EFFECT OF DIFFERENT SILVER NANOPARTICLE COATING METHODS OF POLY(METHYL METHACRYLATE) ON THE SURFACE CHARACTERIZATION AND BACTERIAL ADHESION

THAKSIYA THAKSIN

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science in (Master of Sciences in Dentistry (Prosthodontics) - Type A 2)
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By THAKSIYA THAKSIN

has been approved by the Graduate School as partial fulfillment of the requirements for the Master of Science in Master of Sciences in Dentistry (Prosthodontics) - Type A 2 of Naresuan University

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Title EFFECT OF DIFFERENT SILVER NANOPARTICLE COATING METHODS OF POLY(METHYL METHACRYLATE) ON THE SURFACE CHARACTERIZATION AND BACTERIAL ADHESION

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ABSTRACT

Poly(methylmethacrylate) (PMMA) is an acrylic resin widely used as a denture base material for the Thai elderly for a long time. However, there are still some structural limitations that promote microbial adhesion. Silver nanoparticles (AgNPs) were recently introduced as a surface coating because of their antimicrobial effect. Development of the surface coating with the AgNPs is an attractive strategy to improve the denture's hygiene.

This study aims to compare the effects of PMMA surface coating methods with the AgNPs on PMMA's surface roughness, characteristics of AgNPs, and bacterial adhesion.

Materials and methods: Three different AgNP coating methods (simple immersion, ultrasound irradiation, and microwave irradiation) were used. Six PMMA discs of each method were prepared and compared surface roughness values (R_a) between pre and post-coating and three methods (n=6). The characteristics of AgNPs were also evaluated by using FE-SEM. Fifty PMMA discs were divided into five groups (Negative control, Positive control, Simple immersion, Ultrasound irradiation, and Microwave irradiation) (n=10). The specimens were incubated in *S. aureus* suspension and incubated at 37 ºc for 3 hours. Then bacterial adhesion analysis was

evaluated by the spread plate method.

Results: There were differences in the surface roughness between pre and post-coating and among the three methods. The simple immersion method provided AgNPs with small size and regularly distributed nanospheres. In comparison, the AgNPs from ultrasound and microwave methods were varying size nanospheres and agglomerations. There were differences in the bacterial adhesion between the selected AgNPs coating methods compared to the control. The simple immersion method allowed a better reduction of bacterial adhesion than other methods. Nevertheless, there were no differences in the bacterial adhesion between ultrasound and microwave methods.

Conclusion: Different AgNP coating methods affect different surface roughness of PMMA and the shape, size, and distribution patterns of the AgNPs. Interestingly, the simple immersion method provided the most significant bacterial reduction.

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Chapter I

INTRODUCTION

Background and rationale for the study

Poly(methylmethacrylate) (PMMA) is an acrylic resin widely used as a denture base material for the Thai elderly for a long time. The clear advantages of PMMA to be selected as a denture base include low cost and excellent physical properties, such as rigidity, lightweight, acceptable aesthetics, and handling [1]. However, there are still some structural limitations, including low chemical and wear resistance, low fracture resistance, reduced tongue sensation, and especially microporosity [2]. Such internal inhomogeneity may also lead to fluid absorption that can subsequently deteriorate denture durability and cause dimensional instability. Additionally, functional stress accumulated within the denture may lead to fatigue failure from the microcracks formation [3, 4]. Moreover, the gradual combination of fluid absorption, microporosity, and abraded rough surface of aged PMMA base also promote microbial adhesion and biofilm formation [5].

The prolonged contact with contaminated acrylic denture base may result in complications for a denture wearer such as tissue inflammation, tooth decay, and other microbial infections, including *Candida albicans*, *Staphylococcus aureus*, and *Streptococcus mutans*. Therefore, removing microorganisms and biofilms on the denture base plays a vital role in preventing and reducing microbiological complications [1].

Due to their intrinsic anti-inflammatory and antimicrobial activities, silver nanoparticles were recently introduced and used as drug-delivery formulations, diagnosis platforms, part of regeneration materials, and medical coatings reported in many studies [6-8]. Interestingly, some studies reported that silver nanoparticles could also reduce the accumulation of intraoral microbes and plaque biofilms on the PMMA's surface [2, 9, 10].

However, there is a lack of knowledge regarding nano-synthesizing and incorporating an appropriate silver nanoparticle dose into the denture polymer. Among the various ways of adding, like forming of PMMA nanocomposite, electromagnetic application, the nano surface coating with the solution of silver nanoparticles assisted by ultrasound and microwave irradiation is more practical in terms of investing and further development of clinical applications [11]. Moreover, the characterization of silver nanoparticles after incorporation into the denture base materials is still unclear. By forming a silver-nanocomposite could undoubtedly alter the surface and the intrinsic properties of the PMMA. Reportedly, an experimental nanocomposite of PMMA and silver nanoparticles have higher compressive strength, better heat conductivity, and reduced liquid absorption [3].

Nevertheless, the higher combination load of silver nanoparticles significantly reduced the PMMA's tensile and flexural strength [12, 13]. Also, the distribution of nanoparticles on the denture surface, especially the surface making direct contact with microbes obtained from the mixing method, may become uncertain. The characteristic features of silver nanoparticles, such as size, shape, distribution, and agglomeration, need to be re-evaluated [14]. Previous studies found that the smaller-size, nanoplate-

shape, and regularly distributed from a silver nano layering technique provided a better antimicrobial effect because of the larger surface contact area with stable inherited mechanical properties of PMMA [15, 16]. Therefore, developing a practical silver nanoparticles coating technique synthesized with controlled size, morphology, and distribution may be the goal for the surface improvement of the PMMA [14].

Practically for dentistry, coating silver nanoparticles on the PMMA's surface was performed using ultrasound [17] and microwave irradiation [10, 18]. Nevertheless, there are a few comparative studies on the PMMA's surface characterization and bacterial adhesion with the different silver nanoparticle coating methods. This study aims to compare the surface roughness of the PMMA and the shape, size, and distribution patterns of the silver nanoparticles and bacterial adhesion with different coating methods. Expectedly, further development of the surface coating with the silver nanoparticles is an attractive strategy to improve the denture base's antimicrobial without any changes in the mechanical properties of PMMA [11].

Research question

1. Are there any differences in the surface roughness of PMMA between the groups of selected silver nanoparticle coating methods compared to the controls?
2. Are there any differences in the surface roughness of PMMA among the groups of selected silver nanoparticle coating methods?
3. Do the different silver nanoparticle coating methods affect the silver nanoparticles' shape, size, and distribution patterns on the PMMA's surface?
4. Are there any differences in the bacterial adhesion on the PMMA's surface between the groups of selected silver nanoparticle coating methods compared to the controls?
5. Are there any differences in the bacterial adhesion on the PMMA's surface among the groups of selected silver nanoparticle coating methods?

