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# Effect of Diamond-Like Carbon Thin Film Coated Acrylic Resin on *Candida albicans* Biofilm Formation

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**Keywords**
AFM; complete denture; denture base; DLC; magnetron sputtering; nanoparticles; plasma; profilometry; RBS.

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## Abstract

**Purpose:** The purpose of this study was to evaluate the effect of diamond-like carbon thin films doped and undoped with silver nanoparticles coating poly(methyl methacrylate) (PMMA) on *Candida albicans* biofilm formation. The control of biofilm formation is important to prevent oral diseases in denture users.

**Materials and Methods:** Forty-five PMMA disks were obtained, finished, cleaned in an ultrasonic bath, and divided into three groups: Gc, no surface coating (control group); Gdlc, coated with diamond-like carbon film; and Gag, coated with diamond-like carbon film doped with silver nanoparticles. The films were deposited using a reactive magnetron sputtering system (physical vapor deposition process). The specimens were characterized by optical profilometry, atomic force microscopy, and Rutherford backscattering spectroscopy analyses that determined differences in chemical composition and morphological structure. Following sterilization of the specimens by $\gamma$-ray irradiation, *C. albicans* (ATCC 18804) biofilms were formed by immersion in 2 ml of Sabouraud dextrose broth inoculated with a standardized fungal suspension. After 24 hours, the number of colony forming units (cfu) per specimen was counted. Data concerning biofilm formation were analyzed using ANOVA and the Tukey test ($p < 0.05$).

**Results:** *C. albicans* biofilm formation was significantly influenced by the films ($p < 0.00001$), reducing the number of cfu, while not affecting the roughness parameters ($p > 0.05$). The Tukey test showed no significant difference between Gdlc and Gag. Films deposited were extremely thin (~50 nm). The silver particles presented a diameter between 60 and 120 nm and regular distribution throughout the film surface (to Gag).

**Conclusion:** Diamond-like carbon films, doped or undoped with silver nanoparticles, coating the base of PMMA-based dentures could be an alternative procedure for preventing candidosis in denture users.

Poly(methyl methacrylate's) (PMMA) important characteristics, such as low cost, color manipulation, very low weight, and easy handling, allow this material to be used for denture manufacturing. In contrast, poor chemical and wear resistance, low hardness and high porosity and surface energy limit its lifetime,[1,2] influencing the contact angle, electrostatic interactions and surface roughness, important parameters to biofilm growth.[3,4] Additionally, poor or inappropriate denture hygiene results in scratches, accumulation of debris and biofilm formation, contributing to malodor and causing inflammatory changes in the adjacent mucous membranes.[3,5]

Previous studies demonstrated that a rough surface promotes microbial retention.[6-8] Valleys deeper than 2 $\mu$m interfere in biofilm removal, limiting the clinical use of dentures and the maintenance of oral health,[8,9] and a rough surface could be induced in resin-based dentures due to the common methods of cleaning, such as toothbrushing.[10] Thus, acrylic resin based dentures must be appropriately processed and polished to improve mechanical properties and achieve a smooth surface contributing to oral hygiene and low biofilm retention,[10] since the control of biofilm formation is important to prevention of oral diseases in denture users.

Regarding biofilm, *Candida albicans*, *Staphylococcus aureus*, and *Streptococcus mutans* are the main species colonizing the surface and oral mucosa of denture wearers.[11] A previous study suggested that mixed bacterial-fungal biofilms showed increased resistance to antimicrobial agents.[12] Several attempts to reduce *C. albicans* adherence have been reported in the literature, including the incorporation of chemical compounds into the denture acrylic resin[13,14] and chemical modification of acrylic resin.[15-17] Modifications in the physical properties of the denture surface by plasma treatment[18] and surface coating with Parylene film by chemical vapor deposition method[19] or application of a protective coating of polydimethylsiloxane[15] reduced *C. albicans* biofilm formation. Despite good antifungal activity, the maintenance of biocompatibility and mechanical properties of the resin requires long-term evaluation. Considering that surface characteristics are of utmost importance to *Candida* adherence, modification of the surface properties of resin-based dentures by increasing the resistance to surface wear and/or chemical reactivity of the surface could influence biofilm formation, making it a viable choice for preventing candidosis.

