Antifungal, physical, and mechanical properties of tissue conditioner incorporated with zinc oxide nanoparticles

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Miss Woraporn Homsiang

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คุณสมบัติการยับยั้งเชื้อรา คุณสมบัติทางกายภาพและคุณสมบัติเชิงกลของวัสดุฉาบฐานฟันเทียมชนิดนุ่มที่ใส่อนุภาคนาโนซิงค์ออกไซด์

น.ส.วรพร หอมเสียง

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By	Miss Woraporn Homsiang
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Thesis Advisor	Professor MANSUANG ARKSORNNUKIT, D.D.S., M.S., Ph.D.

Accepted by the FACULTY OF DENTISTRY, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctor of Philosophy

.... Dean of the FACULTY OF DENTISTRY

(Associate Professor Pornchai Jansisyanont, D.D.S., M.S., Ph.D.)

DISSERTATION COMMITTEE

.... Chairman

(Assistant Professor Pisaisit Chaijareenont, D.D.S., M.S., Ph.D.)

.... Thesis Advisor

(Professor MANSUANG ARKSORNNUKIT, D.D.S., M.S., Ph.D.)

.... Examiner

(Assistant Professor ORAPIN KOMIN, D.D.S., Ph.D)

.... Examiner

(Assistant Professor KRID KAMONKHANTIKUL, D.D.S., Ph.D.)

.... Examiner

(NAREUDEE LIMPUANGTHIP, D.D.S., Ph.D.)

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การศึกษานี้ทดสอบคุณสมบัติการยับยั้งเชื้อรา คุณสมบัติทางกายภาพและคุณสมบัติเชิงกลของวัสดุฉาบฐานฟันเทียมชนิดนุ่มที่ใส่อนุภาคนาโนซิงค์ออกไซค์ในปริมาณและระยะเวลาการแช่น้ำที่ต่างกัน (0, 7 และ 14 วัน) ชิ้นงานวัสดุฉาบฐานฟันเทียมชนิดนุ่มที่ใส่อนุภาคนาโนซิงค์ออกไซค์ ร้อยละ 0, 5, 10 และ 15 หรือใส่ยาชนิดนิสแททินร้อยละ 15 โดยน้ำหนักถูกสร้างขึ้น วัสดุฉาบฐานฟันเทียมชนิดนุ่มกลุ่มต่างๆ ถูกนำไปทดสอบการยับยั้งเชื้อราต่อเชื้อราชนิดแคนดิดา อัลบิแคนส์โดยวิธีสัมผัสโดยตรง วิเคราะห์ปริมาณและการกระจายตัวของอนุภาคซิงค์ด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราดร่วมกับเทคนิคการวัดการกระจายพลังงานของรังสีเอกซ์ การทดสอบความลึกของพื้นผิวเมื่อกดและการทดสอบกำลังยึดติดแบบดึงถูกทดสอบตามมาตราฐานองค์การระหว่างประเทศว่าด้วยการมาตราฐานเลขที่ 10139 พบว่า วัสดุฉาบฐานฟันเทียมชนิดนุ่มที่ใส่อนุภาคนาโนซิงค์ออกไซค์ร้อยละ 15 สามารถลดจำนวนเชื้อราได้อย่างมีนัยสำคัญในทุกช่วงเวลา วัสดุฉาบฐานฟันเทียมชนิดนุ่มที่ใส่ยาชนิดนิสแททินร้อยละ 15 สามารถลดจำนวนเชื้อราได้ดีที่สุดที่เวลา 0 วัน ปริมาณอนุภาคซิงค์ที่พบบริเวณพื้นผิวมีความสัมพันธ์ในทิศทางตรงกันข้ามกับจำนวนเชื้อราที่พบ ความลึกของพื้นผิวและแรงยึดติดแบบดึงของวัสดุฉาบฐานฟันเทียมชนิดนุ่มที่ปรับแต่งทุกกลุ่มไม่มีความแตกต่างกันอย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มควบคุมในทุกช่วงเวลา จึงสรุปได้ว่า วัสดุฉาบฐานฟันเทียมชนิดนุ่มที่ใส่อนุภาคนาโนซิงค์ออกไซค์ร้อยละ 15 มีคุณสมบัติการยับยั้งเชื้อราเป็นระยะเวลา 14 วัน โดยไม่ส่งผลเสียต่อคุณสมบัติความลึกของพื้นผิวเมื่อกดและกำลังยึดติดแบบดึง

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This study evaluated the antifungal, physical, and mechanical properties of tissue conditioner incorporated with different amounts of zinc oxide nanoparticles (ZnOnps) at different storage times (0, 7, and 14 days). Specimens of 0, 5, 10, 15 wt% ZnOnps, or 15 wt% nystatin incorporated into tissue conditioner were fabricated. The direct contact test (n=6) was performed to evaluate the antifungal effect against *C. albicans* suspension. Scanning electron microscopy and energy dispersive X-ray spectroscopy (n=6) were used to observe the amount and distribution of superficial zinc element. The penetration depth (n=6) and tensile bond strength (n=8) were evaluated following ISO 13139. The 15Zn group demonstrated a significantly reduced *C. albicans* cell number compared with the control group at all storage times ($p<0.001$). The Nys group had the greatest reduction in *C. albicans* cell number only at day 0. Pearson’s correlation coefficient between the amount of superficial zinc element and the number of *C. albicans* cells showed a significant negative linear correlation ($r=-0.78$, $p<0.001$). The penetration depths and tensile bond strengths of the 5Zn, 10Zn, 15Zn, and Nys were not significantly different compared with control at all storage times ($p>0.05$). In conclusion, the 15Zn provides antifungal effect up to 14 days without adverse effects on penetration depth and tensile bond strength.

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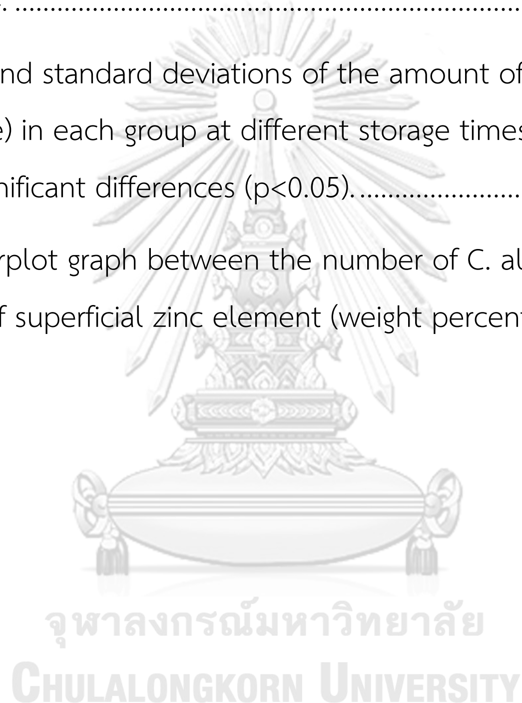
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CHAPTER 1

INTRODUCTION & LITERATURE REVIEW

Background and rationale

Removable denture prostheses are used to rehabilitate patients with tooth loss. Wearing denture can assist in enhancing masticatory performance, solving psychosocial problems, and improving the quality of life. However, using aged denture or having unhygienic denture cares might lead to unintentionally infection, especially in patient who presents with immunocompromised disorder.[1]

Denture stomatitis is one of the most common complications of long-term denture wearers. The etiology of denture stomatitis is multifactorial factors such as poor oral hygiene, ill-fitting denture, denture trauma, nocturnal continuous denture wearing, and smoking.[2, 3] *Candida* species are addressed to be the main contributor of this disease, especially *Candida albicans* (*C. albicans*).[4] *C. albicans* cells often adhere and colonize within the cracks, fissures, and pores of acrylic denture surface.[5] A complex biofilm formation is then formed after bacterial aggregation. The symptoms of denture stomatitis are usually painless and asymptomatic. Other symptoms, such as taste alteration, halitosis, burning sensation and mucosal bleeding, can be individually developed in each patient.[3] This disease is characterized by the inflammation of oral lesion and confirmed by *C. albicans* culture from oral lesion and denture.[6]

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Several methods in treating denture stomatitis, such as topical antifungal therapy, systemic antifungal medication, oral hygiene care, denture irradiation with microwaves, disinfectant soaking and replacement of old denture have been suggested.[7-10] One of the effective methods is to take the denture out of the mouth for a period of time. However, this method might not be applicable in some patients who cannot take denture out for a long period because of esthetic concern and masticatory problem. To eliminate fungal in acrylic denture base, relining procedure with tissue conditioner after grinding tissue surface of denture is generally used.[11] This method disrupts the reinfection cycle and recovers traumatized oral mucosa before making a new denture.

Tissue conditioners, short-term used resilient denture liner, are widely used for conditioning of the oral mucosa. It provides a cushion effect that can distribute occlusal forces and relieve pain from denture soreness. Tissue conditioners consist of polyethyl methacrylate (PEMA) powder, plasticizing agents, and ethyl alcohol.[12] The limitation of these materials is a short period of usage according to leaching out of plasticizing agents and ethyl alcohol. This phenomenon results in loss material's viscoelastic properties. Moreover, the texture of tissue conditioners is susceptible to colonization by microorganism.[13] Several *in vitro* and *in vivo* studies focusing on the incorporation of different antimicrobial agents into the tissue conditioner to disinfect microbes or to prevent microorganism growth.[14-18] However, the incorporation of

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such antimicrobial agents might alter physical and mechanical properties of materials. The antimicrobial effect might also not exist for a long period of time in clinic service.

Zinc oxide (ZnO) have been found to exhibit strong antimicrobial effect against various bacterial and fungal strains. The nanoscale metal oxides are synthesized to improve their ability in material applications. The effectiveness of antimicrobial effect increased with a reduction in particle size of ZnO.[19, 20] Moreover, ZnOnps inhibited *C. albicans* growth with a concentration-dependent manner.[21, 22] To date, there is no study about the optimum amount of ZnOnps incorporated into the tissue conditioner that provided the inhibition of *C. albicans* cells growth and maintained its physical and mechanical properties for duration of use. Besides, superficial distribution pattern of ZnOnps incorporated into tissue conditioner are also limited information.

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Literature review

1. Denture stomatitis

1.1 Epidemiology, symptoms, and diagnosis

Denture stomatitis (denture sore mouth or chronic erythematous atrophic candidiasis) is the most common disorder in long-term denture wearers and characterized as an inflammation reaction beneath a denture.[3] Oral mucosa covered by the denture is susceptible to a reservoir for pathogen growth because of the

reductions of saliva and oxygen flow. This condition is commonly found on maxillary arch more than mandibular arch.[2, 4] Epidemiological studies reported many prevalence values of denture stomatitis, ranged from 15% to over 70%, depended on various population samples in each study. Despite its frequency, denture stomatitis is usually painless and asymptomatic.[3] Some patients may undergo with different symptoms such as mucosal bleeding, halitosis, mouth dryness, taste alteration, burning sensation, itching, or swelling of mucosal tissues covered by denture. Diagnosis of the denture stomatitis begin with clinical observations which occur as a tissue inflammation at the palate. *C. albicans* culture from the denture and underlying mucosa are also evaluated.[7]

1.2 Classification and etiology

The first denture stomatitis classification was classified into three types based on severity and clinical appearance by Newton in 1962. Type one is a localized inflammation or pinpoint hyperemia. Type two, the most common, shows erythema or redness involving palatal mucosal area which is covered by the denture. Type three is an inflammatory nodular or papilla hyperplasia on central of hard palate. The other classification which modified from Newton's classification has been proposed by Barboau *et al*. in 2003. The clinical observations were recorded with more intensive diagram. The presence of plaque and the number of *Candida* species on denture were also evaluated in this classification. The presence of *Candida*

species on the denture was significantly related to the extent of inflammation.[23] The etiology of denture stomatitis has been reported as a multifactorial disease. An ill-fitting denture, denture trauma, nocturnal continuous denture wearing, increased age of denture, poor oral hygiene and smoking were associated with denture stomatitis and accepted as critical risk factors.[3] In contrast, age, sex, retention, stability, cleansing frequency, and palate brushing were not statistically related with denture stomatitis.[23, 24] A prevalence of denture stomatitis may increase when systemic factors of the host present a depression in the defense mechanisms such as diabetes, deficiency of nutritional factors and kidney affections.[25] Nevertheless, *Candida* species are considered as important opportunistic pathogens. Of the many *Candida* species, *C. albicans* is the causative pathogen of denture stomatitis.[4] *C. albicans* is generally presented as a normal oral microflora around 30 to 50% in healthy persons and increased with the age of the patients. In patients over 60 years, the intraoral existence of *C. albicans* is found about 60% in dentate patients.[26] The first step of infection process starts with *C. albicans* adherence to the oral mucosa and hydrophobic acrylic surface of prosthesis.[2, 25] After attaching, *C. albicans* can divide, penetrate, and secrete exopolymeric materials. The bacteria, *Streptococcus* and *Staphylococcus*, are aggregated by using the interactions between proteins and carbohydrates and results in complex biofilm.[25, 27] The differences in biofilm-formatting ability were observed from different isolates recovering from the same patient.[27] A tightly attached biofilm forms on the rough surface of the denture while

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a loosely attached biofilm is found on the smooth surface. It was observed that the microbes on the rough surfaces often colonized within the cracks, fissures, and pores by using scanning electronic microscope (SEM).[5] These cracks, fissures, and pores may become the protective sites for *Candida* cells from denture cleaning.

Macroscopic characteristics of *Candida* species are soft cream-colored colonies under aerobic conditions which have a pH in the range of 2.5-7.5, and a temperature in the range of 20-38°C. Microscopic characteristics of all the *Candida* species are similar. *C. albicans* can classified from other species by fermentation tests, sugar assimilation, and growth characteristics.[26] *C. albicans* exhibits dimorphism: ovoid or spherical blastopores (yeast cells) and parallel-sided hyphae.[28] These forms can switch from one form to another. Hyphal form is considered more pathogenic and higher resistance to antifungal therapy than yeast form. The cell wall of *C. albicans* consists of the polysaccharides such as glucan and chitin. The layers and morphologies of cell wall are different depended on stage of growth, medium, and form. The adhesion of *C. albicans* to host cells involved manner or mannoprotein of cell wall adhered with the fucose or N-acetylglucosamine-containing glycosides of host epithelial cells.[25, 28] An adhesion between *C. albicans* and acrylic denture bases is controlled by Van der Waals force (hydrophobic forces) and electrostatic forces.

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1.3 Treatment of denture stomatitis

The effectiveness of treatment in denture stomatitis is to eliminate a reservoir of infection. The treatment of denture stomatitis is quite difficult and complex due to its multifactorial etiology.[5] The therapeutic strategies are still not definite. There are a large number of methods in treating denture stomatitis as follows;

1. Antifungal drugs and natural products: During the topical application of antifungal drugs, patients are recommended not to wear the denture for at least 14 days.[26] Chlorhexidine, and nystatin are the commonly used as topical agents for prevention and treatment of denture stomatitis.[29] Nystatin, water insoluble polyenes type, binds to ergosterol in cell membrane of *C. albicans*, leads to an increase in cell permeability, and causes cell death.[7] While chlorhexidine, cationic molecule, binds to the phospholipids in call wall of *C. albicans* and changes the cell permeability. The leakage of fluid of cell components results in cell death. The successful topical antifungal drugs in treating of denture stomatitis depends on several factors such as unpleasant taste of drug, maintaining level of drug concentration from saliva and swallowing, patient perception of infection, and continuous denture wearing.[16]

If treating with topical antifungal drugs is unsuccessful, systemic antifungal drugs may be considered. The commonly used systemic antifungal drugs are antifungal suspensions based on amphotericin B, fluconazole, itraconazole, miconazole and clotrimazole. Almost drugs are recommended to be continuously

used for 12-14 days. The risk adverse effects and complications of systemic antifungal drugs for the management of denture stomatitis must be concerned. Amphotericin B can cause nephrotoxicity.[30] Besides, the development of resistant species may occur if systemic drugs are used with a long period.[2] In immunocompromised patients, the responsible failure of treating with fluconazole and itraconazole was found. The tested for drug susceptibility should be done.[30]

Koray *et al* compared the influence of hexetidine mouthrinses, fluconazole 50 mg once a day, and a combination between first and second methods for the management of 61 patients who presents with denture stomatitis. *C. albicans* in mucosa, saliva, and denture after treatment for 14 days showed no statistically significant difference when all groups were compared.[31]

Some studies interested in treating denture stomatitis with natural product such as lemongrass oil, propolis gel (plant product), and Zataria multiflora essential oil.[32-34] The morphology changes of *C. albicans* treated with lemongrass oil was observed with SEM, transmission electronic microscope (TEM) and atomic force microscope (AFM). The changes of *C. albicans* cell morphology and substructure were found.[32] *In vivo* study, the palatal edema and erythema of denture stomatitis patients who applied propolis oil 4 times a day for 7 days had completely healed comparable to micronazole gel.[33] The randomized controlled study of denture stomatitis patients with two treatment protocol was study. The first group was

treated with 2% miconazole gel 4 times a day for 14 days while the second group was treated with 0.1% Zataria multiflora essential oil with similar manner. The erythema surface degree of both groups was decreased but showed no statistically significant difference. However, the number of colony-counts of *C. albicans* on palate in treating with 2% miconazole gel was lower than that of 0.1% Zataria multiflora essential oil at day 0, 7, and 14.[34]

2. Microwave irradiation: Irradiation with microwave has been proposed for the denture disinfection. The irradiation is a high-frequency electromagnetic radiation that promote structural alterations of *C. albicans'* cell wall.[35] Time, power, frequency, and distance from the source of microwaves relate to *C. albicans* viability. The *in vitro* study reported that microwaving denture at 650 W for 6 minutes might be effective method compared to denture soaking for 8 hours in 0.02% sodium hypochlorite. According to the systematic review and meta-analysis, the disinfection effect of microwave irradiation is equivalent to 0.02% sodium hypochlorite, 0.2% chlorhexidine, and 100,000 IU/mL topical nystatin. Besides, microwaves disinfection is more effective than topical micronazole. The advantages of microwaves disinfection are simple, safe, and inexpensive method.[9] Using microwave irradiation at 650 W for 3 minutes does not affect to flexural strength and hardness of acrylic denture bases. Nevertheless, dimensional changes of dentures can be possibly occurred when the irradiation takes place in a wet environment.[36] This method does not remove non-

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visible microorganism from the denture surface demonstrated in SEM.[8] The contraindication of this method is it cannot disinfect the dentures with metal parts.