Research objective

1. To compare the surface roughness of PMMA between before and after performed silver nanoparticle coating in each method
2. To compare the surface roughness of PMMA among different silver nanoparticle coating methods
3. To compare the characterization of the silver nanoparticles coated on the PMMA among different silver nanoparticle coating methods
4. To compare the bacterial adhesion on the PMMA's surface between the groups of selected silver nanoparticle coating methods compared to the controls
5. To compare the bacterial adhesion on the PMMA's surface among different silver nanoparticle coating methods

The scope of the study

1. Laboratory *(in vitro)* study, aim to compare the surface morphology of PMMA between before and after performed silver nanoparticle coating, the surface

characterization of silver nanoparticle coated on the PMMA’s surfaces, and the bacterial adhesion on the PMMA's surface
2. Independent variables: silver nanoparticle coating methods (simple immersion, ultrasound irradiation, and microwave irradiation)
3. Dependent variables: surface roughness, characterization of the silver nanoparticles, and bacterial adhesion
4. Control: size and thickness of specimen, PMMA ratio, silver nanoparticle concentration, and bacterial concentration
5. Location: Research laboratory, the 3rd & 4th Floor, NSU 01 Building, Faculty of Dentistry, Naresuan University

Research hypothesis

1. There is no difference in the surface roughness of PMMA before and after performed silver nanoparticle coating in each method.
2. There is no difference in the surface roughness of PMMA among different silver nanoparticle coating methods.
3. There is no difference in the silver nanoparticles' characterization coated on the PMMA,s surface among different silver nanoparticle coating methods.
4. There is no difference in the bacterial adhesion on the PMMA's surface between the selected silver nanoparticle coating methods compared to the controls.
5. There is no difference in the bacterial adhesion on the PMMA's surface among the groups of selected silver nanoparticle coating methods.

CHAPTER II

LITERATURE REVIEW

The polymer has received attention as an acrylic denture base instead of a vulcanized rubber base since 1937 and developed into PMMA that currently used. PMMA is widely used because of tissue compatibility, good mechanical properties, low price, and ease of manipulation [19]. However, PMMA base stills have some limitations, including low corrosion resistance and high porosity. Liquids can be directly absorbed on the surface, leading to the accumulation of microorganisms and biofilms forming on the denture. The prolonged contact with contaminated acrylic denture base may result in complications for a denture wearer such as tissue inflammation, tooth decay, and other microbial infections, including *Candida albicans*, *Staphylococcus aureus*, and *Streptococcus mutans*. Therefore, removing microorganisms and biofilms on the denture base plays a vital role in preventing and reducing microbiological complications [1].

Staphylococcus aureus (S. aureus) is a gram-positive bacterium and causative infectious oral diseases, such as angular cheilitis, mucositis, periodontitis, and dental implant-associated infections [20, 21]. Moreover, *S. aureus* is one of the main species colonizing and forming a biofilm on denture surface and oral mucosa. [1]

S. aureus forms large yellow or white round colonies with a smooth edge on nutrient-rich agar media. The diameter of the colony ranges from 1-2 mm (Figure 1). Culture media of S. aureus are enriched media such as blood agar (BA), trypticase soy agar (TSA), and brain heart infusion (BHI) agar. Under light microscope, S. aureus cells appear spherical and resemble grape-like cluster after gram staining due to their cells are purple resulting from gram-positive. The diameter of the cell ranges from 0.5 to 1.0 µm [21, 22].

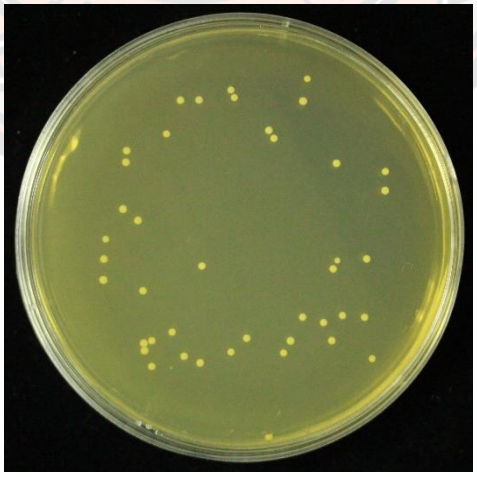

Figure 1 *Colony characteristics of S. aureus on BHI agar*
Yellow round colonies with a smooth edge sized about 1-2 mm

Reportedly, several attempts have been tried for PMMA base to have better mechanical properties and be microbial free by mixing with antimicrobial agents like 2-methacryloyloxyethyl phosphorylcholine [23], chlorhexidine diacetate, nystatin,

and amphotericin B into the PMMA mixture [24]. However, the addition of antimicrobial chemicals may significantly weaken the denture base [25, 26]. Alternatively, the addition of various nanoparticles into the mixture of PMMA or coating of the denture base with the nanoparticles have received more attention [19].

Silver nanoparticles can be synthesized in 3 different ways including,

1. Physical synthesis using evaporation and condensation by an annealing furnace at atmospheric pressure. This method still has many limitations, such as high energy and time consumption and the difficulty of controlling the cooling rate.
2. Chemical synthesis using three main chemical components includes silver salt, reducing agent, and stabilizer. This method is the most popular because it can be synthesized in large quantities, easily control the size and shape of the nanoparticles. However, a combination of the chemicals may harm living organisms as well as the environment.
3. Biological synthesis using bacteria, fungi, yeast, and plant extracts is an alternative that uses a solvent medium instead of the silver salt. The advantage of this method is using a small number of chemicals compared to chemical and physical synthesis. However, it is not easy to control the size and shape of nanoparticles [27].

Silver nanoparticles have received more and more attention in medical applications due to their broad-spectrum, including bacteria, fungi, and viruses, even drug-resistant strains [2]. Besides, there has been no report of drug resistance, which is considered the outstanding advantage of silver nanoparticles [16]. Although the antimicrobial mechanism of using silver nanoparticles is mystified, the studies found that size, shape, and agglomeration are the key to effectiveness. The small-size, nanoplate-shape, and regular distributed silver nanoparticles are preferable [15, 16].

The possible antimicrobial mechanisms of silver nanoparticles could be in several ways. [2, 28]

1. The silver nanoparticles adhere and penetrate the bacterial cell wall.
2. The silver nanoparticles denature ribosomes inhibiting protein synthesis and plasma membrane degradation.
3. The silver nanoparticles interact with sulfur-containing proteins disrupting the microbial cell membrane.
4. The silver nanoparticles attack the respiratory chain in microbial mitochondria by binding with the respiratory enzymes' thiol group.
5. The silver ion inside the microbial cell might create free radicals and induce oxidative stress lead to apoptosis.
6. The silver nanoparticles bind with DNA preventing its replication and multiplication, causing apoptosis.

Regarding the PMMA's surface coating technique with silver nanoparticles, several methods are sol-gel, sputtering, or co-sputtering [29]. Also reported, a nanolayer's recoating with the polymer helps control durability and silver ions' release rate [30]. In many dental studies, the practical silver-nanoparticle coating methods were assisted either by ultrasound irradiation [17] or microwave irradiation [18] (Table 1).

However, the geometrical information of the covered surface, the detailed configuration of the particles, and their bacterial inhibition efficiency developed from

each coating technique still unclear. As an essential background of knowledge before further developing a clinical implication of silver nano-coating technique in dentistry, a well-defined comparative laboratory study regarding the PMMA's surface alteration together with the nano surface characterization of the distribution and geometry of the particles should be first assessed. Additionally, a second part of this study aimed to directly compare the bacterial adhesion effect from the different coated silver nanoparticles' characters from selected coating methods.

