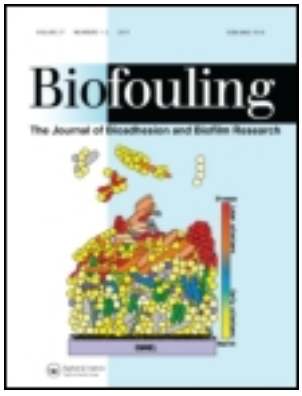


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# Silver colloidal nanoparticles: antifungal effect against adhered cells and biofilms of *Candida albicans* and *Candida glabrata*

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The aim of this study was to evaluate the effect of silver nanoparticles (SN) against *Candida albicans* and *Candida glabrata* adhered cells and biofilms. SN (average diameter 5 nm) were synthesized by silver nitrate reduction with sodium citrate and stabilized with ammonia. Minimal inhibitory concentration (MIC) tests were performed for *C. albicans* ($n = 2$) and *C. glabrata* ($n = 2$) grown in suspension following the Clinical Laboratory Standards Institute microbroth dilution method. SN were applied to adhered cells (2 h) or biofilms (48 h) and after 24 h of contact their effect was assessed by enumeration of colony forming units (CFUs) and quantification of total biomass (by crystal violet staining). The MIC results showed that SN were fungicidal against all strains tested at very low concentrations (0.4–3.3 $\mu g\ ml^{-1}$). Furthermore, SN were more effective in reducing biofilm biomass when applied to adhered cells (2 h) than to pre-formed biofilms (48 h), with the exception of *C. glabrata* ATCC, which in both cases showed a reduction $\sim$90%. Regarding cell viability, SN were highly effective on adhered *C. glabrata* and respective biofilms. On *C. albicans* the effect was not so evident but there was also a reduction in the number of viable biofilm cells. In summary, SN may have the potential to be an effective alternative to conventional antifungal agents for future therapies in *Candida*-associated denture stomatitis.

**Keywords:** silver; nanoparticles; biofilms; *Candida albicans*; *Candida glabrata*



## Introduction

*Candida*-associated denture stomatitis is a common recurring inflammatory process that mainly involves the palatal mucosa of complete denture wearers (Jeganathan and Lin 1992). *Candida albicans* remains the most frequently isolated yeast in the oral cavity but other non-*C. albicans Candida* (NCAC) species have also been isolated and involved in disease (Webb et al. 1998; Ramage et al. 2004; Vanden Abbeele et al. 2008). Several authors (Coco et al. 2008; Vanden Abbeele et al. 2008) reported that the most frequent NCAC species isolated from dentures of elderly patients were *Candida glabrata* (44.1%) and *Candida tropicalis* (19.1%).

One attribute of virulence among *Candida* species is their ability to form biofilms that can develop on oral surfaces including mucosa and acrylic dentures (Hasan et al. 2009). These biofilms are frequently tolerant/resistant to the commonly used antifungal drugs including nystatin (Watamoto et al. 2009), amphotericin B (Hasan et al. 2009; Watamoto et al. 2009), fluconazole (Bagg et al. 2003; Hasan et al. 2009), itraconazole (Bagg et al. 2003), caspofugin (Watamoto et al. 2009), ketoconazole (Watamoto et al. 2009) and flucytosine (Watamoto et al. 2009). Therefore, these *Candida* biofilms, which are resistant to antifungal agents and host immune defenses, are often associated not only with chronic infections but also to failure of prostheses (Samaranayake et al. 2009). Consequently, novel strategies are needed to combat the emergence of antifungal resistance in general.

For many years, silver has been known for its significant broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, protozoa and certain viruses (Balazs et al. 2004), including antibiotic-resistant strains (Melaiye and Youngs 2005; Stobie et al. 2008). Silver, as antimicrobial agent, is used in wound dressings, burn treatments, creams and as coatings on different medical devices (Bjarnsholt et al. 2007; Monteiro et al. 2009). The antimicrobial activities of silver nanoparticles (SN) are related to their size and shape. Baker et al. (2005) observed that smaller particles with a larger surface area available for interaction have a higher bactericidal

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effect than larger particles. Moreover, Pal et al. (2007) found that triangular SN displayed greater biocidal action than rod or spherical nanoparticles. However, the use of SN must be undertaken with caution, because of its concentration-dependent toxicity (Carlson et al. 2008; AshaRani et al. 2009; Panácek et al. 2009). Carlson et al. (2008) found that SN with an average size of 15 nm and 30 nm exhibited significant cytotoxicity at 10–75 $\mu$g ml$^{-1}$, whereas 55 nm nanoparticles required a concentration of 75 $\mu$g ml$^{-1}$ to promote a significant decrease in mammalian cell viability.

