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Silver nanoparticles: influence of stabilizing agent and diameter on antifungal activity against *Candida albicans* and *Candida glabrata* biofilms

D.R. Monteiro[1,2], S. Silva[2], M. Negri[2,3], L.F. Gorup[4], E.R. de Camargo[4], R. Oliveira[2], D.B. Barbosa[1] and M. Henriques[2]

1 Department of Dental Materials and Prosthodontics, Araçatuba Dental School, Universidade Estadual Paulista (UNESP), Araçatuba, São Paulo, Brazil
2 Department of Biological Engineering, Institute for Biotechnology and Bioengineering, University of Minho, Braga, Portugal
3 Faculdade INGÁ, Maringá, Paraná, Brazil
4 LIEC-Department of Chemistry, Federal University of São Carlos (UFSCar), São Carlos, São Paulo, Brazil

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Correspondence
Mariana Henriques, Institute for Biotechnology and Bioengineering, Department of Biological Engineering, University of Minho, 4710-057 Braga, Portugal.
E-mail: mcrh@deb.uminho.pt

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Abstract

Aim: The purpose of this work was to evaluate the size-dependent antifungal activity of different silver nanoparticles (SN) colloidal suspensions against *Candida albicans* and *Candida glabrata* mature biofilms.
Methods and Results: The research presented herein used SN of three different average sizes (5, 10 and 60 nm), which were synthesized by the reduction of silver nitrate through sodium citrate and which were stabilized with ammonia or polyvinylpyrrolidone. Minimal inhibitory concentration (MIC) assays were performed using the microdilution methodology. The antibiofilm activity of SN was determined by total biomass quantification (by crystal violet staining) and colony forming units enumeration. MIC results showed that all SN colloidal suspensions were fungicidal against the tested strains at very low concentrations (0·4–3·3 μg ml^{-1}). With regard to biomass quantification, SN colloidal suspensions were very effective only against *C. glabrata* biofilms, achieving biomass reductions around 90% at a silver concentration of 108 μg ml^{-1}. In general, all SN suspensions promoted significant $\log_{10}$ reduction of the mean number of cultivable biofilm cells after exposure to silver concentrations at or higher than 108 μg ml^{-1}. Moreover, the results showed that the particle size and the type of stabilizing agent used did not interfere in the antifungal activity of SN against *Candida* biofilms.
Conclusions: This study suggests that SN have antifungal therapeutic potential, but further studies are still required namely regarding formulation and delivery means.
Significance and Impact of the Study: SN may contribute to the development of new strategies for the improvement of oral health and quality of life particularly of the complete denture wearers.

Introduction

The emergence of a pathogenic state known as denture stomatitis, which is associated with biofilm formation by *Candida* species, particularly *Candida albicans* and *Candida glabrata* (Coco *et al.* 2008), is common in complete denture wearers. *Candida* infections display increased resistance to antifungal therapy, leading to treatment failure and to recalcitrant infections (Watamoto *et al.* 2009). This fact has stimulated the possibility of using silver nanoparticles (SN) to control *Candida* biofilm formation.

The chemical reaction method is one of the most employed and inexpensive methods to synthesize SN. It is based on the use of a reducing agent (e.g., sodium citrate) to reduce Ag^+ to Ag^0 and a stabilizer [e.g., ammonia (NH_3), polyvinylpyrrolidone (PVP)] to control particle growth and prevent aggregation (Kvítek *et al.* 2008; Gorup *et al.* 2011; Monteiro *et al.* 2012). The SN action mechanism against bacterial and fungal cells is relatively well described (Monteiro *et al.* 2009), and as, according to Allaker (2010), silver may act on a broad range of microbial targets, many mutations need to occur for micro-organisms to resist its antimicrobial effect. Moreover, an inverse relationship between SN size and antimicrobial activity has been demonstrated by Baker *et al.* (2005), who reported that smaller particles, with a larger surface area available for interaction with planktonic microbial cells, were more effective than larger particles.