Table 1 *Reported dental studies regarding specimen's geometry and PMMA's surface coating technique with silver nanoparticles*

Authors, year of publication	**Coating technique**	**Specimen geometry**	**Mixture**
Kotyar et al., 2007 [17]	Ultrasound irradiation	PMMA chips and spheres	Silver nitrate ($AgNO_3$) Ethylene glycol (EG) Ethanol (EtOH) Water (H_2O) Aqueous ammonia (NH_4OH)
Irzh et al., 2007 [18]	Microwave irradiation	PMMA spheres	Silver nitrate ($AgNO_3$) Polyethylene glycol (PEG) Ethanol (EtOH) Water (H_2O) Aqueous ammonia (NH_4OH)
Kamikawa et al., 2014 [10]	Microwave irradiation	PMMA sheets	Silver nitrate ($AgNO_3$) Ethylene glycol (EG) Ethanol (EtOH) Water (H_2O) Aqueous ammonia (NH_4OH)

CHAPTER III

RESEARCH METHODOLOGY

Preparation of self-cured PMMA discs

A self-curing acrylic resin for denture base mixture (lot no.YT1196, V09517, SR Triplex Cold; Ivoclar Vivadent, Liechtenstein) was poured into a cylindrical putty silicone mold (lot no.27-DX418-27/1987, Amcoflex; Amcorp, USA), forming a disc-like specimen with a diameter of 5 and 2 mm thick at a mixing ratio of 23.4g: 10 ml. Then removed the excess polymer, covered the uncured surface with the second glass slide, and pressed 40 kg for 15 minutes. Eighteen acrylic resin discs with a smooth surface were carefully prepared and used in this study (Figure 2).

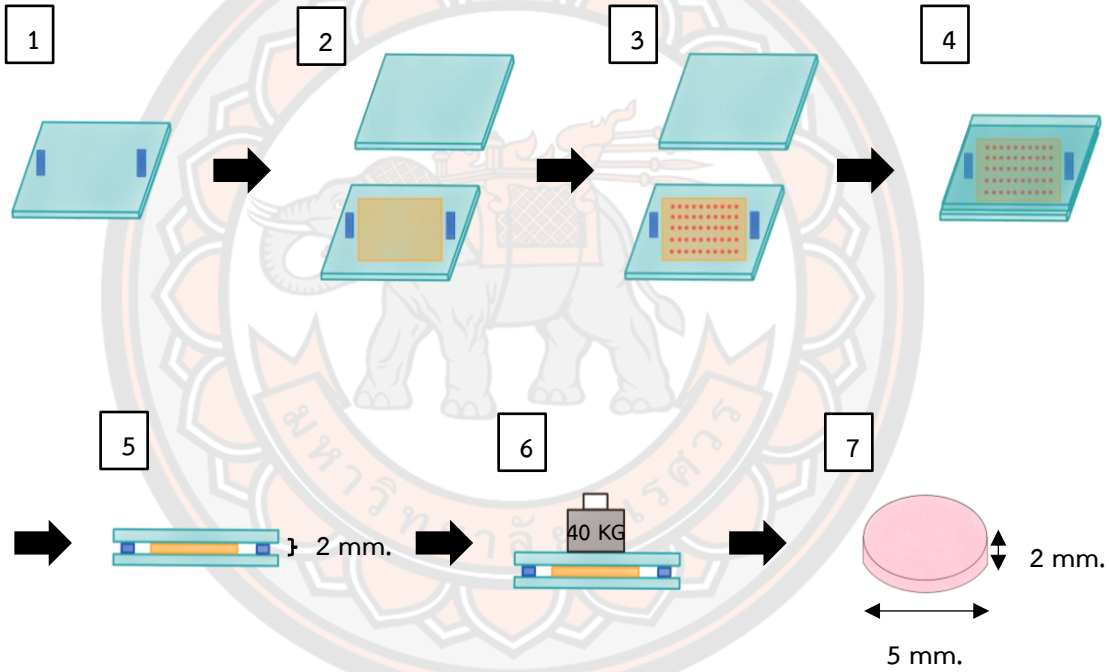

Figure 2 *Schematic drawing of putty silicone molds and self-cure acrylic (PMMA) discs preparation*
(1)-(3) Preparation of 5-mm round symmetrical holes on 2-mm flat silicone (4) Filling up the molds with hand-mixed self-curing PMMA, (5)-(6) Repressing with the second glass plate, loaded with 40 kgs weight for 15 minutes, (7) Archived 5-mm acrylic resin disc with a flat smooth surface with controlled 2 mm thickness.
(Preparation of self-cured acrylic resin specimen; (1) Place 2 mm spacers on the glass slap, (2) Prepare a putty silicone thick 2 mm with two flat glass plates, (3) Prepare symmetrical holes sized 5 mm. diameter, (4) Fill up with self-curing PMMA, (5) Repress with the second glass plate, (6) loaded with 40 kg weight for 15 minutes, (7) The acrylic resin specimen with glassy smooth surface sized 5 mm diameter x 2 mm thickness)

Preparation of silver nanoparticle coating solution (AgNP solution)

A solution mixture applied from the study [18] contained silver nitrate 0.85 ml (lot no.HC90446681, Merck KGaA, Darmstadt, Germany), 50% polyethylene glycol 10 ml (lot no.BCBW6004, Merck KGaA, Darmstadt, Germany), 28% aqueous ammonia 0.7 ml (lot no.A5023-1-2501, Merck KGaA, Darmstadt, Germany), 70% ethanol 10 ml, and sterile water 30 ml. Every preparation step were performed under a controlled environment following a safety laboratory guideline from Naresuan University Institutional Biosafety Committee (NUIBC).

Silver nanoparticle coating method on the surface of acrylic discs (Figure 3)

The prepared PMMA discs were divided into three experimental coated groups following the three methods below.

1. Simple immersion: The acrylic resin disc was immersed in the silver nanoparticle solution for 2 hours.
2. Ultrasound irradiation: The acrylic resin disc was immersed in the silver nanoparticle solution and assisted by ultrasound irradiation for 2 hours. (CPX130; Cole-Parmer, IL, USA, 30 kHz).
3. Microwave irradiation: The acrylic resin disc was immersed in the silver nanoparticle solution and assisted by microwave irradiation for 5 minutes (MS2022D; LG, Thailand, 200 watts), then immersed for 2 hours.

