Evaluating the effect of silver nanoparticles incorporation on antifungal activity and some properties of soft denture lining material

Moamin I. Issa, B.D.S. [(1)]
Nabeel Abdul-Fattah, B.D.S., M.Sc. [(2)]

ABSTRACT

Background: Colonization of soft denture liners by *Candida albicans* and other microorganisms continued to be a serious problem. The aim of this study was to evaluate the effect of incorporating silver nanoparticles into heat cured acrylic-based soft denture liner on the antifungal activity, and on water sorption, solubility, shear bond strength and color change of the soft lining material. Furthermore, evaluating the amount of silver released.

Materials and methods: Silver nanoparticles were incorporated into soft denture liner in different percentages (0.05%, 0.1% and 0.2% by weight). Four hundred and twenty specimens were prepared and divided into five groups according to the test to be performed. The antifungal activity of the soft liner/AgNPs composite was evaluated in three different periods by using (viable count of *C. albicans* and disk-diffusion test). The amount of silver released in artificial saliva was measured by atomic absorption spectroscopy. The water sorptions, solubility, shear bond strength and color change was measured and the results were statistically analyzed.

Results:All experimental groups showed a highly significant decrease in colony forming units of *C. albicans* in comparison to control group. There was no inhibition zone around any test specimen of any test group. There was no silver detected to be released. The addition of AgNPs resulted in a highly significant decrease in water sorption, while only 0.2% group showed highly significant decrease in solubility. Non significant differences in shear bond strength were found. A highly significant increase in light absorption percentage was observed in all experimental groups.

Conclusion: The addition of AgNPs helps to produce soft denture liner with antifungal properties. Silver was not detected to be released. This addition resulted in decrease in water sorption, and did not affect the shear bond strength and it increased the opacity of the material.

Keywords: Soft denture liners, antifungal activity, silver nanopaticles. (J Bagh Coll Dentistry 2015; 27(2):17-23).

INTRODUCTION

Soft denture liners represent polymeric materials which can be placed on the tissue surface of a hard denture base to absorb some of the load resulted from the masticatory forces, and to act as shock absorbers between the hard denture and the underlying supporting oral tissues.[(1)]

One of the main drawbacks associated with using soft denture liners is their susceptibility to be colonized by pathological microorganisms which can be enhanced by increased humidity and high temperature beneath the dentures and by the surface characteristics of the material.[(2)]

Candida albicans was isolated from the surface of soft denture liner and it was considered as one of the etiological factors of denture stomatitis.[(3)]

In addition to that some studies showed that *C. albicans* has the ability to penetrate into different levels of the soft lining materials. This could limit the cleaning efficiency of the available chemical agents.[(4)]

Mechanical and chemical plaque control proceduresare frequently used to prevent subsequent denture stomatitis.

(1) M.Sc. Student. Department of Prosthodontics. College of Dentistry, University of Baghdad.
(2) Professor. Department of Prosthodontics, College of Dentistry, University of Baghdad.

However, to some geriatric or hospitalized patients suffering from cognitive impairment, reduced motor dexterity or memory loss cleaning the denture may be a difficult procedure. In addition to that, these methods can cause substantial damage to the soft lining materials. [(5-7)]

Silver is well known for its antimicrobial activity against different bacteria, fungi and certain viruses [(8)], and recently the antimicrobial properties of nanoparticles have drawn attention of researchers.[(9)] Smaller particle size results in greater surface area to volume ratio, which enhances its chemical and biological activity.[(10)] Particularly, silver nanoparticles with their antimicrobial properties have elicited high interest, and their incorporation into polymers can be beneficial in wound dressings and sutures, venous and urinary catheters, endotracheal tubes, drugs, artificial tendons and orthodontic adhesives. [(11)]

In the present study silver nanoparicles were incorporated in to acrylic-based soft denture liner in an attempt to minimize the microbial growth of *C. albicans*, and evaluating whether this addition would significantly affecting the mechanical and physical properties of the soft lining material ,in addition to evaluate silver release.

MATERIALS AND METHODS

Heat cured acrylic-based soft denture liner (Vertex™ Soft, Vertex-Dental, Netherlands) was used in this study. Silver nanopowder (MK Nano, Canada) was incorporated into the soft liner in different percentages (0.05%, 0.1% and 0.2% by weight). A total of Four hundred and twenty specimens were prepared and divided into five groups according to the test to be performed.

FTIR analysis was performed to determine if there is any chemical reaction between AgNPs and the soft liner.