Although the activity of SN against *C. albicans* and *C. glabrata* adhered cells and biofilms was demonstrated recently (Monteiro *et al.* 2011), research regarding the susceptibility of *Candida* biofilms to SN of different sizes, and synthesized with different stabilizing agents, is unknown at the moment. For this reason, the current study investigated, through the quantification of total biomass and cultivable cells, the size-dependent antifungal activity against *C. albicans* and *C. glabrata* biofilms of SN synthesized with two different stabilizing agents. The null hypotheses were that: (i) there would be no difference among the different sizes of SN in decreasing total biomass and cultivable cells of *Candida* biofilms, and (ii) the type of stabilizing agent would not interfere with SN efficacy.

Materials and methods

Synthesis and characterization of silver colloidal nanoparticles

For the synthesis of silver colloidal nanoparticles, silver nitrate ($AgNO_3$), sodium citrate ($Na_3C_6H_5O_7$) and ammonia (NH_3) were provided by Merck KGaA (Darmstadt; Germany) and PVP was obtained from Sigma-Aldrich, (St Louis, MO). SN of three different sizes: 5 (SN5), 10 (SN10) and 60 (SN60) nm, and stabilized with NH_3 (N) or PVP (P), were prepared according to the procedure reported by Gorup *et al.* (2011). Briefly, 7·5 ml of a solution of sodium citrate at 0·3 mol l^{-1} and five drops of ammonia (1·4 mol l^{-1}) were added to a tri-neck flask containing 150 ml of an aqueous solution of $AgNO_3$ at $5{\cdot}0 \times 10^{-3}$ mol l^{-1} under the temperature of 90°C. The solution was kept stirring at 90°C until it turned yellow, indicating the formation of SN. The silver colloidal nanoparticles were stabilized by adding 7·5 ml of a solution with 1·4 mol l^{-1} of NH_3. The new solution was kept stirring and heating for two more minutes, and the suspension was then allowed to cool at room temperature. In another flask, 1 ml of a solution of 102 g l^{-1} of PVP was added to the colloidal silver after lowering of the temperature. To characterize the silver colloidal nanoparticles suspensions, UV/Visible absorption spectroscopy (Spectrophotometer Shimadzu MultSpec-1501; Shimadzu Corporation, Tokyo, Japan) and Transmission Electron Microscopy (TEM, Electron Microscope FEG-VP Supra 35; Carl Zeiss, Jena, Thüringen, Germany) were performed.

Yeast strains and growth conditions

The nutrient source for biofilm formation was artificial saliva, used to mimic the *in vivo* oral cavity conditions. It was prepared according to Lamfon *et al.* (2003) and its composition per 1 l of deionized water was: 2 g of yeast extract (Liofilchem, Roseto degli Abruzzi, Italy), 5 g of peptone (Liofilchem), 2 g of glucose (AppliChem, Darmstadt, Germany), 1 g of mucin (Sigma-Aldrich), 0·35 g of NaCl (AppliChem), 0·2 g of $CaCl_2$ (Riedel-de Haën, Seelze, Germany) and 0·2 g of KCl (Pronalab, Lisbon, Portugal). The pH was adjusted with NaOH (Pronalab) to 6·8.

Candida strains used for the present experiments were *C. albicans* 324LA/94 (provided by the culture collection of Cardiff Dental School, Cardiff, UK) and *C. glabrata* D1 (obtained from the biofilm group of the Centre of Biological Engineering, University of Minho, Braga, Portugal), both being oral clinical isolates. Yeasts were first subcultured on Sabouraud dextrose agar medium (SDA; Liofilchem) at 37°C for 24 h. A cellular suspension of each yeast strain was prepared in Sabouraud dextrose broth (SDB; Liofilchem) medium and incubated overnight at 37°C and 120 rev min^{-1}. Afterwards, the cells were harvested by centrifugation (6500 ***g*** for 5 min at 15°C), washed twice in phosphate-buffered saline (PBS; pH 7) and adjusted to a concentration of 10^7 cells per ml in artificial saliva, using a Neubauer counting chamber.

Determination of minimum inhibitory concentrations (MICs)

MICs were determined for the six colloidal suspensions of SN against both *Candida* strains in accordance with the guidelines of the Clinical Laboratory Standards Institute (CLSI) (M27-A2), as detailed in a previous study (Monteiro *et al.* 2011).