Diamond-like carbon (DLC) is a metastable form of amorphous carbon with a significant $sp^3$ bond with excellent mechanical and chemical properties, including high mechanical hardness, wear resistance, chemical inertness, and optical transparency.[20] In addition, DLC films are biocompatible and present antibacterial and antifungal properties.[21-23] Physical interaction between carbon-based nanomaterials and bacteria was proposed as the primary killing mechanism,[22-24] since carbon-based nanomaterials can cause irrecoverable damage to the outer membrane of the bacteria studied (*Escherichia coli* and *Shewanella oneidensis*).[24] Moreover, they can be modified by the addition of nanoparticles (silicon, hydrogen, fluorine, nitrogen, oxygen, tungsten, vanadium, cobalt, molybdenium, titanium, platinum, and silver) in the microstructure to improve their properties; e.g., fluorine (F) and silver (Ag) improve the antimicrobial characteristics of these films.[21,22] Previous studies reported enhancements, including improved hardness, wettability and biocompatibility, increased chemical and wear resistance, and decreased friction coefficient and surface energy, in the surface properties of PMMA coated with DLC films.[1,2,21]

The aim of this study was to assess the effect of DLC films, doped and undoped with silver nanoparticles (Ag-NPs), deposited on heat-polymerized acrylic resin on *C. albicans* adherence and biofilm formation. For this research, a reactive magnetron sputtering system was used to grow the films on PMMA substrates. The hypotheses are that DLC film reduces *C. albicans* biofilm formation on PMMA and that Ag-NPs improves this result.

## Materials and methods

### Specimen preparation

Forty-five acrylic resin (Lucitone 550; Dentsply Ind. Com. Ltda, Petrópolis, Brazil) disks (10 mm diameter × 5 mm high) were obtained in accordance with the manufacturer's instructions. Specimens were polymerized in a water bath at 73 °C for 90 minutes, followed by 30 minutes in 100 °C water. The flask was allowed to bench cool for 30 minutes, followed by immersion in running water for 15 minutes. Flash and excess resin were removed by polishing on both sides using 320-, 600-, and 1200-grit silicon carbide paper under water cooling. After polishing, all specimens were stored in distilled water at 37 °C for 48 hours. The specimens were randomly divided into three groups for treatment (n = 15): (a) Gc, no surface coating; (b) Gdlc, surface coated with DLC film; and (c) Gag, surface coated with DLC film doped with Ag-NPs.

### DLC-based film growth

Prior to film deposition, all the specimens were ultrasonically cleaned (Vitasonic, Vita Zanhfabrik, Bad Sackingen, Germany) for 10 minutes in distilled water. The experiments were performed in a cylindrical vacuum chamber evacuated to a background pressure of $5.0 \times 10^{-5}$ Torr (measured by a Penning® gauge, Inficon, Bad Ragaz, Switzerland) by a set of mechanical and diffusion pumps. An electrical discharge (of argon and methane gases) was used to sputter a high-purity carbon target (99.999%) and produce Gdlc films. The working pressure and electrical power of the discharge were maintained constant at 7 mTorr and 120 W, respectively. A previous argon discharge was achieved to remove superficial contamination on the target. For Gag films, a silver wire was positioned on the carbon target. The electric discharge promotes the sputtering of these materials simultaneously, and as a consequence, carbon films doped with Ag-NPs were produced. Depositions were performed for 15 minutes, and the cathode voltage and electric current were measured every 5 minutes to check the process stability.

### Roughness analysis

For the qualitative and quantitative topography and roughness analyses following the coating of PMMA with the films, the specimens were evaluated in a Wyko digital optical profilometer (NT 1100, Veeco, Somerset, NJ) connected to a computer drive containing Vision 32 software (Veeco). The roughness measurement parameters were performed at 20× magnification on five areas of each specimen ($229 \times 301\ \mu m^2$). The roughness parameters evaluated were as follows:

a) Ra, the arithmetical mean of the absolute values of the surface departures from the mean plane within the sampling area. The parameter was measured in $\mu$m, a general and commonly used value;
b) Rz, is the average value ($\mu$m) of the absolute heights of the five highest peaks and the absolute value of the five deepest valleys within the sampling area. This parameter is sensitive to the changes in pronounced topography features.