Evaluating antifungal activity of soft liner /AgNPs specimens using viable count of *C. albicans:*

Specimen fabrication

Specimens with dimensions of (10× 10 × 2.3mm, length, width and thickness respectively) were prepared using plastic patterns to make a silicon-stone mould. The soft lining material was mixed packed and cured according to manufacturer's instructions. For experimental specimens AgNPs were added into the liner monomer and dispersed by using probe sonication apparatus(Soniprep-150, England) for 3 minutes to break them into individual nanoparticles.[12] The mixture was cooled down by placing the container in a cooling bath (ice-water bath), to prevent bulk heating of the liquid during sonication which can cause substantial liquid evaporation, or the degradation of the material.[13]

After complete curing the specimens were finished polished and autoclaved to be sterile.

Isolation of *C. albicans*

C. albicans was isolated from the oral cavity of 18 patients with signs of denture stomatitis and oral thrush,by gentle rubbing of the lesional tissue by a sterile cotton swab, and subsequent culturing on Sabouraud dextrose agar(that was prepared according to manufacturer's instructions) and incubated aerobically at 37°C for 24 - 48 hrs.

Identification of *C. albicans*

It was identified by colony morphology as it develops as creamy, smooth, pasty convex colonies on SDA [14] ,and by microscopical examination using Gram stain method [15], furthermore, germ tube formation procedure was used [16], and the final verification was made by biochemical method by using API Candida system (bioMérieux).

Evaluating viable count of *C. albicans*

To examine the antimicrobial activity of the soft liner/AgNPs composites, *C. albicans* was diluted in 0.9% NaCl, and a yeast suspension of approximately 10^7 CFU/ml (0.5 McFarland standards) was prepared using a McFarland densitometer. Each specimen was placed in a tube containing 9.9 ml of Sabouraud dextrose broth, into which were dispensed 100 µl of the yeast suspension. The final concentration of cells was 10^5CFU/ml. [11] After incubation for 24 hours at 37◦C, 100µl of each mixture was transferred to 9.9ml of NaCl (0.9%) and tenfold dilution was performed. From the second dilution, 100µl was taken and spread on SDA and incubated aerobically for 24hrs at 37ºC (Fig.1).

This procedure had been repeated after 7 days and 30 days of specimens' storage in artificial saliva at 37ºC.

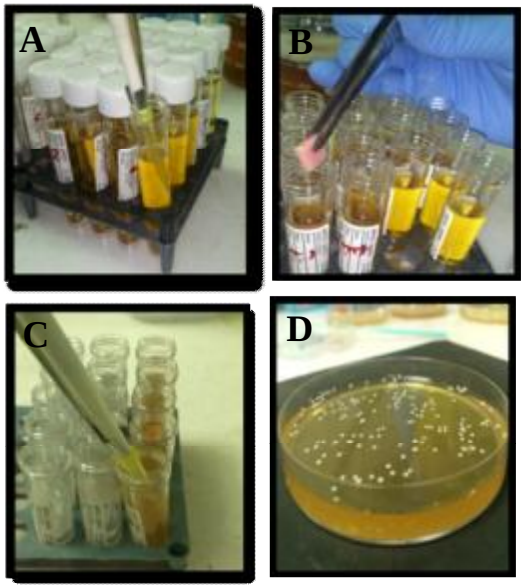

Figure 1: (A)Inoculation of broth with *C. albicans*, (B)Placement of specimen in the broth, (C)Serial dilution,(D) *C. albicans* growth.

Evaluating antifungal activity of soft liner /AgNPs specimens using disk-diffusion test:

Specimens used in this test were (6mm in diameter and 0.5mm in thickness). The culture medium used for this test was Mueller-Hinton agar (that prepared according to manufacturer's instructions) containing 2% glucose and 5µg/ml methylene blue. [17]

Kirby- Baure disk diffusion test was performed. Five-well isolated colonies of *C. albicans* were suspended in 0.85 % sterile normal saline 5 ml to achieve 0.5 McFarland turbidity to yield a yeast stock suspension. A sterile swab was dipped into the inoculum suspension and excess fluid was pressed out. The agar was swabbed in 3 directions to achieve even growth on the surface of the agar plate. [18]

After the agar surface has been left for about 5 minutes, then the soft liner disks (with and without AgNPs) were placed on the agar and the plates were kept at room temperature for 120 min for diffusion of the antimicrobial agents [19], then these agar plates were incubated aerobically for 24 hrs at 37º C. A digital caliper used to measure the inhibition zone that may appear around the disks.

