ADHESION OF *Candida albicans* AND *Streptococcus mutans* TO SILVER NANOPARTICLE-MODIFIED POLYMETHYLMETHACRYLATE

Jorge Méndez-Serrano, Ulises Velazquez-Enriquez, Rosalia Contreras-Bulnes, Isaías De La Rosa-Gómez, Toshiko Sawada and Ryozo Yamaguchi

SUMMARY

This in vitro *study independently evaluated the adhesion of* Candida albicans *(ATCC 18804) and* Streptococcus mutans *(ATCC 25175) to polymethylmethacrylate (PMMA) blocks modified with silver nanoparticles (AgNPs). A total of 160 samples were used, separated into eight groups.* S. mutans *and* C. albicans *were independently cultivated. The blocks were submerged in a solution containing microorganisms radiolabeled with tritium (^3H). For quantitative analysis, the radioactive marker ^3H was used to identify the bacteria that adhered to the surface of the materials previous to colonization at 37°C. Analysis of variance (Sheffè* post hoc*) was used for statistical analysis. Significant differences in microbial adhesion were found among the groups of AgNP-modified PMMA. Heat-curing PMMA showed lower adhesion to* C. albicans *relative to self-curing PMMA. Heat-curing PMMA with AgNPs showed the greatest levels of adhesion to* S. mutans *and* C. albicans*, respectively, similar to those of self-curing PMMA without AgNPs. The groups of heat-curing PMMA were more resistant to bacterial adhesion in spite of the modification with AgNPs.*

Introduction

Microorganisms in the oral cavity have the ability to colonize restorative materials. The accumulation of microorganisms (Aguayo *et al*., 2017) on the soft tissues and teeth causes several diseases, including caries, one of the most common oral diseases. Maintenance of a decent life includes management of oral diseases and good oral rehabilitation (Suo *et al*., 2018). Stomatitis is a frequent oral lesion that mainly affects women and immune compromized patients, as well as children and elderly adults (Namangkalakul *et al*., 2109). A number of dental appliances are regularly manufactured with polymethylmethacrylate (PMMA), which was introduced in 1937 and is an economical alternative to polycarbonate. Notably, PMMA remains the material of choice for denture bases because of its favorable characteristics, processing ease, accurate fit, stability in the oral environment, and superior aesthetics that can be achieved with inexpensive equipment. In spite of these excellent properties, improvement in its biological characteristics (Ladizesky *et al*., 1993) is needed. Microorganisms form a solid surface over the prosthesis in contact with oral tissue and may manifest as cracks and irregularities that occur in some stages of prosthesis preparation. Notably, these defects can act as reservoirs that contribute to adhesion and proliferation of microorganisms, which primarily include *Candida albicans* and *Streptococcus mutans*. Due to the absence of a laboratory polishing procedure, the impression surface or tissue side of the denture base is rough, compared with the outer polished surface; this provides a surface that promotes plaque formation (Ladizesky *et al*., 1993). Hence, prevention of biofilm growth on these materials is important (Stenhagen *et al*., 2019).

S. mutans is one of the most important microorganisms present in oral biofilms. Cariogenic biofilms attached to restorative materials promote the development of caries that reduce the durability of restorations and devices. In recent years, metal oxide nanoparticles have been widely used as additives, on the basis of their antimicrobial activity (Mousavi *et al*., 2018). A comparative study evaluated the antibacterial effects of chlorhexidine, silver titanium dioxide, silicon dioxide, and silver nanoparticles (AgNPs); notably, $AgNO_3$ demonstrated the most efficient bactericidal effect, followed by that of AgNPs, when used as treatment for *S. mutans* (Padovani *et al*., 2015). Moreover, the use of AgNPs in the treatment of cancer is especially attributed to their antitumor properties associated with their oxidative stress induction, resulting in deoxyribonucleic acid (DNA) damage and apoptosis (Noronha *et al*., 2017).