Biofilm formation and treatment with SN

Mature *Candida* biofilms aged 48 h were formed in 96-well microtiter plates according to Silva *et al.* (2010).

Briefly, 200 μl of *Candida* cell suspension (1×10^7 cells per ml in artificial saliva) was placed into each well and incubated at 37°C under 120 rev min^{-1}. Artificial saliva medium was renewed after 24 h. Following *Candida* biofilm formation (48 h), the medium was aspirated and nonadherent cells were removed by washing with 200 μl of PBS. Next, RPMI 1640 medium (200 μl) containing different concentrations (0·4, 1·6, 6·7, 13·5, 27, 54, 108 and 216 μg ml^{-1}) of each SN suspension was then added to the preformed biofilms. The microtiter plates were incubated at 37°C for 24 h in an orbital shaker incubator at 120 rev min^{-1}. After treatment with SN, biofilms were washed once with PBS to remove loosely attached cells. Controls devoid of silver were included in the assay. All assays were performed independently three times in triplicate.

Biofilm quantification

Biomass of *Candida* biofilms treated with SN was evaluated by the crystal violet (CV) staining method. Initially, 200 μl of 99% methanol (Romil, Cambridge, UK) was added to the biofilms for 15 min to allow cell fixation. After, methanol was removed and the microtiter plates were dried at room temperature. Then, 200 μl of CV stain (1% v/v) (Merck) was added into the wells containing biofilms previously fixed. After 5 min, the excess of CV was removed and the plates were washed with deionized water and allowed to dry at room temperature. Afterwards, 200 μl of acetic acid (33% v/v) (Pronalab) was pipetted into each well to dissolve and remove the CV stain from the biofilms. Finally, absorbance was measured at 570 nm. The assays were performed in triplicate and on three separate occasions.

For cultivable counts of *Candida* biofilm cells exposed to SN, biofilms were scraped from the wells and the suspensions obtained were vigorously vortexed for 5 min to disaggregate cells. Serial dilutions (in PBS) of each biofilm cell suspension were prepared and plated on SDA, and the plates were incubated at 37°C. After 24 h, the total number of colony forming units (CFUs) per unit area (log_{10} CFU cm^{-2}) of microtiter plate well was enumerated. For each condition studied, three separate experiments were performed.

Statistical analysis

Biofilm biomass and cultivable cells of the treated groups with SN were analysed using analysis of variance (ANOVA) followed by Bonferroni test. Statistical analyses were performed with the SPSS software (SPSS – Statistical Package for the Social Sciences, Inc., Chicago, IL, USA) with significance of 5%.

Results

Silver colloidal nanoparticles characterization

SN at 540 μg ml^{-1} with different diameters were formed through the reduction of $AgNO_3$ with $Na_3C_6H_5O_7$ followed by stabilization with NH_3 (N) or PVP (P). These procedures led to six different colloidal suspensions of SN, depending on the stabilizing agent used and the particle size: SN5-N, SN5-P, SN10-N, SN10-P, SN60-N and SN60-P. In the UV/Visible analysis (Fig. 1), the absorption peak of SN ranged from 430 to 460 nm, characterizing the nanosized silver particles. The absorption spectra of the SN5-N, SN5-P, SN10-N and SN10-P colloidal suspensions (Fig. 1aI,aII,bI,bII) demonstrated well-defined plasmon bands centred at 430 nm. In the case of SN60-N and SN60-P (Fig. 1cI,cII), the absorption peaks were attained at approximately 460 nm, possibly because of the larger size of the particles. The symmetrical shapes of all plasmon bands in Fig. 1 confirmed the colloidal stability and sharp particle size distribution. These bands have been commonly assigned to nanoparticles having a spherical or spheroidal shape. TEM observations (Fig. 2) indicated that the nanoparticles were well formed, nearly spherical and dispersed, and with mean diameters of 5 (Fig. 2aI,aII), 10 (Fig. 2bI,bII) and 60 nm (Fig. 2cI,cII). Furthermore, it can be noted a few agglomerates of particles mainly when stabilized by PVP (Fig. 2bII,cII).