Silver release test

The amount of silver released was evaluated by using specimens with dimensions of (10mm in diameter and 3mm in thickness)[11]and two atomic absorption spectrophotometers (Phoenix-986/AA Biotech engineering management co., and Shimadzu AA-6800)with a limits of detection of(0.025ppm and 0.01ppb) respectively. All specimens were immersed in 25 ml of artificial saliva inside a plastic plane tubes and kept at 37◦C under agitation for two different periods: T_1 = 7days, T_2 = 30days. The volume of the artificial saliva was reconstituted every 10 days to account for evaporation.

During each period solution of each tube was collected, and the amount of silver releasedwas analyzed by atomic absorption spectroscopy.

Watersorption and solubility test:

Disks measuring 50±1mm in diameter and 0.5±0.05mm in thickness were prepared for experimentaland control specimens according to ADA specifications No.12 [20], by using metal patterns.

All disk-shaped specimens were dried at 37ºC ± 2ºC for 24 hours in a desiccator containing dry silica gel, after that the specimens were removed to room temperature for one hour, and weighed with digital electronic balance with accuracy of (0.0001g).This cycle was repeated untilconstant weight (± 0.5mg) was obtained. This was considered to be the initial weight (W1).

Then specimens were immersed in distilled water for 7 days at 37ºC ± 2ºC. After this period of time, each specimen was removed from the water, wiped with clean, dry hand towel until free from visible moisture, waved in the air for 15 seconds and weighed one minute after removal of water. This weight represents (W2).

After that the specimens dried by the desiccator and they were weighed every 24 hours until a constant weight (± 0.5mg) was obtained, this weight represents (W3).

Water sorption and solubility of each specimen were calculated according to the following formulae:

$$\text{Sorption (mg / cm}^2\text{)} = \frac{W2-W1}{\text{Surface area}}$$

$$\text{Solubility (mg / cm}^2\text{)} = \frac{W1-W3}{\text{Surface area}}$$

Shear bond strength test

To evaluate shear bond strength of soft lining material to acrylic denture base, acrylic blocks with specified dimensions (75mm × 25mm × 5 mm length, width, depth respectively) with stopper of depth about 3mm needed to be made.[21] (Fig.2A).

Heat cured acrylic resin (Spofa dental, Czech) was used. Mixing packing and curing was done according to manufacturer's instructions. Then one block of the acrylic put over the other block leaving a space between them of (25mm × 25mm × 3mm length, width, depth respectively), that filled with wax. Then the whole specimen (the 2 blocks with wax) was invested into silicon material to fabricate a mould for final specimen curing. Wax elimination procedure was done and the formed space (25mm × 25mm × 3mm) was filled with soft lining material and curing was carried out (Fig.2 B&C).

The specimens were tested using Instron testing machine (Instron 1195, England). The maximum load required for failure was recorded in order to calculate the value of shear bond strength for each test specimen according to (ASTM specification D-638m, 1986) formula [22]:

$$\text{Bond strength (N/mm}^2\text{)} = \frac{\text{Maximum load}}{\text{Cross sectional area}} = \frac{F}{A}$$

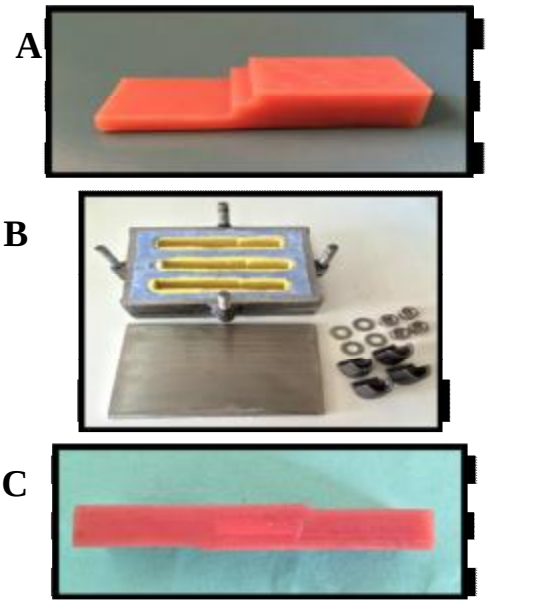

Figure 2: (A) Acrylic block used in test, (B) Custom-fabricated flask with silicon-stone mould. (C) Shear bond strength test specimen.