C. albicans is another representative microorganism frequently found in patients with oral prostheses. Prior analyses have shown that the addition of AgNPs to PMMA even at low concentrations, due to their ability to selectively destroy cellular membranes, has a robust effect, specifically against

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ADHESIÓN DE *Candida albicans* Y *Streptococcus mutans* A NANOPARTÍCULAS DE PLATA-POLIMETILMETACRILATO MODIFICADO

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RESUMEN

Este estudio in vitro evaluó la adhesión bacteriana de C. albicans *(ATCC18804) y* S. mutans *(ATCC25175) de manera independiente en bloques de polimetilmetacrilato (PMMA) modificados con nanopartículas de plata (AgNPs). Se usó un total de 160 muestras clasificadas en ocho grupos.* C. albicans *y* S. mutans *fueron cultivados independientemente. Los bloques fueron sumergidos en una solución conteniendo microorganismos radiomarcados con tritio (^{3}H). Para el análisis cuantitativo el marcador radiactivo ^{3}H fue usado para codificar las colonias bacterianas adheridas a la superficie de los materiales proporcionalmente a la colonización a 37°C. En el análisis estadístico se utilizó análisis de varianza (Sheffè* post-hoc*). Se encontraron diferencias estadísticamente significativas en la adhesión bacteriana entre los grupos de PMMA modificados con AgNPs. Los grupos de PMMA termocurables demostraron baja adherencia a* C. albicans *comparados con los grupos de PMMA autocurables Los grupos de PMMA termocurable resultaron resistentes a la adhesión bacteriana sin importar su modificación con AgNPs.*

ADESÃO DE CANDIDA ALBICANS E STREPTOCOCCUS MUTANS AO POLIMETILMETACRILATO MODIFICADO POR NANOPARTÍCULAS DE PRATA

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RESUMO

Este estudo in vitro *avaliou a adesão bacteriana de* C. Albicans *(ATCC18804) e* S. Mutans *(ATCC25175) de maneira independente em blocos de polimetilmetacrilato (PMMA) modificados com nanopartículas de prata (AgNPs). Foi utilizado um total de 160 amostras classificadas em oito grupos.* C. Albicans *y* S. mutans *foram cultivados independentemente. Os blocos foram submergidos em uma solução contendo microrganismos radiomarcados com trítio (^{3}H). Para a análise quantitativa foi utilizado o marcador radiativo ^{3}H para codificar as colônias bacterianas aderidas na superfície dos materiais proporcionalmente à colonização a 37°C. No estudo estatístico foi utilizada análise de variância (Sheffè* post-hoc*). Encontraram-se diferenças estatisticamente significativas na adesão bacteriana entre os grupos de PMMA modificados com AgNPs. Os grupos de PMMA termocuráveis demostraram baixa aderência a* C. albicans *comparados com os grupos de PMMA autocuráveis. Os grupos de PMMA termocurável resultaram resistentes à adesão bacteriana sem importar sua modificação com AgNPs.*

C. albicans. These results were promising for later clinical use of dental devices comprising PMMA with AgNPs, which aimed at reducing the occurrence of stomatitis (De Matteis *et al*., 2019).

There is also an increasing role for AgNPs in controlling fungal biofilm infestation. The activity of AgNPs against bacterial biofilm has been reported in a study that determined the effect of myogenic AgNPs on *C. albicans* biofilm, using different AgNP concentrations (Hamid *et al*., 2018). The most important parameters to compare the antimicrobial efficacy of a compound are the minimum inhibitory concentration (MIC) and/or minimal bactericidal concentration (MBC) values. However, most of the studies reported the antimicrobial effect of silver-based nanomaterials in terms of zone of inhibition, which is a qualitative measure and, in many cases, the concentration of silver-based nanomaterial is not reported (Durán, 2016). Therefore, preventive modifications of dental materials are needed, such as modifications of PMMA reported in previous studies, in order to increase the resistance to microbial adhesion (Durner *et al*., 2011; Kasraei *et al*., 2014; Neves *et al*., 2014). Several studies have revealed innovative approaches to modify dental materials and oral tissues in order to reduce microbial activity (Besinis *et al*., 2015). In this study we determine the levels of *C. albicans* and *S. mutans* adhered to PMMA, as both microorganisms are regarded as primary causes of dental caries, stomatitis, and other injuries related to bacterial colonization of restorative materials (Aguayo *et al*., 2017).