### Film characterization

Rutherford backscattering spectroscopy (RBS) and atomic force microscopy (AFM) were performed to characterize the elemental chemical composition, thickness (RBS), and the size and spatial distribution of Ag-NPs in the doped film (Gag). For this purpose, the films used in Gdlc and Gag were deposited on silicon wafers (n = 3). The RBS parameters used were 2 MeV He ion beam from a Pelletron accelerator (NEC,

Middleton, WI). The He ion beam irradiated the specimen perpendicular to the specimen surface. Backscattered particles at an angle of 170° were detected and analyzed with a surface barrier detector and conventional electronics. AFM images of file specimens were recorded in contact operation mode on a Shimadzu SPM-9500-J3 AFM (Shimadzu Co., Kyoto, Japan) under ambient conditions. Scanned areas (1 Hz) were perfect squares ($2 \times 2\ \mu m^2$) and were processed with AFM analysis software (Shimadzu Co.).

### Antimicrobial activity assessment

The specimens were sterilized by gamma radiation with cobalt 60 (25 KGy/6 h; Embrarad, Cotia, Brazil). *Candida albicans* ATCC 18804 was grown in Sabouraud dextrose agar (Difco, Detroit, MI) and incubated aerobically for 24 hours at 37 °C. Then, standardized suspensions containing $1 \times 10^6$ cells/ml of *C. albicans* were obtained by spectrophotometry (UV-1203, Shimadzu, Co.) in sterile saline solution (0.85% NaCl). The parameters of optical density and wavelength adopted were: *C. albicans* (0.284/530 nm) as previously established.[25] The specimens were inoculated in vitro by immersion in 2 ml Sabouraud dextrose broth inoculated with 0.1 ml of the standardized suspension. The tests were performed in 24-well plates. The specimens were distributed into the wells in three groups (n = 15), according to surface treatment, as described above.

Following the incubation period of 24 hours at 37 °C under aerobiosis, the surfaces plus biofilm were transferred to tubes containing 10 ml of sterile saline solution (0.85% NaCl), and the adhered cells were dispersed by vortexing. Dilutions of the suspension of $10^{-1}$, $10^{-2}$, and $10^{-3}$ were obtained in 0.85% NaCl. Aliquots of 0.1 ml of these suspensions were plated in duplicate on Sabouraud dextrose agar. Following incubation for 24 hours at 37 °C, the number of colony forming units (cfu) was counted, and the number of cfu/specimen was calculated.

### Statistical analysis

Statistical analyses of the results, expressed in cfu/specimen, Ra, and Rz, were performed using one-way ANOVA and post hoc multiple comparisons by the Tukey's test using Minitab 14 software for Windows (Minitab Inc, State College, PA). The adopted significance level was 5%.

