft lined lower denture and conventional lower denture. Moreover,

fungal growth can be detected not only in the soft lining denture but also at the mucosa of patients.[3, 4] Likewise, the respiratory infection in elderly patients can be resulted from the a colonization of *Staphylococcus aureus* on denture.[5] Therefore, the modification of soft lining material with antimicrobial agents may overcome these problems

A silver nanoparticle consists of various sizes silver atoms or ions in the range from 1 to 100 nm and, in accordance to the small size, this nanoparticle is able to invade bacteria and many organisms.[6] The invasion of the nanoparticles will disrupt structural and morphological integrity of the cells, which can lead cell death. In dentistry, it has been reported that silicone soft lining materials modified with silver nanoparticles can reduce *Candida* colonization and showed a high degree of antimicrobial effects.[7-10] However, the overuse of silver nanoparticle in the soft lining materials may result in a decrease in hardness and bond strength of the soft lining materials, an increase in sorption and solubility during application and a change in the failure type of the samples. [9]

The purpose of this study, therefore, was to evaluate the antimicrobial effects of acrylic soft lining material containing silver nanoprisms against *Candida albicans* and *Staphylococcus aureus*. In addition, we aimed to assess whether the incorporation of silver nanoprisms influences the mechanical and physical properties of acrylic soft lining material.

## Purpose of this study

1. To study antimicrobial effects against *Candida albicans* and *Staphylococcus aureus* of acrylic soft lining material containing silver nanoprisms.
2. To evaluate the physical properties on the water sorption, water solubility and surface hardness of acrylic soft lining material containing silver nanoprisms.

## Significance of the research

The addition of silver nanoprisms to soft lining materials may reduce microbial growth on soft lining denture and improve the mechanical property and physical properties of acrylic soft lining material.

## Limitation of this study

This study is based on laboratory experimental research.

## Definition of terms

Silver nanoprisms

Silver nanoprisms is a blue liquid substance with antimicrobial property. Silver nanoprisms were prepared from silver nitrate ($AgNO_3$), sodium borohydride ($NaBH_4$), soluble starch, trisodium citrate dihydrate, and hydrogen peroxide (($H_2O_2$). The sizes of the silver particles of between 30-120 nm in size with various morphologies including circular disk, truncated triangle and hexagon.

Water sorption

The amount of water which is absorbed by acrylic material if it is immersed in water for a stipulated period of time. The unit of measurement is $mg/cm^2$.

Water solubility

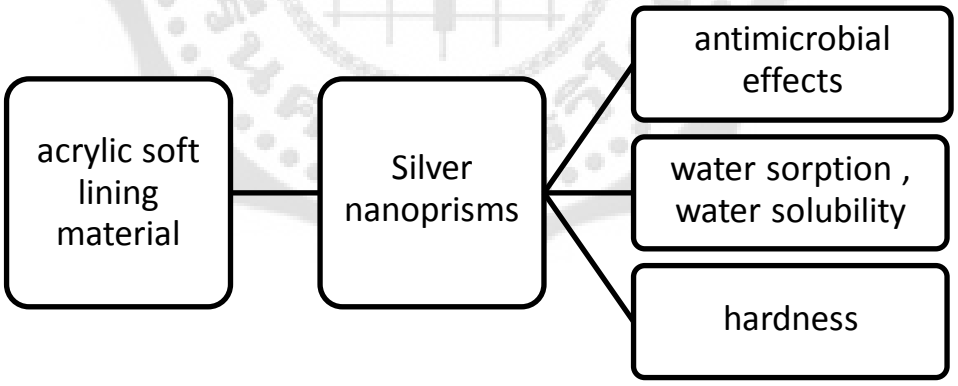
The property of solid, liquid, or gaseous chemical substance that is able to dissolve in a water or solvent. The unit of measurement is $mg/cm^2$.

Hardness

A property of solid material that is able to resist a permanent shape change when a force is applied. The durometer is instrument for measuring the indentation hardness of rubber or rubber-like materials.

## Conceptual framework

The soft lining materials modified by silver nanoprisms have antimicrobial effect. In addition, the soft lining materials containing silver nanoprisms can affect water sorption, water solubility and hardness.




## Variable for this study

In an experiment, the independent variable is the concentration of silver nanoprisms in acrylic soft lining material and dependent variables are antimicrobial effect, water sorption, water solubility and hardness.

## Hypothesis for this study

### 1. Antimicrobial effect

$H_0$ : There is no significant differences on antimicrobial effect between acrylic soft lining material and acrylic soft lining material containing silver nanoprisms.

### 2. Water sorption and water solubility

$H_0$ : There is no significant differences on sorption and solubility between acrylic soft lining material and acrylic soft lining material containing silver nanoprisms.

### 3. Hardness

$H_0$ : There is no significant differences on hardness between acrylic soft lining material and acrylic soft lining material containing silver nanoprisms.

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# CHAPTER 2

# LITERATURE REVIEW

An acrylic resin is a common material for denture base construction because it has many advantages such as esthetic property, ease of use, inexpensive fabrication and maintenance. The denture base made by resin acrylic is so called a conventional hard denture base. Although this hard denture base has been widely accepted for prosthetic treatment, it has shown some drawbacks and undesirable effects. One of the problems is to use with edentulous patients, whose alveolar ridges cannot be tolerate to cushion functional force due to a presence of thin and non-resilient mucosa, severe alveolar resorption and an extensive bony prominence. In addition, a natural resorption of edentulous ridge finally results in loose denture, loss of its stability and denture sore. Accordingly, the soft lining materials are suggested to apply on the mucosal surface of the denture in order to improve fit of the existing denture base. The use of soft relining materials for relining denture is enhance the recovery of denture bearing area from trauma and ridge destruction, as well as, the denture can achieve an accurate adaptation to the edentulous ridge.

## Background knowledge

### 1. Type of soft lining materials

Soft lining materials can be classified into two main categories: (1) temporary soft lining materials and (2) permanent soft lining materials.[1] Both types suite for a different purpose as the temporary material made for a provisional usage during repair or renew of the denture base while the permanent type for lifelong of the denture. The temporary type has only one form, whilst the permanent type constitutes of four subgroups according to composition and processing method: (a) heat polymerized silicone, (b) autopolymerizing

silicone, (c) heat polymerized acrylic resin, and (d) autopolymerizing acrylic resin[10]. This information has shown in Table 1.

Table 1 Soft lining materials [7, 11-17]

| Categories /Processing method | Brand name | Manufacturer |
|---|---|---|
| Temporary soft lining materials (tissue conditioners) | | |
| autopolymerizing acrylic resin | Hydro-Cast | Kay-See dental Mfg.Co.,USA |
| | Visco-Gel | DeTrey Division,Densply,England |
| | GC soft | GC dental Industrial Corp,Japan |
| | Coe soft | GC,Coe Laboratories, Chicago,USA |
| | Dura Conditioner | Reliance Dental Mfg Co, Illinois |
| | Coe comfort | Coe Laboratories Inc,Chicago,Illinois |
| | Ivoseal | Ivoclar AG,Lichtenstein |
| | Shofu Tissue conditioner | Shofu Inc, Kyoto, Japan |
| | Softone | Harry J. Bosworth, Skokie, Illinois |
| Permanent soft lining materials | | |
| Autopolymerizing acrylic resin | Soften | Kamemizu Chem,Japan |
| | Nissin Soft Reverse | Nissin Dental product Inc,Japan |
| | Soft Oryl | Teledyne Dental,Getz-Opotow,Illinois |
| | Permasoft | Myerson,Chicago,Ill |
| | Ever-soft | Myerson, Austenal, Chicago, Illinois |
| Heat polymerized acrylic resin | Coe supersoft | Coe Laboratories Inc,Chicago,Illinois |
| | Vertex soft | Vertex-Dental BV, Zeist, Netherlands |

Table 1 (continued)

| Categories /Processing method | Brand name | Manufacturer |
|---|---|---|
| Autopolymerizing silicone | Mollosil | Detax Karl Huber GmbH,Germany |
| | Evatouch | Neo dental chemical product,Japan |
| | Tokuyama soft relining | Tokuyama Corp,Japan |
| | GC relinine | GC dental Industrial Corp,Japan |
| | Flexibase | Flexico Developments Ltd,London |
| | Silagum Comfort | DMG, Hamburg, Germany |
| | Ufi gel SC | VOCO GmbH, Cuxhaven, Germany |
| Heat polymerized silicone | Molloplast-B | Detax Karl Huber GmbH,Germany |
| | Per-fit | Dental product unlimited, Idaho |
| | Luci-sof | Dentsply, woodbridge, Canada |

2. Composition of soft lining materials

The temporary soft lining is a product of resin acrylic which contains non cross-linked amorphous polymers without initiator and monomer. On the other hand, the permanent soft lining could be compose of silicone or acrylic resin, which is a cross-linked amorphous polymers.

In general, the compositions of acrylic resin and silicone soft lining are different. The acrylic resin soft lining mainly consists of polymer powder (poly ethyl methacrylate), monomer (methyl methacrylate, n-butyl methacrylate, 2-ethoxyethyl methacrylate, lauryl methacrylate, ethyl methacrylate) and plasticizer(di-n-butyl phthalate, Butyl phthalyl butyl gylcollate, 2-ethyl-hexyl di-phenyl phosphate). Meanwhile, silicone soft lining material consists of polymer ($\alpha$-$\omega$-dihydroxy end blocked polydimethyl siloxane), cross-linking agent (triethoxy silanol, ethyl poly silicate, tetraethoxy silane, methyltriacetoxy silane, acryloxyalkyl

silane) and catalyst (dibutyltin dilaurate, stannous octoate, moisture, heat+benzoyl peroxide) [13, 18]

### 3. Properties of soft lining materials

Each type of soft lining has different properties for serving the different purposes. They can be auto or heat polymerized material, or soft, hard and semi-hard. The consideration should decide on both chemical and physical properties. Some soft linings are modified with additive agents to improve basic material quality for specific aim.

A previous study reported that autopolymerized silicone was not suitable for using as a permanent soft lining materials. The disadvantages of autopolymerized silicone is that water absorption is high and its chemical composition can irritate underlying tissue. Furthermore, the autopolymerized silicone has dimensional instability, poor rupture properties and poor wettability. In contrast, a heat polymerized silicone is proper to use as a permanent soft lining materials since the water absorption and water solubility is very low, and have excellent elastic properties. However, the heat polymerized silicone poorly adheres to denture base when it is applied as a liner.[13]

Acrylic resin materials can be used as the temporary materials (tissue conditioners) and the permanent materials. Temporary material remains soft for a limited period (days to weeks) and it is used for recovering soft tissue before fabricating a new denture or rebasing or relining denture. In addition, it can be used for functional impression of immediate denture, immediate surgical splint and cleft palate speech aids.[1]

A different between acrylic-based and silicone-based material is a main component inside the material that modify its property. The plasticizer is added into the acrylic soft lining in order to reduce the transition temperature of the polymer from liquid to solid temperature. It lowers the glass transition temperature of the polymer to a value below mouth temperature,

resulting in greater mobility of the polymeric chains and material flexibility.[19] Consequently, the modulus of elasticity of the resilient material is reduced to a satisfactory level.[20, 21]

Although the soft lining modified by plasticizer becomes semiliquid in the mouth, the leakage and the evaporation of plasticizer leads to hardening of the material. On top of that, the dimensional and mechanical changes also occurs due to water absorption. The acrylic soft lining is, therefore, a provisional material used during denture repair and these materials cannot be considered for permanent soft lining materials. [13, 18, 20] The masticatory effectiveness and maximum biting force of the soft lining were studied. The results showed that 85 percent of person wearing soft lined lower denture preferred them to conventional lower denture. When compared the masticatory effectiveness and maximum biting force for persons wearing soft lined lower denture and persons wearing conventional lower denture found that the difference were not significant[3].