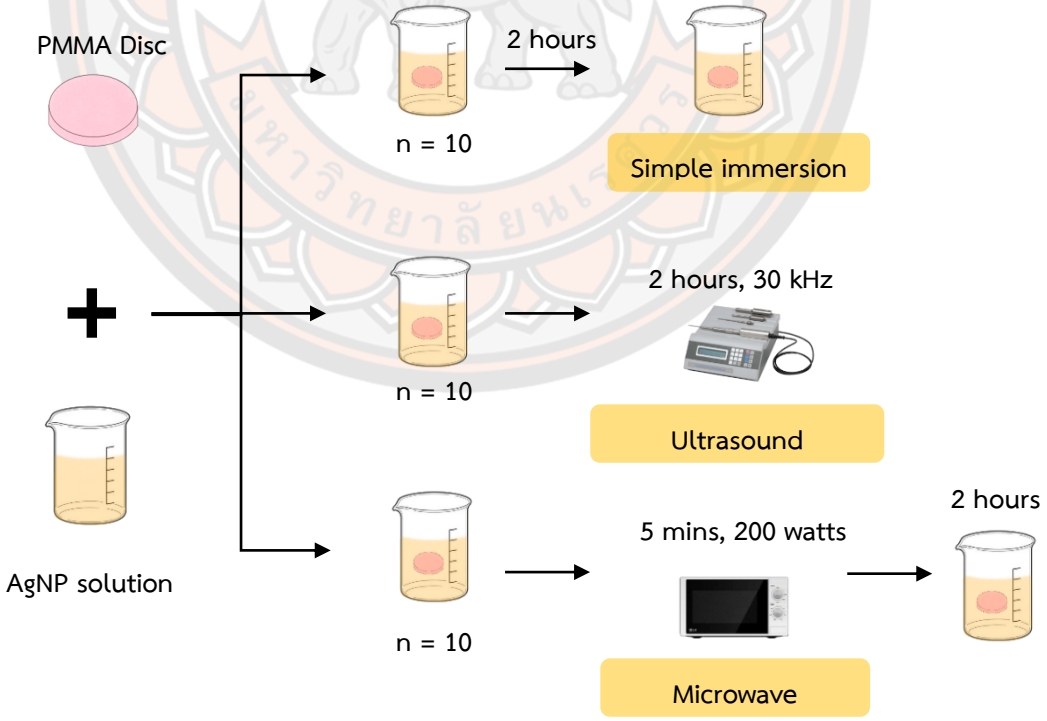

Figure 3 *Three silver nanoparticle coating groups*

Analysis of the PMMA's surface alteration and the characterization of silver nanoparticles

Surface roughness

The precoated specimens' surface roughness ($Ra_{(pre)}$, nm) were initially evaluated by using atomic force microscopy (FlexAFM5; Nanosurf, Liestal, Switzerland) (Figure 4). The surface roughness measurement was performed at the microscale (50x50 μm^2) on the three randomly selected areas at the triangle corners on the specimen surface. The calculated roughness parameter of the precoated surface was the average randomized roughness value ($Ra_{(pre)}$, nm). Comparatively, the average experimental surface roughness ($Ra_{(coat)}$, nm) was also remeasured in the same manner from the specimens (n=6) randomly selected from the three experimental silver nanoparticle coating methods (Table 2).

Table 2 *Grouping of the control and experimental specimens for the surface roughness analysis*

Group	**Sample size (n)**
Control	
Before simple immersion	6
Before ultrasound irradiation	6
Before microwave irradiation	6
Experimental	
After simple immersion	6
After ultrasound irradiation	6
After microwave irradiation	6

Figure 4 *The specimens' nano surface roughness evaluation by atomic force microscopy*

Surface characteristic analysis of the silver nanoparticles

Another duplicated specimen set of three from pre and post coating from each method was used for the surface characteristic analysis. The surface distribution patterns and the silver particles' geometry were evaluated by using a Field-emission scanning electron microscope (FE-SEM) (Apreo 2 S; Thermo Scientific, Massachusetts, USA) with a magnification ratio of 2,000x and 10,000x. Additionally, the elemental analysis was performed by using the energy-dispersive X-ray spectroscopy (EDS) (Ultim Max; Oxford, High Wycombe, UK) to confirm the silver element's presence (Figure 5).

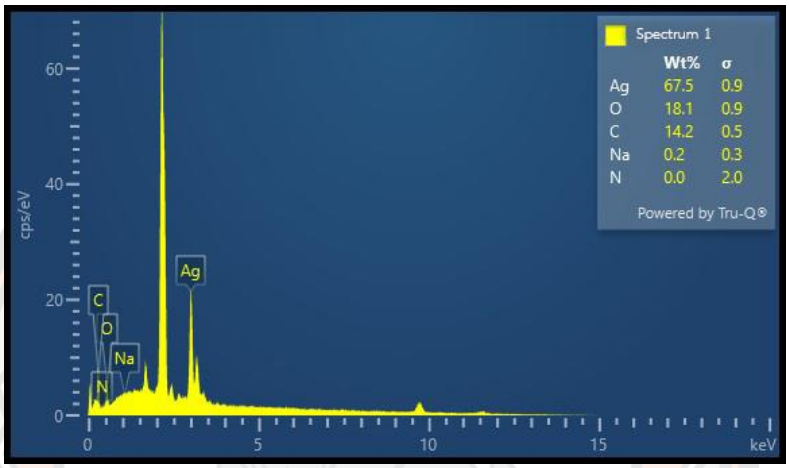

Figure 5 *The elemental analysis was performed by using the energy-dispersive X-ray spectroscopy to confirm the presence of the silver element.*

Statistical analysis of surface roughness

The sample size was calculated by the G*power program (Preface by Russell V. Lenth, Version 3.1.9.4, Iowa, USA). Means and standard deviations were retrieved from the literature [31]. Base on the formula for the paired t-test with a significance level of 5% and a power of 95%, a sample size of at least three specimens per group was required to detect a difference in the PMMA's surface roughness. So the sample size in this study was six specimens per group.

Statistical calculations were performed by the SPSS program (IBM SPSS Statistics for Windows, Version 23.0. IBM Corp, Armonk, NY). Standard descriptive statistics namely, means and standard deviations were calculated for all parameters. Before comparing the average surface roughness, Shapiro-Wilk and Levene's tests were used to determine normality and homogeneity of variances, respectively. A statistically significant level is considered at 0.05. The paired t-test was analyzed to compare surface roughness between before and after performed silver nanoparticle coating in each group. Furthermore, the simple t-test was analyzed to compare surface roughness between the two groups.

Analysis of bacterial adhesion on PMMA's surface

Preparation of bacterial culture solution

S. aureus (ATCC25923) was used in the study. The suspension solution of *S. aureus* was prepared in BHI culture media (lot no.237500, Difco, New Jersey USA) adjusted to a Mcfarland factor of 0.5 (absorbance at 625 nm).

Bacterial adhesion assay and analysis

Fifty PMMA discs were divided into five groups (Negative control, Positive control, Simple immersion, Ultrasound irradiation, and Microwave irradiation) (n=10). PMMA discs without silver nanoparticle coating or bacterial adhesion assay were used as negative control groups. Also, PMMA discs without silver nanoparticle coating were used as positive control groups. PMMA discs with three different silver nanoparticle coating methods were used as experimental groups (Table 3). The specimens were rinsed three times with sterile water, dried at room temperature, packaged in aluminum foil, and autoclaved (SA-300VF; Sturdy, Taipei, Taiwan) at 121 ◦c for 15 minutes.