3. Chemical soaking: A previous study recommended the overnight soaking of acrylic dentures in 0.02% sodium hypochlorite for several weeks effectively reduced the number of *Candida* cells. However, using sodium hypochlorite extensively may lead to prosthesis damage such as discoloration.[7, 8, 25] Systematic review found that immersion of dentures in chlorhexidine or glutaraldehyde was more effective than placebo.[10]

4. Elimination of fungal infection in acrylic denture base: The porous within acrylic denture base can serve as a reservoir of the fungal microorganisms and contribute to reinfection. Grinding tissue surface of denture is generally used for disrupting the reinfection cycle followed by relining procedure with resilient denture liners. The resilient denture liners may be used to cushion the acrylic denture base against oral mucosa and revitalize the tissue. However, the rough surface of this material may be susceptible to the attachment and colonization of *C. albicans*.[3] The incorporation of an antimicrobial/ antifungal agents into resilient denture liners have been studied both *in vitro* and *in vivo*.

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2. Resilient denture liner

2.1 Definition and terminology

According to the ninth edition of the Glossary of Prosthodontic terms (GPT-9), defined resilient denture liner as an interim (ethyl methacrylate with phthalate plasticizers) or definite (processed silicone) liner of the intaglio surface of a removable complete denture, removable partial denture, or intraoral maxillofacial prosthesis. While the definition of tissue conditioner is a resilient denture liner resin placed into a removable prosthesis for a short duration to allow time for tissue healing and can use in functional removable relining procedures to evaluate denture function and patient acceptance prior to laboratory reline processing.[37]

According to ISO 10139-1: 2005, defined temporary soft lining material as the soft lining material for dentures that is intended to be used for a limited period to improve fit, retention, and comfort. While the definition of tissue conditioning material is soft lining material, placed in the fitting surface of a denture, that is intended to be in contact with the denture-supporting mucosa, commonly for a period of up to 7 days, with the aim of assisting its return to a healthy condition.[38]

2.2 Classification, composition, and application

Resilient denture liners can be divided into several types based on different classification criteria which are polymerization process, chemical structure, and duration of use. Based on chemical structure, resilient denture liners can be divided

into 2 groups: acrylic resin-based and silicone-based resilient denture liners. Both groups are available as autopolymerized or heat activated types.[39] Based on duration of use, resilient denture liners can be divided into 2 groups: short-term and long-term resilient denture liners.[12]

According to the viscoelastic properties or cushion like effect of resilient denture liners, they can distribute the mastication forces, evenly absorb energy, and act as a shock absorber.[12] Resilient denture liners are widely used in cases with occurrence of mucosa lesions, severe undercuts of the alveolar bone, and severe or irregular resorption of the alveolar bone with thin mucosa. Immediate denture often requires temporary liners during the wound healing. Resilient denture liners can also be used to modify removable dentures during implant healing and rehabilitate cancer patients requiring obturations. In functional impression taking, some resilient denture liners can be used as an adjunctive technique.[11, 12]

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2.2.1 Short-term resilient denture liner (Tissue conditioner)

Tissue conditioner is short-term autopolymerized acrylic resin-based resilient denture liners. It consists of two parts: powder and liquid. The powder part commonly consists of polyethyl methacrylate (PEMA) or related copolymer without any initiators. The usage of PEMA powder instead of polymethyl methacrylate (PMMA) powder is explained by the solubility parameters of PMMA for strongly bonded solvent is zero. It cannot be dissolved by ethyl alcohol.[40] Moreover, glass

transition temperature (Tg), the temperature at polymeric materials softens when heated above this temperature, of PEMA powder (65°C) is also lower than PMMA powder (100-120°C). (Figure 1) According to the lower Tg value of PEMA, the plasticizing agents in liquid part are less required.

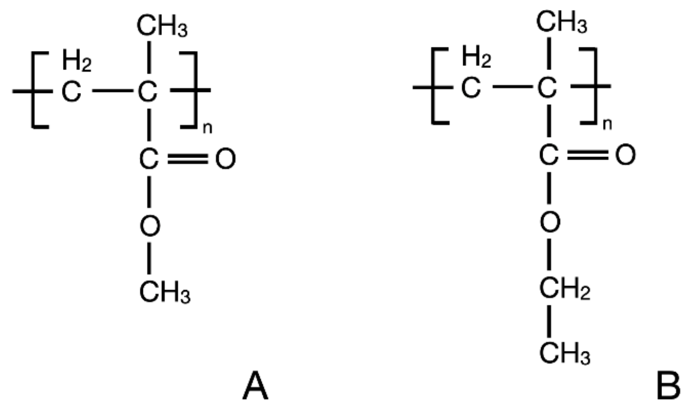

Figure 1: Chemical structures of polymer powder. A: polymethyl methacrylate (PMMA), B: polyethyl methacrylate (PEMA).

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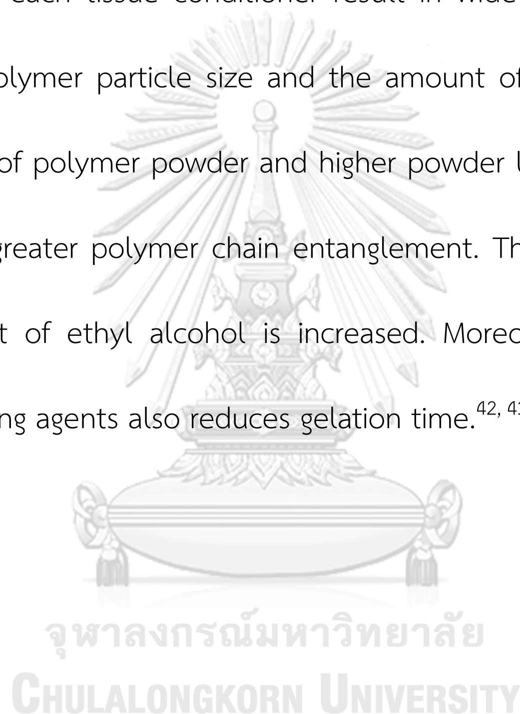
The liquid part consists of ethyl alcohol, highly polar component, and plasticizing agents. Commonly, the amount of an ethyl alcohol is generally between 5 wt% up to 30 wt% depends on the molecular weight of the polymer powder and its particle size. Lower molecular weight or smaller particle size requires less amount of ethyl alcohol.[40] Function of ethyl alcohol is to rapidly swell the polymer particles and allow the plasticizing agent to diffuse between swollen polymer particles resulting in gel formation. Mostly plasticizing agents of tissue conditioners are

aromatic esters such as dibutyl phthalate or DBP (molecular weight (MW) = 278.34), butyl phthalate butyl glycolate or BPBG (MW = 336.38), butyl benzyl phthalate or BBP (MW=312.35), and benzyl benzoate or BB (MW=212.1). Some commercial product (SR Ivoseal, Ivoclar Vivadent) used aliphatic ester which is dibutyl sebacate or DS (MW=314.47), as a plasticizing agent (Figure 2).[41] The various type and amount of composition in each tissue conditioner result in wide range of flow or gelation time. Based on polymer particle size and the amount of ethyl alcohol, the higher molecular weight of polymer powder and higher powder liquid ratio reduce gelation time because of greater polymer chain entanglement. The gelation time decreases when the amount of ethyl alcohol is increased. Moreover, the lower molecular weight of plasticizing agents also reduces gelation time.[42, 43]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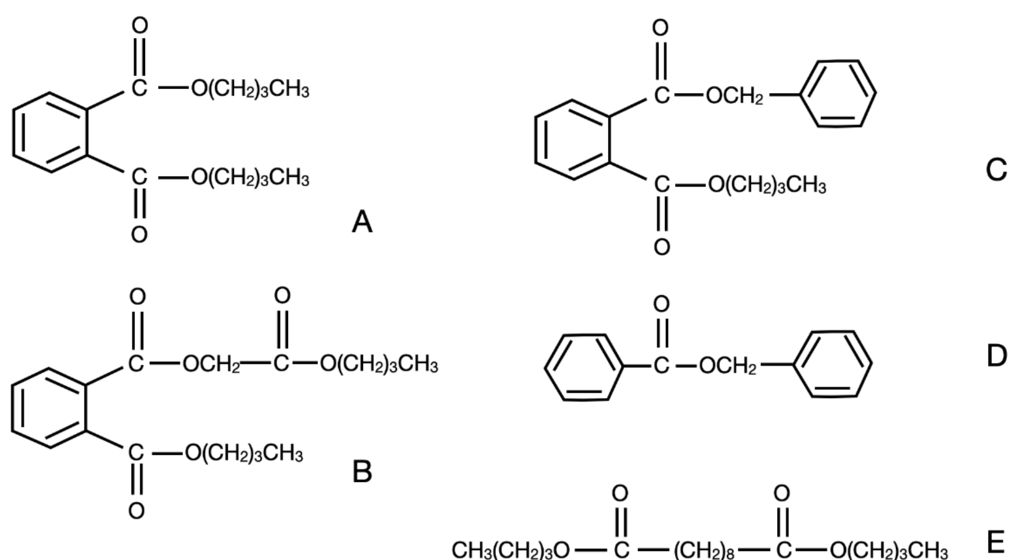

Figure 2: Chemical structures of plasticizing agents. A: dibutyl phthalate, B: butyl phthalate butyl glycolate, C: butyl benzyl phthalate, D: benzyl benzoate, E: dibutyl sebacate.

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The advantage of tissue conditioner is their ability to bond to the PMMA denture resin base because of similar chemical composition. However, the limitation of using tissue conditioner is short period of used. The ethyl alcohol and the plasticizing agents leach out resulting in the loss of viscoelastic properties of tissue conditioner. Jones *et al*. demonstrated that ethyl alcohol was completely lost within 24 h from polymer gel materials stored in water at 37°C.[41] On the other hand, Ellis *et al*. claimed that not all of ethyl alcohol diffused out even after 4 ¾ months conditioning at 37° C.[44] The plasticizing agents were leached out more slowly. The gas chromatography investigation of plasticizing agents' leachability showed that the lower molecular weight esters exhibited the highest loss at day 14.[41] However, the

loss of ethyl alcohol and plasticizing agents of tissue conditioner is quite difficult to estimate because of two-way exchange of fluids as shown in Figure 3. The plasticizing agents and ethyl alcohol leach out while the absorption of water occurs.[41, 45] For remaining of cushioning effect, fresh tissue conditioner should be replaced every 3 to 4 days until full recovery of mucosa has occurred.[12] Delay of tissue conditioner replacement leads to lose surface integrity and increase surface roughness. The rougher surfaces enhance the adhesion of microorganisms onto tissue conditioner and allow fungal growth.

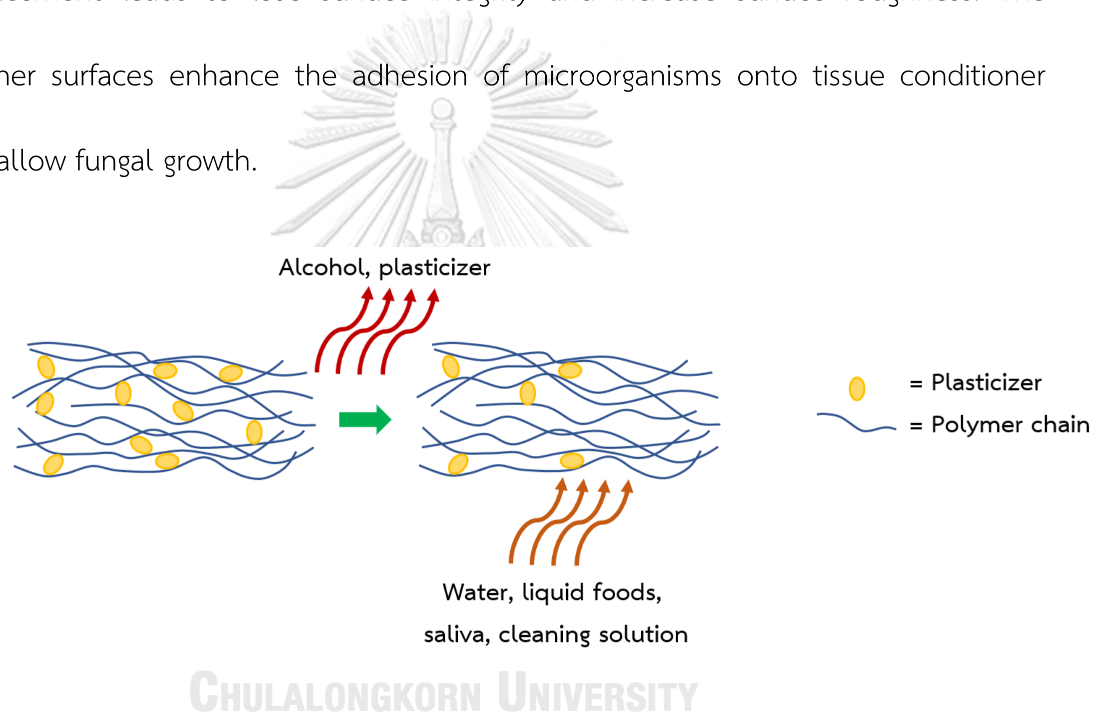

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Figure 3: Two-way exchange of fluids of tissue conditioner.

2.2.1.1 Desirable properties of short-term resilient denture liner

One of the desirable properties of tissue conditioner is viscoelasticity. This material should be able to adapt itself to the irritated tissue for several days and properly distribute occlusal forces. Besides, tissue conditioner should resist to tear,

staining, fungal and bacterial growth. They also have good dimensional stability, proper bond strength to denture base and biocompatibility. According to ISO 10139-1, soft lining materials for short-term use, changing in compliance with age as measured by penetration depth is one of the fundamental properties of soft lining material. The initial resistance to indentation at 2 hours after mixing is the criteria to categorize soft lining materials into two classes. Class 1 is a highly initial compliance which has penetration depth more than or equal 1.5 mm. If the penetration depth is lower than 1.5 mm, the material is classified as class 2, low initial compliance. However, the penetration depth at 7 days of all soft-lining materials shall be more than 0.5 mm.[38]

Table 1: Trade name and composition of the tissue conditioners.

Trade name	Manufacturer	Composition
GC Soft-liner	GC Corp., Tokyo, Japan	Powder: Polyethyl methacrylate Liquid: 15% Ethyl alcohol, 85% Butyl phthalate butyl glycolate
Coe-comfort	GC Corp., Illinois, USA	Powder: Polyethyl methacrylate Liquid: 5-10% Ethyl alcohol, 50-70% Benzyl benzoate, >1% Peppermint oil, >0.5% Butylated hydroxytoluene

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Visco-gel	Dentsply Caulk, Milford, USA	Powder: Polyethyl methacrylate Liquid: 2.5-10% Ethyl alcohol, >50% Butoxycarbonyl methyl butyl phthalate, 2.5-10% dibutyl phthalate
Hydro-Cast	Sultan Healthcare, Pennsylvania, USA	Powder: Polyethyl methacrylate Liquid: 10-20% Ethyl alcohol, 1-5% Acetone, 80-90% Butyl benzyl phthalate
Fitt	Kerr, Salerno, Italy	Powder: Polyethyl methacrylate Liquid: 15-22 % Ethyl alcohol, 75-85% Dibutyl phthalate
Softone	Keystone industries, Pennsylvania, USA	Powder: Polyethyl methacrylate, Pigment, 0.5-5%Titanium dioxide Liquid: ≥10-≤25% Ethyl alcohol, ≥50-≤75% Dibutyl phthalate
Permasoft	Dentsply Sirona, Waverley, Australia	Powder: Ethyl methacrylate homopolymer Liquid: Ethyl alcohol, Ethyl acetate,

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Dibutyl phthalate

2.2.1.2 Antimicrobial agents in tissue conditioner

Several *in vitro* and *in vivo* studies investigated the effectiveness of antifungal agent incorporated with tissue conditioners and other resilient denture liners in different antimicrobial assay.