On top of the soft property, other desirable properties of the soft lining should also include other aspects; a cushioning effect on the mucosa, a permanent resilience, color stability, dimensional stability, and strong adhesion to resin acrylic denture base, as well as, low fluid sorption and solubility, inhibit fungal and bacterial growth, and adequate tear strength.[14, 22, 23]

**4. Indication of soft lining materials**

In prosthetic dentistry, soft lining is usually applied on the mucosal side of dentures for reduce force of patient with thin or a non-resilient mucosal coverage and poor alveolar ridge(atrophic/knife edge). It is often used in the functional impression of immediate denture and congenital or acquired oral maxillofacial defects. For the bony prominent ridge, the use of lining materials can also enhance frictional force of the dentures. Furthermore, the conditions of xerostomia and pain from wearing dentures after radiation can get relieved by applying soft liner. Likewise, the persistent denture sore mouth and pain from superficial

mental nerve lying on the surface of alveolar ridge and denture stomatitis are reduced potentially by using the soft liner. [14, 15, 24]

### 5. Problem of soft lining materials

The main problem of the soft lining materials is the adhesive failure between the resin acrylic denture base and the soft lining materials, especially silicone type. Moreover, the soft lining materials turn to be hard, rough, badly taste or odour and discolored when applying in long term.[3, 24] Although the 2-3mm destruction of denture base can be fixed perfectly with the soft liner, the strength of denture base is reducing.[20, 24] In addition, the soft lining materials are dimensionally unstable because of their water absorption[24, 25].

In addition to physical and mechanical problems, the soft lining materials show to be a place where microorganisms like to grow. As demonstrated by using erythrosin dye, the soft lining surface was reported to be covered with plaque. Such persons wearing soft lined lower denture and conventional lower denture reveal 66 percent of yeast, especially *Candida, Torulopsis* and *Trichosporon* species.[3, 4] Malika and Hopsu-Havu [26] studied about mycotic growth and soft lining materials. The result of this study showed that fungal growth was most present on the soft lined denture, followed by mucosa under the denture, and then the denture without soft lining. [26].

### 6. Maintenance and development of soft lining materials

Denture cleaning can be chemical and mechanical method. However, the mechanical technique by brushing can damages surface of the soft lining materials. Thus, the chemical technique is considered to be a better method.[11] Wright[3] demonstrated that immersion denture cleanser of the alkaline peroxide type can reduced yeast more than cleaning denture by brush with other soap or paste cleansers. However, immersed denture into denture cleanser solution caused the change of surface roughness[12] and color stability.[12, 18]

There were previous studies of the additional of silver nanoparticles to soft lining materials. Vojdani *et al*[27] reported the addition of clotrimazole into silicone soft liner significantly reduced *Candida albicans* growth to the surface of the silicone soft liner. The samples continued to inhibit the fungal growth for 2 months. Rathore *et al*[28]also studied the antifungal activity of copper sulfate and chlorhexidine mixed with resilient liner. Their result showed that the resilient liner containing copper sulfate had better antifungal activity against *Candida albicans* compared to chlorhexidine. In 2011, Nam[5] demonstrated that tissue conditioner containing silver nanoparticles showed microbial inhibitory effect against *Staphylococcus aureus*, *Streptococcus mutans* and *Candida albicans*. In the same year, Chladek *et al* [7] showed the antifungal efficacy of silicone soft lining materials modified with silver nanoparticles. Although the modification seems to improve the major properties of soft liner, when silver nanoparticle concentration increased, the color of soft material became darker. Chladek *et al*[9] also reported that when increase in the nanosilver concentration resulted in decrease in hardness, increase in sorption and solubility, decrease in bond strength. Origanum oil can be used as an additive to tissue conditioner to reduce the adherence of *Candida albicans* without significantly affecting its bond strength to heat-polymerized acrylic.[29]

7. **Denture stomatitis and microorganism**

Denture stomatitis found in complete denture wearers about 50–70% . This pathology is an inflammatory process characterized by homogeneous erythema or red focal areas, especially in the palatal mucosa and usually associated with *Candida* species, particularly *Candida albicans.*[30]

According to Newton (1962), denture stomatitis may be classified as localized simple inflammation or punctiform hyperemia (type I), generalized simple inflammation or diffuse hyperemia (type II), or inflammatory papillary hyperplasia or granular hyperemia (type III).

There are various factors that influence the onset and severity of denture stomatitis such as denture trauma, continuous denture wearing, salivary flow, denture cleanliness, denture base material, denture age, cellular immunity, smoking, dietary factors, pH of the denture plaque and oral microbiota. The oral cavity is a complex environment that has a rich variety of species . In denture stomatitis patients, *Candida albicans* was the most frequently isolated microoranisms, than *Staphylococcus aureus* and *Klebsiella pneumonia* can be isolated from lesions.[31]

*S.aureus* is not generally considered to be a normal oral microbiota but it has detected in the mouths of systemically ill patients and may be a reservoir of this organism among the serious illness.[32] Soft lining materials have complicated molecular structures and are degradable, alongwith being susceptible to bacterial adhesion. The adhered bacteria can be released from denture plaque into saliva and then aspirated into the lower respiratory tract, causing pneumonia, especially in elderly people or those with disabilities.[33] There are potential link between oral colonization with *S.aureus* and severe form of mucositis in elderly. Smith *et al*[34] demonstrated that *S.aureus* has been isolated from infective oral condition, such as angular cheilitis and parotid . Smith *et al*[35] also showed that there were *S.aureus* (95%) among 5,005 specimens from oral rinse, angle of mouth swab, hard palate swab and tongue swab. Additionally, we merely found *S.aureus* in angular cheilitis, erythema/swelling, burning mouth, candida infection and facial abscess. In conclusion, *S.aureus* involvement in oral diseases, such as angular cheilitis, parotiditis, staphylococcal mucositis and periodontitis, and it is a causative bacterium of severe skin and soft tissue infections.

8. **Silver nanoprisms**

Silver (Ag) is a transition metal element having atomic number-47 and atomic mass-107.87. Silver nanoparticles were synthesized by inert gas condensation and co-condensation techniques. Both techniques are based on the evaporation of a metal into an

inert atmosphere with the subsequent cooling for the nucleation and growth of the nanoparticles.[36]

Silver ion has broad spectrum bactericidal activity. It reacts with the thiol group in vital enzymes and inactivates them or interacts with DNA, prevents of DNA replication. Structural changes in the cell envelope and the presence of some small electron-dense granules formed with silver and sulfur have also been demonstrated in bacterial cells.[37] Because of their antimicrobial effect, silver ions have been applied to dental materials.

The dental materials were incorporated with silver ion, such as silver zeolite and silver zirconium phosphate. To produce silver zeolite, alkaline or alkaline earth metal ion complexed with crystal aluminosilicate is partially replaced with silver ion by the ion-exchange method. The silver zeolite has two mechanisms of bactericidal action. One is the action of silver ion itself released from zeolite. Bacterial cells that contact with silver zeolite will take in silver ion. Afterward, the silver ion impedes several functions in the cell and consequently damages cells. Another is that reactive oxygen species generated from silver in the matrix which are produced possibly through the inhibition of a respiratory enzyme(s) by silver ion and attack the cell itself. While oxygen has been reported to be necessary for the bactericidal activity of silver zeolite, silver zeolite has also been reported to be effective on oral bacteria under anaerobic conditions.[37]

The nanotechnology has been applied in dentistry since early 1970s. The beginning era is the microfills but now the nanodentistry is a new generation of technologically advanced materials in prosthodontics. The nanodentistry will make a possible maintenance of comprehensive oral health by employing nanobiomaterials.[6] For example, the silver nanoparticles can be applied as antibacterial agents, and they have been used for hygiene, medical applications, and antibacterial water filters.[33] Furthermore, silver compounds and silver nanoparticles have been studied for applying in dentistry. These dental applications are in various fields, including dental restorative material, endodontic retrofill cement, dental

implants and caries inhibitory solution.[38] The potency of antimicrobial is proportional to the amount of ability to release bioactive silver ions and its availability to interact with bacterial or fungal cell membranes.[6]

Silver is a material that shows to have better antibacterial effects, when the size of the molecules is reduced. Likewise, the nano size, with at least one dimension of 100 nm or less, has the unique physicochemical properties, such as high catalytic capabilities and ability to generate reactive oxygen species that are harmful to bacteria. Silver nanoparticles, therefore, are more toxic to the bacterial cells compared to mere silver ions.[39]

The silver solution, silver diamine fluoride (Ag [$NH_3$] 2F), has also been reported for applying as a caries inhibitor. Principally, fluoride and silver interact synergistically to form fluoropatite, which will not be dissolved easily by acid from bacteria. Moreover, the combination between the experimental composite adhesives and silver nanoparticles (ECAs) showed to inhibit bacterial growth, which help to prevent demineralisation around the enamel's surfaces without compromising their physical properties.[38] Recently, silver nanoparticles have shown superior antibacterial activity compared to that of other silver compounds. The silver nanoparticles posse not only the strong antibacterial activity but also inhibit a broad spectrum of bacteria and fungi[33].

The mechanism of bactericidal actions of silver nanoparticles is still not well understood, while the silver ions have been described clearly. The action of silver nanoparticles is broadly similar to that of silver ion. The silver ions provide a bactericidal effect by interfering the peptidoglycan of cell wall and destructing the plasma membrane.[40] The silver nanoparticles are effective against gram-negative bacteria by creating 'pits' in the cell wall of bacteria. Subsequently, the cell wall with such morphology exhibits a significant increase in permeability.[38] On the other hand, silver ions penetrate into the bacterial cell wall and bind to the phospholipid layer of the cytoplasmic membrane, then bind the bacterial DNA to disrupt DNA replication. Silver ions impair the ability of ribosomes to transcribe

messenger RNA and bind to the sulfhydryl group of the cytochrome b.[39] Silver ions prevent bacterial DNA replication by interacting with the exposed sulfhydryl groups in bacterial proteins, especially with the enzymes involved in vital cellular processes such as the electron transport chain.[40] These interactions effectively produce bacterial death.[38]Silver nanoparticles inhibit a respiratory enzymes, facilitating the generation of reactive oxygen species and consequently damaging the cell.[41]

Some studies found that the antimicrobial activity of silver nanoparticles depended on their sizes.[40, 41] The antibacterial properties were related to the total surface area of the nanoparticles. Smaller particles with a larger surface to volume ratio provided a more efficient means for antibacterial activity.[36, 40] A larger surface area of the silver nanoparticle allows a larger amount of atoms to interact with their surroundings, thus the bactericidal effect of silver nanoparticle is size dependent, where smaller silver nanoparticle are more potent. In addition, the small size of these particles makes penetration through cell membrane easier, thus affecting intracellular process resulting in higher reactivity and antimicrobial activity.[40] The silver nanoparticles were found to exhibit antibacterial effects at low concentrations.[36] The antimicrobial action of silver may be proportional to the amount of released bioactive silver ions ($Ag^{+}$) and their interaction with bacterial cell membranes.[40]

Silver nanoparticles have been incorporated in dental materials to overcome cariogenic bacterial colonization in the marginal gaps and on the material surfaces. Silver nanoparticles exhibit antibacterial effects against a large number of bacterial species, including *S.mutans* and *Lactobacillus spp*., when added to adhesive for bonding orthodontics brackets and to a dental composite.[40] Lee *et al*[33] have reported that silver nanoparticles inhibited the growth of E. coli cells.[33] The previous study showed titanium implants coating with silver nanoparticles had ability to kill all planktonic bacteria in culture medium during the first severals days and the capability to prevent bacterial adhesion is maintained for 30 days.[42] Denture base resin containing silver colloidal nanoparticles had

good efficacy against *C. albicans* [30, 43],however, color change is the problem of modified acrylic resin. [43] In another study, that tested antimicrobial activity of irreversible hydrocolloid Impression containing nano silver against oral microorganisms. The results showed use of silver nanoparticles have antimicrobial effect and theycan be exploited in medicine and dentistry.[44]

Pal *et al*[41] studied antibacterial activity of silver nanoparticles on *E.coli* showed the presence of nanoparticles at a certain level inhibited bacterial growth by more than 90%. However, this level is significantly different for different nanoparticle shapes. For truncated triangular particles, almost complete inhibition of bacterial growth followed spherical nanoparticles and rod-shaped nanoparticles. The uptake of silver can be recognized by irregular pits . Silver has a greater tendency to react with sulfur- or phosphorus-containing soft bases. Thus, sulfur-containing proteins in the membrane or inside the cells and phosphorus-containing elements like DNA was the sites for silver nanoparticle binding. The severely damaged cell membrane has found in an enlarged image of *E.coli* which treated with truncated triangular silver nanoplates. It may be considered that silver nanoparticles with the same surface areas but with different shapes may also have different effective surface areas in terms of active facets.