Antifungal activity of SN has been reported by some authors (Kim et al. 2008, 2009; Monteiro et al. 2009; Panácek et al. 2009; Rai et al. 2009). Panácek et al. (2009) found that SN prepared by the modified Tollens process exhibited inhibitory effect against *Candida* species at a concentration as low as 0.21 $\mu$g ml$^{-1}$. In the study of Kim et al. (2008), SN showed potent activity against clinical isolates and ATCC strains of *Trichophyton mentagrophytes* and *Candida* species (80% inhibitory concentration ($IC_{80}$), 1–7 $\mu$g ml$^{-1}$). The activity of SN was comparable to that of amphotericin B, but superior to that of fluconazole (amphotericin B $IC_{80}$, 1–5 $\mu$g ml$^{-1}$; fluconazole $IC_{80}$, 10–30 $\mu$g ml$^{-1}$ $\mu$) (Kim et al. 2008).

According to some researchers (Sondi and Salopek-Sondi 2004; Lok et al. 2006; Zheng et al. 2008; Kim et al. 2009), SN attach to the sulphur containing proteins of the cell membrane, thereby causing membrane damage and depleting the levels of intracellular ATP of the microorganism. Silver can also interact with the DNA of microorganisms, preventing cell reproduction (Damm et al. 2008). Moreover, Elechiguerra et al. (2005) observed that SN in the 1–10 nm range interacted with gp120 glycoprotein knobs, blocking the HIV-1 virus from binding to host cells.

Although the literature reports some studies related to the antifungal activity of SN, to the authors' knowledge, there are no studies concerning the effect of these particles against adhered cells and biofilms of *Candida* spp. Thus, the aim of the present study was to evaluate the effect of SN against adhered cells and biofilms of *C. albicans* and *C. glabrata* through quantification of the total biomass and cultivable cells. The hypothesis tested was that SN have an antifungal effect against sessile cells of *C. albicans* and *C. glabrata* and this capacity depends on the concentration of SN.

## Materials and methods

### *Synthesis and characterization of silver colloidal nanoparticles*

SN were synthesized by means of the Turkevich et al. (1951) method through the reduction of silver nitrate ($AgNO_3$) with sodium citrate ($Na_3C_6H_5O_7$), as detailed elsewhere (Monteiro et al. 2009), and both chemicals were obtained from Merck KGaA, Darmstadt, Hesse, Germany. The initial concentrations of the reaction components were $5 \times 10^{-3}$ mol l$^{-1}$ of $AgNO_3$ and 0.3 mol l$^{-1}$ of $Na_3C_6H_5O_7$. The aqueous solutions of $AgNO_3$ and $Na_3C_6H_5O_7$ were kept at boiling temperature for $\sim$6 min until the solution turned amber yellow. After that, 7.5 ml of a 1.4 mol l$^{-1}$ solution of ammonia ($NH_3$) (Merck KGaA, Darmstadt, Hesse, Germany) were added. The formation of colloidal SN was confirmed by UV/Visible spectroscopy (Spectrophotometer Shimadzu MultSpec-1501, Shimadzu Corporation, Tokyo, Japan) and, later, by X-ray diffraction (XRD) (Diffractometer Rigaku DMax-2000PC, Rigaku Corporation, Tokyo, Japan). Transmission electron microscopy (TEM, Electron Microscope FEG-VP Supra 35, Carl Zeiss, Jena, Thüringen, Germany) was used in order to further characterize the synthesized SN.

### *Artificial saliva medium*

Artificial saliva was prepared according to Lamfon et al. (2003). The composition per 1 l of deionized water was: 2 g of yeast extract (Liofilchem, Italy), 5 g of peptone (Liofilchem, Italy), 2 g of glucose (AppliChem, Germany), 1 g of mucin (Sigma–Aldrich, USA), 0.35 g of NaCl (AppliChem, Germany), 0.2 g of $CaCl_2$ (Riedel-de Haën, Germany) and 0.2 g of KCl (Pronalab, Portugal). The pH was adjusted with NaOH (Pronalab, Portugal) to 6.8.

