

Addition of Silver Nanoparticles to Composite Resin: Effect on Physical and Bactericidal Properties *In Vitro*

Patrícia Bolzan Agnelli das Neves¹, José Augusto Marcondes Agnelli², Cristina Kurachi³, Clovis Wesley Oliveira de Souza¹

The objectives of this study were to evaluate physical properties and antibacterial activity of a light-activated composite modified with silver nanoparticles. Discs were produced with unmodified resin (control group - CG) and modified resin with silver nanoparticles at two concentrations, 0.3% wt (MR03) and 0.6% wt (MR06). *Streptococcus mutans* and *Lactobacillus acidophilus* biofilms were induced *in vitro* by incubation of discs in a 20% sucrose medium, followed by sonication and counting of viable cells after 1, 4 and 7 days (n=9). The arithmetic roughness of all three groups was evaluated by atomic force microscopy (n=9). Compression assay was conducted in all groups to measure the compressive strength at failure and elasticity modulus (n=5). Data were subjected to ANOVA and Tukey's tests ($\alpha=0.05\%$). At all three time points the number of viable cells was statistically lower for MR03 and MR06 compared with CG, for both specimens. MR03 and MR06 showed no significant differences. Microscopic analysis demonstrated no significant differences for roughness among the three groups ($p>0.05$). The MR03 was stronger to compression than CG, and MR06 was statistically lower than CG and MR03. It was concluded that the MR03 were less conducive to biofilm growth, without compromising the strength in compression and surface roughness.

Introduction

The accumulation of microorganisms on the teeth and soft tissues causes various diseases. Whereas microorganisms can also colonize restorative materials, the prevention of biofilm growth on these materials is also very important (1,2). The cariogenic biofilm attached to the restorative materials or at the tooth-restoration interface can cause caries, reducing the durability of the restoration (3-5). Thus, several studies have attempted to reduce microbial adhesion to restorative materials, and the results are promising. These studies showed the development of resin-based restorative materials with antibacterial effect reached by addition of some reagents as antibacterial monomer, chlorhexidine or titanium dioxide nanoparticles (3-5).

Silver is a metal known for its broad spectrum of activity against bacteria, fungi and certain viruses and in the form of nanoparticles its properties are considerably enhanced (6-8). Silver nanoparticles have affinity with molecular groups that contain sulfur and phosphorus, which are found in bacterial membranes and within bacterial cells. These nanoparticles release silver ions, damaging transmembrane electron transportation and preventing DNA replication (8-10). On the other hand, several studies have demonstrated that silver nanoparticles are non-toxic to both animals and humans cells (11-13). Indeed, nowadays silver nanoparticles are being applied as antimicrobials incorporated in biomaterials and food packaging, and in

coatings for water filters, washing machines, refrigerator and air purifiers, in order to reduce surface microbial biofilm formation (8-10).

However, few studies have investigated dental resins modified with silver nanoparticles and their bactericidal action. One study demonstrated bactericidal activity against *Streptococcus mutans* of a light-activated composite resin modified with silver nanoparticles (14). Others studies reported the antibacterial action of silver-modified adhesives without damaging bond strength (15,16).

The aim of this research was to evaluate the reduction in *S. mutans* and *Lactobacillus acidophilus* biofilm, the surface roughness and the compressive strength on light-activated composite resin when modified with bactericidal silver nanoparticles. The hypothesis was that the antibacterial activity of the silver nanoparticles would be maintained on the surface of the composite after adding them as a new component.

Material and Methods

Sample Preparation

The light-activated composite resin used in this study was Herculite Classic[®] (SDS Kerr, Orange, CA, USA). The silver additive was Nanox Clean[®] (Nanox Technology S.A., São Carlos, SP, Brazil), composed of silver adsorbed on amorphous SiO₂. Silica particle mean diameter is 12 μm , and silver particle mean diameter is 50 nm. Cylindrical specimens

¹Department of Morphology and Pathology, UFSCar - Federal University of São Carlos, São Carlos, SP, Brazil
²Department of Materials Engineering, UFSCar - Federal University of São Carlos, São Carlos, SP, Brazil
³Department of Physics and Materials Science, Physics Institute of São Carlos, USP - University of São Paulo, São Carlos, SP, Brazil

Correspondence: Dra. Patrícia Bolzan Agnelli das Neves, Rodovia Washington Luis, 235, 13565-905, CP: 676, São Carlos, SP, Brasil.
Tel: +55-16-3351-8764. E-mail: patricia_bolzan@yahoo.com.br

Key Words: silver, nanoparticles, composite, bactericidal.