Ekgasit *et al* [45]have been studied $H_2O_2$-triggered shape transformation of silver nanospheres to nanoprisms. A practical procedure for a morphological transformation of silver nanospheres to nanoprisms utilizing $H_2O_2$ is demonstrated. The spontaneous transformation was initiated by feeding $H_2O_2$ into a starch-stabilized silver nanospheres colloid without additional silver ions, reducing agents, or capping agents. The small diameter of silver nanospheres is one of the crucial factors in making the shape transformation possible. The obtained nanoprisms exhibited mixed geometries e.g. hexagonal, truncated triangular, rounded-tip triangular prisms, and circular disks with average bisector lengths of 30 to 120 nm and the thickness of 10 to 20 nm

Nanoparticles must selectively kill bacteria without imparting toxicity on mammalian cells. Studies demonstrated the positive correlation between silver nanoparticles concentration and cytotoxic effects. Nevertheless, an absence of cytotoxity against human cells was demonstrated when 0.05–0.70% concentrations of silver nanoparticles were incorporated in polymers.[40]

Cytotoxic of silver tissue conditioner was evaluated by MTT assay and human gingival fibroblasts(HGF) were used to tested. Cytotoxicity was evaluated by the cell viability. Kim *et al* [46] have studied the subchronic oral toxicity and tissue accumulation of Ag in rats for 90 days and found that Ag have significant toxic effects on cells that primarily occur in a dose-dependent manner. The results indicated that 2.0% Ag group was expressed as highly cytotoxic effect to HGF at 24 and72 hours incubation periods while control (0%) and 0.1% and 0.5% groups were graded as non-cytotoxic at all incubation periods. Furthermore, the quantities of released $Ag^+$ were dose dependent and decreased in all tested specimens as aging time. The toxic effects of silver nanoparticles are proportional to the activity of free $Ag^+$ ions released by the nanoparticles.[38] Exposure to silver nanoparticles has been associated with tissue damage especially in liver. In rats, a No Observable Adverse Effect Level NOAEL) of 30 mg/kg and the Lowest Observable Adverse Effect Level (LOAEL) of 125 mg/kg has been determined for silver nanoparticles. Initially, in order to avoid the toxicity of these materials, silver nanoparticles can be used for temporary periods in the dental field.[38]

The previous study has reported that the amount of eluted $Ag^+$ followed a steady state of approximately 0.08 mg/L in 0.5% of Ag and 0.21 mg/L in 1.0% of Ag after 48 hours storage. In 2.0% of Ag-tissue conditioner, eluted $Ag^+$ was detected approximately 0.49 mg/L at 72 hours storage with steeper decrease as compared to other.[33] Torres *et al*[47] have tested cytocompatible antifungal of acrylic resin containing silver nanoparticles for dentures. The results show that PMMA-silver nanoparticle discs significantly reduce adherence of *C. albicans* and do not affect metabolism or proliferation. They also appear not to cause

genotoxic damage to cells. Hadrup and Lam[48] investigated an anti-smoking mouthwash containing silver nitrate as the active ingredient. In rats, the LD50 was found to be 280 mg of silver/kg of bw following oral administration. In rabbits, the LD50 was found to be 800 mg of silver/kg of bw/day. The previous study tested that orally administered nanoparticulate silver was not toxic to guinea pigs at acute doses of up to 5000 mg/kg of bw/day.[49]In another study, Abe *et al*[50] have examined cytotoxicity of antimicrobial tissue conditioners containing silver-zeolite (0,2,5% by dry weight). The results showed individual compounds eluted from tissue conditioners and silver ions released from silver-zeolite might cause such cytotoxicity. Cell viabilities for tissue conditioners containing silver-zeolite no significant difference decreased with increasing silver-zeolite content.

# CHAPTER 3

# MATERIALS AND METHODS

## Materials

Acrylic soft lining material in this study is Visco-gel(Dentsply, USA, listed in Table 2). Silver nanoprisms is fabricated from Sensor Research Unit, Department of Chemistry, Faculty of Science, Chulalongkorn University. Three moulds were used in this study, including Silicone mould (disc-shaped for antimicrobial activity), Stainless steel mould (disc-shaped for water sorption and water solubility) and PVC mould (cylindrical-shaped for hardness test).

Table 2 Details of materials used in the experiment : Visco-gel

| | |
|---|---|
| Brand of material | Visco-gel |
| Manufacturer | Dentsply, USA |
| Type | Soft lining materials |
| Compositon | Powder: Polyethylmethacrylate<br>Liquid: Ethyl alcohol<br>Citrate ester plasticizer<br>Separator : Mineral oil, spearmint oil |
| Method to application | Powder/liquid : 3 g / 2 ml |

## Preparation of test materials

In this study, acrylic soft lining materials (Visco gel) and silver nanoprisms (Fig.1) (Sensor Research Unit, Department of Chemistry, Faculty of Science, Chulalongkorn University) were combined together in a various concentration. The soft lining was a prompt kit for clinical use that contains powder and liquid part, whilst the silver nanoprisms are derived from a process of morphological transformation utilizing $H_2O_2$ to convert from nanoparticles to nanoprisms. The specimens were fabricated by mixing the powder part with the liquid part that pre-load with a gradient of silver nanoprisms. Five concentrations of the silver nanoprisms were prepared with the liquid part as described in the Table 3,4.


Fig 1 (a) acrylic soft lining materials :Visco gel, (b) silver nanoprisms

Table 3 The classification of experimental ratio type1

| group | Concentration of silver nanoprisms in material (ppm) | Volume of modified liquid=2ml. | | Mixing ratio |
|---|---|---|---|---|
| | | volume of silver nanoprisms 1000 ppm (ml) | volume of liquid (ml) | |
| 1 | 0 | 0 | 2 | Powder : modified liquid 3 g : 2 ml |
| 2 | 25 | 0.05 | 1.95 | |
| 3 | 50 | 0.1 | 1.9 | |
| 4 | 100 | 0.2 | 1.8 | |
| 5 | 200 | 0.4 | 1.6 | |

Table 4 The classification of experimental ratio type2

| group | Concentration of silver nanoprisms in material (ppm) | Volume of modified liquid=2ml. | | Mixing ratio |
|---|---|---|---|---|
| | | volume of silver nanoprisms 1000 ppm (ml) | volume of liquid (ml) | |
| 1 | 0 | 0 | 2 | Powder : liquid 3 g : 2 ml |
| 2 | 25 | 0.05 | 2 | |
| 3 | 50 | 0.1 | 2 | |
| 4 | 100 | 0.2 | 2 | |
| 5 | 200 | 0.4 | 2 | |

## Antimicrobial activity test

Two microorganisms were selected for the antimicrobial activity test of the soft lining loaded with silver nanoprisms. The culture plates were prepared from the standardized suspension of the microorganisms. Firstly, *Staphylococcus aureus* was grown in tryptone soya broth (Oxoid LTD., Basingstoke, Hampshire, England) and on to Mueller Hinton agar plates (Oxoid LTD., Basingstoke, Hampshire, England) at 37ºC for 24 hrs. Secondly,

*Candida albicans* was grown in tryptone soya broth (Oxoid LTD., Basingstoke, Hampshire, England) and on to Sabouraud agar plate (Oxoid LTD., Basingstoke, Hampshire, England) at 37ºc overnight. To standardize the inoculum suspension, a spectrophotometer was used to adjust the density of the suspension. The suspensions were measured the optical density(OD) at 600 nm was adjusted to 1.0 and then the suspension was diluted with phosphate-buffered saline (PBS pH 7.4) to a 1:100 concentration.

The 120 disc-shaped specimens, 10 mm in diameter and 3 mm thick (Fig.2), were prepared by the silicone mould. There were four groups, and each group (30 specimens) were immersed in distilled water 1,4,7 and 14 days. Each group was divided equally for testing with *Staphylococcus aureus* and *Candida albicans* and were sterilized with ethylene oxide gas prior the test. Each specimen was placed in 24-well plate where microbial suspension (100µl) in tryptone soya broth(1ml) were inoculated to each well, and the plate was incubated at 37ºC for 24 hrs. (Fig.3a,b) The antimicrobial properties were determined via turbidity test using spectrophotometer at 530 nm. (Fig.3c) The percentage of microbial reduction was calculated from a proportion of the declined OD value of the specimen suspension to the OD value of the control suspension. The control group were tested for evaluate the influence of the color of specimen on the optical density. These control groups were represented by specimens that did not have any *S. aureus* or *C. albicans* in tryptone soya broth. The optical density was determined at 530 nm. Data was statistically analyzed by using two-way ANOVA with LSD's multiple comparison test and independent t-test.


Fig 2 mould(a) and specimen(b) for antimicrobial activity test : Disc-shaped specimen

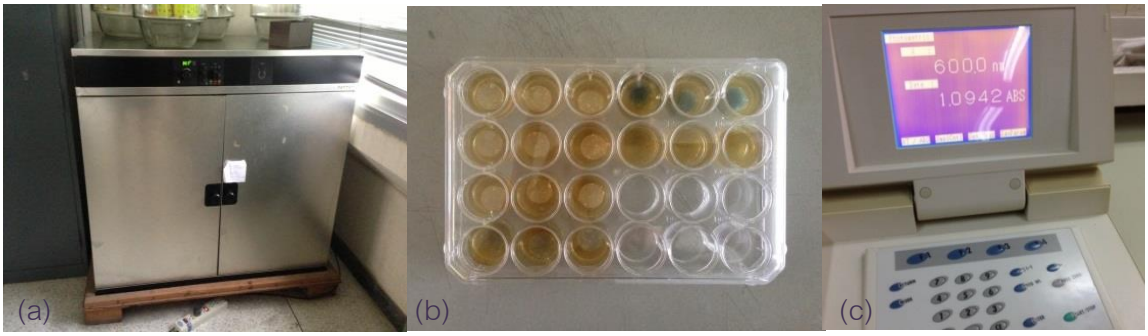


Fig 3 (a)Incubator ,(b) disc sample in plate with microbial suspension, (c)spectrophotometer

## Water sorption and solubility test

Water sorption and solubility were following the method described in International Organizationfor Standardization (ISO) Standard No. 1567. Specimen disks, 50 mm in diameter and 0.5 mm thick, were prepared by the stainless steel mould (Fig.4).