Materials and Methods

PMMA sample preparation

The AgNPs used in the study had an average size of 15-20nm, diluted in double distilled water solution. A total of 240 blocks, 4×4×1mm in size, were made using a Teflon mold that comprised four containers filled with PMMA. The experimental groups comprised PMMA with AgNPs (1.25ml AgNPs solution per 12.5g PMMA), while the control groups included PMMA without AgNPs. The AgNPs solution was added to the polymer, mixed in four groups of PMMA, and filled a Teflon mold that was covered with a microslide glass. After filling the molds, the excess polymer was cut and the blocks were polished with 1,500- and 2,000-grit sandpaper sheets, cleaned ultrasonically, and sterilized with ethylene oxide gas.

The test samples comprised eight groups. The first four experimental groups included AgNPs and four control groups were without AgNPs, as follows; GI: Arias® self-curing PMMA with AgNPs, GII: Nic Tone® heat-curing PMMA with AgNPs, GIII: Nic Tone® self-curing PMMA with AgNPs, GIV: Arias® heat-curing PMMA with AgNPs, GV: Arias® self-curing PMMA, GVI: Nic Tone® heat-curing PMMA, GVII: Nic tone®

self-curing PMMA, and GVIII: Arias® heat-curing PMMA.

Energy dispersion scanning

The EDS (Oxford Abingdon, UK) attached to SEM microscope (JEOL, JSM-6510LV at 20kV, Japan) was performed to carry out analysis of characterization, recording means of energy dispersion, with a resolution of 137eV.

Radio labeled bacteria and culture conditions

S. mutans ATCC 25175 and *C. albicans* ATCC 18804 were maintained as frozen stock cultures and were cultured anaerobically at 37°C in a semisolid trypticase soy broth (TSBY; BBL, Cockeysville, MD, USA) with yeast extract (Difco Laboratories, Detroit, MI, USA) for 18h. Then, the microorganisms were anaerobically inoculated, using the TSBY semisolid cultures, in 150ml of TSBY liquid with a radioactive marker 74kBq of [6-^{3}H] thymidine in order to identify the microorganisms; they were cultured for 18h at 37°C. Next, the bacteria were collected by centrifugation at 12,000rpm for 15min into 0.05M phosphate-buffered saline (PBS, pH 7.0) and were washed three times with PBS. The concentrations of *S. mutans* and *C. albicans* were adjusted to 10^5 colony-forming units/ml.

Sample analysis

The PMMA specimens were suspended from the cap of a glass mold and submerged in 150ml of a solution containing radiolabeled *S. mutans* (120 samples, prepared as above) or radiolabeled *C. albicans* (120 samples, prepared as above) in two rounds. In the first round, specimens were submerged in a solution containing radiolabeled *S. mutans* (60 samples) or radiolabeled *C. albicans* (60 samples) at 37°C for 2h with constant movement. One week later, the process was repeated with an additional 60 samples per microbe (60 specimens each were submerged in a solution containing radiolabeled *S. mutans* or radiolabeled *C. albicans*) for a second round. To remove non-adherent bacteria, PMMA samples were removed from the glass mold and were washed three times with PBS.

The radiolabeled bacteria that adhered to PMMA specimens were harvested using automatic sample combustion equipment, and the score was measured using a liquid scintillation counter (LSC-900, Aloka, Japan), with values recorded in disintegrations per min (dpm). To ensure the reliability of the results, this measurement was repeated three times.