Most studies attempted to incorporate different antifungal drugs, both of topical and systemic drugs, into the tissue conditioners or resilient denture liners. Chow *et al*. studied the incorporation of nystatin, fluconazole, itraconazole into Coe-Soft, Visco-gel and Fitt and evaluated the antifungal effect against *C. albicans* by using modified disc diffusion method. The result showed that itraconazole had greater fungicidal activity than fluconazole. While nystatin demonstrated the least fungicidal activity when compared to the others.[14] Chopde *et al*. compared the antifungal effect of two tissue conditioners, Visco-gel and GC-Soft liner, incorporated with nystatin, miconazole, and fluconazole by using disc diffusion method. This result confirmed with the previous study that nystatin showed the least antifungal effect.[46] Bueno *et al*. studied the antifungal effect of five drugs (nystatin, micronazole, ketonazole, itraconazole and chlorhexidine diacetate) incorporated into tissue conditioner (Softone) and resilient denture liner (Trusoft) on the *C. albicans* biofilm. Cell viability was evaluated by the tetrazolium salt reduction assay (XTT) followed by

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confocal laser scanning microscopy (CLSM) analysis. The result showed that all drugs incorporated into both materials were inhibited *C. albicans* growth more than 90%.[16] Some *in vitro* studies[14, 47] focused on the effective concentration of each drug in antifungal effect. The higher concentration of nystatin resulted in higher antifungal effect.[47] However, this study recommended that the amount of added drug should be selected by case severity. One percentage by weight of itraconazole incorporated with tissue conditioner was suitable for a case of Newton's type I. While in Newton's type III case, the concentration of itraconazole should be increased to 5% by weight.[14]

The disadvantages of antifungal drugs incorporated into tissue conditioner were changing of tissue conditioner's properties and duration of antifungal effect. Nystatin (500,000 and 1,000,000 U) incorporated into Softone demonstrated higher hardness value than control group in 24 hours, 7 days, and 14 days. While 5 and 10 % of Chlorohexidine incorporated into Softone showed an increase in roughness.[48] Moreover, water sorption of Softone with chlorhexidine increased when compared to control group.[49] Truhlar *et al*. reported that the fungicidal effect of Nystatin (100,000 U to 1,000,000U) incorporated into Visco-gel and Lynal significantly lost at day 2.[47]

A few studies focused on inorganic antimicrobial agents incorporated in tissue conditioners or resilient denture liners such as triclosan, cetylpyridinium chloride (CPC), silver-zeolite, silver nanoparticles, combined zinc oxide with silver

nanoparticles (ZnO-Ag), magnesium oxide (MgO), and ionic-liquid. Triclosan incorporated into Permasoft material (1-part triclosan per 80-parts Permasoft) could reduce the adherence of *C. albicans* on Permasoft surface. However, the reduction level was not significant difference when compared with Permasoft alone.[50] The CPC, antibacterial substance, are commonly used in soap and bath products. When the CPC was incorporated into tissue conditioner, the antibacterial effect against *S. aureus* was sustained for 3 weeks and the antifungal effect against *C. albicans* was remained for only 7 days.[51] Matsuura *et al*. found that tissue conditioners containing 2 wt% silver-zeolite had antimicrobial effects for 4 weeks against *C. albicans* and nosocomial respiratory infection-causing bacteria in *in vitro* saliva.[18] Similarly, Nikawa *et al.* combined 1-5 wt% silver-zeolite with GC-Soft liner and examined the antifungal effects on the *in vitro* growth and acid production of *C. albicans* on protein-free and saliva-coated specimen. The result of this study demonstrated that the silver-zeolite had concentration-dependent inhibitory effects and could delay the decline of pH.[52]

Nam and Lee found that 0.1 wt% silver nanoparticles incorporated into GC-Soft liner showed a great bactericidal and fungicidal activity, the amount of *S. mutans* and *S. aureus* cell reduction of 99.9%. Moreover, up to 0.5% of silver nanoparticles showed no cytotoxicity influence of human gingival fibroblast.[53] The ZnO-Ag nanoparticles were synthesized for combining of the strength of both

material, strong antibacterial activity of silver ions and fascinating properties of ZnO. Then, the ZnO-Ag nanoparticles were incorporated into GC Soft-liner in 0.625, 1.25, 2.5, 5,10, and 20 wt% concentration and tested with 3 bacterial species (*S. aureus, P. aeruginosa*, and *E. faecalis*) and 1 fungal species (*C. albicans*). The results showed that 10 and 20 wt% ZnO-Ag concentration completely inhibited of *C. albicans* growth. While the ZnO-Ag inhibited concentration of bacterial species was lower than fungal species.[54]

Organic or natural agents were used to incorporate with tissue conditioners or resilient denture liners such as neem leaf, origanum oil, terpenes, and melaleuca oil.[55-59] Some of natural agents are completely soluble in water. Thus, the disc diffusion method was usually selected to evaluate antifungal effect and reported as a mean of inhibition zone. Barua DR *et al*. reported that at 24 hours, 15 wt% neem leaf extract incorporated with Visco-gel showed greater of inhibition zone against *C. albicans* than 10 wt% chlorhexidine diacetate. However, this inhibition zone of 15 wt% neem leaf extract was still less than 5 wt% ketoconazole or 5 wt% nystatin with Visco-gel at 24 hours and day 7.[55] Srivatstava *et al*. mixed the origanum oil with tissue conditioner (Visco-gel) from 10 to 65 wt%. The inhibition zone against *C. albicans* of 10 to 30 wt% was not significantly different. This research suggested to mix 60 wt% origanum oil into Visco-gel for 7 days to obtain antifungal effect. However, the tensile strength of visco-gel was altered because of large quantity of

added agents.[56] According to incorporation of melaleuca oil into tissue conditioner, Sharma S and Hedge V found that the inhibition zone against *C. albicans* of 30 wt% melaleuca oil incorporated with Visco-gel was comparable to 5 wt% fluconazole. Moreover, the antifungal effect of 30 wt% melaleuca oil was greater than that of 5 wt% fluconazole at day 7.[57] *In vivo* study, the patient treated with 20 % melaleuca oil (by volume) incorporated with tissue conditioners (Coe-comfort) showed a significant decrease of palatal inflammation compared to treated with issue conditioner alone.[58]

The changes of *C. albicans* cellular structure was observed with SEM and TEM after combining of organic agents with tissue conditioners. Martinez et al found that a cell wall disorganization, irregular shaped cell, and increased cell size of *C. albicans* in the terpene blends mixed with tissue conditioner (Coe-comfort).[59]

Several studies focused on the incorporation of chitosan, a polysaccharide derived from chitin, with tissue conditioner on antifungal properties.[60-63] Mousavi *et al.* found that 5 wt% of chitosan incorporated into tissue conditioner completely inhibited *C. albicans* growth at 24 and 48 hours.[60] Saeed *et al.* synthesized chitosan oligosaccharide from commercial chitosan and, mixed with tissue conditioner (GC Soft-liner). The result that the tissue conditioner modified with synthesized chitosan oligosaccharide decreased the number of *C. albicans* cells growth for 3 days.[61] A study evaluated the antimicrobial effect of tissue conditioner incorporated with

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synthesized particle which consisted of a combination between Ag/ZnO nanoparticles and chitosan. The 0.625, 1.25, 1.5, 2.4, 5, 10, and 20 wt% of synthesized particle was homogenously mixed into the tissue conditioner and tested with 3 bacterial species (*S. mutans, P. aeruginosa*, and *E. faecalis*) and 1 fungal species (*C. albicans*). At 24 and 48 hours, the minimum concentration of synthesized particle incorporated into tissue conditioner which completely inhibited the *C. albicans* growth was 2.5 wt%. Whereas 5 wt% of synthesized particle incorporated into tissue conditioner completely inhibited all tested microorganism.[62] Another study modified tissue conditioner (GC Soft-liner) with grafted 2-[(acryloyloxy)ethyl] trimetyl ammonium chloride (AETMAC) onto chitosan. Five wt% of modified tissue conditioner reduced the number of *C. albicans* cells without adverse effects on cell cytotoxicity on gingival fibroblast and tensile bond strength to acrylic denture base.[63]

To date, there was no consensus on preventive protocol or treatment regimen using any antifungal agent in soft lining material against *Candida* infection without jeopardizing the physical properties of tissue conditioner.

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2.2.2 Long-term resilient denture liner

The indication of long-term resilient denture liners is quite similar with tissue conditioner and commonly used after tissue recovery from using tissue conditioner. However, long-term resilient denture liners are mostly used in patients who cannot tolerate the stresses from the denture because of sharp ridges, thin-resilient oral

mucosa, submucosal exposure of the inferior alveolar nerve, severe bony undercuts, or tissue defects.[12]

Long-term resilient denture liners are available as silicone-based or acrylic-based resilient denture liner in autopolymerized or heat activated types. Long-term acrylic-based resilient denture liner is often known as soft liner. The powder part of soft liner is PEMA powder with or without initiators. The liquid part is ester plasticizing agents and methacrylate monomer such as ethyl, n-butyl, and 2-ethoxyethyl methacrylate. The liquid part of soft liner can provide greater durability than tissue conditioner. The acrylic-based type provides good adhesion to the denture base due to their similarity in chemical structure. Long-term silicone-base resilient denture liner uses condensation reaction between polydimethyl siloxane and polyethyl silicate similar with silicone impression materials. This material consists of only 2 components, paste and catalyst, without any plasticizing agents. Heat activated silicone-base resilient denture liner achieve more degree of crosslinking polymer than autopolymerized type. Both polymerized types of silicone-base resilient denture liner provide longer resilience and elasticity than the acrylic-based type. However, the major problem of silicone is loss of adhesion between liner and denture base especially around the border of denture. A primer application is required before relining to prevent adhesive failure. Surprisingly, several studies found that long term acrylic-based type showed lower bond strength than silicone-based type.[64-66] It can

be explained by incomplete penetration of monomer into the high-density cross-linked denture base polymer. Moreover, adhesive system of the silicone-based type, a polymeric substance in volatile solvents, has been improved resulted in better bond strength.[66]

Table 2: Trade name and composition of the long-term resilient denture liners.

Trade name/ Type of based material	Type of polymerization	Manufacturer	Composition
Flexacryl Soft/ Acrylic- based resilient denture liner	Autopolymerized type	Lang Dental Mfg. Co., Illinois, USA	Powder: <99% Polymer, <2% Benzoyl peroxide Liquid: <50% Citric Plasticizer, <50% N-Butyl methacrylate, <5% N, N-Dimethyl-p-Toluidine, <5% Trimethylolpropane trimethacrylate
Super-Soft/ Acrylic-based resilient denture liner	Heat activated types	GC Corp., Illinois, USA	Powder: 90-100% Polyethyl methacrylate Liquid: 50-70% Dibutyl phthalate, 25-50% Isodecyl

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			methacrylate, 10-20% Methyl methacrylate
Vertex Soft/ Acrylic-based resilient denture liner	Heat activated types	Henry Schein Dental, Auckland, New Zealand	Powder: Methyl methacrylate, <1% Dibenzoyl peroxide, <1% Barbituric acid Liquid: >80% Plasticizer, <20% Methyl methacrylate, <5% Ethylenglycol dimethacrylate
Mucopren Soft/ Silicone-based resilient denture liner	Autopolymerized type	Kettenbach GmbH & Co. KG, Eschenburg, Germany	Silicone polymers, Fillers with platinum-catalyst
Sofreliner tough M/ Silicone-based resilient denture liner	Autopolymerized type	Tokuyama Dental Corporation, Tokyo, Japan	Silicon dioxide 10-30%, α, ω-Divinyl polydimethylsiloxane 50-80%
Ufi Gel P/ Silicone-based resilient	Autopolymerized type	Voco GmbH, Cuxhaven,	Modified polydimethylsiloxane (A-

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denture liner		Germany	silicone), platinum catalyst*
GC reline soft/ Silicone-based resilient denture liner	Autopolymerized type	GC Corp., Illinois, USA	5-10% Methylhydrogen dimethylpolysiloxane
Luci-Sof/ Silicone-based resilient denture liner	Heat activated types	Dentsply Sirona, *Waverley, Australia*	1-10% Dimethylsiloxane, methylhydrogen, 1-5% Dibenzoyl peroxide
Molloplast B/ Silicone-based resilient denture liner	Heat activated types	Detax GmbH & Co. KG, Ettlingen, Germany	Polydimethylsiloxane, 1-<5% Benzoyl peroxide, <0.5% Dodecaemthylcyclohexasiloxane, <1% Dichloromethane

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* A material safety data sheet (MSDS) does not provide the information of Ufi Gel P composition. This information is provided from a journal article.[67]

2.2.2.1 Desirable properties of long-term resilient denture liner

Since the purpose of using resilient denture liner is in more permanent situation, good dimensional stability, permanent softness, color stability, and low degradation would be primary concern.[68] The other desirable properties are

biocompatibility, low water sorption and water solubility, good wettability by saliva, adequate abrasion resistance and tear resistance, unaffected by aqueous environment and cleanser, easy to clean, simple to manipulate and good esthetics, and inhibits colonization of fungi and other microorganisms.

Adequate bond strength between denture liner and denture base throughout working time is another important property. The bond strength of each resilient denture liner was reported significantly lower overtime.[69] According to ISO 10139-2, soft lining materials for long-term use, the bond strength of soft lining material that shore A hardness is lower or equal 55 shore A units shall be at least 1.0 MPa. While the bond strength of soft lining material that shore A hardness is lower or equal 35 shore A units shall be at least 0.5 MPa.[70]

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3. Zinc oxide nanoparticles

3.1 General information

In recent years, nanometer-scales metal oxides (1-100 nm) are interested and considered to use in several applications. Zinc oxide, white insoluble powder in water, exists in two forms: hexagonal wurtzite and cubic zinc blende. Hexagonal wurtzite structure is more stable in ambient conditions than another form. ZnOnps in wurtzite structure can present various growth morphology such as nanorings, nanohelixes, nanocombs, and nanowires.[71] With a various of morphology, ZnOnps have been widely used for applications in field of biology and medicine (bioimaging, drug and gene delivery, antitumor and antimicrobial activity), cosmetic industry (UV filters in sunscreens, mineral cosmetics), manufacturing industry (antimicrobial food packaging, antimicrobial textiles), and energy and electronics (low-cost solar cells, chemical sensors based on ZnO nanowires). Moreover, ZnOnps have been found to exhibit antimicrobial activity against various bacterial (gram-positive and gram-negative) and fungal strains such as Escherichia *coli, Pseudomonas fluorescens,* Staphylococcus *aureus,* Salmonella *typhimurium, C. albicans,* and Aspergillus *fumigatus*.[72, 73]

There are several methodologies for synthesizing ZnOnps such as physical methods (physical vapor deposition, thermal evaporation, ultrasonic irradiation, and arc plasma method), chemical methods (microemulsion, hydrothermal synthesis, sol-gel synthesis, spray pyrolysis, and precipitation), and biological methods (plant

extraction, microorganism, and biochemistry method).[71] The different synthesis methods result in an individual characteristic of particles in terms of size, shape, and spatial structure. However, the chemical methods showed the better control of the ZnOnps particle size and morphology. Several studies founded a correlation between the antimicrobial effect and particle size of inorganic antimicrobial agents. These studies concluded that the antimicrobial effect increased with a reduction in particle size of inorganic antimicrobial agents including ZnO particles.[19, 74] Moreover, Lipovsky *et al*. reported that ZnOnps inhibited *C. albicans* growth with a concentration-dependent manner under normal ambient lighting conditions.[21, 22]

3.2 Antimicrobial mechanisms of ZnOnps

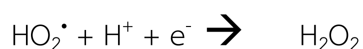
Although several studies focused on the antimicrobial effect of ZnOnps, the antimicrobial effect is still obscure. Numerous studies suggested mechanism of the antibacterial effect of ZnO associated with the generation of reactive oxygen species from water and oxygen. The reactive species are hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), and superoxide anion ($^{\bullet}O_2^-$).[74] These molecules are very reactive and powerful oxidizing agents. ZnOnps can generate ROS in concentration-dependent manner when exposed to UVA radiation and also generate ROS in dark conditions. Sawai *et al*. hypothesized the chemical formulas in producing reaction of hydrogen peroxide which generated from ZnO powder slurry using the oxygen electrode method as indicates below:[75]

$$ZnO + h\upsilon \rightarrow e^- + h^+$$

$$h^+ + H_2O \rightarrow {}^{\bullet}OH + H^+$$

$$e^- + O_2 \rightarrow {}^{\bullet}O_2^-$$

$$O_2^- + H^+ \rightarrow HO_2^{\bullet}$$


$$HO_2^{\bullet} + H^+ + e^- \rightarrow H_2O_2$$

The superoxide anion and hydroxyl radicals cannot penetrate through cell membrane due to their negative charges. Both molecules are found on the outer surface of the bacteria. The hydrogen peroxide, the most powerful reactive molecule, can pass through the bacterial cell wall, destruct the microorganism cell components (lipid, DNA, and proteins) and cause cell death.[72]