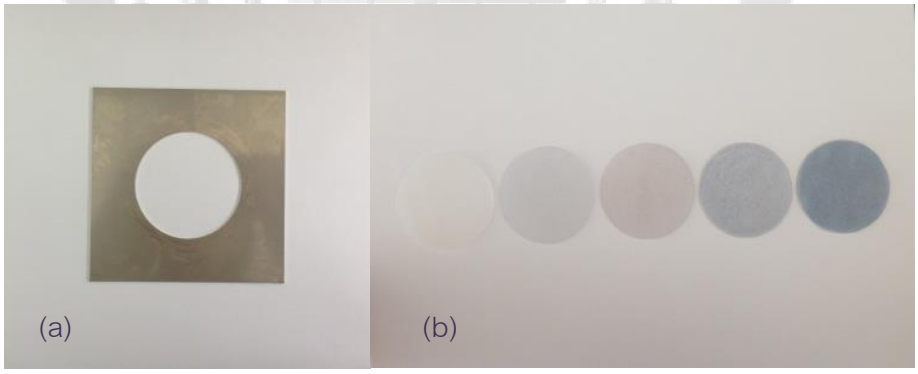


Fig 4 (a) stainless steel mould, (b) specimen for sorption and solubility test

All materials were mixed and manipulated according to the manufacturer's instruction. Five specimens of each group were conditioned to a constant mass in a desiccator at 37 ± 2 ºC for 24 hours, removed to desiccator at room temperature for 1 hour and then weighed using an analytical balance with a precision of ±0.2 mg. (Fig.5). This cycle

should be repeat until constant mass is attained. This weight value was considered the initial weight of the specimen (W1).

Fig 5 analytical balance

The specimens were immersed in distilled water at 37±1ºC for 24 hours. Specimens were then removed from the water, dried with absorbent paper and weighed (W2). The value of water sorption should be calculated as follows :

$$\text{Sorption (mg/cm}^2\text{)} = \frac{W_2 - W_1}{\text{Surface area}}$$

The second technique(net sorption) should be calculated as follows:[51]

$$\text{Percent sorption} = \frac{W_2 - W_3}{W_1} \times 100\ \%$$

Reconditioned to constant mass in the desiccator and weighed again (W3). Water solubility were calculated as follows :

$$\text{Solubility (mg/cm}^2\text{)} = \frac{W_1 - W_3}{\text{Surface area}}$$

The second technique should be calculated as follows: [51]

$$\text{Percent solubility} = \frac{W_1 - W_3}{W_1} \times 100\ \%$$

W1: initial weight of specimen after first drying

W2: weight of specimen after immersed in distilled water

W3: weight of specimen after second drying

Surface area of specimen = Areas of top and bottom +Area of the side

$= 2\pi r^2 + 2\pi rh$

## Hardness test

Five cylindrical specimens of each group were made of the dimensions 28 mm × 6 mm (according to ASTM: D-2240) with PVC mould(Fig.6a). Applied separator on the mould for easy removal of the specimens. Base of the mould was placed on a glass slab. Soft lining materials were manipulated according to manufacturer's instructions and pressed into the mould. The mould was then covered the top by glass slab was pressed against the mould(Fig.6b). Removed the specimens from the mould and excess was trimmed with blade(Fig.6c). Specimens were stored in 37ºC distilled water and tested at 1,4,7,14 day. The specimens were tested using a Shore A Durometer. Five readings were noted for each sample (Fig.7a,b).

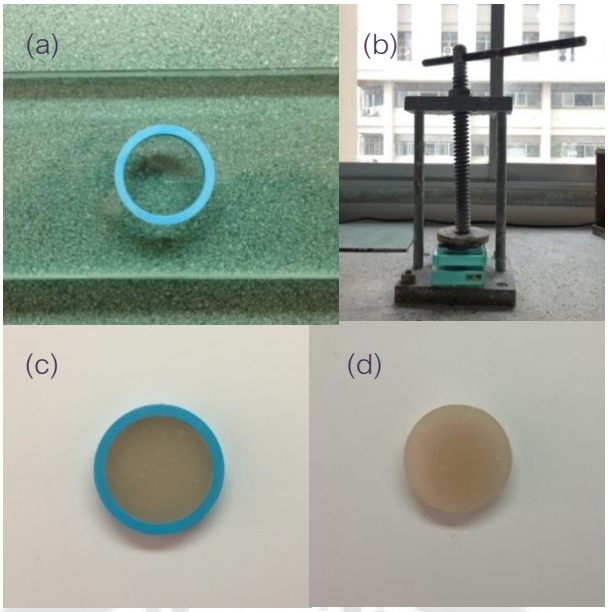


Fig 6 (a)PVC mould, (b,c)preparation of specimens, (d)specimen for hardness test

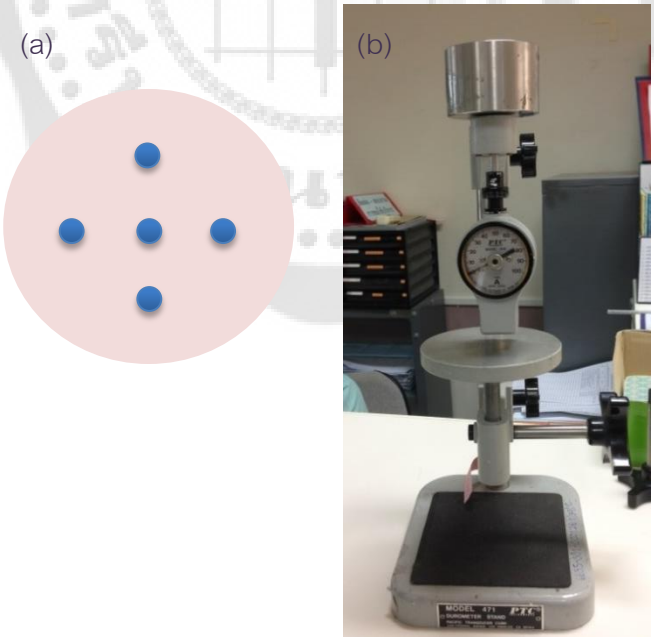


Fig 7 (a)Five positions on the specimen for hardness test, (b)Shore A Durometer

## Color stability

The disc-shaped specimens, 10 mm. in diameter and 3 mm. thick were prepared by the silicone mould. The color change of soft lining materials were determined after 1,4,7 and 14 days storage in distilled water. Color stability evaluated by visual method.

## Scanning electron microscopy evaluation

To evaluate the silver nanoprisms in soft liners, specimens were prepared for SEM(Fig.8). Samples were examined using an acceleration voltage of 5 kV at 5000× magnification.

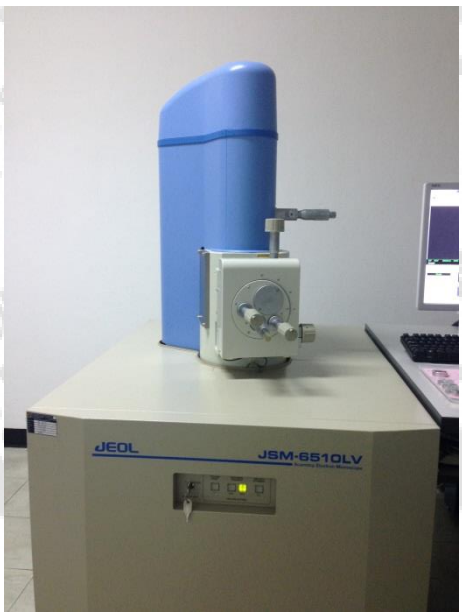


Fig 8 Scanning electron microscopy

# CHAPTER 4
# RESULTS

## Antimicrobial effect

The antimicrobial effect of silver nanoprisms containing acrylic soft liner (experimental ratio type1) was detected by percentage of microbial reduction after four periods. Data was analyzed by two-way ANOVA and Tukey HSD test. The silver nanoprism concentration and time had significant influence on the antimicrobial effect.

The average percentage of *S. aureus* reduction is showed in Table 5, Fig9. The results showed that the differences were significant ($P<0.05$), the antibacterial effect of 50ppm group was highest followed by 25ppm, 100ppm, 200ppm and 0ppm group, respectively. The percentage of *S. aureus* reduction showed maximum antibacterial efficacy were 1day group followed by 4,14 and 7 days, respectively.

The average percentage of *C. albicans* reduction results are shown in Table6, Fig. 10. The concentration of silver nanoprisms combined acrylic soft liner demonstrated maximum antifungal efficacy was 50 ppm and minimum antifungal efficacy was 0ppm. There was significant different on the antifungal efficacy of study group after the test period ($P<0.05$). The percentage of *C. albicans* reduction showed maximum antifungal efficacy were 1day group followed by 4,7 and 14 days, respectively.

The average optical density (OD) in control group after 1,4,7 and 14 days were showed in Table 7. Data was analyzed by two-way ANOVA. The average optical density ranged from 0.427-0.453. The results showed that there is no statistically significant differences between group ($P<0.05$).

Table 5 The average percentage of *S. aureus* reduction after 1,4,7 and 14 days (experimental ratio type1), standard deviation*

| Silver nanoprism (ppm) | percentage of *S. aureus* reduction | | | |
|---|---|---|---|---|
| | after 1 day | After 4 days | after 7 days | after 14 days |
| 0ppm | 22.93(9.5)$^{A,a}$ | 20.60(14.4)$^{A,a}$ | 6.15(4.5)$^{B,a}$ | 8.49(16.8)$^{B,a}$ |
| 25ppm | 35.30(19.3)$^{A,bc}$ | 35.72(19.3)$^{A,a}$ | 24.31(13.2)$^{A,bc}$ | 26.20(22.3)$^{A,a}$ |
| 50ppm | 43.14(13.6)$^{A,b}$ | 31.58(15.8)$^{A,a}$ | 34.06(25.6)$^{A,b}$ | 22.59(17.8)$^{A,a}$ |
| 100ppm | 30.61(8.1)$^{A,ac}$ | 25.32(12.5)$^{A,a}$ | 17.88(8.7)$^{A,ac}$ | 25.81(15.7)$^{A,a}$ |
| 200ppm | 22.76(6.2)$^{A,a}$ | 19.79(12.4)$^{A,a}$ | 20.35(15.0)$^{A,ab}$ | 26.51(11.3)$^{A,a}$ |

* Groups with the same uppercase superscript letters; (A–B) for each row and lowercase superscript letters;(a–c) for each column are not significantly different at the $p > 0.05$ level.

Table 6 The average percentage of *C. albican* reduction after 1,4,7 and 14 days (experimental ratio type1), standard deviation*

| Silver nanoprism (ppm) | percentage of *C. albican* reduction | | | |
|---|---|---|---|---|
| | after 1 day | After 4 days | after 7 days | after 14 days |
| 0ppm | 50.35(22.9)$^{A,a}$ | 25.78(7.6)$^{B,a}$ | 12.84(17.4)$^{B,a}$ | 14.01(9.7)$^{B,a}$ |
| 25ppm | 48.81(27.4)$^{A,a}$ | 35.77(7.7)$^{AB,bc}$ | 25.04(13.4)$^{B,ab}$ | 25.02(13.6)$^{B,a}$ |
| 50ppm | 58.52(20.1)$^{A,a}$ | 40.46(5.1)$^{B,c}$ | 32.79(7.7)$^{B,b}$ | 17.77(8.3)$^{C,a}$ |
| 100ppm | 50.92(24.9)$^{A,a}$ | 36.46(6.5)$^{AB,bc}$ | 25.08(15.0)$^{BC,ab}$ | 19.96(10.9)$^{C,a}$ |
| 200ppm | 45.24(21.2)$^{A,a}$ | 32.48(10.3)$^{AB,ab}$ | 32.47(12.1)$^{AB,b}$ | 22.61(11.8)$^{B,a}$ |

* Groups with the same uppercase superscript letters; (A–C) for each row and lowercase superscript letters;(a–c) for each column are not significantly different at the $p > 0.05$ level.