### *Organisms and growth conditions*

Two *Candida* species and two different strains of each species were used in this work. Regarding *C. albicans*, one strain was from the American Type Culture Collection, ATCC 10231, and the other was an oral clinical isolate (strain 324LA/94) from the culture collection of the Cardiff Dental School (Cardiff, UK). For *C. glabrata* one strain was from the American Type Culture Collection, ATCC 90030 and the other was an oral isolate (strain D1) obtained from the biofilm group of the Centre of Biological Engineering, University of Minho (Braga, Portugal). All *Candida* strains were subcultured on Sabouraud dextrose agar medium (SDA; Liofilchem, Italy) at 37°C for 24 h. An inoculum of each yeast strain, obtained from SDA plates, was suspended in 30 ml of Sabouraud dextrose broth (SDB; Liofilchem, Italy) and incubated at 37°C for 20–24 h under agitation (120 rpm). After incubation, the cells were harvested by centrifugation at 8000 rpm for 5 min at 15°C. Thereafter, the pellet was washed twice with 30 ml of phosphate buffered saline

(PBS; pH 7), and the yeasts were enumerated using a Neubauer counting chamber and adjusted to a concentration of $10^7$ cells $ml^{-1}$ in artificial saliva.

### *Minimum inhibitory concentration (MIC)*

MIC was determined using the microdilution method in accordance with the guidelines of the Clinical Laboratory Standards Institute (CLSI) (M27-A2). The colloidal suspension of SN was first diluted in deionized water in a geometric progression, from 2 to 1024 times. Then, each silver concentration obtained previously was diluted (1:5) in RPMI 1640 medium (Sigma-Aldrich, USA). The final concentrations of SN in the dispersion ranged from 54 $\mu g\ ml^{-1}$ to 0.1 $\mu g\ ml^{-1}$ (54; 27; 13.5; 6.75; 3.38; 1.69; 0.84; 0.42; 0.21; 0.1 $\mu g\ ml^{-1}$). Inocula from 24 h yeast cultures on SDA were adjusted to a turbidity equivalent to a 0.5 McFarland standard in saline solution (0.85% NaCl). The yeast suspension was diluted (1:5) in saline solution and afterwards diluted (1:20) in RPMI 1640. Each yeast inoculum (100 $\mu l$) was added to the respective well of microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium) containing 100 $\mu l$ of each specific concentration of SN colloidal suspension. Controls devoid of SN were also included. The microtiter plates were incubated at 37°C, and the MICs were determined visually as the lowest concentration of SN showing no yeast growth after 48 h. As a control, the influence of the stabilizers ($Na_3C_6H_5O_7$ and $NH_3$) used for synthesizing the SN was also tested. For that, the solution was diluted in RPMI 1640 medium in a microtiter plate to the same concentrations used when diluting the suspension of silver colloidal nanoparticles. All assays were repeated in duplicate on three different occasions.

### *Application of SN in adhered cells and biofilms*

#### *Adhesion assay*

An aliquot of yeast cell suspension (200 $\mu l$ of $10^7$ cells $ml^{-1}$ in artificial saliva) was added to each well of a 96-well microtiter plate and incubated for 2 h at 37°C in an orbital shaker incubator at 120 rpm. After this, the cell suspensions were aspirated, and each well washed once with 200 $\mu l$ of PBS to remove loosely adherent cells.

#### *Biofilm formation*

*Candida* biofilms were developed in the 96-well microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium) using the methodology described by Silva et al. (2010). Cell suspensions (200 $\mu l$ of $1 \times 10^7$ cells $ml^{-1}$ in artificial saliva) were pipetted into each well and incubated for 48 h at 37°C in a shaker at 120 rpm. After 24 h, 100 $\mu l$ of artificial saliva medium were removed and an equal volume of fresh artificial saliva was added. After biofilm formation for 48 h, the medium was aspirated and each well was rinsed once with 200 $\mu l$ of PBS to remove non-adherent cells.

#### *Treatment with SN*

In this study, SN were added to adhered yeast cells (2 h) and to mature biofilms (48 h) and their effect was assessed after contact for 24 h. Stock suspensions of silver colloidal nanoparticles were serially diluted with deionized water and RPMI 1640 to obtain concentrations ranging from 54 to 0.1 $\mu g\ ml^{-1}$, similar to the procedure for MIC determination. Each dilution (200 $\mu l$) was added to the columns of a 96-well microtiter plate, in decreasing concentrations, containing adhered cells or biofilms (prepared as described previously). For controls, a column of each plate was handled in an identical way except that no SN suspension was added. The plates were incubated at 37°C for 24 h in an orbital shaker incubator at 120 rpm. After treatment with SN, adhered cells and biofilms were washed once with PBS to remove loosely attached cells prior to analysis of biomass and cultivable cells.

All assays were performed in triplicate and on three separate occasions.