Color change test:

Disk-shaped specimens with 50 ± 1mm in diameter and 0.5 ± 0.05mm in thickness (in accordance with ADA specifications No.12) [20] were prepared to be used for color change measurements by using UV-visible spectrophotometer (UV-160AShimadzu, Japan) that evaluates color change by measuring the absorbed light percentage.

RESULTS

FTIR analysis showed that there was no chemical interaction between the soft lining material and AgNPs. All experimental groups (0.05%, 0.1% and 0.2% AgNPs) showed a highly significant decrease in colony forming units of *C. albicans* in comparison to control group with more decrease as the incubation time in artificial saliva increase (Table 1&2,Fig. 3).

Disk-diffusion test didn't show any inhibition zone around test specimens of any test group. There was no silver detected to be released in artificial saliva at any incubation period. The addition of AgNPs resulted in a highly significant decrease in water sorption mean value (Table 3&4), while only 0.2% group showed highly significant decrease in solubility (Table 5&6). Non significant differences in shear bond strength found among all test groups (Table 7). A highly significant increase in light absorption percentage observed in all experimental groups (Table 8 & 9).

Table 1: Descriptive statistics and one-way ANOVA of viable count of *C.albicans* for all study groups and for different periods.

Incubation period	Groups	Mean	S.D.	ANOVA F-test
Before incubation in saliva	Control	262.7	10.57	279.268 (HS)
	0.05% Ag	160	10.60	
	0.1% Ag	158.1	9.05	
	0.2% Ag	181.3	6.43	
After 7 days of incubation in saliva	Control	249.8	7.66	306.291 (HS)
	0.05% Ag	149.6	9.12	
	0.1% Ag	145.6	8.09	
	0.2% Ag	164.6	10.29	
After 30 days of incubation in saliva	Control	245.7	10.02	485.996 (HS)
	0.05% Ag	139.8	6.61	
	0.1% Ag	136.1	8.75	
	0.2% Ag	130.5	5.52	

Table 2: LSD test between viable count means.

Incubation period	Groups		MD	P-value
Before incubation in saliva	Control	0.05%	102.7	0.000 (HS)
		0.1%	104.6	0.000 (HS)
		0.2%	81.4	0.000 (HS)
	0.05	0.1%	1.9	0.651 (NS)
		0.2%	-21.3	0.000 (HS)
	0.1%	0.2%	-23.2	0.000 (HS)
After 7 days of incubation in saliva	Control	0.05%	100.2	0.000 (HS)
		0.1%	104.2	0.000 (HS)
		0.2%	85.2	0.000 (HS)
	0.05%	0.1%	4	0.319 (NS)
		0.2%	-15	0.001 (HS)
	0.1%	0.2%	-19	0.000 (HS)
After 30 days of incubation in saliva	Control	0.05%	105.9	0.000 (HS)
		0.1%	109.6	0.000 (HS)
		0.2%	115.2	0.000 (HS)
	0.05%	0.1%	3.7	0.303 (NS)
		0.2%	9.3	0.013 (S)
	0.1%	0.2%	5.6	0.123 (NS)

MD = Mean difference

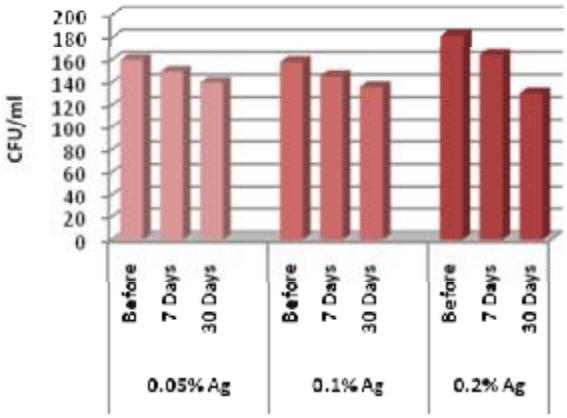

Figure 3: Bar chart showing mean values of CFU/ml at different periods of the study for each experimental group.

Table 3: Descriptive statistics and one-way ANOVA of water sorption test results.

Groups	Mean	S.D.	ANOVA F-test	P-value
Control	0.806	0.137	8.973	0.000 (HS)
0.05% Ag	0.643	0.095		
0.1% Ag	0.628	0.083		
0.2% Ag	0.601	0.057		

Table 4: LSD test between water sorption study groups.