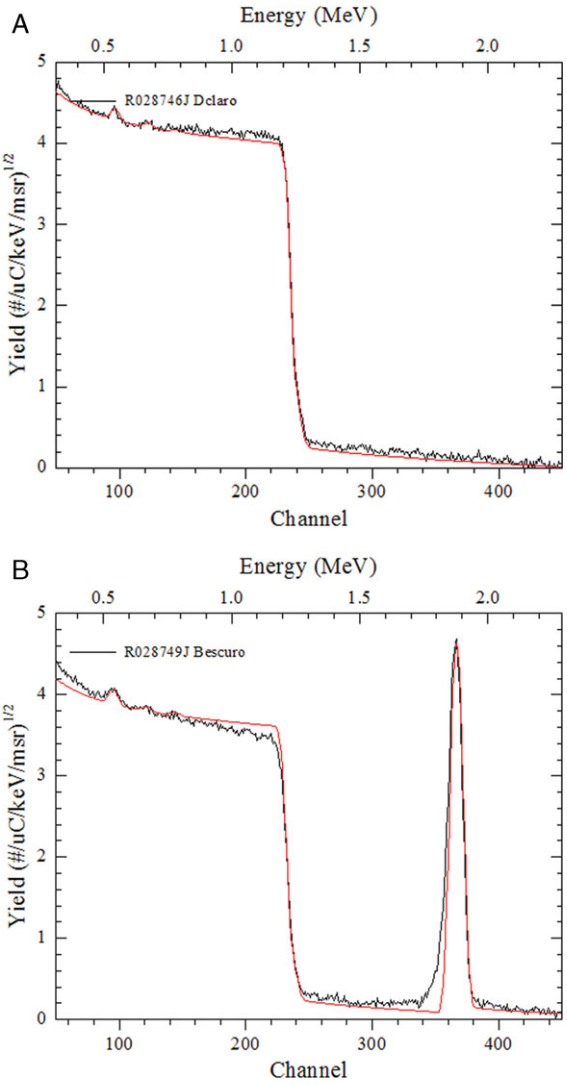


**Figure 1** RBS (black) of films used in (A) Gdlc and (B) Gag and simulation (red) performed to characterize the elemental chemical composition (energy, x-axis) and thickness of films (measured by the energy width of the C peak divided by the energy loss of the incident ion particle per unit depth). Note the presence of a peak of carbon (~0.5 MeV) to both spectra and the peak of silver (~1.9 MeV) to Gag. The y-axis is related to counts of atoms.

## Results

RBS was used to analyze the atomic composition of the DLC thin films (doped and undoped with Ag-NPs). Figure 1 shows spectra with a band related to carbon atoms over the silicon substrate in Gdlc and Gag and a corresponding peak of silver atoms in Gag. Based on the RBS spectra, the film has the presence of carbon atoms in both groups (Gdlc and Gag) and silver atoms in Gag.

The elemental concentration of the films is shown in Table 1. The thicknesses of the films in Gdlc and Gag were 33 nm and 42 nm, respectively, and the silver particles presented a diameter between 60 and 120 nm and were regularly distributed throughout the film surface (Fig 2).

ANOVA (Table 2) revealed that Ra and Rz did not significantly affect the number of adhered microorganisms. The results for the roughness (Ra parameter) in $\mu$m (mean ± SD) were: Gc (0.14 ± 0.02); Gdlc (0.14 ± 0.02); Gag (0.15 ± 0.02). The results for roughness (Rz parameter) in $\mu$m (mean ± SD) were: Gc (1.1 ± 0.3); Gdlc (1.0 ± 0.2); Gag (1.3 ± 0.3); however, the surface treatment significantly reduced *C. albicans* counts (Table 2). The results of cfu/specimen (mean $\times 10^6 \pm$ standard deviation) were as follows: Gc (69.4 ± 16.4); Gdlc (18.2 ± 7.2); Gag (26.1 ± 11.9). Tukey's test verified no significant difference between Gdlc and Gag (groups with film deposition) (Table 3).

**Table 1** Elemental chemical composition of the film in Gdlc and Gag, provided by RBS analysis

| Groups | C | Ag | H | O | N |
|---|---|---|---|---|---|
| Gdlc | 63.1 | - | 18.4 | 5.4 | 13.0 |
| Gag | 67.6 | 9.6 | 9.0 | 3.0 | 10.8 |

Gdlc, surface coated with DLC film; Gag, surface coated with DLC film doped with Ag-NPs.

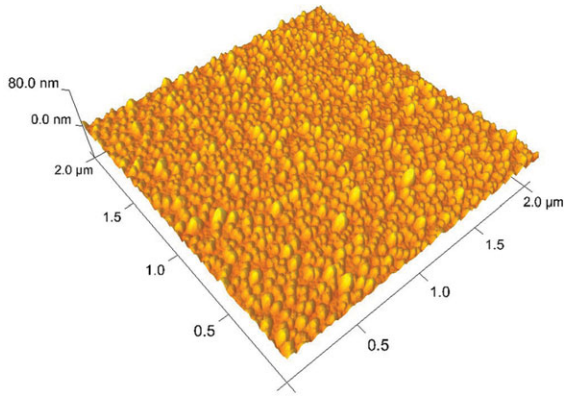


**Figure 2** AFM image showing the film topography used Gag. Note the regular distribution of Ag-NPs (peaks) throughout the film surface.