Statistical Analysis

Parametric tests with descriptive mean and variance statistics for quantitative variables were used. Analysis of variance (ANOVA) was performed with the Sheffè *post hoc* test for multiple comparisons of similarity of distribution; differences with $p \leq 0.05$ were considered to be significant.

Results

Adhesion of S. mutans

The adhesion of radiolabeled *S. mutans* to PMMA samples significantly differed among the groups ($p \leq 0.05$). The scores in dpm are shown in Table I. The value obtained for GI was significantly lower than that obtained for all the other groups; it was followed by the value for GV. Moreover, the values obtained for GI, GII, GV, GVI, GVII, and GVIII were not significantly different. In contrast, the value for GIV was significantly higher than that obtained for all the other groups.

Adhesion of C. albicans

The adhesion of radiolabeled *C. albicans* to PMMA samples significantly differed among the groups ($p \leq 0.05$). The scores in dpm are shown in Table II. The value obtained for GVI was significantly lower than that obtained for all the other groups; it was followed by the value for GIII. The values obtained for GI, GIV, and GVII were found not significantly different. Furthermore, the value obtained for GV was significantly higher than that obtained for all the other groups.

TABLE I
QUANTITATIVE TEST OF *Streptococcus mutans* BY RADIOLABELED ^{3}H

PMMA	DPM (mean)	SD	*ANOVA
GI	1648,1427	599,01333	A
GII	1863,5980	880,19597	A
GIII	1905,9693	612,21821	B
GIV	1914,7657	832,85071	B
GV	1681,4263	402,02124	A
GVI	1689,8990	405,50411	A
GVII	1702,5863	333,50053	A
GVIII	1721,0007	386,65655	A

PMMA: polymethylmethacrylate, DPM: disintegrations per minute, SD: standard deviation.

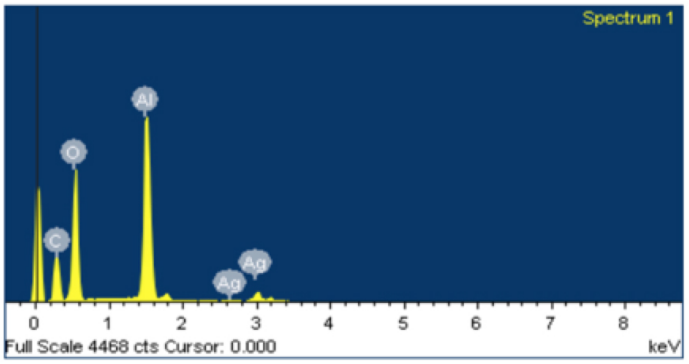
*ANOVA analysis of variance with different letters are significantly different from each other.

EDS

The characterization technique was consistent with the elemental chemical mapping analysis of the surfaces of PMMA samples, showing absortion peaks of silver (Ag) between 1.86, 2.66 and 2.83 weight % (Figures 1, 2 and 3).

Discussion

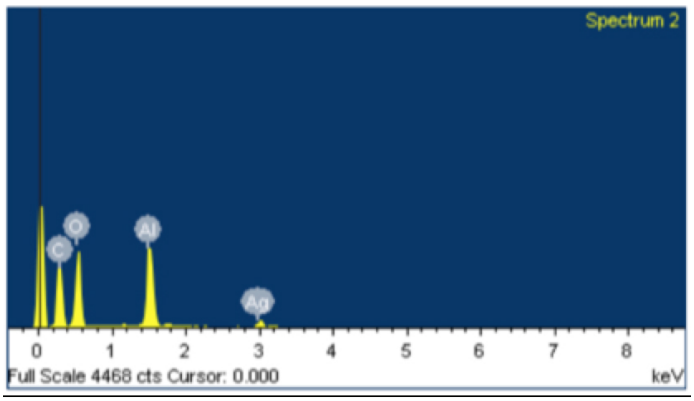
In the present study, the samples were not coated with saliva. This did not significantly alter the adhesion pattern of *S. mutans*, a phenomenon that is consistent with the findings of other studies, which indicated that the presence or absence of saliva coating did not significantly alter the adhesion of streptococcito underlying materials (De Matteis *et al*, 2019).