### *Adhered cells and biofilm quantification*

#### *Biomass quantification by crystal violet staining*

For fixation of the adhered cells and biofilms, 200 $\mu l$ of 99% methanol (Romil, UK) were added to each well (containing adhered cells or biofilms treated with SN, as described previously), after 15 min the methanol was removed and the plates were allowed to dry at room temperature. Then, 200 $\mu l$ of crystal violet stain (CV; 1%, v/v) (Merck, Germany) were added to all wells. After 5 min, the excess of CV was removed and the plates were gently washed in water. Finally, 200 $\mu l$ of acetic acid (33%, v/v) (Pronalab, Portugal) were added to all wells to dissolve the CV stain and the absorbance was measured at 570 nm. The assays were performed in triplicate and on three separate occasions.

#### *Quantification of cultivable cells*

The walls of each well, containing adhered cells or biofilms treated with SN, were scraped and the suspensions obtained were vigorously vortexed for 5 min to disaggregate cells. Serial decimal dilutions (in PBS) were plated on SDA. Agar plates were incubated

for 24 h at 37°C, and the total number of colony forming units (CFUs) per unit area (Log CFU $cm^{-2}$) of microtiter plate well were enumerated. The assays were performed on three separate occasions.

### *Statistical analysis*

Statistical analysis was performed using SPSS software (SPSS-Statistical Package for the Social Sciences, Inc., Chicago, USA) and the results were compared using a one-way ANOVA followed Bonferroni test. The significance level was set at $P < 0.05$.

## Results

### *Synthesis and characterization of silver colloidal nanoparticles*

In this work, the silver colloidal nanoparticles were stabilized using $NH_3$ in order to prevent aggregation. $NH_3$ plays an important growth moderating role, making it possible to stabilize metallic silver nanoparticles, since free silver ions, which are responsible for particle growth and the formation of new nuclei, are trapped by the formation of diammine silver (I) complexes (Gorup et al. 2011). The absorption spectrum of the colloidal suspension displayed in Figure 1A shows a well-defined plasmon band centered at 430 nm, characteristic of nanosized silver. Indeed, the symmetrical shape of the plasmon band in Figure 1A confirms the colloidal stability and sharp particle size distribution. The TEM image (Figure 1B) of colloidal SN revealed the presence of nearly spherical particles with average size of 5 nm. Moreover, the concentration of the resultant colloidal suspension was determined to have 540 $\mu$g of Ag $ml^{-1}$. The characteristic XRD pattern (Figure 1C) also confirmed the presence of metallic SN, with a cubic crystalline structure (JCPDS 04-0783). The diffraction peaks assigned with Ag at 38.5°, 44.5°, 64.8° and 78° can be attributed to the (1 1 1), (2 0 0), (2 2 0) and (3 1 1) crystallographic planes of metallic Ag, respectively.

### *Minimum inhibitory concentration (MIC)*

The results showed that the SN were fungicidal against all of the tested yeasts at very low concentrations and the fungicidal activity was dependent on the yeast species and strains tested (Table 1). These results were confirmed by plating the content of each well on SDA, and there was no growth for any of the strains resultant from the MIC point. The lowest MICs of SN, at a silver concentration of 0.4–0.8 $\mu$g $ml^{-1}$, were obtained against *C. albicans* 324LA/94 and *C. glabrata* ATCC 90030. On the other hand, *C. albicans* ATCC 10231 and *C. glabrata* D1 were less sensitive, with MIC values equal to 0.8–1.6 $\mu$g $ml^{-1}$ and 1.6–3.3 $\mu$g $ml^{-1}$, respectively. The control solution without silver ($Na_3C_6H_5O_7 + NH_3$) did not reveal any effect on the tested yeasts.

### *Effect of SN on biofilm biomass*

Figure 2A presents the results of biomass reduction of biofilms formed 24 h after the application of SN to adhered cells. These data show that SN were effective in inhibiting biofilm development by all the tested yeasts. When compared to the control group (biofilms without SN), *C. albicans* ATCC 10231 showed significant biomass reduction (~60%) at silver con-

Table 1. Minimum inhibitory concentrations (MIC) of silver nanoparticles against the tested yeasts.

| Tested yeasts | MIC ($\mu$g $ml^{-1}$) |
|---|---|
| *C. albicans* ATCC 10231 | 0.8–1.6 |
| *C. albicans* 324LA/94 | 0.4–0.8 |
| *C. glabrata* ATCC 90030 | 0.4–0.8 |
| *C. glabrata* D1 | 1.6–3.3 |