Raghupathi *et al*. studied the transcription analysis of cells treated with ZnOnps and found no enhanced expression of specific genes against ROS. Thus, the ROS might not be the only factor in the antibacterial activity of ZnOnps.[22] The other mechanism has been proposed for antimicrobial activity of ZnOnps that is direct interaction of ZnOnps with microbial cells. The direct interaction between ZnOnps and microbial cell resulted in the disruption of cellular function and disorganization of cell membrane which can be observed by field emission scanning electron microscope (FESEM).[76]

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Another antimicrobial mechanism is a presence of soluble zinc ions when ZnOnps is suspended in water. The zinc ions can react with negative charge ions of lipopolysaccharide in cell membrane of microorganisms. Nevertheless, Applerot *et al*. studies the antimicrobial mechanism of zinc ions by dissolving precursor $Zn(Ac)_2 \cdot H_2O$ in water. Even though, this substance can produce the amount of zinc ions more than 5 times compared to the amount of zinc ions releasing by ZnO, the great amount of zinc ions did not significantly reduce the viable count of *E. coli* and *S. aureus*.[72] Similarly, Raghupathi *et al*. determined the amount of free zinc ions by atomic absorption spectrophotometry (AAS) and found that the release amount of free zinc ions formed in the ZnO suspension is very low. The antibacterial activity of synthesized ZnOnps is mainly due to the action of ZnOnps rather than free zinc ions.[22]

3.3 Antimicrobial methods

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3.3.1 Agar disc-diffusion test

Agar disc-diffusion test is a standard method for evaluating the antimicrobial behavior of dental and medical materials especially antibiotic drugs. The petri dishes are prepared using agar then microbials suspension are spread on the top of the plates using sterile glass rod. After allowing the suspension to dry, disc-shaped samples (about 6 mm in diameter) containing various concentration of antimicrobial agents are separately placed on the petri plate. The petri dishes are incubated under

suitable condition. The zone of inhibition, the surrounding area of the disc-shaped sample that demonstrates no growth of microorganism, is measured after incubation. The advantage of this method is the test simplicity, easy interpretation, and low cost.[77, 78]

3.3.2 Agar well diffusion test

The procedure of the agar well diffusion test is quite similar with the agar disc-diffusion test. After preparing of microbial petri dishes, a hole with 6 to 8 mm in diameter is punched aseptically with sterile tip. The antimicrobial agent solution (about 20-100 μL) is dropped into the prepared hole. The petri dishes are incubated, and the inhibition zones are measured. Both of agar disc-diffusion and agar well diffusion methods commonly used for antimicrobial screening of plant extracts, essential oils, and drugs.[77]

3.3.3 Broth dilution test

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Broth dilution test is basic antimicrobial susceptibility testing method that used for the determination of minimum inhibitory concentration. Two-fold serial dilution of antimicrobial agents (e.g., X, 2X, 4X, and 8X) in liquid growth medium dispensed in tested tube are prepared. The antimicrobial tubes are inoculated with 0.5 McFarland standard microbial suspension. After incubation under suitable condition, the tubes are examined for visible microbial growth as evidenced by turbidity. The lowest concentration of antibiotic that can completely inhibit microbial

growth is minimum inhibitory concentration. The weakness of this method is high technical training requirement.[78]

3.3.4 Direct contact test

Direct contact test is performed to determine the antimicrobial activity which measured direct and close contact between the tested materials and the tested microorganism.[79, 80] This method is suitable for evaluating of solid materials that have low soluble components. The antimicrobial suspension is dropped on the disc-shaped specimens and left to evaporate in the incubator. Evaporation of the suspension ensures a direct contact between microorganism and the tested materials. After that, broth is added to each well and incubated at 37 ^{o}C overnight. The small amount of suspension in each well is serial-diluted and sub-cultured onto the agar medium to determine the amount of microorganism. Nonetheless, this method cannot determine the amount of adherent microorganism inside the specimens.

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4. Characteristics instruments

4.1 Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) is the widely used technique for observing of the surface morphology by scanning the sample with a high energy beam of electrons. The images form SEM have high magnification (5x to 1,000,000x) and great resolution with a three-dimensional quality. The SEM consists of 2 parts:

the column and cabinet. The column is the part that the high energy of electron beam (5-100 keV) traverses from their emission until interacts with the constituent atoms of the sample in sample chamber. When the electron beam contacts with the sample, the beam of electron will produce secondary electrons, backscattered electrons, and characteristic X-ray. The secondary electrons mode is the most common type of signal used for SEM image.[81] The electron detectors will capture the signal, transform the signal into an electrical signal, and send to cabinet part. The cabinet part will transform the electrical signal into analyzable information which are the images and graphics.[82, 83] Both the electron column and sample chamber are vacuumed through a turbomolecular pump to create the environment for electron travelling.

For SEM imaging, the specimens must be electrically conductive at surface for producing the artifact-free images. Nonconductive specimens (ceramics, polymers, and biomaterials) might be coated with a thin layer of conducting material (5-20 nm), commonly carbon, gold, or platinum. The metal selected for conductive coating should not present within the sample.[84] However, the thick metal coating results in a poor representation of surface geometry. Alternatively, electrical insulating samples can be analyzed without coating in low-voltage SEM with accelerating voltage lowered to approximately 1 keV.[83]

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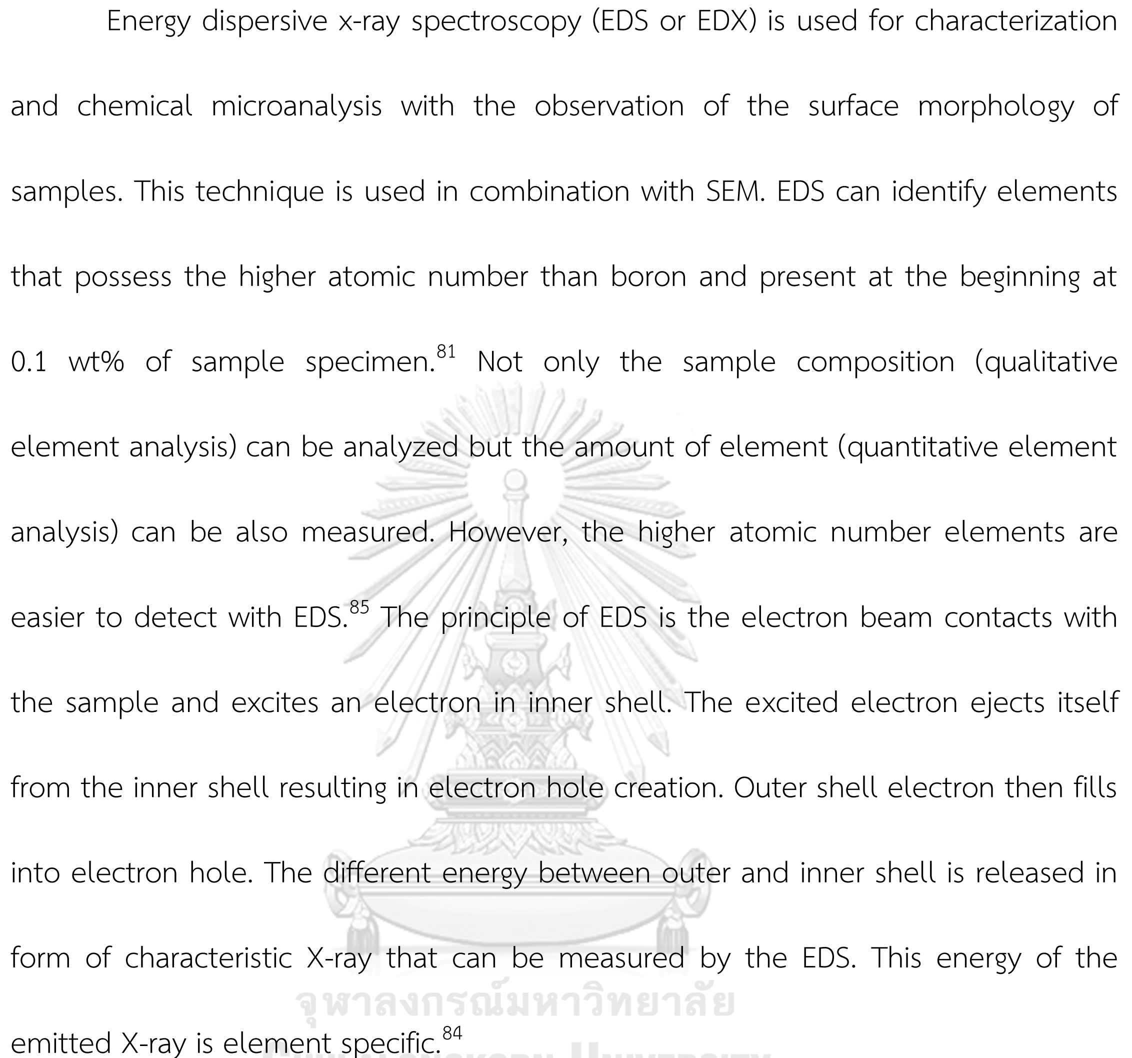
4.2 Energy dispersive x-ray spectroscopy (EDS or EDX)

Energy dispersive x-ray spectroscopy (EDS or EDX) is used for characterization and chemical microanalysis with the observation of the surface morphology of samples. This technique is used in combination with SEM. EDS can identify elements that possess the higher atomic number than boron and present at the beginning at 0.1 wt% of sample specimen.[81] Not only the sample composition (qualitative element analysis) can be analyzed but the amount of element (quantitative element analysis) can be also measured. However, the higher atomic number elements are easier to detect with EDS.[85] The principle of EDS is the electron beam contacts with the sample and excites an electron in inner shell. The excited electron ejects itself from the inner shell resulting in electron hole creation. Outer shell electron then fills into electron hole. The different energy between outer and inner shell is released in form of characteristic X-ray that can be measured by the EDS. This energy of the emitted X-ray is element specific.[84]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CHAPTHER 2

RESEARCH QUESTION, RESEARCH OBJECTIVES, RESEARCH HYPOTHESES, EXPECTED BENEFITS, AND LIMITATIONS

Research question

Does the incorporation of zinc oxide nanoparticles into the tissue conditioner exhibit *in vitro* antifungal effect against *C. albicans* without jeopardizing the physical and mechanical properties at different storage times (0, 7, and 14 days)?

Research objectives and research hypotheses

1. To find the optimum amount of ZnOnps incorporated into the tissue conditioner which exhibited antifungal effect against *C. albicans* at different storage times (0, 7, and 14 days).

First hypothesis

H_0: There is no significant difference on antifungal effect between modified tissue conditioner (5Zn, 10Zn, 15Zn, and Nys) and control at different storage times (0, 7, and 14 days).

H_1: There is a significant difference on antifungal effect between modified tissue conditioner (5Zn, 10Zn, 15Zn, and Nys) and control at different storage times (0, 7, and 14 days).

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2. To measure the penetration depth of all tested groups at different storage times (0, 7, and 14 days).

Second hypothesis

H_0: There is no significant difference on penetration depth between modified tissue conditioner (5Zn, 10Zn, 15Zn, and Nys) and control at different storage times (0, 7, and 14 days).

H_1: There is a significant difference on penetration depth between modified tissue conditioner (5Zn, 10Zn, 15Zn, and Nys) and control at different storage times (0, 7, and 14 days).

3. To measure the tensile bond strength of all tested groups at different storage times (0, 7, and 14 days).

Third hypothesis

H_0: There is no significant difference on tensile bond strength between modified tissue conditioner (5Zn, 10Zn, 15Zn, and Nys) and control at different storage times (0, 7, and 14 days).

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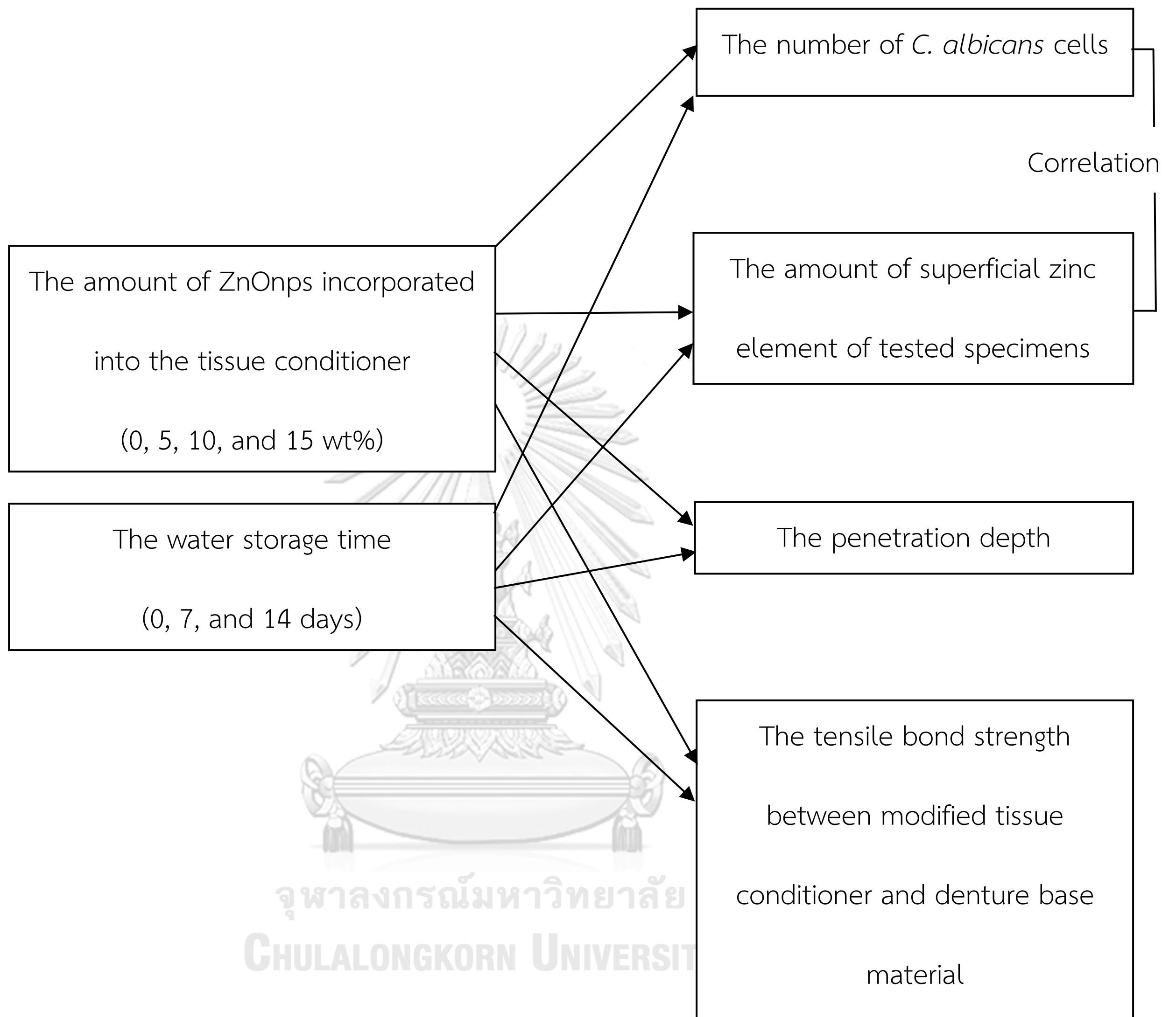
H_1: There is a significant difference on tensile bond strength between modified tissue conditioner (5Zn, 10Zn, 15Zn, and Nys) and control at different storage times (0, 7, and 14 days).

Conceptual framework



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Expected benefits

This research is the frontier in incorporation of ZnOnps into the tissue conditioner. This modified tissue conditioner exhibited an antifungal effect against *C. albicans* throughout 14 days. While the physical and mechanical properties of this material, evaluated by penetration depth and tensile bond strength, are within the ISO standard threshold. The result of this study could supplement the treatment protocol of denture stomatitis by promoting proper environment for healing, decreasing the number of *C. albicans* cells, and reducing the frequency of replacing with new tissue conditioner.

Limitations

This experimental design limited only *in vitro* study. Type of microorganisms on mixed biofilms of each patient might exhibit different results. The safety of incorporated of ZnOnps into the tissue conditioner should be address because of the direct contact between the tissue conditioner and oral mucosa. *In vitro* and *in vivo* of cell cytotoxicity or biocompatibility test of this modified tissue conditioner should be further investigated.

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CHAPTHER 3
MATERIAL AND METHODS

GC Soft-liner was used in this study. All tested materials, batch number, and their compositions are listed in Table 3.

Table 3: Materials used in this study and their chemical compositions.