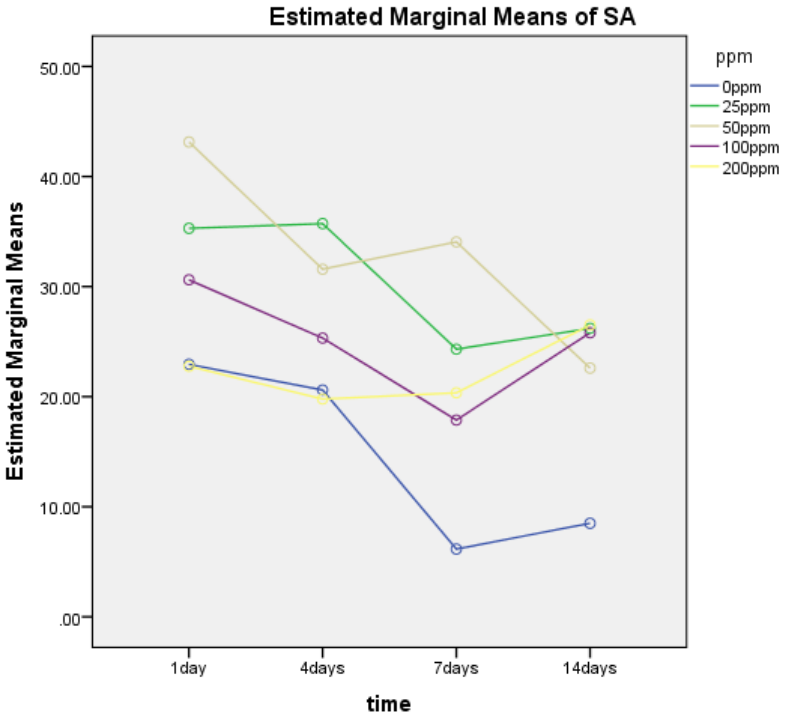


Fig 9 The percentage of *Staphylococcus aureus* reduction after 1,4,7 and 14 days.

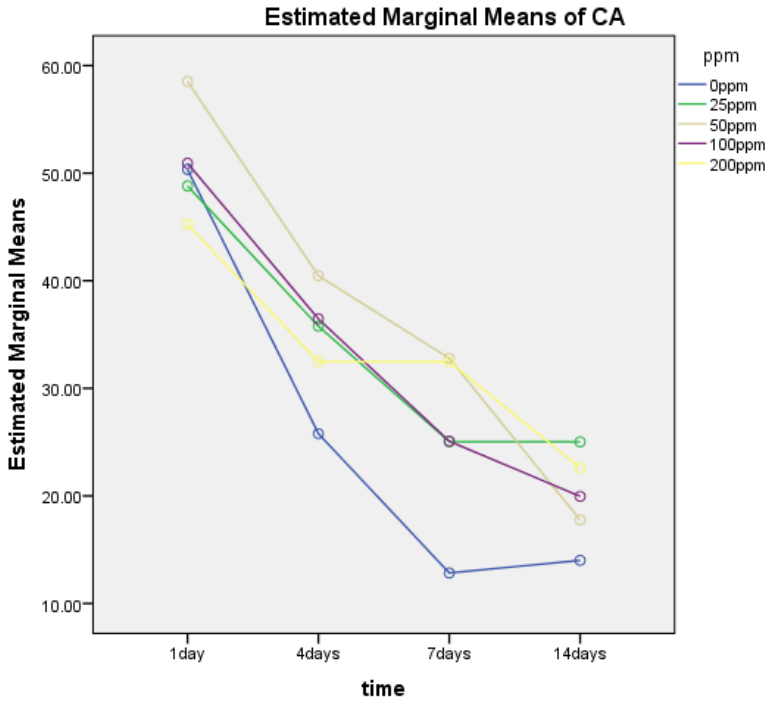


Fig 10 The percentage of *Candida albicans* reduction after 1,4,7 and 14 days.

Table 7 The average optical density (OD) in control group after 1,4,7 and 14 days (experimental ratio type1), standard deviation

| Silver nanoprism (ppm) | optical density (OD) | | | |
|---|---|---|---|---|
| | after 1 day | After 4 days | after 7 days | after 14 days |
| 0ppm | 0.445 (0.023) | 0.427 (0.036) | 0.433 (0.016) | 0.450 (0.014) |
| 25ppm | 0.434 (0.025) | 0.445 (0.040) | 0.435 (0.031) | 0.453 (0.026) |
| 50ppm | 0.449 (0.031) | 0.430 (0.012) | 0.438 (0.025) | 0.444 (0.028) |
| 100ppm | 0.447 (0.023) | 0.433 (0.034) | 0.433 (0.005) | 0.451 (0.009) |
| 200ppm | 0.444 (0.015) | 0.447 (0.046) | 0.451 (0.016) | 0.446 (0.021) |

## Water sorption and water solubility

The data of water sorption and water solubility was analyzed by one-way ANOVA and Tukey HSD test. The result of water sorption and solubility studies (experimental ratio type1) are summarized in Table8,9. Water sorption and solubility values ranged from 1.72-2.99 $mg/cm^2$ and from 0.62-1.53 $mg/cm^2$, respectively. Percent sorption and percent solubility values ranged from 4.984-10.404% and from 1.308-3.694%, respectively.

For water sorption, control group (0 ppm of silver nanoprisms) exhibited highest mean value (2.99 $mg/cm^2$) though 200 ppm of silver nanoprism group exhibited least mean value (1.72 $mg/cm^2$). There were significantly decrease in water sorption among 50,100,200 ppm of silver nanoprisms group and control group (Fig.11). There were differences in water sorption between study groups, as following: 0ppm, 25ppm, 100ppm, 50ppm and 200ppm, respectively in descending order. There were no significant differences ($p>0.05$) in the mean water sorption values between 0 ppm and 25 ppm group, among 50 ppm,100 ppm and 200 ppm group.

For water solubility, 25 ppm of silver nanoprisms group exhibited highest mean value (1.53 $mg/cm^2$) though 200 ppm of silver nanoprisms group exhibited least mean value (0.62$mg/cm^2$). There were significantly decrease in water solubility among 200 ppm of silver nanoprisms group and control group (Fig.12). There were differences in water solubility between study groups, as following: 25 ppm, 0 ppm, 50 ppm, 100 ppm and 200 ppm, respectively in descending order. There was no statistically significant among 0 ppm, 25 ppm and 50 ppm group, and among 0 ppm, 50 ppm and 100 ppm group.

Table 8 Mean values and standard deviations for sorption and solubility of experimental ratio type1, value with dissimilar letter are significantly different from each other *(P<0.05)*

| Concentration of silver nanoprisms in material (ppm) | Sorption ($mg/cm^2$) | Solubility ($mg/cm^2$) |
|---|---|---|
| 0 | 2.993(0.28) $^{a}$ | 1.426 (0.17) $^{a,b}$ |
| 25 | 2.778(0.47) $^{a}$ | 1.527(0.23) $^{a}$ |
| 50 | 1.787(0.23) $^{b}$ | 1.231(0.21) $^{a,b}$ |
| 100 | 1.939(0.14) $^{b}$ | 1.153(0.07) $^{b}$ |
| 200 | 1.724(0.37) $^{b}$ | 0.617 (0.12) $^{c}$ |

Table 9 Mean values and standard deviations for percent sorption and percent solubility of experimental ratio type1, value with dissimilar letter are significantly different from each other *(P<0.05)*

| Concentration of silver nanoprisms in material (ppm) | Percent Sorption | Percent Solubility |
|---|---|---|
| 0 | 10.067(0.83) $^{a}$ | 3.262 (0.55) $^{a,b}$ |
| 25 | 10.404(1.57) $^{a}$ | 3.694(0.66) $^{a}$ |
| 50 | 6.873(0.87) $^{b}$ | 2.802(0.48) $^{a,b}$ |
| 100 | 6.862(0.58) $^{b}$ | 2.564(0.29) $^{b}$ |
| 200 | 4.984 (0.89) $^{b}$ | 1.308 (0.22) $^{c}$ |

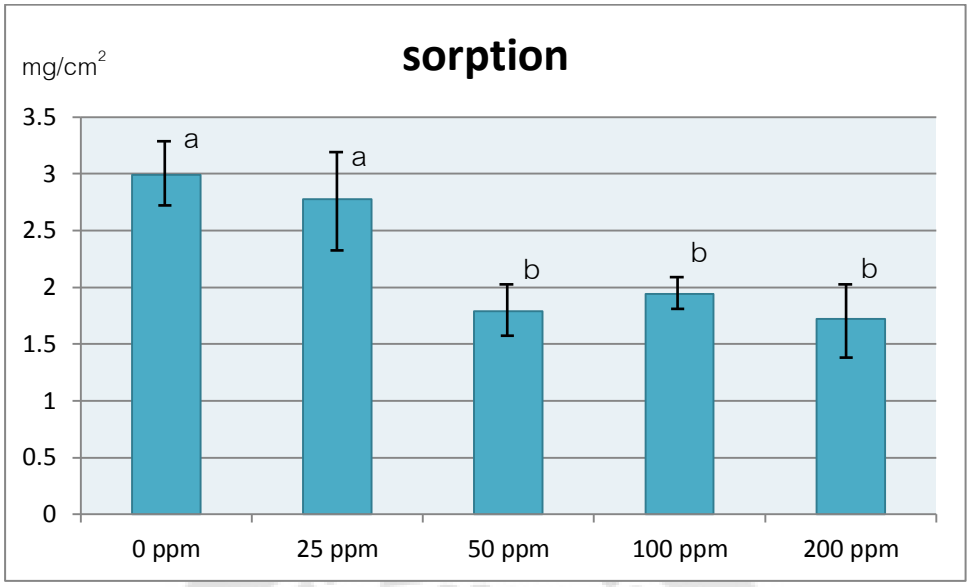


Fig 11 Mean values for sorption of experimental ratio type1, value with dissimilar letter are significantly different from each other *(P<0.05)*

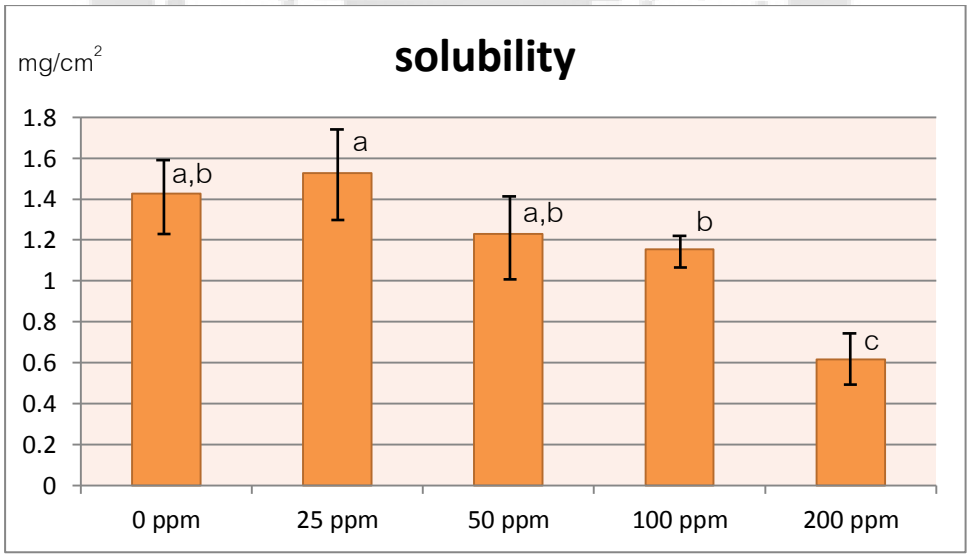


Fig 12 Mean values for solubility of experimental ratio type1, value with dissimilar letter are significantly different from each other *(P<0.05)*

The result of water sorption and solubility studies (experimental ratio type2) are summarized in Table10,11. Water sorption and solubility values ranged from 2.031-2.903 $mg/cm^2$ and from 0.604-1.237 $mg/cm^2$, respectively. Percent sorption and percent solubility values ranged from 7.826-11.730 % and from 1.754 -3.475 %, respectively.