Table 3 *Grouping of the control and experimental specimens for bacterial adhesion on PMMA's surface analysis*

Group	Materials	Sample size (n)
Negative control	PMMA disc without silver nanoparticle coating BHI culture media	10
Positive control	PMMA disc without silver nanoparticle coating BHI culture media S. aureus suspension	10
Simple immersion	PMMA disc with silver nanoparticle coating BHI culture media S. aureus suspension	10
Ultrasound irradiation	PMMA disc with silver nanoparticle coating BHI culture media S. aureus suspension	10
Microwave irradiation	PMMA disc with silver nanoparticle coating BHI culture media S. aureus suspension	10

Bacterial adhesion was performed by placing the specimen in a 5 ml tube containing 1 ml of *S. aureus* suspension (10^6 cells/ml) (Figure 6), then incubated (1535-2E; Shellab, Oregon, USA) at 37 ◦c for 3 hours. Then, removed from the *S. aureus* suspension, rinsed with phosphate-buffered saline (PBS) to remove non-adherent *S. aureus*, and transmitted to the new sterile test tubes containing 1 ml of PBS. Adherent *S. aureus* was subjected to ultrasonic vibration (Sonorex Super 10 P;

Bandelin, Berlin, Germany) for 10 minutes, followed by vortexing (VM 300; Gemmy, Taipei, Taiwan) for 1 minute.

A 100-µl solution from each test tube was inoculated on BHI agar by the spread plate method then incubated at 37 ◦c for 24 hours. The quantification of *S. aureus* adhesion on PMMA's surface would be counted by colony-forming units (CFUs).

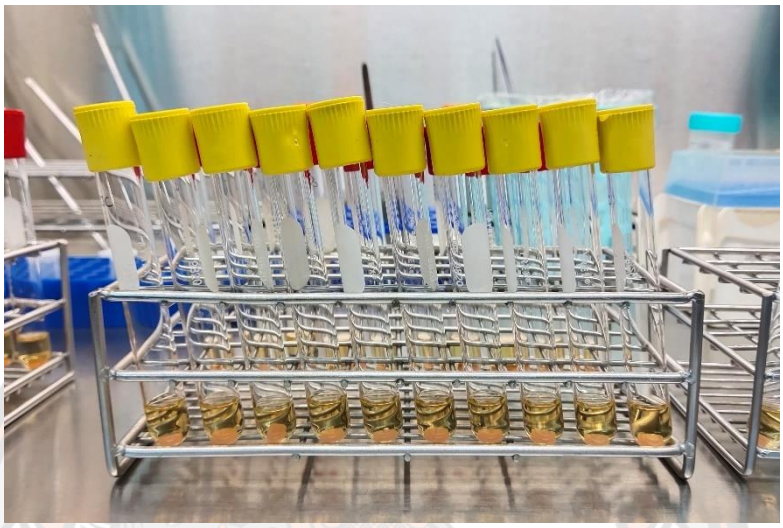

Figure 6 *Bacterial adhesion was performed by placing the specimens in 5 ml tubes containing 1 ml of S. aureus suspension.*

Statistical analysis of bacterial adhesion on PMMA's surface

The sample size was calculated by the G*power program (Preface by Russell V. Lenth, Version 3.1.9.4, Iowa, USA). Means and standard deviations were retrieved from the literature [10]. Base on the formula for t-test with a significance level of 5% and a power of 95%, a sample size of at least three specimens per group was required to detect a difference in bacterial adhesion. So the sample size in this study was ten specimens per group.

Statistical calculations were performed by the SPSS program (IBM SPSS Statistics for Windows, Version 23.0. IBM Corp, Armonk, NY). Standard descriptive statistics, namely means and standard deviations were calculated for all parameters. Before comparing bacterial adhesion, Shapiro-Wilk and Levene's tests were used to determine normality and homogeneity of variances, respectively. A statistically significant level is considered at 0.05. The simple t-test and Mann-Whitney U test were analyzed to compare bacterial adhesion between the two groups.

CHAPTER IV
RESULTS AND DISCUSSION

Results

Surface roughness

The descriptive statistics of the surface roughness are given in Table 4. The mean surface roughness ($Ra_{(pre)}$, nm) of the precoated specimens from simple immersion, ultrasound irradiation, and microwave irradiation groups were 17.99±2.69 nm, 15.55±4.00 nm, and 17.67±6.65 nm. In comparison, the values of the coated groups ($Ra_{(coat)}$, nm) were 14.80±2.02 nm, 24.47±2.78 nm, and 35.55±2.57 nm, respectively.

Table 4 *The descriptive statistics, paired t-test and simple t-test of the surface roughness*

Group	Sample size (n)	Mean (nm)	SD (nm)	Paired t-test & Simple t-test
Control				
Before simple immersion	6	17.99	2.69	a
Before ultrasound irradiation	6	15.55	4.00	a
Before microwave irradiation	6	17.67	6.65	a
Experimental				
After simple immersion	6	14.80	2.02	b
After ultrasound irradiation	6	24.47	2.78	c
After microwave irradiation	6	35.55	2.57	d

For all variables with the same letter, the difference between the means is not statistically significant.

From the paired t-test, there were statistically significant differences in the surface roughness between before and after silver nanoparticle coating. While the simple immersion method provided a smoother surface than the control group, ultrasound and microwave irradiation methods provided a rougher surface than the uncoated group.

Among the three coated groups, from the simple t-test, there were statistically significant differences in the surface roughness between every coating method.

Surface characteristics of the silver nanoparticles

The SEM images of the precoated PMMA's surface at the low and high magnification (Figure 7a and 7b) showed the surface geometry and the smoothness of the polished PMMA's surface. Also, the elemental analysis from the energy-dispersive X-ray spectroscopy showed the absence of the silver element. While the SEM images of simple immersion groups present fine nanospheres, sized approximately 10 nm, regularly deposited on the PMMA's surface and the elemental analysis showed the presence of the silver element (47 Wt%) (Figure 8a and 8b). Interestingly, when using ultrasound irradiation, the silver nanoparticles deposited on the PMMA's surface with the mixing of small silver nanospheres (~10 nm) and approximately 400 nm silver's agglomeration on the surface. Besides, the elemental analysis shows the larger weight percent of the silver element compared to simple immersion (62.4 Wt%) (Figure 9a and 9b). It is worth highlighting that for the microwave irradiation groups, a fine densely distributed silver nanoparticles ~100 nm and the largest agglomeration size ~1000 nm regularly deposited on the PMMA's surface together with the elemental analysis shows the largest amount of the silver element (67.5 Wt%) (Figure 10a and 10b).

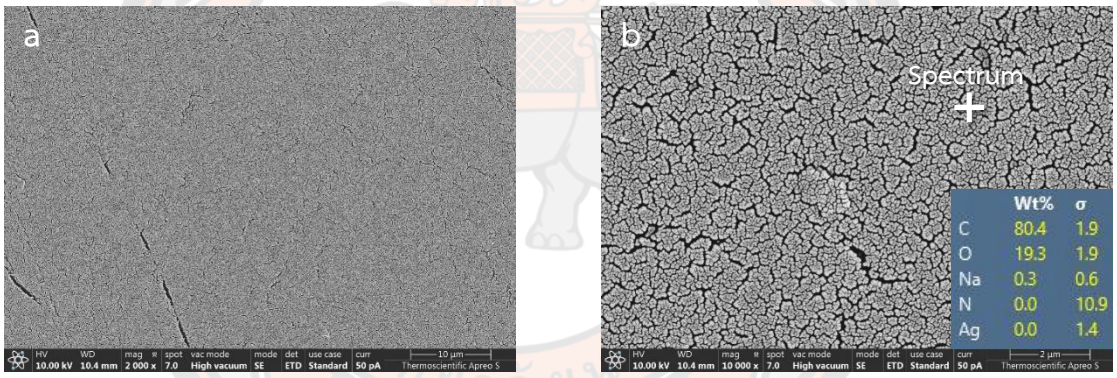

Figure 7 *SEM images of uncoated PMMA's surface*
(a) observation at 2,000x and (b) at 10,000x and the elemental analysis shows the absence of the silver element.