Element	Weight %	Atomic %
C K	28.33	37.62
O K	52.23	52.07
Al K	16.78	9.92
Ag L	2.66	0.39

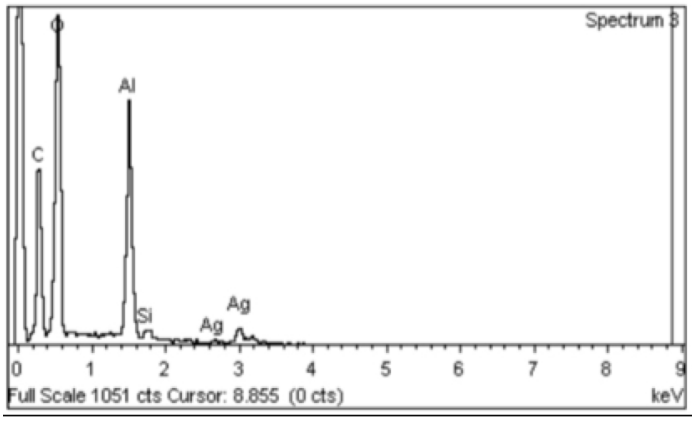
Figure 1. Representative images obtained by EDS elemental analysis of PMMA with presence of silver.

Higher dpm values indicated higher radioactivity, which was consistent with increased adhesion of radiolabeled microorganisms. In contrast, lower values of dpm indicated reduced adhesion of radiolabeled microorganisms (Velazquez-Enriquez *et al*, 2012; Iavicoli *et al*, 2016). The results (Tables I and II) showed that the GI and GVI permitted the lowest adhesion. Analysis of *S. mutans* adhesion (Table I) revealed that GIII and GIV permitted significantly the highest levels of adhesion, in contrast, GIV and GV permitted the lowest levels of adhesion. Analysis of *C. albicans* adhesion (Table II) showed that GVI permitted significantly lower levels of adhesion, relative to those of other groups (Fragkou *et al*, 2016).



Element	Weight %	Atomic %
C K	39.00	48.82
O K	48.03	45.14
Al K	10.14	5.65
Ag L	2.83	0.39

Figure 2. SEM-EDS elemental analysis of characterization of PMMA surface.



C K	34.44	43.22
O K	54.63	51.46
Al K	8.67	4.84
Si K	0.41	0.22
Ag L	1.86	0.26

Figure 3. EDS of the AgNPs was confirmed in the presence of elemental silver signal.

In this study, variations in the amount of microbial adhesion may have resulted from the diverse surface characteristics of each type of PMMA. Surface characteristics of materials are known to influence their permissiveness for microbial adhesion, based on surface roughness; in the present study, GI (PMMA with AgNPs) and GV (PMMA without AgNPs) were not significantly different in the permissiveness of *S. mutans* adhesion. This may be due to the addition of the AgNP solution, which increased surface porosity and served as a reservoir for microorganisms. Therefore, although all surfaces were polished, PMMA resin samples showed surface irregularities, which were associated with variable microbial adhesion (Birnbaum and Gutknetch, 2010; Acosta *et al*., 2019). The PMMA heat-curing groups permitted lower *C. albicans* adhesion, because the material was less modified by the incorporation of AgNPs; thus, the sample surface was likely to be more polished and contain no pores.