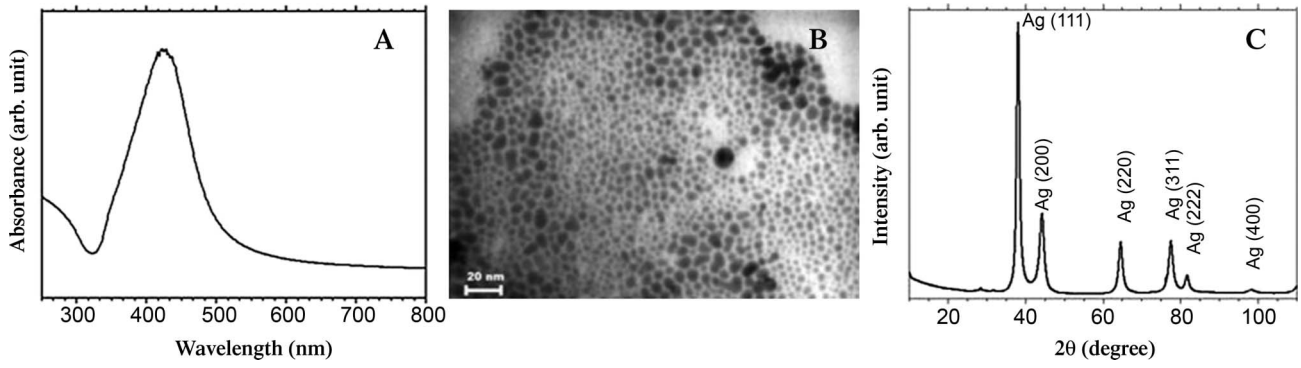


Figure 1. (A) UV–Vis absorption spectrum; (B) transmission electron microscope image (magnification: 880 KM); (C) X-ray diffraction pattern of SN synthesized through the reduction of silver nitrate with sodium citrate.

centrations $> 1.6\ \mu g\ ml^{-1}$ ($P < 0.001$), while for *C. albicans* 324LA/94 ($P = 0.009$), *C. glabrata* ATCC 90030 ($P < 0.001$) and *C. glabrata* D1 ($P < 0.001$) this effect was only significant for silver concentrations $> 3.3\ \mu g\ ml^{-1}$. In general, *C. glabrata* strains showed a higher percentage of biomass reduction ($> 90\%$) for silver concentrations above $3.3\ \mu g\ ml^{-1}$ than *C. albicans* strains (Figure 2A) and independently of the silver concentration increase. However, *C. albicans* ATCC 10231 and *C. albicans* 324LA/94 showed a higher silver concentration dependent biomass reduction, rising to $\sim 85\%$ above a silver concentration of $6.7\ \mu g\ ml^{-1}$ (Figure 2A).

Interestingly, SN had a similar effect in reducing the biofilm biomass of *C. glabrata* strains when applied to adhered cells (2 h), due to inhibition of biofilm formation, and on pre-formed biofilms (48 h), which in this case represents a true biomass reduction. However, on *C. albicans*, silver particles were more effective when applied to adhered cells (Figure 2A and B). Furthermore, biomass reduction of *C. glabrata* ATCC 90030 (Figure 2B) was statistically significant only for the highest SN concentration (reduction of 97.12%; $P = 0.028$).

### *Effect of SN on biofilm cultivable cells*

Mean and standard deviation (SD) values of the $\log_{10}$ CFU $cm^{-2}$ obtained in the two experimental conditions tested for each *Candida* strain are shown in Figure 3.

After contact of the adhered cells with SN for 24 h a reduction in the number of cells was observed, expressed as $\log_{10}$ CFUs, dependent on the silver concentration (Figure 3A), with the exception of *C. albicans* ATCC 10231. The other yeast strains, *C. albicans* 324LA/94, *C. glabrata* ATCC 90030, and *C. glabrata* D1 showed a significant reduction in the number of CFUs for silver concentrations in the range $3.3\ \mu g\ ml^{-1}$ to $54\ \mu g\ ml^{-1}$ (Figure 3A). However, *C. glabrata* biofilm cells were more sensitive to silver than those of *C. albicans* 324LA/94. At a silver concentration of $3.3\ \mu g\ ml^{-1}$ there was a 1.5-$\log_{10}$, 6.5-$\log_{10}$ and 4.0-$\log_{10}$ reduction in the number of CFUs for *C. albicans* 324LA/94 ($P = 0.003$), *C. glabrata* ATCC 90030 ($P < 0.001$) and *C. glabrata* D1 ($P < 0.001$), respectively, compared to the control values. These findings agree with the results of the CV staining assay which also show a significant biomass reduction at a silver concentration of $3.3\ \mu g\ ml^{-1}$ (Figure 2A). For *C. albicans* ATCC 10231, although there was an approximately 1.1-$\log_{10}$ reduction in the