(2 mm high and 4 mm diameter) were produced with unmodified resin (control group - CG) and modified resin with silver nanoparticles at two different concentrations, 0.3% wt (MR03) and 0.6% wt (MR06). Metal matrix (base 2 cm diameter, central hole 4 mm diameter, height 2 mm) was used to accommodate the material and standardize the size of all specimens used in the biofilm experiment and surface roughness analysis (171 resin discs in total).

During preparation of discs with unmodified resin (CG), the resin was placed in increments within the matrix. After filling, two glass plates were placed below and above the matrix to result in flat circular faces. Then, the resin was cured by light exposure for 40 s with Optilight LD III (15 VA power, 50 Hz frequency; Gnatus, Ribeirão Preto, SP, Brazil) and removed for a new matrix filling, repeatedly until achieving the necessary number of discs.

To incorporate nanoparticles within the composite resin, the required amount of nanoparticles was weighed for every 1 g of resin in order to provide the desired concentration of modified resin. A previous merger between the two materials was made with a metal spatula on a glass plate. After, a manual mixing was performed for 1 min. The modified resin was protected from light inside a small darkened box to prevent premature polymerization until placement within of matrix. The resin was taken in increments from the darkened box and placed within the matrix. Glass plates were placed below and above the matrix. Then the resin was cured and removed for a new matrix filling. The procedures were repeated until obtaining the desired number of discs. Finally, all resin discs were polished with Enhance silicone tips (Dentsply, Adlestone, Surrey, UK).

Biofilm Formation

S. mutans strain ATCC 25175 was cultivated in 10 mL of the culture medium MRS broth (Oxoid, Basingstoke, HAM, UK), modified with 20% sucrose. The culture was incubated at 37 °C for 24 h. After incubation a bacterial suspension was prepared, with standardized absorbance. The culture was centrifuged at 3000 rpm for 20 min (Excelsa II centrifuge, 206-BL model; FANEM, São Paulo, SP, Brazil). Next the supernatant was discarded and the precipitated biomass was resuspended in phosphate buffered saline (PBS) until it reached an absorbance corresponding to McFarland scale 0.5 (number of cells in the order of 10⁸ colony-forming units -CFU/mL).

For *L. acidophilus* strain ATCC 4356 the same procedures were performed, but

the culture was incubated for 48 h within the anaerobic jar containing a microaerophilic atmosphere generator (Microaerobac, Probac, São Paulo, SP, Brazil) at 37 °C, and after a bacterial suspension was likewise prepared. Microaerobac generates an atmosphere with 10% CO₂ and 5% O₂. The ampoules with strais were acquired from Oswaldo Cruz Foundation (FIOCRUZ, Rio de Janeiro, RJ, Brasil).

The resin discs were placed in a 24-well polystyrene microtiter plate (Nunclon™, Nunc, Rochester, NY, USA). The wells were filled with 1 mL of 20% sucrose MRS and 200 µl of standardized bacterial suspension in PBS. Biofilms were grown on nine discs for each time point from all three groups, for both species. To induce biofilm formation the plates were incubated at 37°C, and the time points chosen for sonication of biofilms were 1, 4 and 7 d. Only microtiter plates with *L. acidophilus* were incubated within jar with 10% CO₂. In order to avoid nutrient depletion, the medium in all wells was changed after the first 48 h to sterilized culture medium, and then changed successively every 48 h.