Groups		Mean Difference	P-value
Control	0.05%Ag	0.163	0.001 (HS)
	0.1% Ag	0.178	0.000 (HS)
	0.2% Ag	0.204	0.000 (HS)
0.05% Ag	0.1% Ag	0.015	0.737 (NS)
	0.2% Ag	0.041	0.351 (NS)
0.1% Ag	0.2% Ag	0.026	0.548 (NS)

Table 5: Descriptive statistics and one-way ANOVA of solubility results.

Groups	Mean	S.D.	ANOVA F-test	P-value
Control	0.223	0.037	24.359	0.000 (HS)
0.05% Ag	0.202	0.035		
0.1% Ag	0.243	0.036		
0.2% Ag	0.130	0.007		

Table 6: LSD test between solubility study groups.

Groups		Mean Difference	P-value
Control	0.05%	0.021	0.141 (NS)
	0.1% Ag	-0.020	0.158 (NS)
	0.2% Ag	0.093	0.000 (HS)
0.05% Ag	0.1% Ag	-0.042	0.006 (HS)
	0.2% Ag	0.071	0.000 (HS)
0.1% Ag	0.2% Ag	0.113	0.000 (HS)

Table 7: Descriptive statistics and one-way ANOVA of shear bond strength test results.

Groups	Mean	S.D.	ANOVA F-test	P-value
Control	0.534	0.071	0.253	0.859 (NS)
0.05% Ag	0.529	0.071		
0.1% Ag	0.548	0.042		
0.2% Ag	0.551	0.081		

Table 8: Descriptive statistics and one-way ANOVA of color change test results.

Groups	Mean	S.D.	ANOVA F-test	P-value
Control	0.120	0.013	70.664	0.000 (HS)
0.05% Ag	0.144	0.012		
0.1% Ag	0.198	0.014		
0.2% Ag	0.236	0.033		

Table 9: LSD test between color change study groups.

Groups		Mean Difference	P-value
Control	0.05% Ag	-0.024	0.009 (HS)
	0.1% Ag	-0.078	0.000 (HS)
	0.2% Ag	-0.116	0.000 (HS)
0.05% Ag	0.1% Ag	-0.054	0.000 (HS)
	0.2% Ag	-0.092	0.000 (HS)
0.1% Ag	0.2% Ag	-0.038	0.000 (HS)

DISCUSSION

In this study AgNPs were added into soft denture liner in an attempt to improve the antimicrobial properties of the liner against *C. albicans* yeast which is one of the main causative factors of denture-induced stomatitis.

The results of this study showed a statistically highly significant decrease in colony forming units/ml of *C. albicans* after incorporating the soft denture liner with AgNPs. The antimicrobial efficacy seemed to be concentration dependant. The antifungal activity of the tested soft liner /AgNPs composite seemed to increase with the increase of incubation time in artificial saliva.

The explanation for that could be due to the presence of specimens in aqueous environment for longer period, so there was a greater possibility of AgNPs oxidation and Ag+ formation which enhance the antimicrobial activity , because the silver ions are the main active and reactive species of silver.[23] In addition to that movement of some AgNPs from the bulk of specimen to the surface might occur with increase in storage time, however silver or its ions were not detected to be released.[11] These phenomena together with decrease in water sorption could explain the lower level and later improvement of antifungal activity in (0.2% group).

For disk-diffusion test, no inhibition zone was detected around specimens for any AgNPs percentage used even after incubating the specimens in artificial saliva. This could be explained by absence of silver ions release from soft liner/AgNPs composite which was verified by the results of this study.

These findings indicated that the antifungal activity was achieved by contact kill; no Ag+ leached out of the copolymer. [24]

This was also confirmed by the results of this study as no silver or silver ions were detected in artificial saliva using atomic absorption spectroscopy at any incubation period. Previous studies were made to evaluate silver release from different polymeric materials, and some of them confirmed the results of the present study [11], while others disagreed by detecting different concentrations of silver released. [25, 26]

This could be explained by differences in the type of polymeric materials and their polymerization methods, in addition to differences in AgNPs incorporation methods.

Water sorption and solubility were evaluated simultaneously through water gain and loss of soluble components. The resulted decrease in water soption mean values could be attributed to addition AgNPs, with their hydrophobic nature, so

the number of PEMA molecules which would be available on the surface of the specimen which would allow water diffusion reduced, this is in accordance with Arora et al. [27]. Furthermore, the addition of AgNPs resulted in decrease of microporosity that resulted after polymerization process and subsequently reducing the water sorption.