Minimum inhibitory concentration

MICs of the six SN colloidal suspensions determined against planktonic cells of *Candida* are shown in Table 1. It was possible to observe that the two species of *Candida* were susceptible to all the SN tested in very low concentrations. The lowest MIC values were against *C. albicans* 324LA/94, using SN5-N, SN60-N and SN60-P (0·4–0·8 μg ml^{-1}). Additionally, between the two *Candida* spp. tested, *C. glabrata* D1 was the least susceptible to SN, with MIC values ranging from 0·8 to 3·3 μg ml^{-1}. With regard to the particle size and the stabilizing agents, interestingly, MIC values for each *Candida* species remained almost the same. Solutions of $Na_3C_6H_5O_7$, NH_3 and PVP, used as controls, did not exhibit any antifungal activity on the *Candida* spp. assayed.

Biofilm quantification

The results displayed in Fig. 3a show that SN were not effective in reducing total biomass of *C. albicans* 324LA/94 biofilms. Interestingly, it was possible to note that, at concentrations ranging from 0·4 to 108 μg ml^{-1},

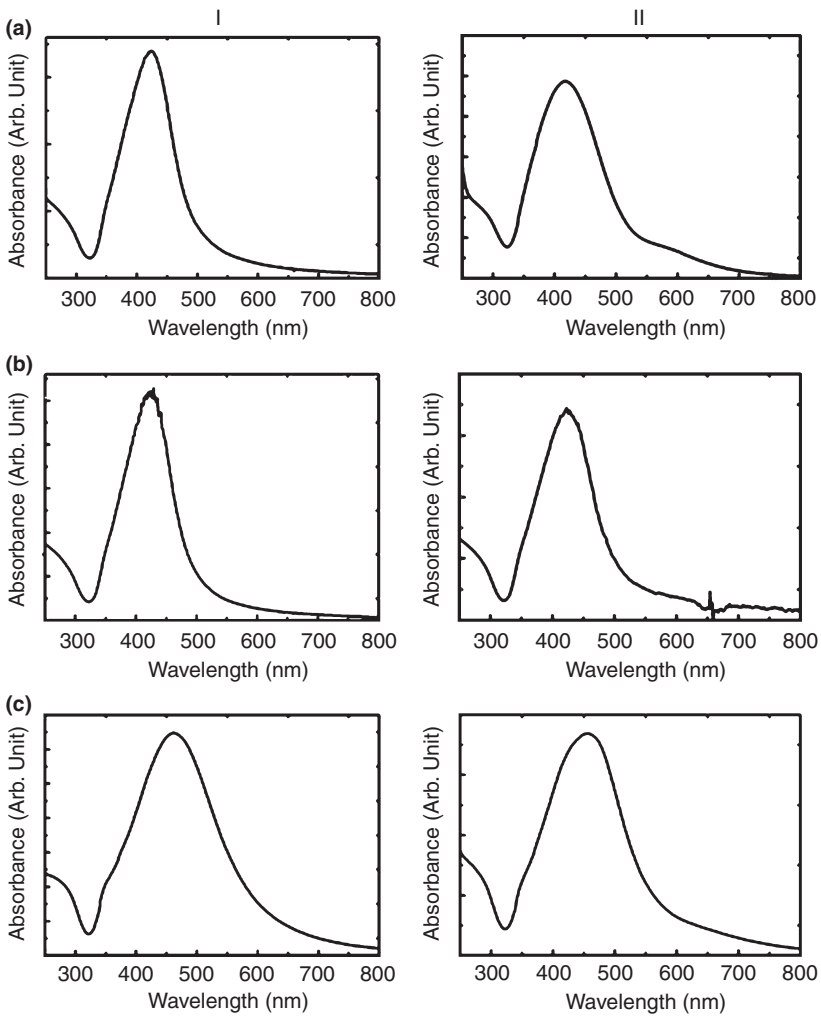

Figure 1 UV-visible spectra of silver nanoparticles (SN) colloidal suspensions with 5 (a), 10 (b) and 60 nm (c), stabilized with ammonia (I) and polyvinylpyrrolidone (II).

all SN colloidal suspensions promoted an increase in biofilm biomass regardless of the silver concentration tested, except for SN5-N. When compared to the control values, the biomass increase was significant for SN10-N at concentrations of 1·6 ($P = 0.001$), 6·7 ($P = 0.003$), 13·5 ($P = 0.004$), 27 ($P = 0.019$) and 216 μg ml^{-1} ($P = 0.002$), and for SN60-N at a concentration of 216 μg ml^{-1} ($P = 0.032$). Curiously, the comparisons of different colloidal suspensions at the same silver concentration showed no statistical differences ($P > 0.05$).