Biofilm Quantification

After each incubation period, the discs of all groups were washed three times with sterilized PBS to remove loosely adherent cells, then placed in tubes with 10 mL of PBS. These tubes were shaken for 1 min and placed in an ultrasonic tank for 8 min (Digital Ultrasonic Cleaner, with cleaning power of 160 W; Kondortech, São Carlos, SP, Brazil). This procedure causes the biofilm disaggregation and releases the bacterial cells adherent to the discs. The viable cells in the resulting suspension were quantified

Table 1. Viable *Streptococcus mutans* and *Lactobacillus acidophilus* cells recovered from the biofilm adhered to the surfaces of specimens after 3 incubation periods, expressed as colony forming units per mL of culture medium (CFU/mL)

	1 day	4 days	7 days
<i>S. mutans</i>			
Control group	2.2×10 ⁶ ± 0.9×10 ⁶ Ca	1.7×10 ⁷ ± 0.7×10 ⁷ Ba	4.6×10 ⁷ ± 1.4×10 ⁷ Aa
MR03	1.3×10 ⁶ ± 0.5×10 ⁶ Cb	4.3×10 ⁶ ± 0.9×10 ⁶ Ab	2.8×10 ⁶ ± 1.3×10 ⁶ Bb
MR06	1.1×10 ⁶ ± 0.3×10 ⁶ Cb	4.5×10 ⁶ ± 0.9×10 ⁶ Ab	2.3×10 ⁶ ± 1.1×10 ⁶ Bb
<i>L. acidophilus</i>			
Control group	1.6×10 ⁵ ± 0.5×10 ⁵ Ca	5.0×10 ⁶ ± 0.8×10 ⁶ Ba	7.4×10 ⁶ ± 0.8×10 ⁶ Aa
MR03	7.8×10 ⁴ ± 0.9×10 ⁴ Cb	6.3×10 ⁵ ± 0.8×10 ⁵ Ab	3.1×10 ⁵ ± 0.9×10 ⁵ Bb
MR06	7.9×10 ⁴ ± 0.8×10 ⁴ Cb	6.4×10 ⁵ ± 0.9×10 ⁵ Ab	2.8×10 ⁵ ± 0.6×10 ⁵ Bb

Control Group, MR03 and MR06 represent unmodified resin, and modified resins with 0.3% and 0.6% of silver nanoparticles by weight, respectively. The values are presented as means and standard deviations (n=9). Different uppercase letters in rows and lowercase letters in columns indicate statistically significant difference at 5% (ANOVA and Tukey's test).

after disaggregation, by counting of colony-forming units (CFU). Five dilutions were made from this initial suspension.

In the first, 100 μL from the initial suspension was inoculated into 900 μL of PBS (10^{-1} dilution), with the final dilution being 10^{-5} . From each dilution, 100 μL was pipetted on to MRS agar on a Petri dish (90 x 15 mL), and this inoculum was spread over the surface with a Drigalsky handle. The Petri dishes with *S. mutans* were incubated directly in the oven at 37°C for 48 h and dishes with *L. acidophilus* were incubated under microaerophilic atmosphere (10% CO_2) at 37°C for 48 h. After incubation, dishes with 30-300 colonies were selected for counting. This number was multiplied by the dilution factor and then by 10 in order to obtain the number of CFU present in 1 mL of the initial suspension. Tables were structured with the CFU number per mL. Statistical analysis was performed by two-way analysis of variance and Tukey's test pairwise comparison ($\alpha=0.05\%$).

Surface Roughness Analysis

Atomic force microscopy analysis (AFM) was performed to measure surface roughness (R_a , arithmetic roughness) at three sites (10 μm^2) of three discs from each group and the values were statistically analyzed by ANOVA one-way and Tukey's test ($\alpha=0.05\%$). Surfaces were analyzed using a Nanoscope V Multimode microscope (Bruker, Billerica, MA, USA). The resin discs previously formed did not need to be cut to fit this microscope. To operate the microscope, the method chosen was tapping mode, in air. NanoScope analysis[®] (Version 1.40, Bruker Corporation, Billerica, MA, USA) is a specific software for the analysis of data provided by Bruker for scanning probe microscopes.