**Table 2** Results of one-way ANOVA for comparison of *C. albicans* counts (cfu/specimen), Ra and Rz parameter data (*$p < 0.05$)

| | cfu/specimen | Ra | Rz |
|---|---|---|---|
| *p* | 0.00001* | 0.491 | 0.106 |

**Table 3** Mean of cfu/specimen in the groups tested and the Tukey test results ($p < 0.05$) (same letters indicate homogeneous subsets)

| Groups | cfu/specimen | Tukey's test |
|---|---|---|
| Gc | $0.69 \times 10^8$ | A |
| Gdlc | $0.18 \times 10^8$ | B |
| Gag | $0.26 \times 10^8$ | B |

Gc, no surface coating; Gdlc, surface coated with DLC film; Gag, surface coated with DLC film doped with Ag-NPs.

## Discussion

The properties of an interface and its interactions with other molecules and cells may be obtained by surface modifications using advanced techniques. These modifications are considered good options for controlling microbial adhesion.[4] This research aimed to evaluate *C. albicans* biofilm formation on PMMA following the deposition of different DLC films (doped and undoped with Ag-NPs). Both films were effective in *candidal* biofilm control. No additional activity could be attributed to Ag-NPs addition. The hypotheses were partially accepted.

In recent studies, different methods have been suggested to prevent *Candida* adhesion and the development of denture-induced stomatitis.[13-18] Minimal evidence concerning the effect of these methods on the mechanical properties of the denture surface is available; however, the benefits and deposition techniques of DLC film on PMMA have been reported.[1,2,21]

A rough material has a larger surface area that increases surface energy and facilitates microbial colonization and biofilm formation. In agreement with a previous study,[26] the pattern of roughness did not change after the film deposition process, even for the Rz parameter, which is sensitive to detecting spot defects. Thus, roughness had no decisive effect on biofilm formation, suggesting that the reduced *C. albicans* biofilm formation in Gdlc and Gag was due to the chemical modification of the PMMA surface promoted by DLC film coating, such as hydrophobicity and low surface energy properties provided by DLC films.[2,23,26]

Other advantages of DLC films have been reported. The chemical resistance of DLC thin film coating on PMMA substrate to various organic solvents and acid and alkali solutions has been tested, and the results verified that coating provided a protective layer for polymer-based microfluidic devices.[1] Other related properties of DLC films were high hardness and low friction coefficients.[20,22] These features could preserve PMMA topography over time, decreasing denture surface degradation by wear and facilitating the cleaning of denture bases, making DLC films candidates as biocompatible coatings for complete dentures and other acrylic resin-based intraoral devices used in orthodontics, prosthodontics, and temporomandibular joint disorder treatments.

Previous studies showed that silver exhibited very strong bactericidal activity against both Gram-positive and Gram-negative bacteria, including multi-resistant strains from denture biofilm, such as *S. aureus*.[21-23,27] Antifungal and anti-inflammatory activity has also been previously described.[21,28,29] In addition, reduction of silver particle size improved its biocompatibility.[27] Lower cytotoxicity to fibroblasts when compared to conventional antifungal drugs and enhancement of fluconazole activity[29] have been cited as potential advantages of Ag-NPs.[28] Interestingly, in this study no additional effect on biofilm formation was observed with the addition of silver to DLC. Despite the promising results observed in relation to the control group, future studies using other culture media (i.e., RPMI), highly filamentous isolate (such as SC5314), different Ag-NPs concentrations, and long-term evaluation should be performed to evaluate the performance of the material under different challenges.

Regarding the clinical application, even with the different film composition in Gdlc and Gag, both were extremely thin (~50 nm) and probably would not promote any misfit between the base of resin-based dentures and oral tissues.

Despite the good results shown by DLC-based films on *C. albicans* biofilm formation, further studies are required to validate the clinical use of these materials. Irrespective of the potential of such films, the plasma technique is not a common process in dentistry, and the cost of its implementation in a prosthetics laboratory remains high.

## Conclusions

DLC thin films significantly diminished *C. albicans* biofilm formation on the resin surface compared with the control group.

The films undoped and doped with silver nanoparticles presented similar behavior. DLC thin films coating a denture base could be an alternative procedure for preventing candidosis.

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