Material	Product name	Manufacturer	Batch number	Composition
Tissue conditioner	GC Soft-liner	GC Corp., Tokyo, Japan	1806052	Powder: polyethyl methacrylate (PEMA) Liquid: ethyl alcohol 15%, *butyl phthalyl butyl glycolate 85%*
Zinc oxide nanoparticles	ZoNoP	Nano Materials Technology Co., Ltd., Chonburi, Thailand	190411	Zinc oxide: (purify>99.5%, average particles size 20-40 nm)

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Heat-cured denture base material	SR Triplex Hot	Ivoclar Vivadent, Schaan, Liechtenstein	XT 0628	Powder: polymethyl methacrylate (PMMA) Liquid: methyl methacrylate 50-100%, ethylene glycol dimethacrylate 3-<10%
Nystatin oral suspension	Nyst Oral	Continental-Pharm Co., Ltd., Bangkok, Thailand	014	Nystatin 100,000 unit

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Sample size calculation

The sample size was determined by the pilot study's result by using G-power software v3.1.9.2 (Franz Faul, Universität Kiel, Germany) with the significance level set at 0.05 and the power at 0.80.

Part 1 Antifungal evaluation

Specimen preparation

The tested groups consist of 5 groups that were 0, 5, 10, 15 wt% ZnOnps, and 15 wt% of nystatin (Control, 5Zn, 10Zn, 15Zn, and Nys). A control group was a pure

mixture between PEMA powder and liquid part at a powder/liquid ratio of 2.2 g/ 1.8 g following the manufacturer instruction. The added ZnOnps powder, calculated from the weight of the PEMA powder. The ratio of powder (ZnOnps powder and PEMA powder) and liquid was kept constant of 2.2 g/ 1.8 g as shown in Table 4. A mixture of PEMA powder and liquid part mixed with 15 wt% of nystatin was served as positive control. The 15 wt% nystatin, calculated from the weight of the PEMA powder (2.2 g), was added into the liquid part. The PEMA powder was increased from 2.2 g to 2.6 g for maintaining powder/liquid ratio.

Table 4: The amount of PEMA powder, ZnOnps, and liquid part in each tested group.

Group	PEMA powder (g)	ZnOnps (g)	Nystatin (g)	Liquid (g)
Control	2.2	0	-	1.8
5 Zn	2.2	0.11	-	1.89
10 Zn	2.2	0.22	-	1.98
15 Zn	2.2	0.33	-	2.07
Nys	2.6	-	0.33	1.8

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A square-shaped metal mold with an internal round-shaped hole (12 mm in diameter and 2 mm in diameter) was prepared and placed on a flat glass plate. Eighteen tested specimens per group were fabricated in sterile condition of laminar flow cabinet. Five, 10, 15 wt% ZnOnps, and 15 wt% nystatin was separately mixed into the liquid part of tissue conditioner for 30 seconds to make a suspension in a glass jar. After that, the PEMA powder was mixed in the prepared suspension and uniformly stirred for 90 seconds. The mixture was poured into the hole of prepared metal mold and covered with an un-plasticized polyester film. A flat glass plate was placed on top to press out the excess material. Two-kg-weight load was placed for 10 minutes until the material set. The disc-shaped specimen was push out of the hole of metal mold. The excess was trimmed with sterile scissors. A photograph of a specimen in each group is shown in Figure 4.

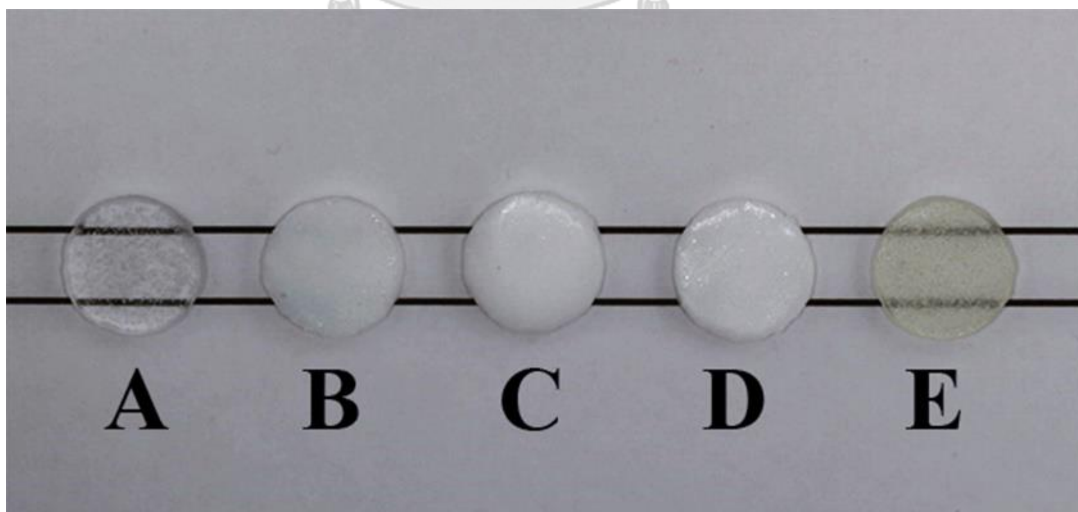

Figure 4: Representative photograph of the specimens in each group in the antifungal evaluation. A: control, B: 5Zn, C: 10Zn, D: 15Zn, and E: Nys.

Microbial suspension preparation

C. albicans, ATCC 90028, cells were obtained from the Department of Microbiology, Faculty of Dentistry, Chulalongkorn University. The inoculum was prepared by picking 3 isolated colonies of 1 mm in diameter from 24 hours cultured on Sabouraud Dextrose agar (SDA) (HiMedia Laboratories Pvt. Ltd., Mumbai, India). The colonies were transferred into 3 mL of Sabouraud Dextrose broth (SDB) (HiMedia Laboratories Pvt. Ltd., Mumbai, India) of a fresh glass tube. The suspension was homogenously vortexed for 15 seconds and incubated for at 37°C. After 24 hours, the suspension was vortexed and diluted with SDB to obtain the suspension that optical density was equal to a 0.5 McFarland standard solution at 530 nm wavelength by using an UV-vis spectrophotometer (Nicolet Evolution 500, Thermo Electron Corp., Madison, WI, USA).[86] The stock suspension, consisting of approximately 1.0-1.5x10^6 cells/mL, was diluted 100 times with SDB to achieve the final concentration of 1x10^4 cells/mL. One hundred microliters of diluted suspension were spread on SDA plates to confirm the initial number of *C. albicans* cells before testing as shown in Figure 5.

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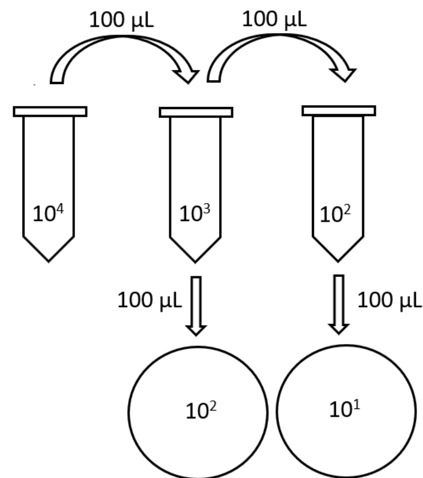

Figure 5: Diagram of serial dilution and spread plate method for confirming initial number of C. albicans cells.

Antifungal assay

The antifungal assay was modified from a previous study.[87] Eighteen disc-shaped specimens per group (Control, 5Zn, 10Zn, 15Zn, and Nys) were randomly divided into 3 subgroups (n=6) based on storage times (day 0, 7, and 14). Each disc-shaped specimen was placed at the bottom of 24-well cell culture plate wells (Coster, Corning, NY, USA). For the day 7 and 14 tested groups, each specimen was stored in 2 ml of deionized water in each well of 24-well cell culture plate, shaken with an orbital shaker at 100 rpm, and kept in a 37±1°C incubator. Two mL of new fresh deionized water was changed daily. After storing for 7 and 14 days, the deionized water was completely aspirated before the antifungal assay was performed.

A 50 µL of *C. albicans* suspension was dropped on the surface of each specimen in each cell culture plate well. The culture plate lid was slightly opened and left in the 37±1°C incubator for 120 minutes. After that, a 950 µL of SDB was added to each well. A SDB alone served as a negative control while a mixture between the *C. albicans* suspension and SDB served as a positive control. (Figure 6) The 24 well-plate was shaken with an orbital shaker at 100 rpm in a 37±1°C incubator for 24 hours. A 100 µL of fungal suspension in each well was serially diluted, spread on an agar plate in triplicate, and incubated for 24 hours at 37°C to determine the *C. albicans* colony-forming unit (CFU) number. The fungal colonies on the agar plates with 20–200 CFUs were counted. The number of *C. albicans* cells was converted to logarithm colony-forming units (log CFU/mL).

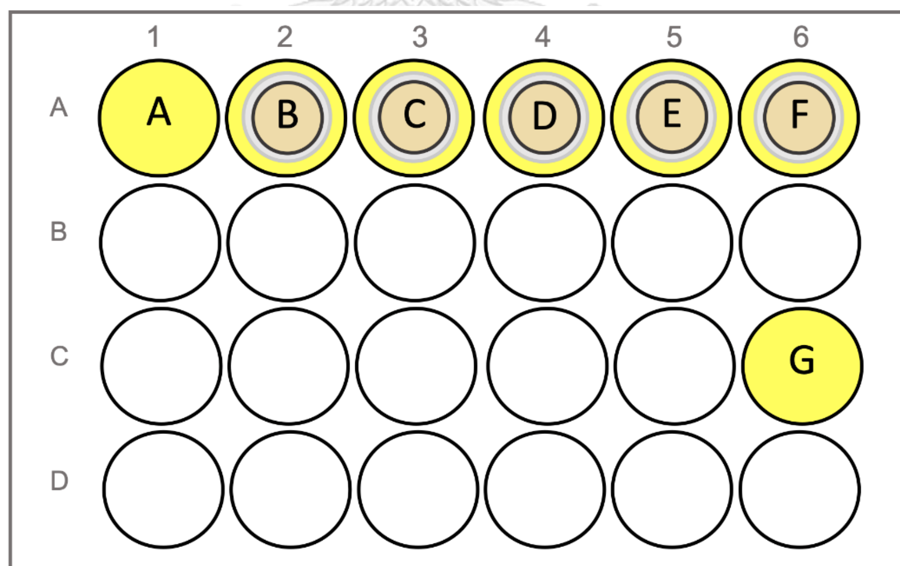

Figure 6: Diagram of a labelled 24-well plate A: SDB, B: control, C: 5Zn, D: 10Zn, E: 15Zn, F: nystatin, G: C. albicans suspension in SDB.

Statistical analysis

The number of *C. albicans* cells data was analyzed for normal distribution by using the Shapiro-Wilk test. The number of *C. albicans* cells was analyzed by two-way ANOVA followed by Tukey's *post-hoc* test for multiple comparison ($p<0.05$). All statistical analyses were performed using SPSS 22.0 (IBM Corp., Armonk, NY, USA).

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Part 2 Scanning electron microscopy and energy dispersive X-ray spectroscopy (SEM-EDS) analysis

Specimen preparation

Eighteen plastic collar (12 mm in internal diameter and 2 mm in height) per group was placed on a flat plane. The PEMA powder and liquid were homogenously mixed according to the manufacturer's recommendation at a powder/liquid ratio of 2.2 g:1.8 g and served as the control group. Five, 10, and 15 wt% ZnOnps was separately incorporated into the tissue conditioner as aforementioned in antifungal part, then poured into a plastic collar and covered by an un-plasticized polyester film. A glass plate (50x50x6 mm^3) was placed on top of the polyester film, followed by a 2-kg-weight load for 10 minutes. After the material set, the load and the un-plasticized polyester film were removed. The specimens were stored in the deionized water at 37±1°C for 1, 7, and 14 days (n=6) similar with the antifungal part.

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Scanning electron microscopy and energy dispersive X-ray spectroscopy (SEM-EDS) analysis

The specimens were taken out of the water and returned to ambient conditions. The superficial distribution of ZnOnps of specimens were observed by using SEM (Quanta 250; FEI Company, Elindhoven, the Nertherlands). Each specimen was measured 3 different positions which apart from each point by 120° by using EDS analysis (JSM-5410LV; JEOL Ltd., Tokyo, Japan). The images were obtained from the

specimen surfaces at an accelerating voltage of 15 kV with 1,000x magnification. The spot-EDS analyses were performed under 50-second scans.

Statistical analysis

The amount of superficial zinc element data was analyzed for normal distribution by using the Shapiro-Wilk test. The amount of superficial zinc element data was analyzed by two-way ANOVA followed by Tukey's *post-hoc* test for multiple comparison ($p<0.05$). The Pearson's correlation coefficient was used to analyze correlation between the amount of superficial zinc element and the number of *C. albicans* cells from part 1: antifungal evaluation ($p<0.05$).

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Part 3 Penetration depth test

The penetration depth test was performed following ISO specification 10139-1:2005.[38]

Specimen preparation

Thirty plastic rings (30 mm in internal diameter and 3 mm in height) and 30 plastic plates (50x50x4 mm^3) were prepared. A plastic ring was placed on a plastic plate on a flat plane. The PEMA powder and liquid were homogenously mixed according to the manufacturer's recommendation at a powder/liquid ratio of 2.2 g:1.8 g and served as the control group. Five, 10, 15 wt% ZnOnps, and 15 wt% nystatin was separately incorporated into the tissue conditioner as aforementioned in antifungal part, then poured into a plastic ring and covered by an un-plasticized polyester film (n=6). A glass plate (50x50x6 mm^3) was placed on top of the polyester film, followed by a 2-kg-weight load for 10 minutes. After the material set, the load and the glass plate were removed. All specimens were stored in the deionized water at 37±1°C for 2 hours.

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Penetration depth test

The specimens were taken out of the water, returned to ambient conditions, and the polyester film was removed. The cylindrical penetrator with a 1-mm-diameter vertical rod, with a total mass of 0.5 N was dropped onto the specimen surface for 3±0.1 seconds. (Figure 7.) Each specimen was measured at 3 different

positions that were 5 mm apart from the edge of the plastic ring and apart from each point by 120°. The penetration depth was recorded in millimeters and averaged. The specimen was then immediately stored in deionized water at 37±1°C after measurement. The penetration depth test was repeated at day 7 and day 14 after immersion in deionized water at 37±1°C at different positions as shown in Figure 8.

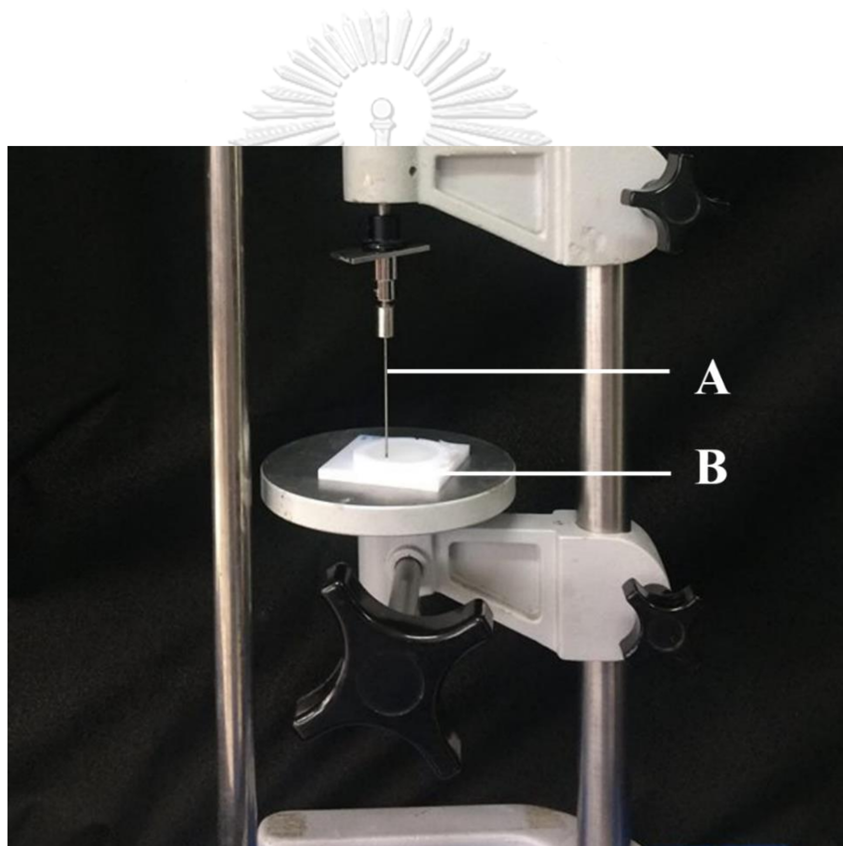

Figure 7: Representative photograph of the penetration depth test. A: 1-mm-diameter vertical rod, B: tested specimen.

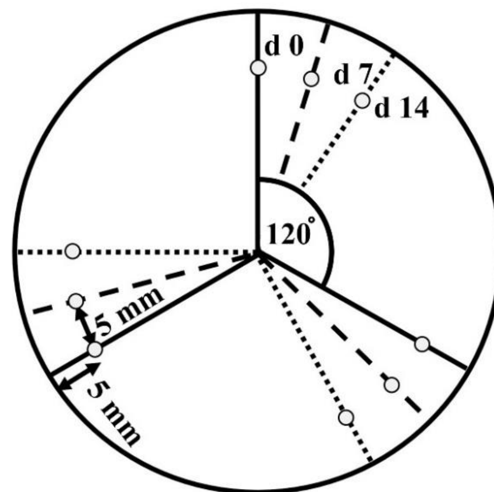

Figure 8: Diagram of different positions in penetration depth test on day 0, day 7 and day 14.