There were significantly decrease in water sorption among 25,50,100,200 ppm of silver nanoprisms group and control group(0 ppm) (Fig.13). There were significantly decrease in water solubility among 50,100,200 ppm of silver nanoprisms group and control group(0 ppm) (Fig.14).

Table 10 Mean values and standard deviations for sorption and solubility of experimental ratio type2, value with dissimilar letter are significantly different from each other *(P<0.05)*

| Concentration of silver nanoprisms in material (ppm) | Sorption ($mg/cm^2$) | Solubility ($mg/cm^2$) |
|---|---|---|
| 0 | 2.903 (0.10) $^{a}$ | 1.237 (0.41) $^{a}$ |
| 25 | 2.367 (0.11) $^{b}$ | 0.955 (0.14) $^{a,b}$ |
| 50 | 2.151 (0.19) $^{b,c}$ | 0.714 (0.13) $^{b,c}$ |
| 100 | 2.241 (0.07) $^{b,d}$ | 0.606 (0.04) $^{c}$ |
| 200 | 2.031 (0.28) $^{c,d}$ | 0.604 (0.23) $^{c}$ |

Table 11 Mean values and standard deviations for percent sorption and percent solubility of experimental ratio type2, value with dissimilar letter are significantly different from each other *(P<0.05)*

| Concentration of silver nanoprisms in material (ppm) | Percent Sorption | Percent Solubility |
|---|---|---|
| 0 | 11.730 (1.11) $^{a}$ | 3.475 (1.02) $^{a}$ |
| 25 | 9.253 (0.70) $^{b}$ | 2.664 (0.47) $^{b}$ |
| 50 | 7.864 (1.03) $^{c}$ | 1.938 (0.25) $^{b,c}$ |
| 100 | 8.387 (1.14) $^{b,c}$ | 1.785 (0.24) $^{c}$ |
| 200 | 7.826 (0.67) $^{c}$ | 1.754 (0.58) $^{c}$ |

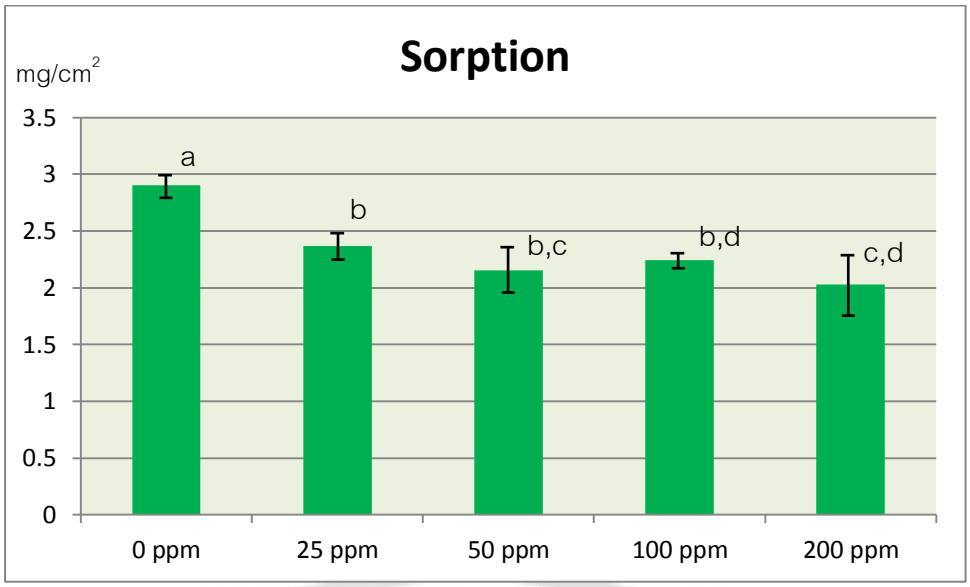


Fig 13 Mean values for sorption of experimental ratio type2, value with dissimilar letter are significantly different from each other *(P<0.05)*

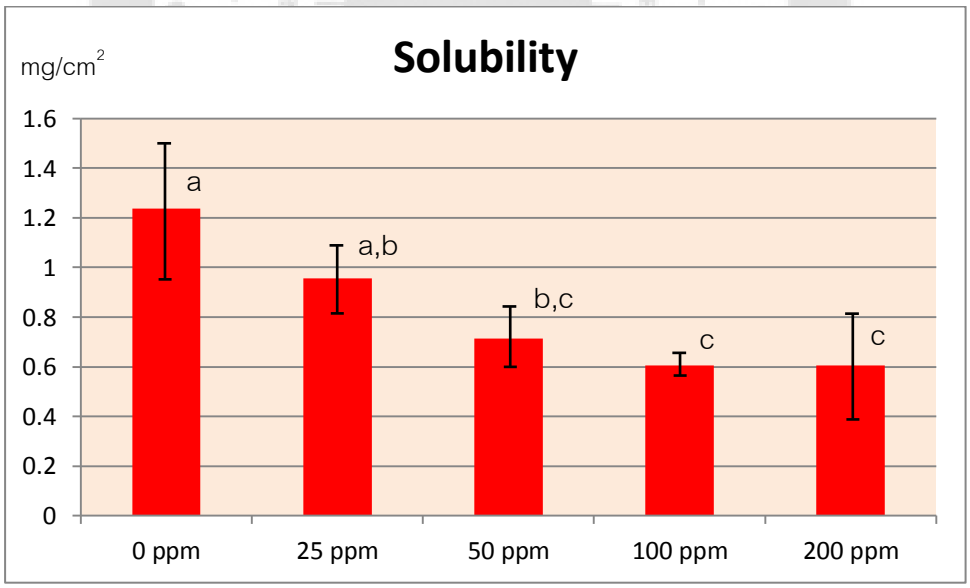


Fig 14 Mean values for solubility of experimental ratio type2, value with dissimilar letter are significantly different from each other *(P<0.05)*

## Hardness

The hardness of silver nanoprisms containing acrylic soft liner was detected by shore A hardness test during four periods. Data was analyzed by two-way ANOVA Tukey HSD test The silver nanoprism concentration and time had significant influence on hardness. The addition of silver nanoprisms were significantly increase in mean hardness values for period of time. The specimens showed an increase in hardness with time (Table12-13, Fig.15-16).

Table 12 Mean values and standard deviations of hardness. (experimental ratio type1)*

| Concentration of silver nanoprisms (ppm) | Hardness, Shore A Unit | | | |
|---|---|---|---|---|
| | 1 day | 4 days | 7 days | 14 days |
| 0 | 13.80(1.0)$^{A,a}$ | 15.44(1.1)$^{A,a}$ | 25.20(2.3)$^{B,a}$ | 34.84(2.4)$^{C,a}$ |
| 25 | 16.56(2.8)$^{A,a}$ | 20.48(2.1)$^{A,b}$ | 26.48(1.6)$^{B,a}$ | 38.60(3.2)$^{C,a}$ |
| 50 | 17.52(0.8)$^{A,a}$ | 24.12(1.1)$^{B,c}$ | 31.92(0.9)$^{C,b}$ | 44.08(1.8)$^{D,b}$ |
| 100 | 27.76(3.8)$^{A,b}$ | 34.84(1.9)$^{B,d}$ | 41.36(2.3)$^{C,c}$ | 50.52(2.7)$^{D,c}$ |
| 200 | 33.52(3.6)$^{A,c}$ | 37.32(1.3)$^{AB,d}$ | 42.16(3.0)$^{B,c}$ | 55.68(3.5)$^{C,c}$ |

* Groups with the same uppercase superscript letters; (A–D) for each row and lowercase superscript letters;(a–d) for each column are not significantly different at the $p > 0.05$ level.

Table 13 Mean values and standard deviations of hardness.(experimental ratio type2)*

| Concentration of silver nanoprisms (ppm) | Hardness, Shore A Unit | | | |
|---|---|---|---|---|
| | 1 day | 4 days | 7 days | 14 days |
| 0 | 16.32(1.4)$^{A,a}$ | 20.40(2.0)$^{B,a}$ | 29.88(2.5)$^{C,a}$ | 32.76(2.4)$^{C,a}$ |
| 25 | 19.72(0.9)$^{A,ab}$ | 28.64 (2.8)$^{B,b}$ | 29.44(1.8)$^{B,a}$ | 33.32(1.9)$^{C,a}$ |
| 50 | 21.84(4.2)$^{A,b}$ | 27.76(3.1)$^{B,b}$ | 33.60(2.4)$^{C,a}$ | 36.00(2.0)$^{C,ab}$ |
| 100 | 20.52(3.0)$^{A,ab}$ | 31.16(4.0)$^{B,b}$ | 32.12(2.9)$^{B,a}$ | 34.04(2.0)$^{B,a}$ |
| 200 | 23.48(1.7)$^{A,b}$ | 32.64(2.4)$^{B,b}$ | 33.44 (1.3)$^{B,a}$ | 38.40(2.2)$^{C,b}$ |

* Groups with the same uppercase superscript letters; (A–C) for each row and lowercase superscript letters;(a–b) for each column are not significantly different at the $p > 0.05$ level.

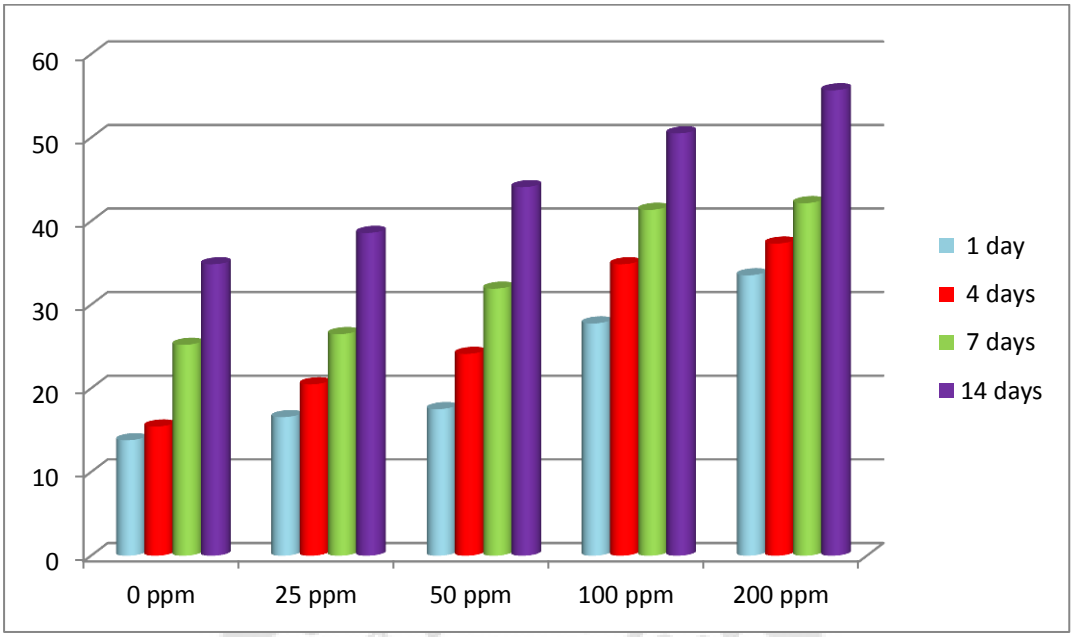


Fig 15 Mean values of Shore A hardness of silver nanoprisms containing soft liner (experimental ratio type1)