However, on *C. glabrata* D1 (Fig. 3b), all SN colloidal suspensions were very effective in reducing total biomass after 24 h of treatment of mature biofilms (formed for 48 h). When compared to the control groups (biofilms without SN) SN5-N, SN5-P, SN10-N, SN10-P, SN60-N and SN60-P showed significant biomass reduction at silver concentrations greater than or equal to 1·6 ($P = 0.001$), 6·7 ($P = 0.011$), 13·5 ($P = 0.002$), 6·7 ($P = 0.001$), 1·6 ($P = 0.011$) and 6·7 μg ml^{-1} ($P < 0.001$), respectively. In general, *C. glabrata* D1 demonstrated a biofilm biomass reduction dependent on silver concentration, achieving biomass reductions greater than 50% at a concentration of 13·5 μg ml^{-1} and around 90% at a concentration of 108 μg ml^{-1}, for all colloidal suspensions tested.

Regarding the effect of SN on biofilm cultivable cells, Fig. 4 presents mean values and standard deviations of $\log_{10}$ CFU cm^{-2} for both *Candida* species. For *C. albicans* 324LA/94 (Fig. 4a), after 24 h of biofilms treatment with SN colloidal suspensions, it was noted that the smaller particles (SN5-N and SN5-P) had the antifungal activity significantly increased from a concentration of 27 ($P = 0.006$) and 13·5 μg ml^{-1} ($P < 0.015$), respectively. In addition, using SN5-N and SN5-P at a silver concentration of 216 μg ml^{-1}, there was 3·51-$\log_{10}$ ($P < 0.001$) and 3·36-$\log_{10}$ ($P < 0.001$) reduction in the number of CFUs, respectively, compared to the control groups. However, SN10-N similarly to SN10-P, and SN60-N similarly to SN60-P, started significant decrease in the number of CFUs in con-

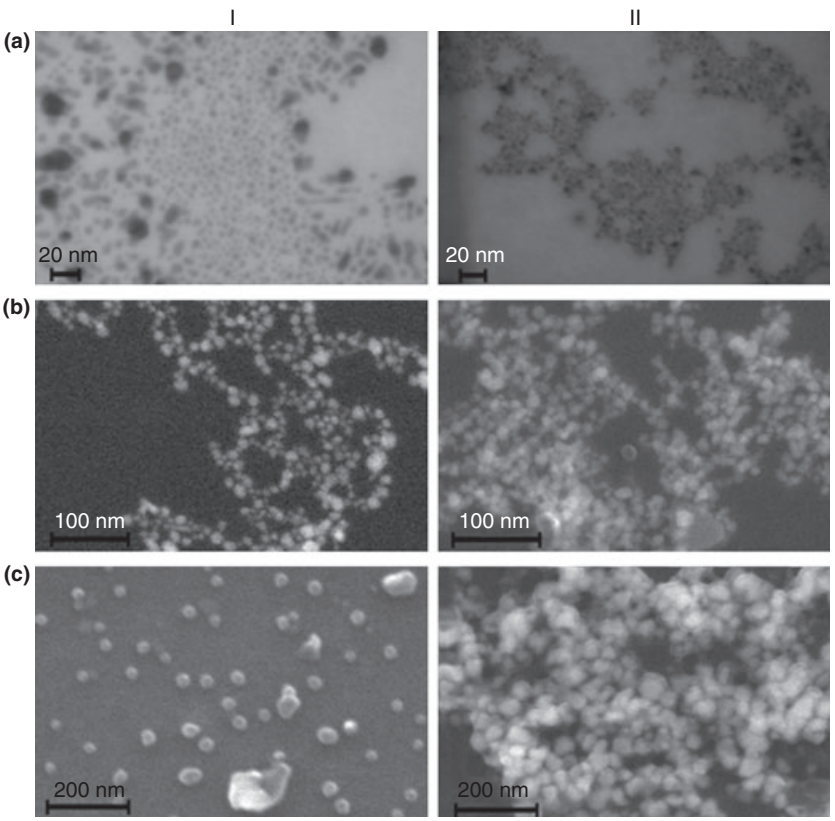

Figure 2 Transmission electron microscopy images of silver nanoparticles (SN) with 5 (a), 10 (b) and 60 nm (c), stabilized with ammonia (I) and polyvinylpyrrolidone (II).