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Statistical analysis

The penetration depth data was analyzed for normal distribution by using the Shapiro-Wilk test. The penetration depth data were analyzed by two-way repeated measures ANOVA followed by Bonferroni *post-hoc* test for multiple comparison ($p<0.05$).

Part 4 Tensile bond strength test

The tensile bond test was performed following ISO specification 10139-2:2016.[70]

Specimen preparation

Two hundred-forty wax patterns (25x25x3 mm^3) were prepared. The wax patterns were invested in dental flasks with dental stone. After the dental stone set, the dental flasks were opened, and the wax patterns were washed out with boil water. The powder and liquid part of heat-cured denture base material (SR Triplex Hot, Ivoclar Vivadent, Schaan, Liechtenstein) were mixed according to the manufacturer's recommendation at a powder/liquid ratio of 23.4 g:10 mL and left for 10 minutes for reaching the dough stage of material. The mixtures were placed into the flask and hydraulic pressed using a conventional method. The flasks were placed in a water bath, heated up to boiling point at 100°C, and let boil for 45 minutes. After polymerization, 240 acrylic resin plates were deflasked and wet-polished using 600-grit silicon carbide paper with a polishing machine (Nano 2000, Pace Technologies, Tucson, AZ, USA). The dimension of the acrylic resin plates was measured with a digital vernier caliper. All acrylic resin plates were stored in deionized water at 37±1°C for 7 days.

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A plastic collar (10 mm in internal diameter and 3 mm in height) was placed at the center of the prepared acrylic resin plate. The PEMA powder and liquid of the tissue conditioner were homogenously mixed according to the manufacturer's

recommendation at a powder/liquid ratio of 2.2 g:1.8 g and served as the control group. Five, 10, 15 wt% ZnOnps, and 15 wt% nystatin was separately incorporated into the tissue conditioner as aforementioned in antifungal part. The mixture of each group was poured into the plastic collar. Another prepared acrylic resin plate was placed on top of the collar, followed by a 2-kg-weight load for 10 minutes. After material set, the load was removed. The bonded specimens in each group were equally divided into 3 subgroups (n=8) and stored in deionized water at 37±1°C for 1, 7 and 14 days before testing.

Tensile bond strength

The specimens were taken out of the deionized water, returned to ambient conditions. The upper and lower acrylic resin plate of each specimen were attached to the clamps of the universal testing machine (EZ-SX, Shimadzu, Kyoto, Japan) without torsion forces. The specimens were pulled apart at a crosshead speed of 10 mm/min until failure (Figure 9). The bond strength was calculated from the maximum force (N) divided by the cross-sectional area (mm^2) and recorded in megapascals (MPa). The failure modes were observed and classified as adhesive (total debonding at the interface between the lining material and acrylic resin plate), cohesive (total rupture within the tissue conditioner) and mixed mode.

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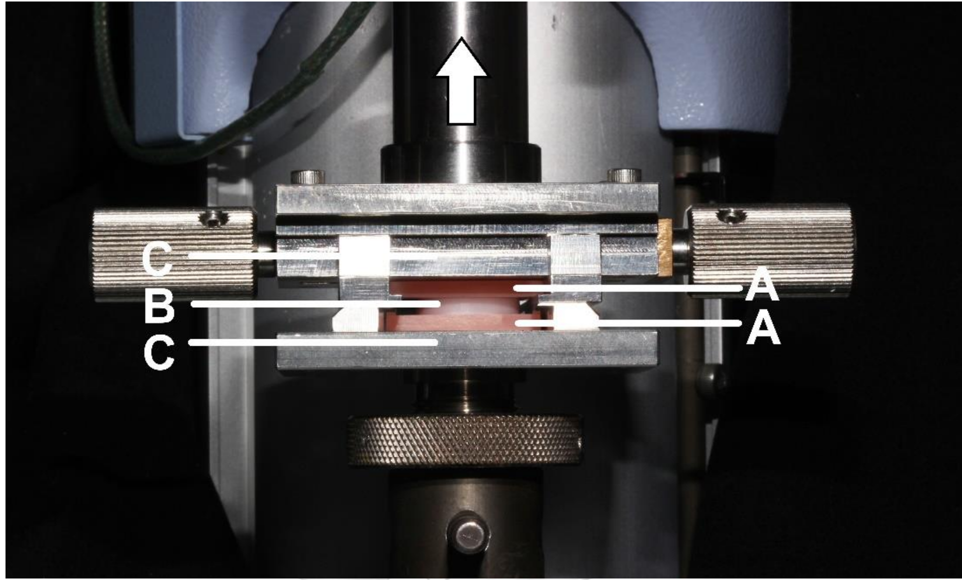

Figure 9: Representative photograph of the tensile bond strength test. A: acrylic resin plates, B: plastic collar, C: upper and lower clamp.

Statistical analysis

The tensile bond strength data was analyzed for normal distribution by using the Shapiro-Wilk test. The tensile bond strength data was analyzed by two-way ANOVA followed by Tukey's *post-hoc* test for multiple comparison ($p<0.05$). Descriptive analysis of the failure mode was performed.

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CHAPTHER 4

RESULTS

Part 1 Antifungal evaluation

The result of two-way ANOVA of the antifungal assay is shown in Table 5. Two main factors (group and storage time) and their interaction were significantly different ($p<0.001$). The means and standard deviations of the number of *C. albicans* cells (log CFU/mL) are summarized in Figure 10. Within day 0 of water immersion, ZnOnps incorporated into the tissue conditioner dose-dependently reduced *C. albicans* cell number. The antifungal effect in the 15Zn group resulted in a significant reduction in *C. albicans* cell number compared with the control group at all storage times ($p<0.001$). The antifungal effect in the Nys group generated the greatest reduction only at day 0 ($p<0.001$) while, the antifungal effect in the Nys group at day 7 ($p=0.594$) and day 14 ($p=0.156$) were not significantly different compared with the control group.

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Table 5: Two-way ANOVA test for comparison of the number of C. albicans cells

Factor	Sum of squares	*df*	Mean Square	F	*P*
Group	64.894	4	16.224	578.195	<0.001
Time	133.305	2	66.653	2375.456	<0.001
Group x time	120.873	8	15.109	538.478	<0.001

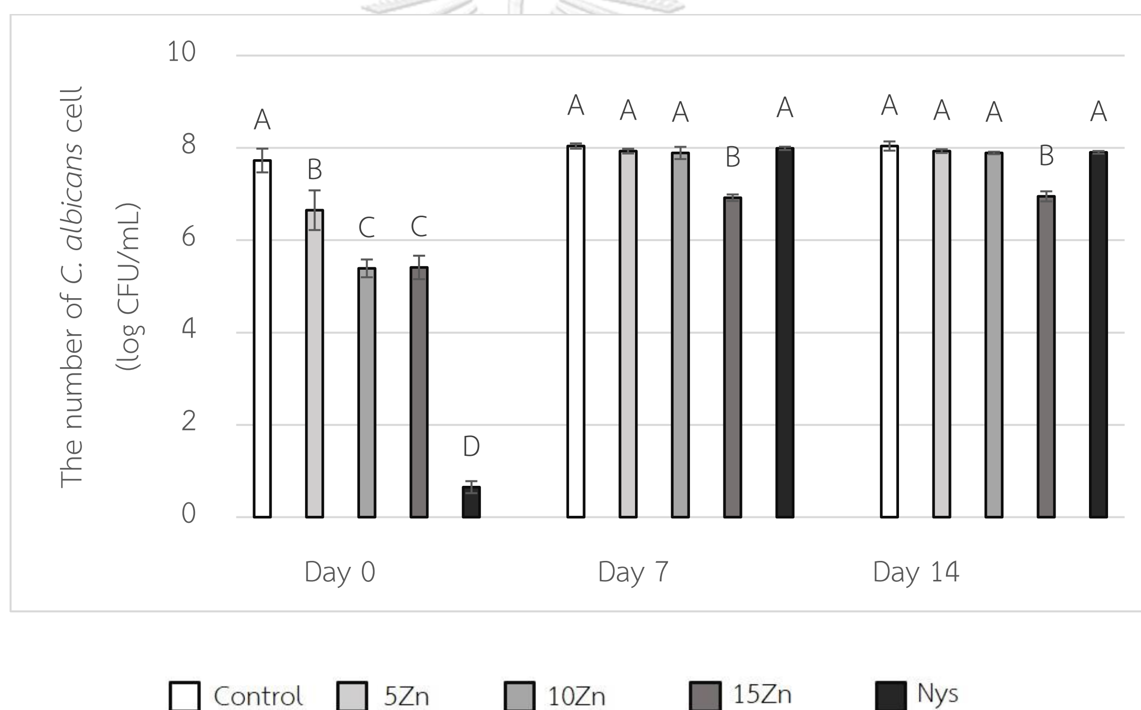
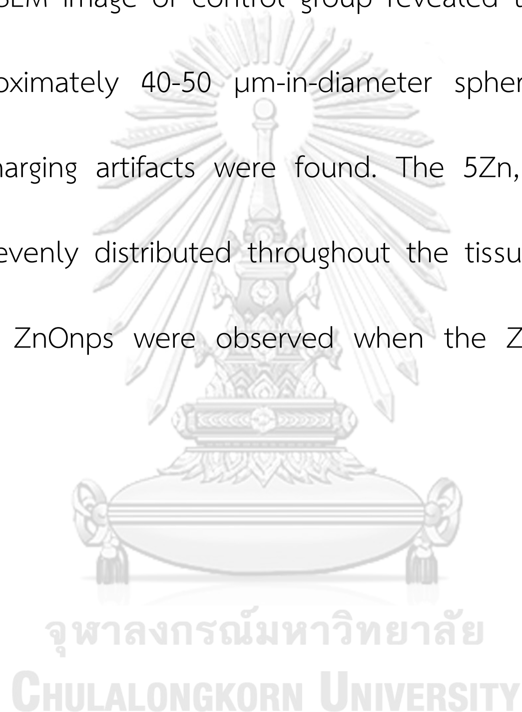


Figure 10: Means and standard deviations of the number of C. albicans cells (log CFU/mL) in each group at different storage times. Bar with the different letters indicate significant differences (p<0.05).

Part 2 Scanning electron microscopy-energy dispersive X-ray spectroscopy (SEM-EDS) analysis

Representative superficial zinc element patterns of all experimental groups in day 0, 7, and 14 examined by SEM are shown in Figure 11. The differences of surface integrity in each experimental group at all time points were not found by an observation. The SEM image of control group revealed that PEMA beads in tissue conditioner, approximately 40-50 µm-in-diameter spherical particles, and small porosities with charging artifacts were found. The 5Zn, 10Zn, and 15Zn groups showed ZnOnps evenly distributed throughout the tissue conditioner matrix. The white clusters of ZnOnps were observed when the ZnOnps incorporation was increased.



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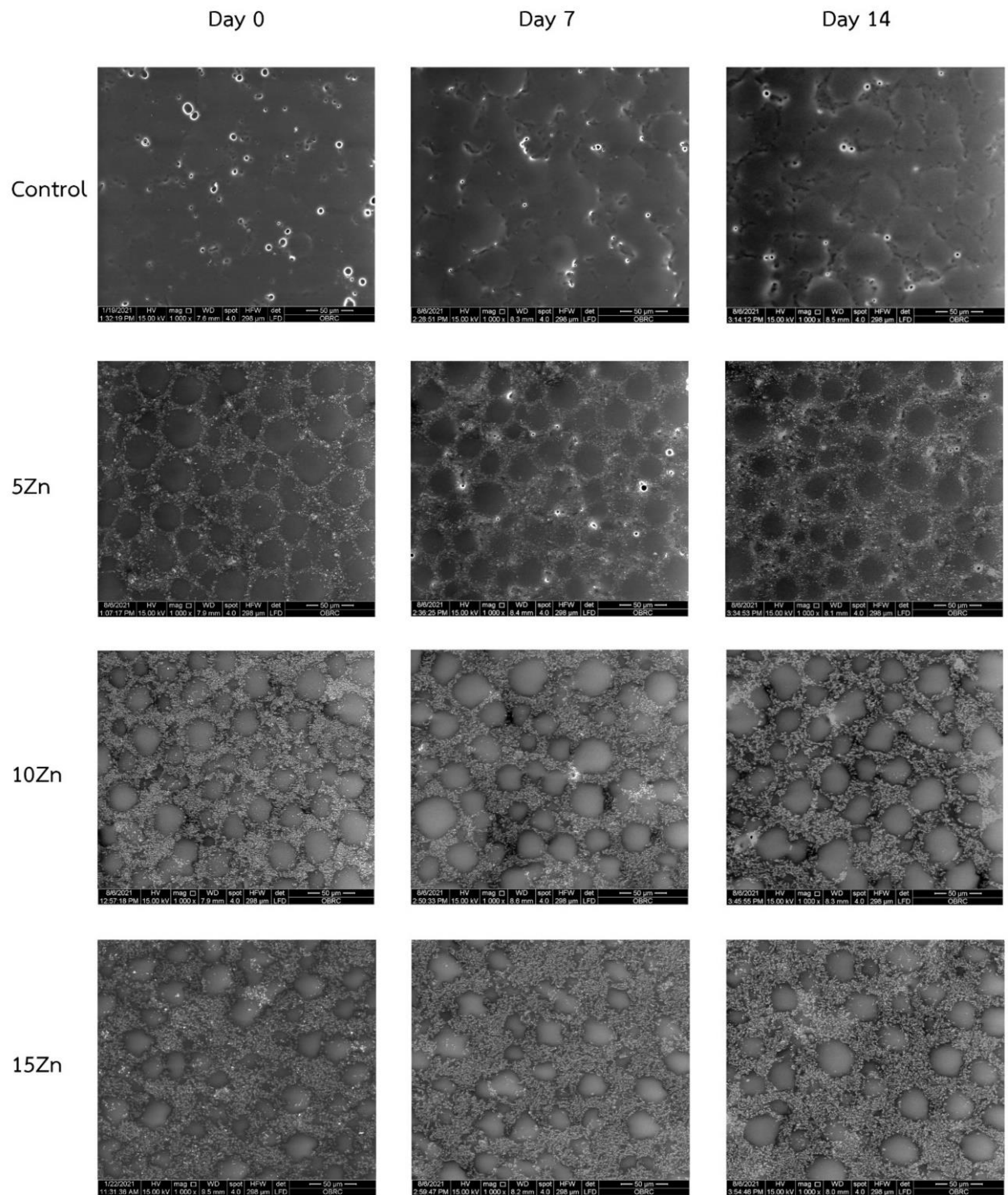

Figure 11: Scanning electron microscope images of four experimental groups at different storage times. (Magnification x1,000)

The EDS spectrum of control group at day 0, 7, and 14 are shown in Figure. 12. Only carbon (67-70 wt%) and oxygen (30-33 wt%) elements are identifiable. The EDS spectrum of 5Zn, 10Zn, and 15Zn groups at day 0, 7, and 14 are shown in Figures 13-15, respectively. The majority of element in EDS spectrum are carbon and oxygen, while the minority of element is zinc. The result of two-way ANOVA of the amount of superficial zinc element is shown in Table 6. Two main factors (group and storage time) and their interaction were significantly different (p<0.001). The means and standard deviations of superficial zinc element in modified tissue conditioner (wt%) are shown in Figure. 16. Among the storage times, 5Zn, 10Zn, and 15Zn groups demonstrated the highest in amount of superficial zinc element at day 0 (2.31± 0.23, 5.46± 0.29, and 7.88± 0.16, respectively). The amount of superficial zinc element of tested groups at day 7 and 14 were significantly lower compared to day 0 (p<0.05) Pearson's correlation coefficient between the amount of superficial zinc element and the number of *C. albicans* cells showed a significant negative linear correlation (r=-0.78, p<0.001) (Figure. 17).