Compression Assay

Compression assay was carried out in order to check whether there was damage to the mechanical strength of the modified resin. Five cylindrical specimens with circular base 1 cm diameter and height 1 cm were produced for each experimental group. The specimens had to be larger for a

good fit in the testing machine. One polytetrafluoroethylene matrix was machined with cylindrical perforations of desired dimensions. The composite resin was condensed in small increments in the spaces of the matrix and then polymerized. The compression tests were performed using an Instron 5569 universal testing machine (Instron Corp., Norwood, MA, USA). The procedure adopted was based on ASTM D 695-10 guideline (17), at compression speed 1.3 mm/min and load cell 10 KN. Two parameters were measured for all samples: compressive strength at failure and elasticity modulus in compression. Statistical analysis was performed by one-way ANOVA and Tukey's test ($\alpha=0.05\%$).

Results

Viable Cell Count

Table 1 shows the viable cells of *S. mutans* and *L. acidophilus* recovered from the biofilms adherent to the surfaces of specimens after the three incubation periods, expressed as CFU/mL. The results showed that for the three incubation periods, CFU numbers from biofilms were significantly lower in MR03 and MR06 than in CG, for both studied species ($p<0.05$). MR03 and MR06 showed no statistically significant differences ($p>0.05$). Within each material group, CFU numbers were significantly different for the three incubation periods, for both species ($p<0.05$).

Surface Roughness Analysis

Table 2 shows the values for surface roughness obtained by NanoScope software expressed as arithmetic roughness (R_a). The result demonstrates that there was no difference among the three material groups according to statistical analysis ($p>0.05$).

Compression Assay

The values of compressive strain at failure and elasticity modulus in compression are shown in Table 3 for the three groups. The table demonstrates that compressive strength

Table 2. Values of surface roughness (R_a)

Material group	Roughness (nm)
Control group	12.5 \pm 2.1 ^a
MR03	12.1 \pm 2.1 ^a
MR06	11.8 \pm 2.6 ^a

The values are presented as means and standard deviations (n=9). Same letters indicate no statistically significant difference at 5% (ANOVA and Tukey's test). Control Group, MR03 and MR06 represent unmodified resin, and modified resins with 0.3% and 0.6% of silver nanoparticles by weight, respectively.

Table 3. Values of compression assay

Material group	Strength at failure (MPa)	Elasticity modulus (MPa)
Control group	85.4 \pm 5.3 ^b	1466.0 \pm 72.1 ^b
MR03	96.0 \pm 4.9 ^a	1725.0 \pm 54.0 ^a
MR06	72.8 \pm 4.2 ^c	1205.0 \pm 84.9 ^c

Control Group, MR03 and MR06 represent unmodified resin, and modified resins with 0.3% and 0.6% of silver nanoparticles by weight, respectively. The table presents the means and standard deviations of two parameters: compressive strength at failure and modulus of elasticity in compression (n=5). The assay was based on ASTM D 695-10 standard. Different letters indicate statistically significant difference at 5% (ANOVA and Tukey's test).

at failure and elasticity modulus in compression were statistically different among the three groups ($p < 0.05$).

Discussion

The biofilm reduction *in vitro* verified on the modified resins is an important result because a composite resin with these properties will enable lower bacterial accumulation on the restored areas of the teeth, helping to prevent recurrent caries. After the 3 incubation periods, CFU counts from biofilms were similar in MR03 and MR06, and lower in these groups than in CG, for both species studied. This result supports the hypothesis that the antibacterial action of silver nanoparticles, previously demonstrated in the literature (8-10), remains occurring after mixing with the composite resin and affects the microorganisms that are in contact with the modified material, similarly to others *in vitro* investigations (14-16).

In addition, the roughness results obtained by AFM analysis demonstrate that there was no significant difference among the three material groups. Therefore, the surface roughness of composite resin was not increased by the presence of silver nanoparticles, in both concentrations of modified resin tested. These findings are important because a change in the roughness of modified resin could increase the biofilm accumulation. In accordance with this, several studies have shown that a rougher surface favors the biofilm growth on restorative materials (18-20). In contrast, an experimental composite adhesive containing silver nanoparticles that also presented bactericidal property showed rougher surface than conventional adhesive (15).