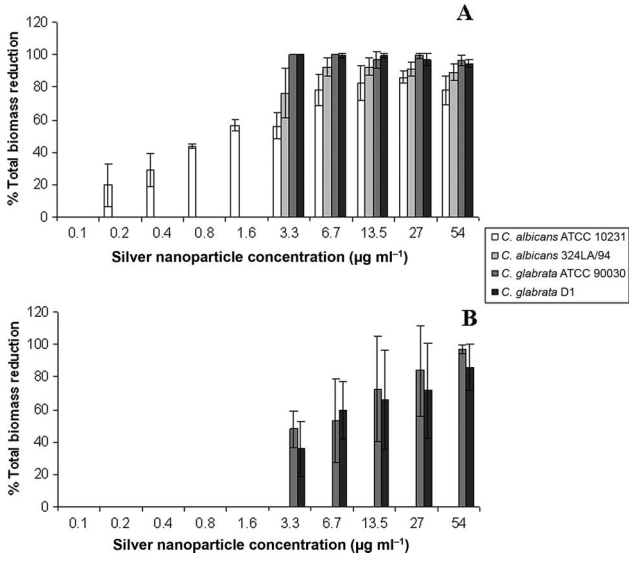


Figure 2. (A) Percentage of total biomass reduction of adhered cells of *C. albicans* and *C. glabrata* obtained with the CV staining assay; (B) Their mature biofilms after treatment for 24 h with different SN concentrations. Error bars indicate the SDs of the means. Note: there was no total biomass reduction in pre-formed biofims of *C. albicans* strains.

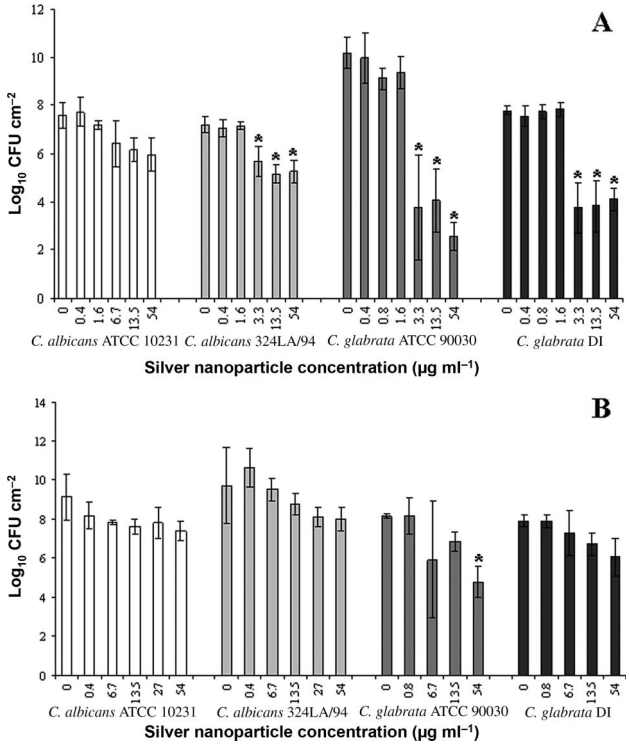


Figure 3. Mean values of the logarithm of colony forming units normalized by area of adhesion ($\log_{10}$ CFU $cm^{-2}$) of adhered cells of *C. albicans* and *C. glabrata* (A), and biofilms (B) after treatment for 24 h with different SN concentrations. Error bars indicate the SDs of the means. *Indicates $P < 0.05$, as compared to the control group, using a one-way ANOVA with the Bonferroni test.

number of CFUs at a silver concentration of 6.7 $\mu$g ml$^{-1}$, this difference was not statistically significant when compared to the control group ($P = 0.496$; $P > 0.05$).