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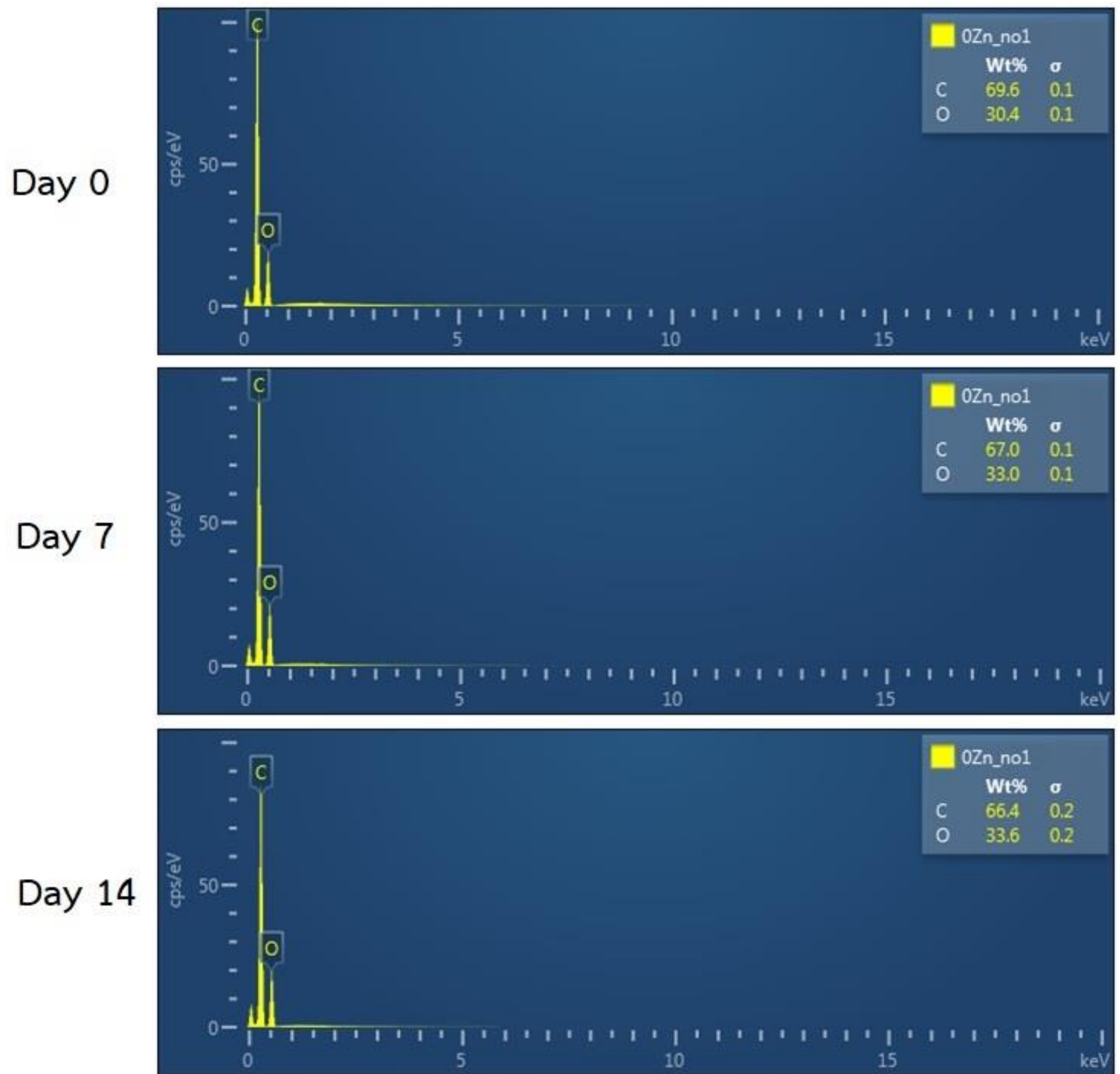

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Figure 12: Element identified by energy dispersive X-ray spectroscopy of control group at day 0, 7, and 14.

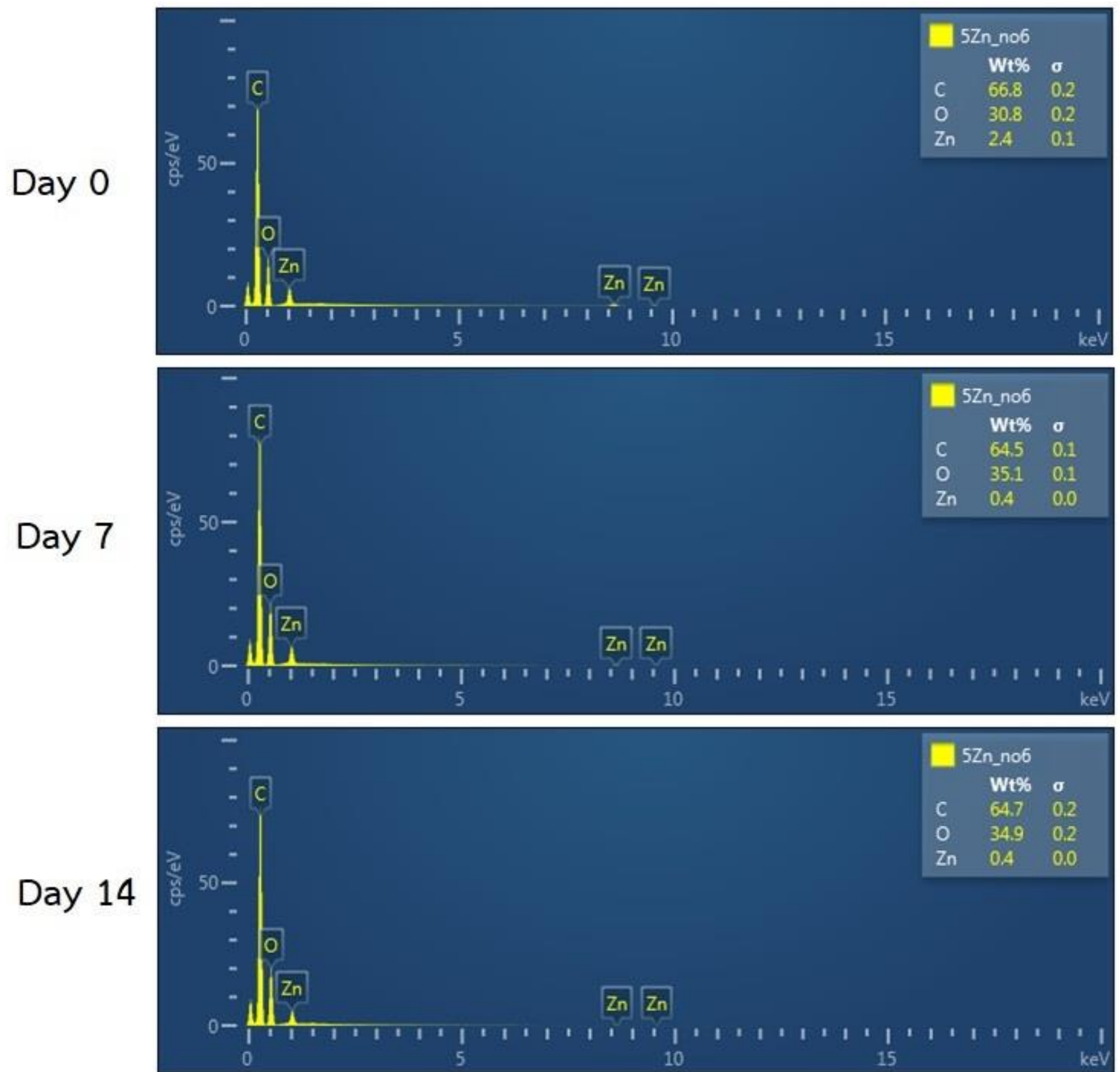

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Figure 13: Element identified by energy dispersive X-ray spectroscopy of 5Zn group at day 0, 7, and 14.

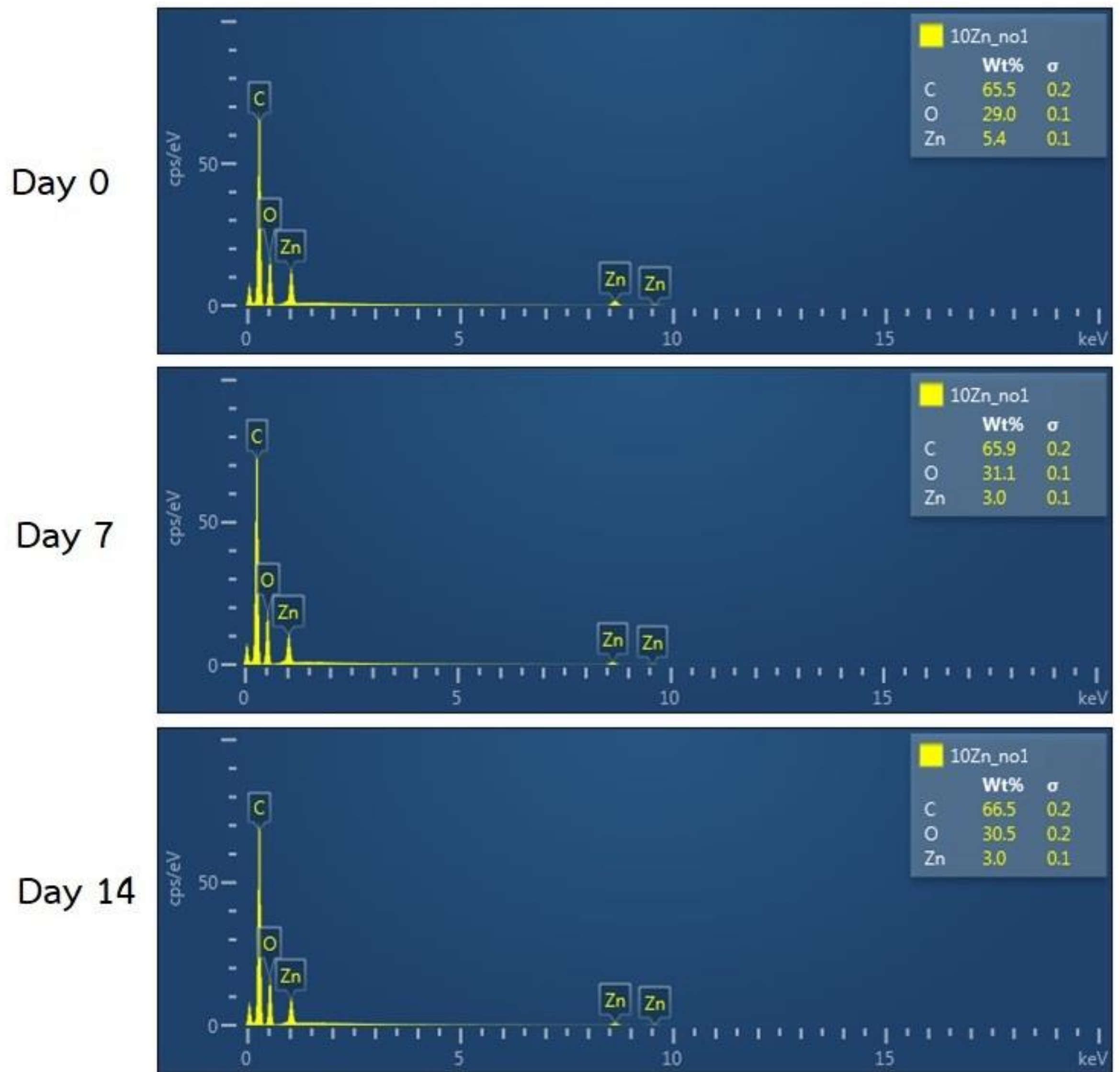

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Figure 14: Element identified by energy dispersive X-ray spectroscopy of 10Zn group at day 0, 7, and 14.

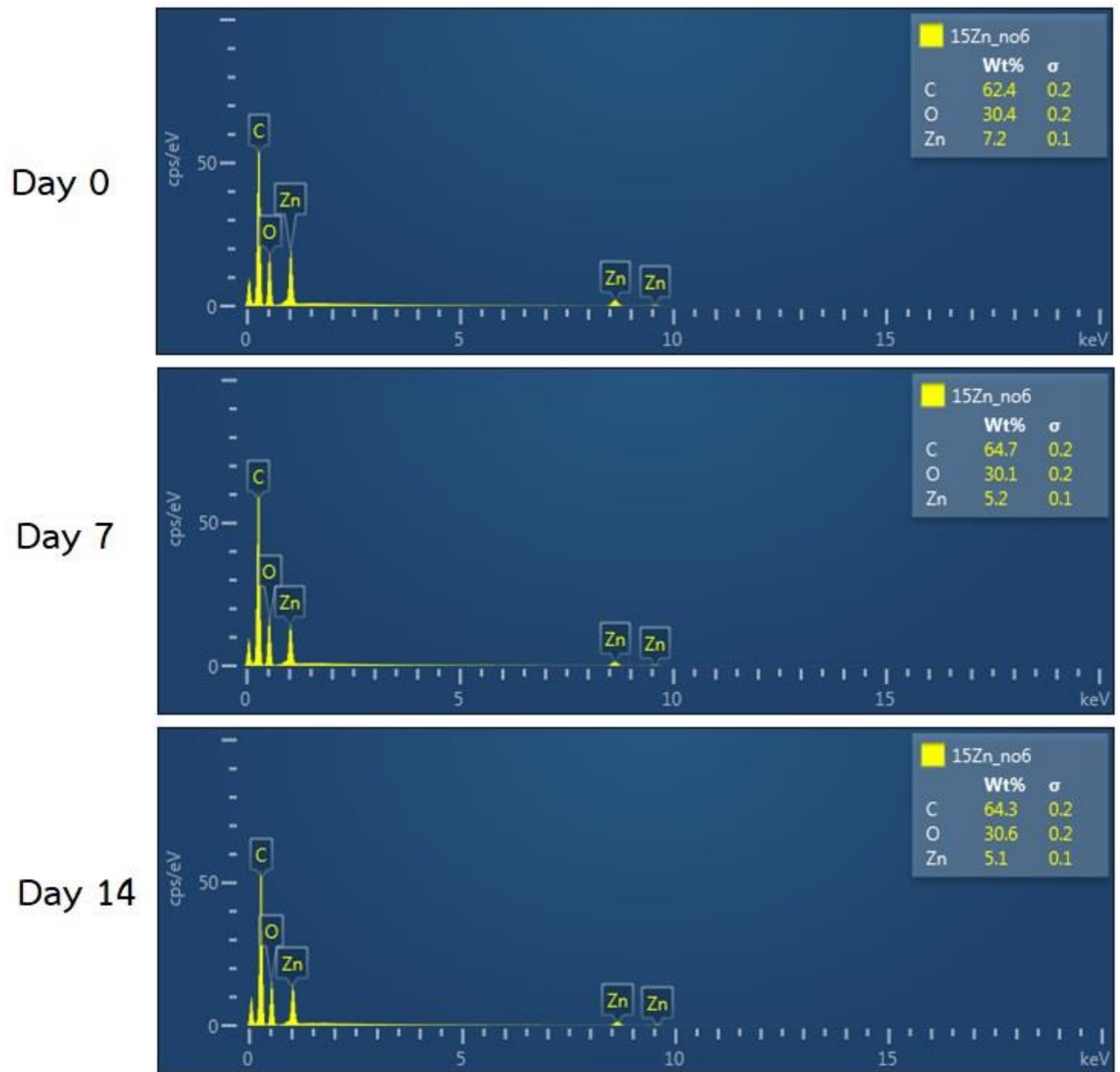

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Figure 15: Element identified by energy dispersive X-ray spectroscopy of 15Zn group at day 0, 7, and 14.

Table 6: Two-way ANOVA test for comparison of the amount of superficial zinc element

Factor	Sum of squares	*df*	Mean Square	F	*P*
Group	470.143	3	156.714	900.820	<0.001
Time	36.416	2	18.208	104.662	<0.001
Group x time	12.643	6	2.107	12.112	<0.001

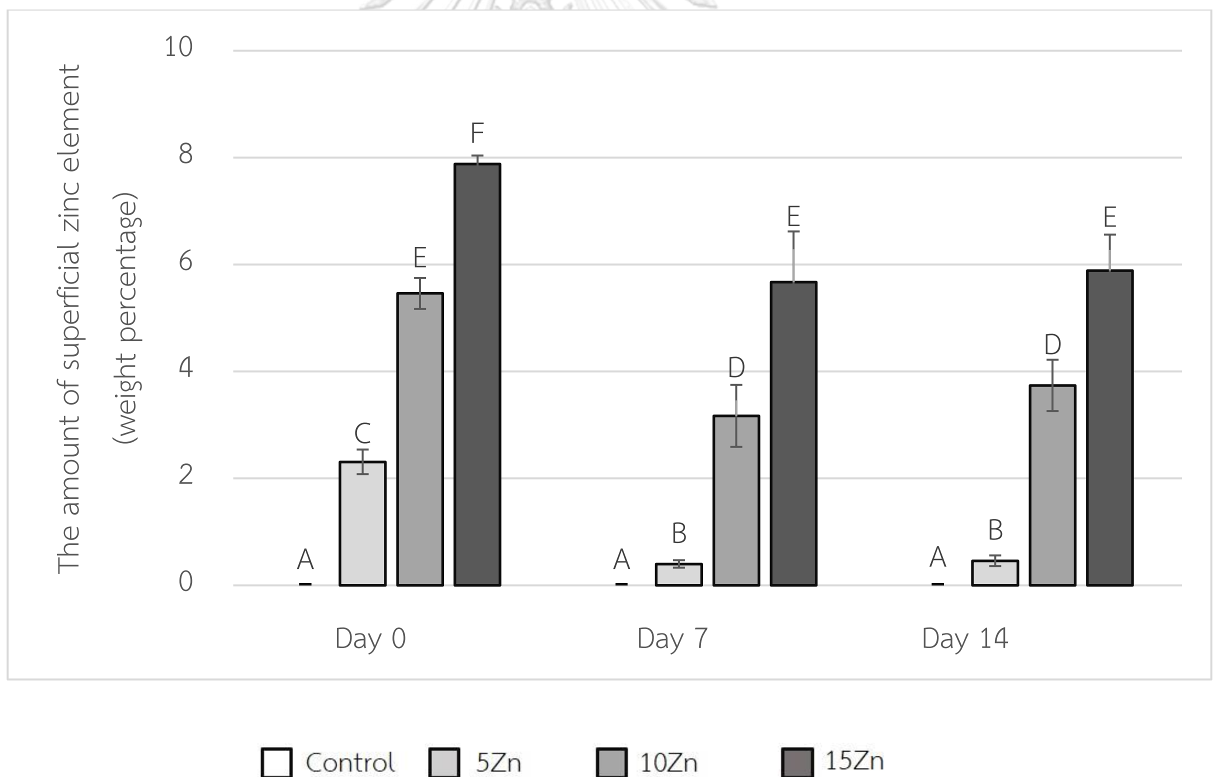

Figure 16: Means and standard deviations of the amount of superficial zinc element (weight percentage) in each group at different storage times. Bar with the different letters indicate significant differences (p<0.05).