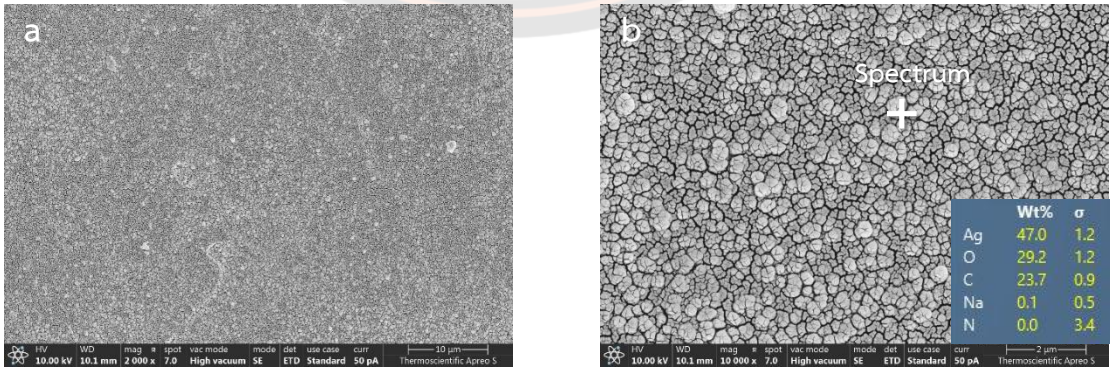

Figure 8 *SEM images of PMMA's surface with silver nanoparticle coating by simple immersion*

(a) observation at 2,000x and (b) at 10,000x shows fine silver nanoparticles deposited on the PMMA's surface (nanosphere, size ~10 nm, regular distribution) and the elemental analysis shows the presence of the silver element.

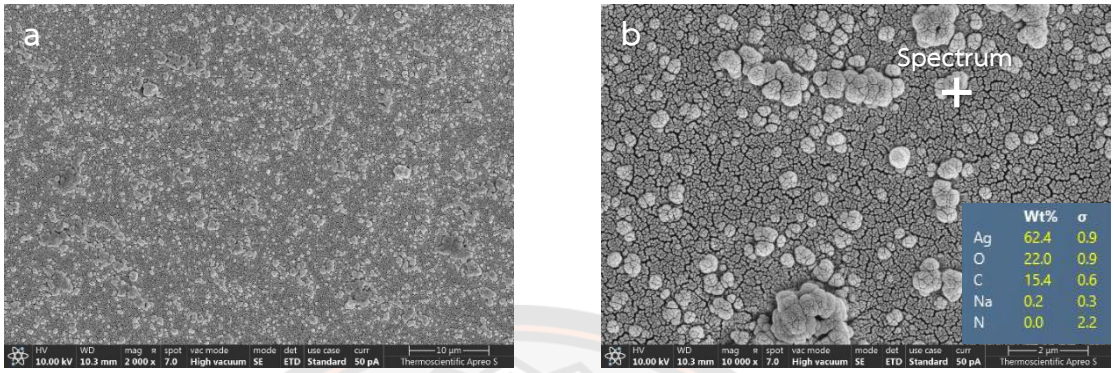

Figure 9 *SEM images of PMMA's surface with silver nanoparticle coating by ultrasound irradiation*
(a) observation at 2,000x and (b) at 10,000x shows silver nanospheres and small agglomeration deposited on the PMMA's surface (nanosphere, size ~10-400 nm, agglomeration) and the elemental analysis shows the presence of the silver element.

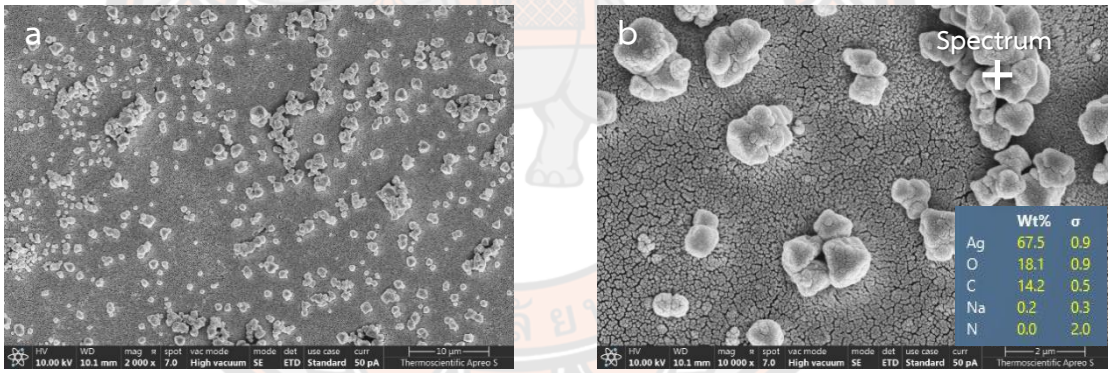

Figure 10 *SEM images of PMMA's surface with silver nanoparticle coating by microwave irradiation*
(a) observation at 2,000x and (b) at 10,000x shows a background of fine silver nanospheres and the largest agglomeration deposited on PMMA's surface (nanosphere, size ~100-1,000 nm, agglomeration) and the elemental analysis shows the presence of the silver element.

Bacterial adhesion

The descriptive statistics of the bacterial adhesion are given in Table 5. The mean colony-forming units (CFUs) of positive control, simple immersion, ultrasound irradiation, and microwave irradiation groups were 56.00±11.86 cfu, 3.60±0.90 cfu, 7.30±1.34 cfu, and 11.80±2.38 cfu, respectively.

There were statistically significant differences in the bacterial adhesion between positive control and experimental groups from the Mann-Whitney U test. All three different silver nanoparticle coating methods could significantly reduce bacterial adhesion (Figure 11).

Among the three coated groups, there were statistically significant differences in the bacterial adhesion between simple immersion and the other two unique activations (ultrasound and microwave irradiation) from the simple t-test. In contrast, there were no statistically significant differences between ultrasound irradiation and microwave irradiation groups.

Table 5 *The descriptive statistics, Simple t-test, and Mann-Whitney U test of the colony-forming units (CFUs)*

Group	Sample size (n)	Mean (cfu)	SD (cfu)	Simple t-test & Mann-Whitney U test
Negative control	10	0	0	-
Positive control	10	56.00	11.86	a
Simple immersion	10	3.60	0.90	b
Ultrasound irradiation	10	7.30	1.34	c
Microwave irradiation	10	11.80	2.38	c

For all variables with the same letter, the difference between the means is not statistically significant.