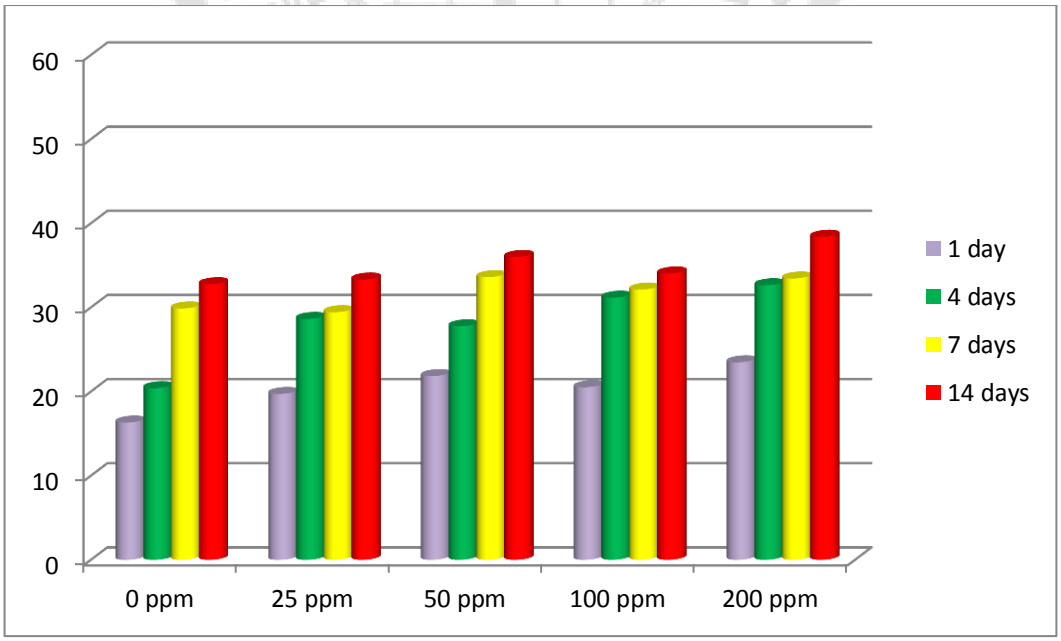


Fig 16 Mean values of Shore A hardness of silver nanoprisms containing soft liner (experimental ratio type2)

## Color stability

Color stability were evaluated by visual method. The shade of specimens at 4,7,14 days were fader than specimens at 1 day. There were no differences in color among specimens at 4,7,14 days(Fig.17).

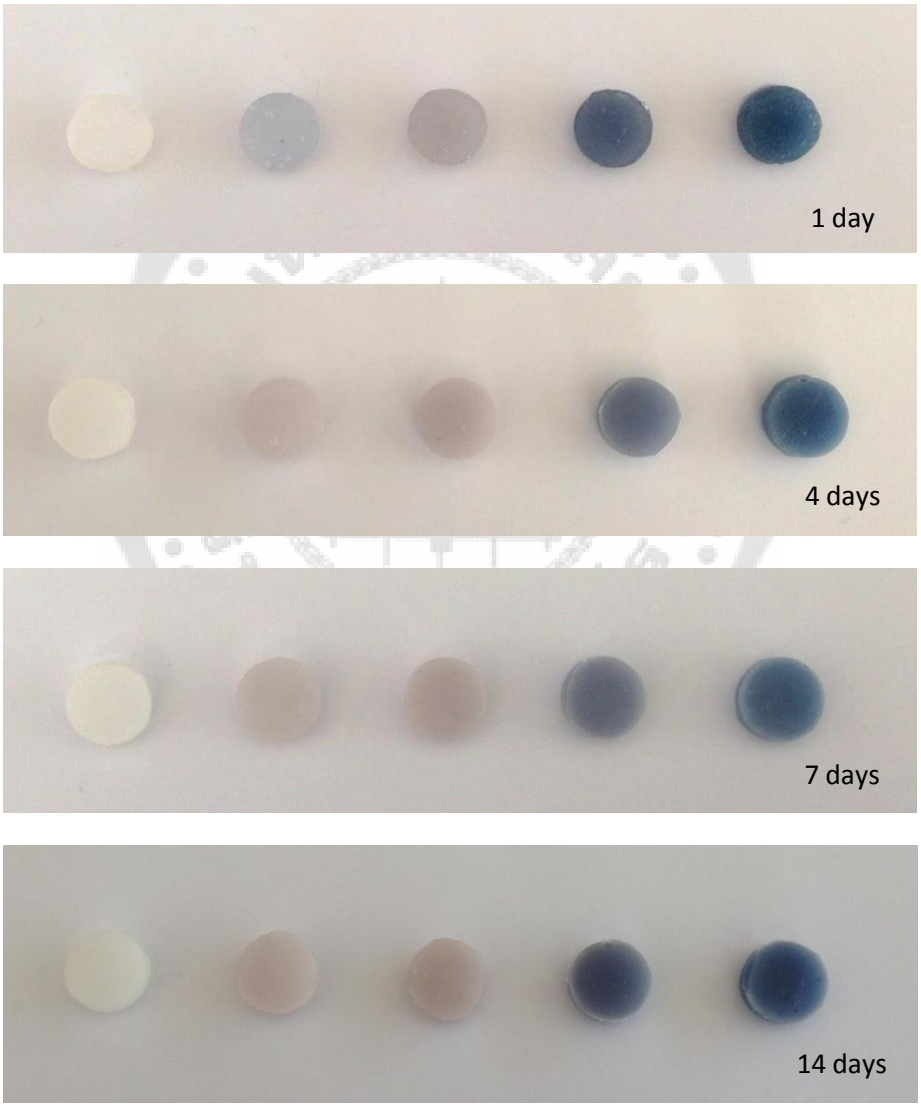


Fig 17 color of experimental specimen after 1,4,7 and 14 days

## Scanning electron microscopy evaluation

SEM images (Figure 18) showed silver nanoprisms(200ppm) containing acrylic soft liner at base and top part. The top of specimens have more nanoprism particles than base of specimens.

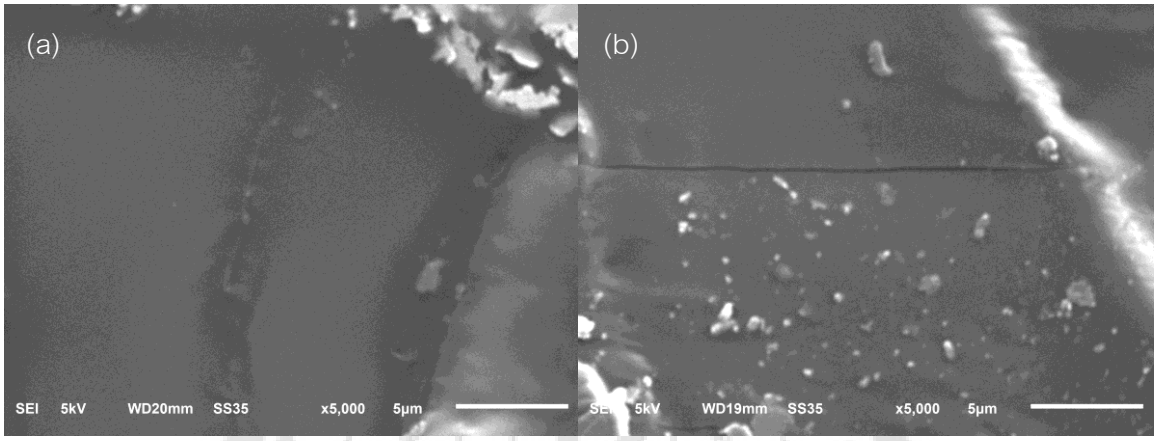


Figure 18 SEM images (5000X) of soft liner with silver nanoprisms (200ppm) at base (a) and top (b) of specimens. This shows silver nanoprisms particles in the top(b) of specimens.

# CHAPTER 5
# DISCUSSION

This study demonstrates that silver nanoprisms-containing acrylic soft lining materials have antimicrobial effect on *Staphylococcus aureus* and *Candida albicans*. *S. aureus* was detected in the mouths of systemically ill patients and may be reservoir of this organism among the serious ill. There are potential link between oral colonization with *S.aureus* and severe form of mucositis in elderly.[32] *S.aureus* has been isolated from infective oral condition, such as angular cheilitis and parotid. [34] *C. albicans* can be found in denture stomatitis about 50–70% . This pathology is an inflammatory process characterized by homogeneous erythema or red focal areas, especially in the palatal mucosa and usually associated with *Candida* species, particularly *Candida albicans.*[30]

The additional component, silver nanoprism to soft lining materials can be antimicrobial material. Methods of denture cleaning to eliminate the organisms can be chemical and mechanical. However, brushing or mechanical technique can damages surface of soft lining materials. Thus, chemical technique is proper method.[11] However, immersed denture into denture cleanser solution caused the change of surface roughness[12] and color stability.[12, 18] The enhancement of antimicrobial properties of soft lining material is the alternative approach to overcome these problems. There were previous studies of the additional component, silver nanoparticles to soft lining materials.[5, 7, 9]

The 1,4,7 and 14 days were chose for period to evaluate antimicrobial activity due to soft lining materials remain soft for a limit period (2 weeks). These materials were used for recovering soft tissue before fabricating a new denture or rebasing or relining denture. Plasticizer reduce the transition of the polymer from liquid to solid below mouth temperature and reduce the modulus of elasticity of soft lining materials.[20] Soft lining material is semiliquid in the mouth. However, leaching and evaporation of plasticizer leads to hardening of the material and water are absorbed into materials cause dimensional and mechanical

changes. Thus, these materials cannot be considered for permanent soft lining materials. [13, 18, 20]

The mechanism of bactericidal actions of silver nanoparticles is still not well understood. The action of silver nanoparticles is broadly similar to that of silver ion. Silver nanoparticles provide the bactericidal effect by interactions with the peptidoglycan cell wall and the plasma membrane. Silver nanoparticles prevent bacterial DNA replication by interacting with the exposed sulfhydryl groups in bacterial proteins, especially with the enzymes involved in vital cellular processes such as the electron transport chain.[40] Silver nanoparticles inhibit a respiratory enzymes, facilitating the generation of reactive oxygen species and consequently damaging the cell.[41]

The morphologies of silver nanoprisms were circular disk, truncated triangle, and hexagon after injection $H_2O_2$ into silver nanospheres colloid, these shapes promote antimicrobial effect. The inhibited bacterial growth level is significantly different for different nanoparticle shapes. For truncated triangular particles, almost complete inhibition of bacterial growth followed spherical nanoparticles and rod-shaped nanoparticles.[41]

According to the average percentage of *S. aureus* reduction results (Table5) showed the silver nanoprisms-containing acrylic soft lining material have ability to inhibit the growth of *S. aureus*. The addition of 25, 50 and 100 ppm of silver nanoprisms effected antimicrobial activity. There was significant difference on the antibacterial efficacy when compared with control group ($P<0.05$).In accordance with previous study, Nam(2011)[5] demonstrated that tissue conditioner containing silver nanoparticle showed microbial inhibitory effect against *S. aureus*.

The present study demonstrated the average percentage *C. albicans* reduction results (Table6) showed the silver nanoprisms-containing acrylic soft lining material have ability to inhibit the growth of *C. albicans*. The concentration of 50 ppm of silver nanoprisms effected antifungal activity. There was significant difference when compare with control

group (0 ppm) after period of time ($P$<0.05). These results correspond with other authors, Nam(2011)[5] demonstrated that tissue conditioner containing silver nanoparticle showed microbial inhibitory effect against *Candida albicans*. Chladek (2011)[7] showed the antifungal efficacy of silicone soft lining materials modified with silver nanoparticles , however, when silver nanoparticle concentration increased, the color of soft material became darker. The present study demonstrated the average optical density in control group results (Table7) showed that there is no statistically significant differences between group. These results is no influence of the color of specimen on the optical density.