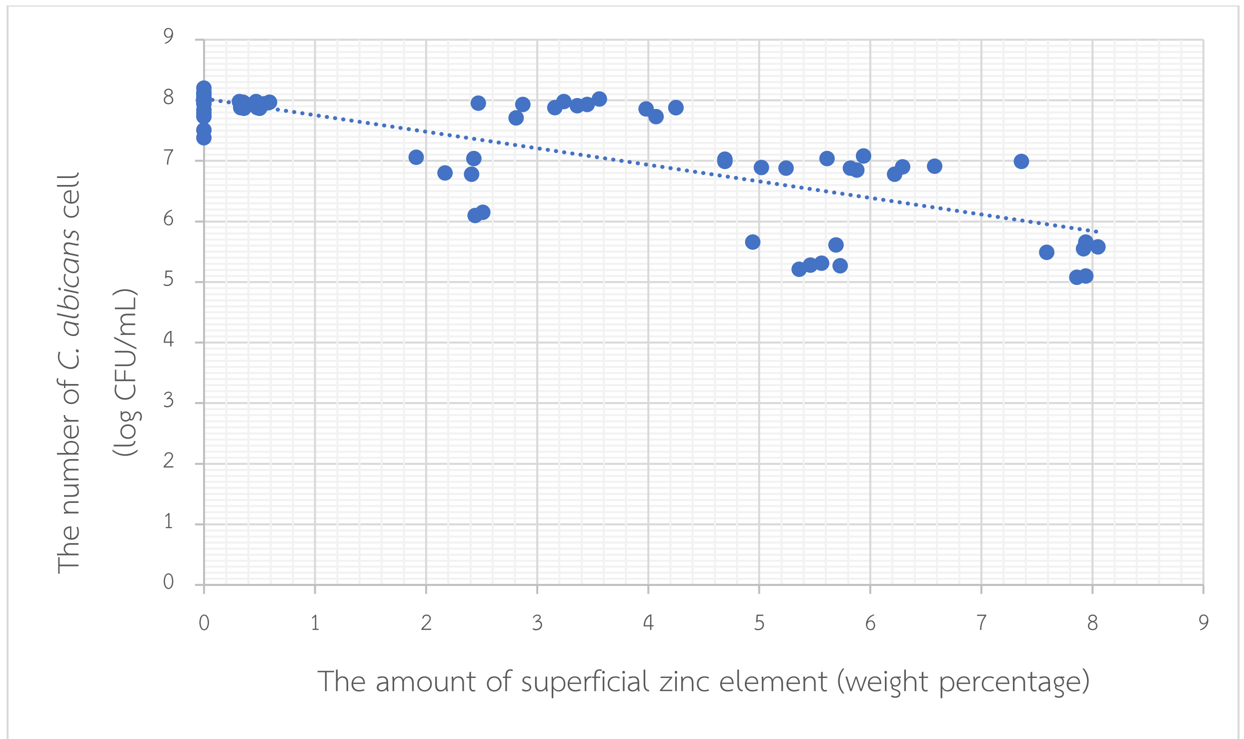

Figure 17: A scatterplot graph between the number of C. albicans cell (log CFU/mL) and the amount of superficial zinc element (weight percentage) (r=-0.78, p<0.001).

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Part 3 Penetration depth test

The result of two-way repeated measures ANOVA of the penetration depth is shown in Table. 7. The main factor of storage time of the penetration depth was significantly different (*p*<0.001). However, the main factor of group (*p*=0.055) and the interaction between group and storage time (*p*=0.527) were not significantly different. The means and standard deviations of the penetration depths (mm) are summarized in Table 8. The penetration depth of all experimental groups significantly reduced over time (*p*<0.001).

Table 7: Two-way repeated measures ANOVA test for comparison of the penetration depths

Factor	Sum of squares	*df*	Mean Square	F	*P*
Group	0.007	1.750	0.004	4.235	0.055
Time	6.6605	1.186	5.567	7097.192	<0.001
Group x time	0.002	3.628	0.001	0.807	0.527

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Table 8: The means and standard deviations of the penetration depth (mm) in each group at different storage times

Group	Storage time		
	Day 0	Day 7	Day 14
Control	1.22 (0.03)	0.70 (0.01)	0.64 (0.01)
5Zn	1.24 (0.02)	0.70 (0.02)	0.62 (0.02)
10Zn	1.23 (0.02)	0.69 (0.02)	0.62 (0.01)
15Zn	1.24 (0.02)	0.69 (0.01)	0.62 (0.01)
Nys	1.21 (0.02)	0.67 (0.03)	0.61 (0.02)
Average (95% CI)	1.226 ^{a} (1.217–1.235)	0.686 ^{b} (0.675–0.697)	0.622 ^{c} (0.617–0.626)

Values with the different superscript letters indicate significant differences between storage times ($p<0.05$). 95% CI=95% Confidence interval.

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Part 4 Tensile bond strength

The result of two-way ANOVA of the tensile bond strength is shown in Table 9. The main factor of storage time of the tensile bond strength was significantly different (p<0.001). However, the main factor of group (p=0.052) and the interaction between group and storage time (p=0.781) were not significantly different. The means and standard deviations of the tensile bond strengths are summarized in Table 10. The mean tensile bond strength of all groups at day 7 and 14 were significantly higher compared to day 0 (p<0.001). In addition, all specimens demonstrated a cohesive mode of failure.

Table 9: Two-way ANOVA test for comparison of the tensile bond strength

Factor	Sum of squares	*df*	Mean Square	F	*P*
Group	0.008	4	0.002	2.429	0.052
Time	0.020	2	0.010	13.007	<0.001
Group x time	0.004	8	0.000	0.593	0.781

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Table 10: The means and standard deviations of the tensile bond strength (MPa) in each group at different storage times

Group	Storage time		
	Day 0	Day 7	Day 14
Control	0.25 (0.02)	0.26 (0.04)	0.29 (0.03)
5Zn	0.26 (0.03)	0.26 (0.03)	0.28 (0.03)
10Zn	0.24 (0.02)	0.26(0.02)	0.27 (0.04)
15Zn	0.24 (0.02)	0.26(0.03)	0.28 (0.02)
Nys	0.26 (0.03)	0.29 (0.02)	0.29 (0.02)
Average (95% CI)	0.250 ^{a} (0.241–0.259)	0.268 ^{b} (0.259–0.277)	0.282 ^{b} (0.273–0.290)

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Values with the different superscript letters indicate significant differences between storage times (p<0.05). 95% CI=95% Confidence interval.

CHAPTHER 5

DISCUSSION AND CONCLUSION

Discussion

This study evaluated the antifungal, physical and mechanical properties of tissue conditioner incorporated with different amounts of ZnOnps and different storage times. Statistical analysis revealed that there were significant differences on two main factors (group and storage time) and their interaction in the antifungal effect. There were significant differences only between storage times in the penetration depth and tensile bond strength. Therefore, the null hypotheses were partially rejected.

In the present study, GC Soft- liner, tissue conditioner, was chosen because it is distributed globally and popular in Thailand. Tissue conditioner is recommended to be changed every few days, commonly for a period up to 7 days[38], with the aim of revitalizing the oral mucosa to a healthy condition and prevention itself from serving as reservoir of microorganism. However, the maximum of 14-day period of water immersion was selected in the present study because this interval mimics the protocol set in treating denture induced stomatitis using nystatin.[88] ZnOnps was selected due to its interesting properties especially white appearance and antimicrobial effect against various stains.[72] The concentration of ZnOnps incorporated into the tissue conditioner was increased up to 15 wt% since

this concentration was the minimum concentration which still provided antifungal effect after 14-day water immersion in the pilot study.

Several methods in evaluating the antimicrobial medication test were recommended. The agar disc-diffusion and agar well diffusion tests are well-known procedure due to its simplicity, easy interpretation, and low cost.[78] However, the agar diffusion tests are limited to the solubility of tested materials in diffusing into the surrounding aqueous media such as essential oils, plant extracts, and drugs.[77] Therefore, these methods are not compatible to evaluate antimicrobial effect of ZnOnps. The direct contact test was chosen in this study because it is suitable to evaluate the antimicrobial effect of close contact between tested materials and microorganism for solid or low soluble materials.

An increased amount of ZnOnps incorporated into tissue conditioner resulted in an increased in reduction amount of *C. albicans* cell at day 0. This finding is consistent with the previous studies that found that ZnOnps is a concentration-dependent antimicrobial effect.[21, 87] The 5 Zn and 10Zn groups showed significant reduction in *C. albicans* cells only at day 0 (89% and 99%, respectively). After 7 and 14-day water immersion, 5Zn and 10 Zn groups did not demonstrate a significant reduction in *C. albicans* cells compared to control group. This phenomenon might be attributed to the leaching out of ZnOnps into the water which resulted from the lack of chemical bond between ZnOnps and any compositions in tissue conditioner. The high porosity of tissue conditioner might

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also enhance the ZnOnps particle diffusion.[89] However, the 15Zn group showed a significant reduction in *C. albicans* cells at day 0 (99 %), 7 (93 %) and 14 (92 %), respectively, compared with control group at each storage time. It should be considered that the antifungal effect of the 15Zn group at day 7 and 14 was modest compared with its effect at day 0. Thus, the reduced *C. albicans* cell number *in vitro* might not warrant performing *in vivo* study. Clinical studies are needed to find the effective degree of antifungal effect of tissue conditioner.

SEM was performed to observe the surface morphology and the distribution of ZnOnps within the tissue conditioner matrix. The PEMA particles, approximately 40-50 μm in diameter, with small porosities in the matrix were generally found in control group. While the SEM images of 5Zn, 10Zn, and 15Zn groups demonstrated the PEMA particles are encompassed with densely accumulated ZnOnps which distributed throughout the tissue conditioner matrix. An increase in incorporated ZnOnps resulted in more white cluster particles. Although the SEM images of each storage time within the same ZnOnps concentration were not different by an observation, the amount of superficial zinc element by the EDS analysis of each group at day 7 and 14 were significantly lower compared to day 0. The decrease of amount of superficial zinc element occurred by releasing of outermost layer of the ZnOnps into the environment (deionized water). From the present study finding, the amount of released ZnOnps from day 0 to day 7 was higher than that from day 7 to day 14. Statistical analysis showed no significant differences in the amount of zinc

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element in each group between 7 and 14-day water immersion. These findings associated with the antimicrobial effect which showed no significant difference in the number of *C. albicans* cell in each group between 7 and 14-day water immersion. The Pearson's correlation coefficient between the amount of superficial zinc element and the number of *C. albicans* cells showed a significant negative linear correlation (r=-0.78, p<0.001). This implies that the amount of superficial zinc element plays a major role in the antimicrobial effect. This finding supports the mechanism of the antimicrobial effect associating with the direct interaction of ZnOnps with the microbial cells.[76] It is noticeable that, although, the amounts of superficial zinc element of 15Zn group at day7 and day 14 were comparable with that of 10Zn group at day 0, the antimicrobial effects between those groups were not similar. This result indicated that the antimicrobial effect of ZnOnps might possibly relate with the ROS generation by ZnOnps which might release for a short period due to their short half-life.[72, 90]

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Nystatin is a highly effective antifungal agent in treating denture induced stomatitis by direct topical application to the oral lesions 3 to 5 times daily.[91] The antifungal mechanism of nystatin resulted from the binding of nystatin to the ergosterol on cellular membrane of *Candida* species, leading to an increase in the permeability and causing cell death. This study clearly demonstrated that Nys group caused the highest reduction of *C. albicans* cells only at day 0. The inhibition of *C. albicans* cells of Nys groups at day 7 and 14 was not significantly different

compared with control group as also shown by Geerts et al.[17] The deterioration of antifungal effect of nystatin might occur from two reasons: surrounding factors and solubility of nystatin. Dobias and Hazen stated that the antifungal effect of nystatin depends on some surrounding factors.[91] The effect of nystatin can be fully exhibited at a pH of 2 to 3, at a temperature of 30°C. In the present study, the specimens at day 7 and 14 were stored in the daily fresh deionized water, which was neutral pH. This might lead to a decrease in the antifungal effect of nystatin. Moreover, the water solubility of nystatin (0.36 g/L at 24°C)[92] might be greater than that of ZnOnps (0.0042 g/L at 18 °C).[93] These might lead to the sustainable effect of ZnOnps compared to nystatin.

The penetration depth test and the bond strength test were selected to evaluate whether the addition of ZnOnps would affect the physical and mechanical properties of tissue conditioner. The penetration depth test was recommended to evaluate the softness of soft lining material.[38] An adequate bond strength between soft lining material and denture base is an important property for longevity of material. Various methods have been used to evaluate the bond strength such as shear bond strength test, peel test, and tensile bond strength test.[94, 95] However, the tensile bond strength test is the most preferred method.[96] The increased amounts of ZnOnps up to 15 wt% into the tissue conditioner did not affect the penetration depth and the tensile bond strength compared control group at each storage time.

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This might be due to the small amount of additive and nanosized particle of ZnO incorporated into the tissue conditioner. Moreover, the cylindrical penetrator with a 1-mm-diameter vertical rod in the penetration depth test might not be able to detect the differences of each group containing nanosized particles of ZnO at the same storage time.

The mean penetration depth of all groups showed a decrease in value over time. However, the penetration depth of all groups after 7-day water immersion was over 0.5 mm which was within the ISO criteria.[38] The mean tensile bond strength of all groups at day 7 and 14 were significantly higher when compared to day 0. The results of both penetration depth and tensile bond strength might be attributed to the leaching out of ethyl alcohol from the tissue conditioner, especially at day 7, leading to the hardening of tissue conditioner over time.[97] All means of the experimental groups could not represent the actual tensile bond strength between tissue conditioner and denture base because the failure of all the experimental groups were classified as cohesive failure in tissue conditioner as previously demonstrated by Schneid.[98] This suggested that cohesive force between the molecules of tissue conditioner was lower than the tensile bond strength at the interface between tissue conditioner and acrylic denture base. The strong mechanical retention at the interface between the tissue conditioner and acrylic denture base results from the chemical components of the tissue conditioner. The

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ethyl alcohol in liquid part of tissue conditioner swells the polymer particles of the acrylic denture base and allows the plasticizer to penetrate between the swollen particles.[97] Moreover, the powder of tissue conditioner is methacrylate-based component, similar with denture base material.

This study is the pioneer research focusing on ZnOnps incorporated into tissue conditioner to enhance the antifungal property. By adding 15 wt% ZnOnps, the antifungal property of tissue conditioner can be extended for a longer period, while the physical and mechanical properties are sustained without adverse effect. This new knowledge could help denture wearers who suffered from denture induced stomatitis by reducing the amount of pathogen, promoting a proper environment for healing, decreasing the frequency of appointments that need for changing denture soft lining material. Even though, the usage of systemic or topical antifungal drugs such as ketoconazole, nystatin is a standard treatment. This modified material might be used as an adjuvant treatment. However, the limitations of the present study are deserved to be addressed for future studies in terms of type of microorganisms and tissue conditioner. The effect of ZnOnps against mixed biofilms that mimics an oral environment might exhibit different results. The antifungal effect of tissue conditioner incorporated with ZnOnps might deliver in prolonged periods in xerostomic patients due to a decrease of saliva secretion. The safety of ZnOnps incorporated into tissue conditioner should be addressed because tissue conditioner

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made direct contact with oral mucosa. The cytotoxicity of tissue conditioner containing ZnOnps both of *in vitro* and *in vivo* should be further investigated.

Conclusion

Within the limitations of this *in vitro* study, it was concluded that 15 wt% ZnOnps incorporated into the tissue conditioner provides an antifungal effect up to 14 days without adverse effects on the penetration depth and tensile bond strength of the tissue conditioner.

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Chulalongkorn University

VITA

NAME	Woraporn Homsiang
DATE OF BIRTH	12 March 1990
PLACE OF BIRTH	Chiangmai
INSTITUTIONS ATTENDED	Faculty of Dentistry, Chiangmai University
HOME ADDRESS	88 Maneenoparat Road, Sriphum, Muang, Chiangmai 50200