Figure 3B displays the number of 24 h biofilm cultivable cells after contact with different SN concentrations for pre-formed biofilms (48 h). Although at high silver concentration (54 $\mu$g ml$^{-1}$), there was a reduction in the $\log_{10}$ of the mean number of CFUs of 1.73-$\log_{10}$ for *C. albicans* ATCC 10231 ($P = 0.112$), 1.73-$\log_{10}$ for *C. albicans* 324LA/94 ($P = 0.800$) and 1.87-$\log_{10}$ for *C. glabrata* D1 ($P = 0.055$), these reductions were not significant when compared to the control groups. However, at the same silver concentration, *C. glabrata* ATCC 90030 biofilms showed a significant $\log_{10}$ CFU reduction compared to the control group (3.39-log reduction; $P = 0.003$). Finally, the comparison of biofilm biomass (Figure 2B) and biofilm cultivable cells (Figure 3B) clearly shows that the highest reductions in total biomass and number of $\log_{10}$ CFUs occurred for *C. glabrata* ATCC 90030 at the silver concentration of 54 $\mu$g ml$^{-1}$.

## Discussion

The present study evaluated the effect of SN against adhered cells and biofilms of *C. albicans* and *C. glabrata*, through quantification of their biomass and cultivable cells. The research hypothesis was accepted since SN showed activity against the tested yeasts and this capacity was dependent on the silver concentration.

In the MIC tests, performed according to CLSI methodology (Table 1), both strains from each species were susceptible to SN. SN promoted 100% reduction in the planktonic growth of the yeasts in concentrations ranging from 0.4 $\mu$g ml$^{-1}$ to 3.3 $\mu$g ml$^{-1}$. Panácek et al. (2009) observed that the lower MIC values for *C. albicans* occurred at a SN concentration of 0.052 $\mu$g ml$^{-1}$ to 0.1 $\mu$g ml$^{-1}$, while Kim et al. (2008) reported values of 2–4 $\mu$g ml$^{-1}$ for *C. albicans* and 1–7 $\mu$g ml$^{-1}$ for *C. glabrata*. These differences may be explained by differences in the nanoparticle synthesis methods. Kim et al. (2008) dissolved solid silver in nitric acid and the $Ag^+$ ions were reduced by sodium chloride, while Panácek et al. (2009) used a modified Tollens reaction to prepare SN, in which $Ag^+$ ions are reduced by saccharides in the presence of ammonia. Subsequent stabilization of SN by sodium dodecyl sulfate was performed in the study of Panácek et al. (2009). In addition, the differences in the MICs of the SN probably result from differences in the strains tested.

Nevertheless, SN had already been shown to be effective against planktonic *Candida* cells, so the more important question was whether treatment with these particles could eradicate adhered cells and inhibit mature biofilms.

Regarding the results obtained with the CV staining and CFU enumeration assays for adhered cells (Figures 2A and 3A), in general, a significant reduction in biomass and in the number of CFUs was observed for silver concentrations at or higher than 3.3 $\mu$g ml$^{-1}$. Therefore, for all tested strains, SN showed an ability to inhibit biofilm formation when applied to already adhered cells. SN present a large surface area, which provides better contact with microorganisms (Rai et al. 2009). According to Kim et al. (2009), SN affect yeast cells by attacking their membranes, thus disrupting the membrane potential. These authors observed, by transmission electron microscopy, the formation of 'pits' on the membrane surfaces of *C. albicans* and finally the formation of pores and subsequent cell death. Moreover, the effective silver concentration found in the present study was lower than in previous reports concerning the toxic concentration of SN *in vitro* against human cells (Carlson et al. 2008; Panácek et al. 2009).

Another interesting observation from the present study is that SN were more effective in reducing total biomass and CFUs when applied to adhered cells (2 h) (Figures 2A and 3A), than to pre-formed biofilms (48 h) (Figure 3A and 3B). Such results are in good agreement with those published by Chandra et al. (2001), who found that the progression of drug resistance in *C. albicans* biofilms was associated with the concomitant increase in metabolic activity of developing biofilms. When compared to young and mature *Candida* biofilms of 24–48 h, the adhesion phase contains a lower cell mass (Seneviratne et al. 2009), due to the incipient production of the extracellular matrix, one of the most specific traits of biofilms. Nevertheless, adhesion phase cells seem to be in a metabolically excited state compared with their older counterparts (Seneviratne et al. 2009) in well established biofilms. This may indicate that the observed decrease in susceptibility to SN was a reflection of a lower metabolic activity of cells in mature biofilms.

However, the experiments on pre-formed biofilms (Figure 3B) showed that at the highest silver concentration (54 $\mu$g ml$^{-1}$) there was a reduction in the $\log_{10}$ of the mean number of CFUs for all *Candida* strains, but this reduction was only significant for *C. glabrata* ATCC 90030, when compared to the control group (without silver). This effect of SN on pre-formed biofilms may be due to the presence of water channels throughout the biofilm. Since water channels are present in all biofilms for nutrient transportation, SN may directly diffuse inside the matrix layer through the

pores and may impart an antifungal function (Kalishwaralal et al. 2010).