In our study, the silver nanoprism concentration(50,100,200 ppm) resulted in significantly decrease in water sorption. The silver nanoprism concentration (200 ppm) resulted in significantly decrease in water solubility. These findings not in agreement with Chladek *et al*[9], who evaluated effect of silver nanoparticle incorporation into soft liner material on sorption and solubility. The difference may be result of differences in soft liner type, silicone-based liner was used in study. The silicone-based materials generally exhibit much lower sorption and solubility than acrylic-based lining materials because they do not contain components such as plasticizers that are rinsed out by water and consequently allow water absorption. The water sorption and solubility in our study were disagreement with Lima *et al*[52], who evaluated the addition of antifungals for *Candida albicans* biofilm on the water sorption and solubility of interim denture resilient liners. The difference may be result of differences in acrylic lining material and the addition in soft liner. The incorporation of antifungals (nystatin, ketoconazole and chlorhexidine diacetate) into the polymeric matrix may interfere with the material structure after polymerization is complete, causing an increase in material softness and ability to absorb water.

Water sorption depends on the degree of hydrophobicity and porosity of the material. The presence of a cross-linking agents has also been found to decrease water sorption and solubility.[53] Highly cross-linked materials showed lower water sorption than

non-cross-linked reline acrylic materials.[54] In addition, plasticizers in acrylic soft liner and the loss of ethanol, are thought to be the causes for the water sorption and solubility behavior. The previous study stated that ethanol diffused out of the liner more rapidly than water was absorbed. [54] The acrylic soft lining material with silver nanoprisms, with their hydrophobic nature, would allow water diffusion reduced. The silver nanoprisms containing soft liner resulted in decrease porosity then reduced water sorption. Furthermore, reduction of diffused water will reduce the leach out of soluble component from soft liner.

In our study, effect of aging time had significant increase on the mean hardness. This finding corroborates those of other studies of Mese and Guzel[21], who determined the effect of storage duration on the hardness of acrylic resin- and silicone-based resilient liners. The results of the current study are also in agreement with those of other researchers, who reported the hardness of acrylic soft liner increased over time.[19, 55-57]

When the acrylic soft lining materials immersed in water, two processes occurred. The leaching of plasticizers and other soluble materials into the water and the absorption of water by other polymer. The initial softness of the plasticized acrylic resins results from the plasticizer, which is responsible for maintaining material softness. Leaching of plasticizers causes hardening of acrylic soft liner. Factors such as processing methods, material type, beverages and thermocycling may affect hardness value. Mese *et al*[21] demonstrated that autopolymerized resilient liners are affected more hardness by immersion compared with heat-polymerized resilient liner materials. In addition, the hardness values of the acrylic resin-based showed greater changed than silicone-based liner. Safari *el al*[58] reported that the hardness values of the soft liners stored in beverages(Coca-Cola, 8% and 50% ethanol) would be lower than those of specimens immersed in distilled water. Qudah *et al*[57] have tested that thermocycling had a deleterious effect on the softness of soft lining materials. The addition silver nanoprism resulted increased hardness could be explained by decrease porosity and reduced water sorption subsequently hardnening.

This study compared hardness, water sorption and water solubility between different ratio of mixing. (experimental ratio type1 and experimental ratio type2). For water sorption (experimental ratio type1), the results were no significant differences in the mean water sorption values between 0 ppm and 25 ppm group. There were significantly decrease in water sorption among 50,100,200 ppm of silver nanoprisms group and control group. For experimental ratio type2, there were significantly decrease in water sorption among 25,50,100,200 ppm of silver nanoprisms group and control group(0 ppm). For water solubility(experimental ratio type1), there were significantly decrease in water solubility among 200 ppm of silver nanoprisms group and control group. There was no statistically significant among 0 ppm, 25 ppm and 50 ppm group, and among 0 ppm, 50 ppm and 100 ppm group. For experimental ratio type2, there were significantly decrease in water solubility among 50,100,200 ppm of silver nanoprisms group and control group(0 ppm).

The hardness of silver nanoprisms containing acrylic soft liner was detected by shore A hardness test during four periods. The addition of silver nanoprisms were significantly increase in mean hardness values for period of time. The specimens showed an increase in hardness with time. For experimental ratio type1, there were significantly increase in hardness among all groups. For experimental ratio type2, there were significantly increase in hardness among 25,50,100,200 ppm and control groups(0 ppm). There were no significant differences ($p>0.05$) in the mean hardness among 25,50 ppm and 100 ppm group, between 50 ppm and 200 ppm group.

The color of specimens changed after 4 days and no differences in color among specimens at 4,7,14 days. SEM images (5000X) of soft liner with silver nanoprisms (200ppm) shows silver nanoprisms particles in top of specimens. The silver nanoprisms particles at surface of contact mucosa cause antimicrobial activity. The optical density in control group(no microbial test )showed that there is no statistically significant differences. There is no influence of the color of specimens on the optical density test.

# CHAPTER 5
# CONCLUSION

From this study indicated that silver nanoprisms-containing acrylic soft lining material demonstrated ability to inhibit the growth of *Staphylococcus aureus* and *Candida albicans.* The addition of 25 ppm of silver nanoprisms effected antimicrobial activity. An increase in silver nanoprisms concentration resulted in an increase hardness, a decrease water sorption and water solubility.

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# APPENDIX

# Statistical analysis

## 1. Antimicrobial activity

Table1 Two-way Analysis of Variance for *S.aureus* test

Tests of Between-Subjects Effects

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 13260.057[a] | 19 | 697.898 | 3.073 | .000 |
| Intercept | 112542.522 | 1 | 112542.522 | 495.565 | .000 |
| ppm | 7444.059 | 4 | 1861.015 | 8.195 | .000 |
| time | 3024.751 | 3 | 1008.250 | 4.440 | .005 |
| ppm * time | 2791.248 | 12 | 232.604 | 1.024 | .429 |
| Error | 36335.874 | 160 | 227.099 | | |
| Total | 162138.453 | 180 | | | |
| Corrected Total | 49595.931 | 179 | | | |

Table2 Two-way Analysis of Variance for *C.albicans* test

Tests of Between-Subjects Effects

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 29383.664[a] | 19 | 1546.509 | 6.738 | .000 |
| Intercept | 191522.879 | 1 | 191522.879 | 834.463 | .000 |
| ppm | 2578.720 | 4 | 644.680 | 2.809 | .027 |
| time | 24429.598 | 3 | 8143.199 | 35.480 | .000 |
| ppm * time | 2375.346 | 12 | 197.945 | .862 | .586 |
| Error | 36722.604 | 160 | 229.516 | | |
| Total | 257629.147 | 180 | | | |
| Corrected Total | 66106.268 | 179 | | | |

## 2. Water sorption and solubility test

Table 3 One-way Analysis of Variance

ANOVA

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| sorption | Between Groups | 7.093 | 4 | 1.773 | 17.277 | .000 |
| | Within Groups | 2.053 | 20 | .103 | | |
| | Total | 9.145 | 24 | | | |
| solubility | Between Groups | 2.502 | 4 | .625 | 21.620 | .000 |
| | Within Groups | .579 | 20 | .029 | | |
| | Total | 3.080 | 24 | | | |

Table 4 Multiple comparisons with Tukey HSD

Multiple Comparisons

Tukey HSD

| Dependent Variable | (I) ppm | (J) ppm | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| sorption | 0ppm | 25ppm | .215312 | .202609 | .823 | -.39097 | .82159 |
| | | 50ppm | 1.205945* | .202609 | .000 | .59966 | 1.81223 |
| | | 100ppm | 1.054078* | .202609 | .000 | .44779 | 1.66036 |
| | | 200ppm | 1.269389* | .202609 | .000 | .66311 | 1.87567 |
| | 25ppm | 0ppm | -.215312 | .202609 | .823 | -.82159 | .39097 |
| | | 50ppm | .990633* | .202609 | .001 | .38435 | 1.59692 |
| | | 100ppm | .838766* | .202609 | .004 | .23248 | 1.44505 |
| | | 200ppm | 1.054078* | .202609 | .000 | .44779 | 1.66036 |
| | 50ppm | 0ppm | -1.205945* | .202609 | .000 | -1.81223 | -.59966 |
| | | 25ppm | -.990633* | .202609 | .001 | -1.59692 | -.38435 |
| | | 100ppm | -.151867 | .202609 | .942 | -.75815 | .45442 |
| | | 200ppm | .063444 | .202609 | .998 | -.54284 | .66973 |
| | 100ppm | 0ppm | -1.054078* | .202609 | .000 | -1.66036 | -.44779 |
| | | 25ppm | -.838766* | .202609 | .004 | -1.44505 | -.23248 |
| | | 50ppm | .151867 | .202609 | .942 | -.45442 | .75815 |
| | | 200ppm | .215312 | .202609 | .823 | -.39097 | .82159 |
| | 200ppm | 0ppm | -1.269389* | .202609 | .000 | -1.87567 | -.66311 |
| | | 25ppm | -1.054078* | .202609 | .000 | -1.66036 | -.44779 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | 50ppm | -.063444 | .202609 | .998 | -.66973 | .54284 |
| | | 100ppm | -.215312 | .202609 | .823 | -.82159 | .39097 |
| | | 50ppm | -.063444 | .202609 | .757 | -.48608 | .35919 |
| | | 100ppm | -.215312 | .202609 | .301 | -.63795 | .20732 |
| solubility | 0ppm | 25ppm | -.100912 | .107569 | .879 | -.42280 | .22098 |
| | | 50ppm | .194830 | .107569 | .395 | -.12706 | .51672 |
| | | 100ppm | .272761 | .107569 | .122 | -.04913 | .59465 |
| | | 200ppm | .808792* | .107569 | .000 | .48691 | 1.13068 |
| | 25ppm | 0ppm | .100912 | .107569 | .879 | -.22098 | .42280 |
| | | 50ppm | .295741 | .107569 | .081 | -.02615 | .61763 |
| | | 100ppm | .373673* | .107569 | .018 | .05179 | .69556 |
| | | 200ppm | .909704* | .107569 | .000 | .58782 | 1.23159 |
| | 50ppm | 0ppm | -.194830 | .107569 | .395 | -.51672 | .12706 |
| | | 25ppm | -.295741 | .107569 | .081 | -.61763 | .02615 |
| | | 100ppm | .077932 | .107569 | .948 | -.24395 | .39982 |
| | | 200ppm | .613963* | .107569 | .000 | .29208 | .93585 |
| | 100ppm | 0ppm | -.272761 | .107569 | .122 | -.59465 | .04913 |
| | | 25ppm | -.373673* | .107569 | .018 | -.69556 | -.05179 |
| | | 50ppm | -.077932 | .107569 | .948 | -.39982 | .24395 |
| | | 200ppm | .536031* | .107569 | .001 | .21414 | .85792 |
| | 200ppm | 0ppm | -.808792* | .107569 | .000 | -1.13068 | -.48691 |
| | | 25ppm | -.909704* | .107569 | .000 | -1.23159 | -.58782 |
| | | 50ppm | -.613963* | .107569 | .000 | -.93585 | -.29208 |
| | | 100ppm | -.536031* | .107569 | .001 | -.85792 | -.21414 |

## 3. Hardness test

Table 5 Two-way Analysis of Variance

**Tests of Between-Subjects Effects**

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 13353.966[a] | 19 | 702.840 | 126.802 | .000 |
| Intercept | 99919.210 | 1 | 99919.210 | 18026.847 | .000 |
| ppm | 5775.284 | 4 | 1443.821 | 260.486 | .000 |
| time | 7453.268 | 3 | 2484.423 | 448.225 | .000 |
| ppm * time | 125.414 | 12 | 10.451 | 1.886 | .049 |
| Error | 443.424 | 80 | 5.543 | | |
| Total | 113716.600 | 100 | | | |
| Corrected Total | 13797.390 | 99 | | | |

# VITAE

# VITAE

| | |
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