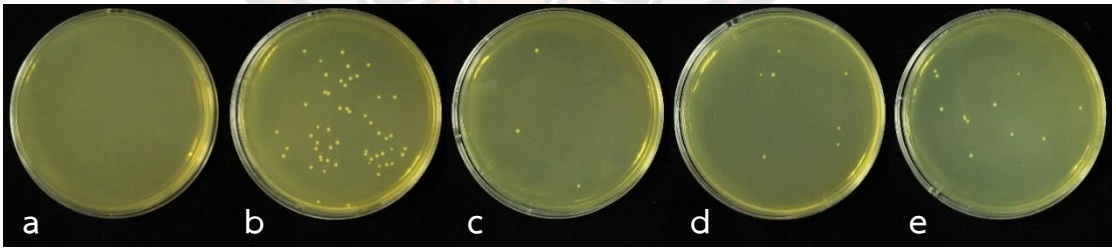

Figure 11 *Macroscopic observation of S. aureus on BHI agar after incubated at 37 ºc for 24 hours*
(a) Negative control shows S. aureus colony's absence. (b) Positive control, (c) Simple immersion, (d) Ultrasound irradiation, and (e) Microwave irradiation shows the presence of the S. aureus colonies

Discussion

This study compared the surface characteristics of acrylic denture base material, namely PMMA discs modified by two practical nano surface coating techniques assisted by ultrasound and microwave irradiation with the simple immersion in silver nanoparticles solution. After performing silver nanoparticle synthesis, it is necessary to identify the silver nanoparticle presentation and the nano surface characterization, significantly impacting their antimicrobial effect. As a preclinical trial before further clinical application development, the nanoparticle distribution and particles' geometry after the surface coating onto a flat PMMA specimen were firstly explored.

There were statistically significant differences in the surface roughness between the surface roughness analysis before and after performed silver nanoparticle coating in every method. Interestingly, while simple immersion tends to achieve a slightly smoother surface (table 2), both ultrasound irradiation and microwave irradiation methods tend to deliver a slightly rougher surface compared to the precoat surfaces. Therefore, the different silver nanoparticle coating methods affect the surface roughness of PMMA. However, such differences among the coating methods are within the nanoscale range (~7.9 - 25.34 nm), which might not be of clinical relevance in terms of plaque accumulation or adaptation of the denture base. As been mentioned in the literature, the Ra that significantly increases plaque formation was above 200 nm [32-34].

Many studies used the microwave irradiation method to modify PMMA beads for a denture base with inorganic nanoparticles, including silver, platinum, and gold [10, 18]. Reportedly microwave irradiation at 650 watts for 5 minutes did not affect flexural strength, impact strength, hardness, surface roughness, and denture base adaptation. While microwave irradiation at 690 watts for 6 minutes could damage denture base, changed surface roughness, and worsen denture adaptation [35, 36]. Therefore, the microwave irradiation power used in this study was under 650 watts for 5 minutes, which administered a sufficient amount of the nanoparticles on the PMMA's surface (Figure 10).

The silver nanoparticles' characterization, seemingly the small-size (approximately 10 to 500 nm), nanoplate-shape, and regular distributed silver nanoparticles had a better antimicrobial effect because of the larger surface contact area [14-16]. Consequently, the development of a silver nanoparticle coating method with well-controlled structures with uniform size, morphology, and distribution is essential [14].

From the surface microanalysis, a simple immersion method provided regular, uniformly distributed nanospheres. In comparison, the silver nanoparticles from ultrasound and microwave irradiation provide varying size nanospheres and agglomerations. The size of silver nanoparticles from a simple immersion method is the smallest, size ~10 nm. In contrast, the nanoparticles from the ultrasound irradiation were denser and vary in size between approximately 10 to 400 nm. Also, silver nanoparticles from the microwave irradiation method provided the largest size vary between ~100-1000 nm because of agglomeration.

Previous studies reported several factors that affect silver nanoparticles' size and agglomeration, such as the type of medium solution, the preparation method, and temperature [15, 37, 38]. Polyethylene glycol was used as a stabilizer in this study to

slow down the silver nanoparticles [18]. Moreover, Increasing the molecular weight of PEG could also enhance silver nanoparticles' stability [39]. Expectedly, this study selected PEG6000 to produce small and stable silver nanoparticles.

Temperature is an additional factor that may affect the size and agglomeration of the silver nanoparticles. At the lower temperatures, the reaction rate was reportedly slower, resulting in smaller and fine particles, while at the higher temperatures, the particle size and agglomeration significantly increased [37]. Accordingly, the silver nanoparticles form ultrasound, and microwave irradiation, which may generate heat during the preparation, produced the more extensive and higher agglomerated particles than the simple immersion. Because each coating method produces different sizes of silver nanoparticles on PMMA, the surface roughness of PMMA has statistically significant differences in all coating methods.

However, this study prepared the coating solution that followed Irzh's study [18] and used the PEG instead of EG as the stabilizing agent since the higher viscosity of PEG could delay the silver nanoparticle's growth and provide a smaller particle's size than EG. Interestingly, the study used the microwave irradiation method provided a particle size of 124 nm that smaller than what we found in this study. The temperature shift from the different microwave's power might be an additional factor that may affect the resulting silver nanoparticles' sized agglomeration. Moreover, adding poly(vinyl pyrrolidone) as a surfactant or replacing silver nitrate with silver acetate, which may acetate ions and acts as an additional surfactant, may produce an adequate particle size and no agglomeration [18]. Therefore, to achieve the control particle's size and reduce the nano agglomeration by microwave-assisted silver nanoparticle coating, the coating solution may be modified by adding poly(vinyl pyrrolidone) and replacing the PEG and silver acetate instead of EG and silver nitrate.

Several techniques are available for bacterial adhesion inspection, including physical, chemical, microscopy, and microbiological methods [40]. Physical methods using weight are easy to perform but difficult to interpret and have low sensitivity and accuracy. Chemical methods using microtiter plate dye-staining could apply to a broad range of microorganisms, but its drawbacks are lack of reproducibility and low sensitivity. Microscopy method using confocal scanning laser is also an excellent way to assess the quality and quantity of bacterial adhesion. Nevertheless, sample preparation, inability to obtain a large survey scan area and high cost are limitations. The microbiological method using colony-forming units is the most widely used technique due to its ease of performance, availability in every microbiology laboratory, and acceptable accuracy. However, it is time-consuming and limited to microorganisms that develop colonies on agar plates [40, 41]. Since the only bacteria species, namely *S. aureus,* was used in this experiment and *S. aureus* can develop colonies on BHI agar and quickly evaluate bacterial adhesion quantity with acceptable accuracy. This study selected a microbiological method using colony-forming units.