The compression assay showed that MR03 achieved the best mechanical performance for two measured parameters, and MR06 the worst, with a statistical difference. MR03's better performance in the compression assay compared with CG suggests that the 0.3% silver nanoparticle concentration should be within the ideal range of modification for optimization of the composite mechanical properties on compression. About the change of filler volume-fractions in composite resins, Masouras et al. (21) found a correlation of inorganic filler content and elastic properties of fifteen trademarks of composite resins, considering three parameters. Elasticity modulus varied with statistical significance and a positive correlation occurred between modulus and inorganic filler fraction in the materials evaluated.

On the other hand, the results of the compressive assay for MR06 indicate that the concentration of nanoparticles was higher than that appropriate for maintaining the efficiency of resin reinforcement. The main limitation of the compression assay is that most assays about composite resin resistance have not used the same norm. This fact

impairs the comparison of values obtained here with the values already reported about the minimum acceptable resistance values. Nevertheless, the comparison of the experimental groups with the control group was carried out, and no reduction in resistance to compression is desired.

In terms of bacterial effect, the modified resins used in MR03 and MR06 would be advantageous. Thus, MR03 is more appropriate for clinical application because it requires fewer nanoparticles and has lower cost. Furthermore, the results of the compression assay suggest that MR06 is more prone to suffer impairment of physical properties than MR03. This research shows promising outcomes, but further investigation are necessary in order to determine whether a new dental restorative material can be used in the oral cavity. Future studies will be performed to quantify the release of silver to saliva. They will be references for comparison with studies that described a non-toxic amount of silver nanoparticles to human cells (11-13). Additional studies will also be conducted to assess the cytotoxicity of modified resin through contact with living tissue in comparison with unmodified resin, and it will be essential to investigate other physical properties and color change.

In conclusion, the experimental composite resins with 0.3% and 0.6% wt of silver nanoparticles were less conducive to biofilm growth on their surfaces than unmodified resin and suffered no increase of surface roughness. Moreover, the modified resins with 0.3% wt of silver nanoparticles achieved improvement of compression resistance. Continuation of this study is very important because a restorative composite with these properties may benefit patients by helping maintaining good oral hygiene along with regular toothbrushing and by assisting in the prevention of caries recurrency.

Resumo

Os objetivos do estudo foram avaliar as propriedades físicas e a atividade antibacteriana de uma resina composta fotopolimerizável modificada com nanopartículas de prata. Discos foram produzidos com resina não modificada (grupo controle - GC) e resina modificada com nanopartículas de prata em duas concentrações 0,3 e 0,6% em massa (MR03 e MR06, respectivamente). Biofilmes de *Streptococcus mutans* e *Lactobacillus acidophilus* foram induzidos *in vitro* pela incubação dos discos em meio líquido com 20% de sacarose, seguida pela sonicação e contagem de células viáveis após 1, 4 e 7 dias. A rugosidade aritmética de todos os grupos foi avaliada por microscopia de força atômica (MFA). Os dados foram submetidos à ANOVA e ao Teste de Tukey ($\alpha = 0,05\%$). Nos três períodos de tempo, o número de células viáveis foi estatisticamente mais baixo para MR03 e MR06, em comparação com GC, para ambas as espécies. MR03 e MR06 não apresentaram diferenças significantes. A análise por meio de MFA demonstrou que não houve diferença significativa para a rugosidade entre os três grupos. MR03 apresentou-se mais resistente em compressão do que GC, e o desempenho em compressão em MR06 foi estatisticamente pior do que em GC e MR03. Foi possível concluir que as resinas modificadas de MR03 inibiram o

crescimento dos biofilmes, sem comprometimento da resistência à compressão e da rugosidade superficial.

Acknowledgements

This research was financially supported by the Brazilian Government research funding agency CAPES.

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Received February 6, 2014

Accepted March 21, 2014