Despite the reduction in the number of CFUs at the highest silver concentration (Figure 3B), it was found that a progressive increase in silver concentration did not provide significant reductions in the number of CFUs, characterizing a relatively weak dose-response. Perhaps increasing the silver concentration above 54 $\mu$g ml$^{-1}$ would result in a better dose-response. Furthermore, when compared with the antifungal drug concentrations used in some studies, the silver concentrations tested in this study can be considered low. Vandenbosch et al. (2010) evaluated the fungicidal activity of miconazole against mature *Candida* biofilms and found that a 24 h-treatment with miconazole resulted in a significant reduction (ranging from 89.3% to 99.1%) in the number of CFUs for all strains investigated (*C. albicans, C. glabrata, C. Krusei, C. parapsilosis* and *C. tropicalis*). However, it should be noted that the antifungal concentration used in this study (2081 $\mu$g ml$^{-1}$) was higher than the common concentration used *in vivo*. Tobudic et al. (2010) verified that posaconazole at concentrations of 2 and 256 $\mu$g ml$^{-1}$ failed to significantly reduce the CFUs of biofilms of *C. albicans*, compared with the untreated control ($< 1$ $\log_{10}$ CFU ml$^{-1}$). However, the combination of 1 $\mu$g ml$^{-1}$ of amphotericin B and 2 $\mu$g ml$^{-1}$ of posaconazole showed the greatest decrease in the CFUs ($> 2$ $\log_{10}$ CFU ml$^{-1}$). Thus, the most traditional antifungals applied alone seem to have a low effect against biofilms of *Candida* species compared to the effect of SN as observed in the present study.

With regard to biomass reduction in pre-formed biofilms (Figure 2B), the SN were effective only for *C. glabrata* strains. *C. albicans* forms larger and more complex biofilms than *C. glabrata* (Samaranayake et al. 2005). In the studies of Samaranayake et al. (2005) and Seneviratne et al. (2010), *C. glabrata* biofilms presented reduced thickness, were less profuse, and were devoid of hyphal elements, when compared with *C. albicans* biofilms. The morphogenic transition of yeast to hyphae has been shown to play an important role in biofilm formation in *C. albicans* (Samaranayake et al. 2005). Probably, hyphal elements facilitate the exuberant architecture of *C. albicans* (Seneviratne et al. 2009) biofilms, making them more difficult to eliminate. Moreover, a relationship between biofilm thickness and resistance to antibiotics has been observed (Mah and O'Toole 2001). Thus, all these factors may explain the higher activity of SN against *C. glabrata* biofilms, which is still of major importance since this species is known to be very resistant to common antifungal agents, making it very difficult to eliminate.

Several mechanisms have been proposed to explain the resistance of *Candida* biofilms to antifungal agents, viz. the robust biofilm architecture, a decreased metabolic activity, altered gene expression, the extracellular matrix, the presence of 'persister cells' and higher anti-oxidative capacities (Mah and O'Toole 2001; Ramage et al. 2005; Seneviratne et al. 2010). Harrison et al. (2006) suggest that *Candida* biofilms may adsorb metal cations from their surroundings and that sequestration in the extracellular matrix may contribute to resistance. However, the exact mechanism by which fungi in the biofilm mode can acquire resistance remains to be elucidated.

Finally, in clinical terms, the *in vitro* data obtained in the present study demonstrate that SN may have an important role in preventing *Candida* biofilm formation in the oral cavity. However, more studies are required to investigate the morphology and the matrix composition of *Candida* biofilms in the presence of SN. Further studies on the development, the antifungal properties, the cytotoxicity and genotoxicity of SN with different sizes and with stabilizing agents are also necessary for *in vivo* experimentation with such antifungal agents. Such studies would stimulate the development of antifungal drugs based on SN working to prevent *Candida*-associated denture stomatitis.

## Conclusions

SN exhibit fungicidal activity against all the tested yeasts at very low concentrations (0.4–3.3 $\mu$g ml$^{-1}$). These nanoparticles were more effective in inhibiting biofilm formation than in controlling mature biofilms. However, their antifungal activity was higher against *C. glabrata* than against *C. albicans*. The significance of this work is that this is the first report concerning the effect of SN against adhered cells and biofilms of *Candida* spp. Moreover, the fact that the SN used here can easily be prepared in a cost-effective manner is also an important factor. Thus, it can be expected that SN may have potential as an antifungal agent to prevent *Candida*-associated denture stomatitis.

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