There is minimal evidence regarding the ideal concentration of AgNPs to be added to polymers or monomers of methacrylate to achieve optimal antibacterial properties (Fragal *et al*., 2016; Shen *et al*., 2017). For several types of cells, AgNPs are reportedly cytotoxic *in vitro* (Giannunzio and Speerli, 2008); however, the toxic mechanism involving AgNP exposure is not fully elucidated. This cytotoxic effect is similar to bacterial death, in that it changes the permeability of the cell membrane by blocking ion channels, thereby causing mitochondrial dysfunction and producing oxidative stress (Iavicoli *et al*, 2016; Kadiyala *et al*, 2016; Metin-Gürsoy and Taner, 2017). Other studies have suggested that the antibacterial effectiveness of AgNPs is dependent on the release of silver ions, as well as direct contact with the bacteria; materials with increased concentrations of nanosilver release additional nanosilver ions and show a greater inhibitory effect (Saku *et al*., 2010). *In vitro* studies have shown the antimicrobial effectiveness of nanosilver-coated materials, (Jasso-Ruiz, 2019) with and without the release of nanosilver ions (Metin-Gürsoy and Taner, 2017). In the present study, the nanosilver was present both inside the material and as a coating on the surface; the concentration of AgNP solution added to polymer was 20ml/100g. This polymer was then added to the monomer to begin the polymerization reaction; hence, further studies are necessary to determine whether the addition of AgNPs to the

TABLE II
QUANTITATIVE TEST OF *Candida albicans* BY RADIOLABELED ^{3}H

*PMMA	DPM (mean)	SD	*ANOVA
GI	1380.1960	760.00977	A
GII	1040.0193	389.24180	B
GIII	1033.3233	664.27690	B
GIV	1258.8800	596.16628	A
GV	1706.1760	1031.81856	C
GVI	822.8663	435.66791	D
GVII	1327.7823	770.40476	A
GVIII	1077.6263	418.22628	B

PMMA: polymethylmethacrylate, DPM: disintegrations per minute, SD: standard deviation, ANOVA: analysis of variance.

*PMMA with different letters are significantly different from each other.

monomer would yield enhanced antibacterial properties, as well as to determine the ideal concentration of AgNPs. In the present study, two types of PMMA were used: heat-curing and self-curing. The addition of AgNPs modified the characteristics of the material, creating a porous material that fostered microbial adhesion; therefore, the self-curing PMMA (Groups I, III, V, and VII) showed greater adhesion to *C. albicans*, regardless of its AgNP content. *S. mutans* adhesion did not show the same pattern. Therefore, we suggest conducting further studies wherein the AgNP solution is added as a coating on PMMA blocks to avoid porosity, changes in material structure and other factors caused by the addition of the AgNP solution in the polymer (Zhang *et al*., 2012). This study showed that heat-curing PMMA exhibited lower adhesion to *C. albicans*, relative to that of self-curing PMMA. Moreover, PMMA with AgNPs, tested in groups IV and V (without AgNPs), showed the greatest levels of adhesion to *S. mutans* and *C. albicans*, respectively. PMMA without modifications, tested in groups VIII (heat-curing) and V (self-curing), showed the greatest levels of adhesion to *S. mutans*. PMMA with AgNPs, tested in groups I and III, showed the lowest levels of adhesion to *S. mutans* and *C. albicans*, respectively. Finally, PMMA without modifications, tested in groups V (self-curing) and VI (heat-curing), showed the lowest levels of adhesion to *S. mutans* and *C. albicans*, respectively.

These findings will aid in identifying suitable types of PMMA for making dental devices and prostheses with reduced risks for development of caries and prosthetic stomatitis.

Conclusions

The findings of this research provide information about the suitable type of PMMA to be used in dentistry, the heat cured PMMA modified with AgNPs presented the best antimicrobial properties that have a minor risk for adhesion. Furthermore, the aggregation of AgNPs within PMMA proved to have potential to reduce the microbial activity; thus, it could reduce the risk of oral stomatitis. For that reason, this material could be used to elaborate several prosthetic devices; nevertheless, it is necessary to carry out further research about another properties of this modified material in relation to color, hardness and stability before being tested in the buccal environment.

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