Table 1 Minimum inhibitory concentrations (MICs) of silver nanoparticles (SN) stabilized with ammonia (NH_3) and polyvinylpyrrolidone (PVP) against *Candida* spp.

	MIC (μg ml^{-1})					
	SN stabilized with NH_3			SN stabilized with PVP		
Candida spp.	5 nm	10 nm	60 nm	5 nm	10 nm	60 nm
Candida albicans 324LA/94	0·4–0·8	0·8	0·4	0·8	0·8–1·6	0·4–0·8
Candida glabrata D1	1·6–3·3	1·6	0·8–1·6	1·6–3·3	3·3	1·6–3·3

centrations over 54 ($P = 0.002$; $P = 0.007$) and 27 μg ml^{-1} ($P = 0.027$; $P = 0.015$), respectively, suggesting the type of stabilizing agent was not decisive for antifungal activity against *C. albicans* 324LA/94 biofilms. Comparing the different SN colloidal suspensions within the same silver concentration, no significant statistical differences ($P > 0.05$) were found in the average number of $\log_{10}$ CFUs.

For *C. glabrata* D1 (Fig. 4b), when each SN colloidal suspension was evaluated separately and compared to the control groups, the data showed that the SN5-N ($P = 0.015$) and SN10-P ($P < 0.010$) colloidal suspensions induced a significant decrease in the number of biofilm cells at silver concentrations above 54 μg ml^{-1} while for SN5-P ($P < 0.001$), SN10-N ($P = 0.006$), SN60-N ($P = 0.010$) and SN60-P ($P = 0.009$) this activity was statistically significant for silver concentrations greater than or equal to 27 μg ml^{-1}. SN5-P and SN60-P at silver concentration of 216 μg ml^{-1} exhibited the highest reductions in the average number of CFUs, with reductions of 4·24-$\log_{10}$ ($P < 0.001$) and 3·72-$\log_{10}$ ($P < 0.001$), respectively, when compared to the control groups. On the other hand, comparisons of the different SN colloidal suspensions within the same silver concentration did not indicate significant differences ($P > 0.05$). Thus, in general, according to the biofilm quantification results obtained in this study, the particle size and the type of SN stabilizing agent did not affect the antifungal activity of SN against *Candida* biofilms.