Regarding bacterial adhesion assessment, microbial reduction percent relating to the coated silver nano surface characters was calculated. In previous studies that used silver nanoparticles incorporated with PMMA, the percentages of microbial reduction were approximately 40.74-83.33% [42-44]. However, the study reported the adverse mechanical consequence from incorporating more than 1%wt of silver nanoparticles with flexural strength decreased significantly. Alternatively, for the surface coating techniques developed in this study could be more effective surface

disinfectant, the percentages of microbial reduction of the three methods ranged from 78.89- 93.57%. Interestingly, the simple immersion method provided the most significant microbial reduction (Table 5, Figure 11). This result is consistent with the result of the characterization of silver nanoparticles since the silver nanoparticles from the simple immersion method are the smallest and uniformly distributed. Although the mechanism of action of silver nanoparticles is still unclear, based on the plausible theory, it may be related to the smaller silver nanoparticles have more surface contact area and more easily penetrate the cell wall of bacteria [14-16, 28].

Even though there are many studies concerning the antimicrobial effect of PMMA enhancing silver nanoparticles, the degree of microbial reduction was not consistent across studies with the same coating technique (78.93% of microbial reduction for microwave irradiation and those reported in a previous study (99.94%)) [10]. The reason may be the fact that it was using different microbial species. The previous study used *C albicans*, a fungi kingdom member, while this study used *S. aureus*, a bacteria kingdom member. Both are opportunistic pathogenic microbes usually found in the denture wearer's oral cavity [45]. While the microorganism intraorally is a symbiotic community, the reduction potential represented from forming plaque biofilm using one bacteria species in an in-vitro study might not represent the real clinical situation.

Although there is no clear mechanism information regarding the retentive force between silver nanoparticles and the PMMA's surface, their plausible adhesional strength may be a consequence of the interfacial van der Waals force, surface roughness, and chemical bonding [46]. Reportedly, for the polyimide coating, the most significant silver nanoparticle adhesion factor, namely van der Waals forces, may depend on substrate hardness [47]. Decreasing substrate hardness may promote silver nanoparticle adhesion because of the more surface deformation of the substrate, the more nanoparticles' tight contact from the van der Waals force. In contrast, surface roughness and chemical bonding are secondary factors. It might be the fact that a microscale range of the surface roughness stills wide-reaching than the silver nanoparticle size, which is much smaller on a nanoscale and does not affect silver nanoparticle adhesion [47]. In comparison, the chemical interaction between the inert polymer surface and the nanoparticles is still mystified.

Besides, the solution's stability might affect coating efficacy when stored in a different storage condition. A previous study found that the solution using borohydride as a reducing agent and citrate as a stabilizer agent was more stable for at least one year in terms of silver ratio and particle size and shape when stored in the dark 4.0±2 ºC. On the contrary, the silver nanoparticle solution stored in the dark and at higher room temperature encountered a dramatic change in particle size and shape [48]. While our study used aqueous ammonia as a reducing agent and polyethylene glycol as a stabilizer agent, the solution's stability might behave differently depending on the effects of oxidant, reducing agent, stabilizer, and temperature [49].

Additionally, there was still no study comparing the antimicrobial effect of the different storage conditions of silver nanoparticle solution. Therefore, before developing the clinical application, the critical information of the solution stability and optimal interaction between the particle retention and the disintegration risk of silver nanoparticles from a daily removal home cleaning devices should be the main focus of the further study.

There are still some limitations to this study. Firstly, only one type of PMMA used in this study, while other PMMA suppliers may produce a complex chemical composition and a variation of some additives to make a wide range of polymer-based products. This chemical dissimilarity might affect a charge alteration on the precoat surface and directly related to the silver nanoparticles' binding efficiency. Although there might also be a wide range of precoat surface roughness, the coated PMMA's surface geometry trend would still be the same, depending on how the nanoparticles form on the surface. Secondly, to closely evaluate the nano surface geometry and identify the silver nanoparticles' presentation required a combination of SEM and EDS. Due to the fact that PMMA showed some distortion and burning from the heat generated from SEM and EDS, this is more significant when the magnification is more than 10,000x (Figure 12). Additionally, the silver nanoparticles in the round single-particle (size varied from 10 to 50 nm) or in the accumulation type (size varied from 300 to 500 nm) showed the same contrast with the PMMA background, which was difficult to distinguish in the SEM images. Consequently, quantitative comparisons of the nanoparticle on the surface between the experimental groups were obstructively impossible. Lastly, this study's bacterial cultivation is only one strain of *S. aureus*, while the oral cavity is dynamic and highly diverse, including bacteria, fungi, viruses, archaea, and protozoa [50]. Furthermore, denture plaque is the accumulation of a large number of microbes. Various microbial strains have been identified in denture biofilms [23]. It might be insufficient to compare microbial adhesion and biofilm from the oral cavity with highly complex communities.

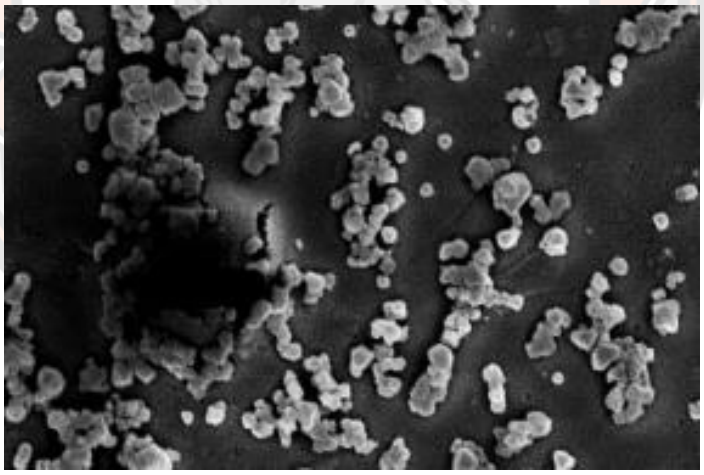

Figure 12 *SEM image of the burning area on PMMA's surface with silver nanoparticle coating at magnification 12,000x*

Seemingly, the three coating methods could generate different coated surface architectures and heterogeneous. Moreover, the three different coating methods could reduce bacterial adhesion on the PMMA's surface. The Simple immersion seems to be an innovation of antimicrobial dental prostheses applicable in minor surface alteration, consistent fine production of the particles, and reduce bacterial adhesion. Further study would firstly focus on evaluating the biocompatibility, toxicity, and the coating solution's stability because the chemical reactions might reduce over time, especially after cleaning with the solvent or mechanical cleaning devices.

CHAPTER V
CONCLUSIONS

Conclusions

Within the limitation of this study, these conclusions can be drawn as follow:

1. There are differences in the surface roughness of PMMA between before and after performed silver nanoparticle coating in each method.
2. There are differences in the surface roughness of PMMA among simple immersion, ultrasound irradiation, and microwave irradiation methods.
3. There are differences in the size, shape, distribution, and agglomeration patterns of the silver nanoparticles on the PMMA's surface, depending on the activation methods.
4. Temperature affects the shape, size, and distribution patterns of the silver nanoparticles.
5. There are differences in the bacterial adhesion on the PMMA's surface between the selected silver nanoparticle coating methods compared to the controls.
6. There are differences in the bacterial adhesion on the PMMA's surface between simple immersion and ultrasound irradiation groups.
7. There are differences in the bacterial adhesion on the PMMA's surface between simple immersion and microwave irradiation groups.
8. There are no differences in the bacterial adhesion on the PMMA's surface between ultrasound irradiation and microwave irradiation groups.

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