While for solubility test only (0.2% group) showed a highly significant decrease in solubility. This could be attributed to the decrease in water sorption properties of the soft lining material with the increase in the amount of AgNPs added, as indicated by this study. This limitation in the diffused water will reduce the possibility of molecular flexibility and the leach out of soluble constituents from the polymer mass.

About the shear bond strength, the non significant change that resulted could be explained by the compatibility and high degree of similarity in chemical structure between polymethyl methacrylate denture base material and polyethyl methacrylate soft liner which would result in a chemical bonding between the two materials. However, FTIR analysis showed that no chemical interaction was detected between AgNPs and soft liner. In addition to that the flowability of the soft lining material; which allows the material to readily adapt to the bonding surfaces and creates an intimate union, didn't seem to change subjectively.

Color change test results showed that there was a statistically highly significant increase in light absorption percentage with the increase in AgNPs amount which was added to the soft lining material. This could be explained by the presence AgNPs in the polymer matrix, as the silver nanoparticles have extraordinary efficient ability to absorb and scatter light due to their optical properties [28], and a single silver nanoparticle can interact with light more efficiently than any known organic or inorganic colored particle with same dimensions [29], so AgNPs absorb more light energy than polymer matrix and appear more opaque. In addition to that AgNPs will act as fillers which tend to fill any spaces or voids within the polymer, thereby increasing the amount of scattered and absorbed light by the specimen and decrease the amount of transmitted light.

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الخلاصة

الخلفية: إن عملية استيطان بطانة طقم الاسنان اللينة من قبل المبيضات البيض والكائنات الدقيقة الاخرى لايزال يمثل مشكلة جدية ،كان الغرض من هذه الدراسة هو تقييم تأثير ادماج الفضة النانوية في مادة التبطين الاكريليكية المعالجة بالحرارة اللينة لطقم الاسنان على النشاط المضاد للفطريات (ضد المبيضات البيض) ،وعلى امتصاص المياه، قابلية الذوبان، قوة الربط القصية والتغير اللوني لمواد التبطين اللينة. علاوة على ذلك، تقييم كمية الفضة المتحررة من مركب البطانة اللينة مع الفضة النانوية.

المواد وطرق البحث: تم دمج الفضة النانوية مع مادة التبطين الاكريليكية اللينة لطقم الاسنان بنسب وزنية مختلفة (0.05% ، 0.1% و0.2%).تم اعداد اربع مائة وعشرين عينة وتم تقسيمها الى خمس مجموعات وفقا لنوع الاختبار المراد اجرائه. تم تقييم نشاط مزيج مادة التبطين اللينة مع الفضة النانوية ضد الفطريات على ثلاثة فترات مختلفة وباستخدام طريقتين: تعدادالمبيضات البيض القابلة للحياة واختبار انتشار القرص. وقد تم قياس كمية الفضة المتحررة في اللعاب الاصطناعي بواسطة التحليل الطيفي للامتصاص الذري. تم قياس قابلية امتصاص الماء , قابلية الذوبان ,قوة الربط القصية والتغير اللوني.وتم تحليل النتائج احصائيا.

النتائج: اظهرت نتائج جميع المجموعات التجريبية انخفاضا كبيرا للغاية في عدد مستعمرات المبيضات البيض مقارنة بالمجموعة الضابطة. لم يكن هنالك اي منطقة تثبيط حول اي عينة في اي مجموعة من مجاميع الاختبار. لم يتم الكشف عن اي اثر للفضة المتحررة. اظهرت النتائج ان اضافة الفضة النانوية ادت الى انخفاض ملحوظ بدرجة كبيرة في قابلية امتصاص الماء، في حين اظهرت مجموعة 0.2% فقط انخفاض كبير جدا في قابلية الذوبان. تم العثور على اختلافات غير ملحوظة في قوة الربط القصية لجميع المجموعات، وقد لوحظ زيادة كبيرة جدا في نسبة امتصاص الضوء في جميع المجموعات التجريبية.

الاستنتاج: ان اضافة الفضة النانوية الى مادة التبطين الاكريليكية اللينة لطقم الاسنان يساعد على انتاج مادة تبطين لينة مع خصائص مضادة للفطريات. لم يتم اكتشاف اي فضة متحررة. وقد اسفرت هذه الاضافة عن انخفاض في قابلية امتصاص الماء ولم تؤثر على قوة الربط القصي لمادة التبطين، في حين ادت الى تغير لون المادة من خلال زيادة التعتيم.

الكلمات الرئيسية: بطانة طقم الاسنان اللينة, النشاط المضاد للفطريات, الفضة النانوية.