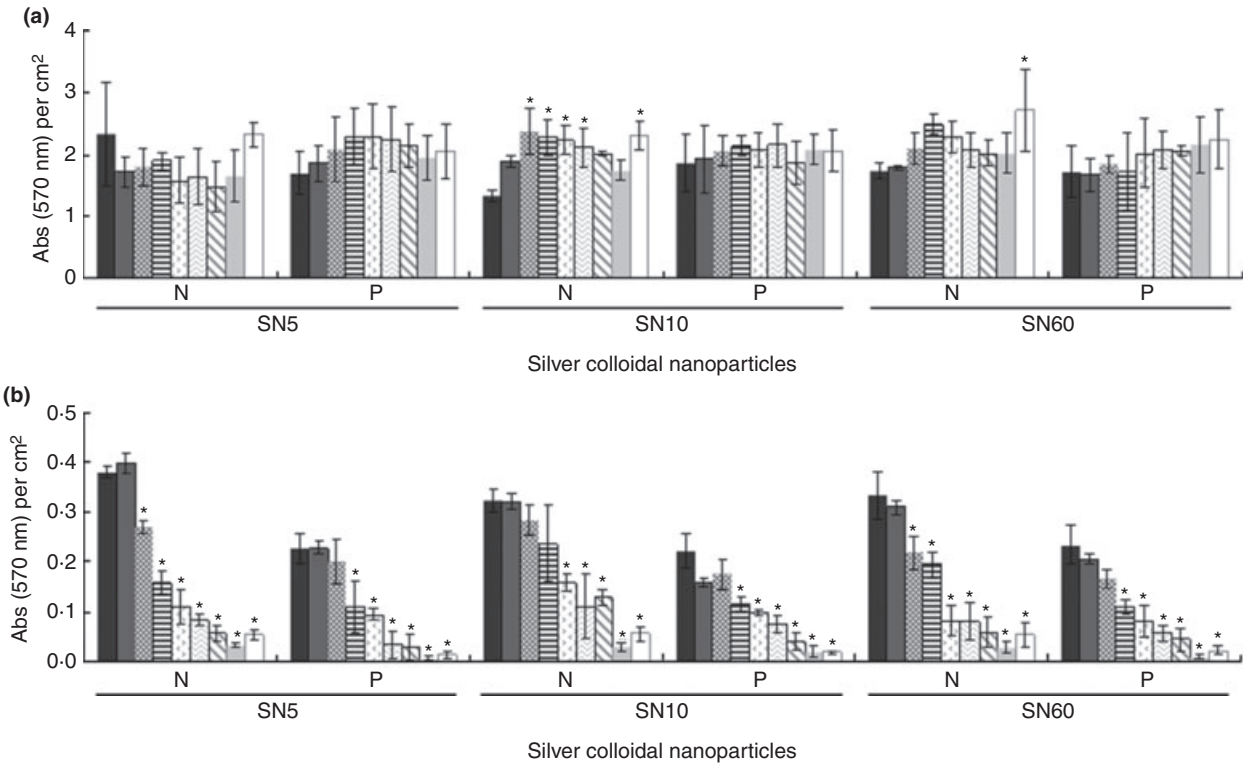

Figure 3 Absorbance values per cm^2 obtained with crystal violet staining assay for *Candida albicans* 324LA/94 (a) and *Candida glabrata* D1 (b) mature biofilms after 24 h of treatment with silver nanoparticles (SN) with 5 (SN5), 10 (SN10) and 60 nm (SN60), which were stabilized using ammonia (N) and plyvinylpyrrolidone (P). Error bars indicate the standard deviations of the means. *$P < 0{\cdot}05$, as compared to the control groups by using a one-way anova followed by Bonferroni test. (a) Silver concentration ($\mu g\ ml^{-1}$): (■) 0; (■) 0·4; (▨) 1·6; (▤) 6·7; (▩) 13·5; (▩) 27; (▧) 54; (▣) 108 and (□) 216. (b) Silver concentration ($\mu g\ ml^{-1}$): (■) 0; (■) 0·4; (▨) 1·6; (▤) 6·7; (▩) 13·5; (▩) 27; (▧) 54; (▣) 108 and (□) 216.

Discussion

Denture stomatitis is a pathogenic state common in complete denture wearers, and its infective causes include some micro-organisms, mainly *C. albicans* and *C. glabrata* (Coco *et al.* 2008). Therefore, the ability of SN to inhibit *Candida* biofilms was evaluated against the aforesaid micro-organisms. The results of the present study did not allow the rejection of the null hypotheses, which are that the nanoparticle size and the type of stabilizing agent would not interfere with SN efficacy in decreasing total biomass and cultivable cells of *Candida* biofilms.

Because SN may not only act in the biofilm cells, but also in their matrix, CV staining assay was performed to quantify the total biomass of *Candida* biofilms. This assay does not allow differentiation between living and dead cells, so it cannot be used for susceptibility testing of biofilms. CV staining was used as a complement to CFU enumeration. From Fig. 3, it is possible to observe that the different SN were very effective against *C. glabrata* D1 biofilms, with significant biomass reduction at silver concentrations greater than or equal to 1·6 $\mu g\ ml^{-1}$ for all conditions (diameter and solution) assayed. For this species, all SN colloidal suspensions showed biomass reductions around 90% at a silver concentration of 108 $\mu g\ ml^{-1}$. However, in general, for *C. albicans* 324LA/94 biofilms, there was some increase in biofilm biomass when compared to untreated biofilms, including different particle sizes and solutions. These differences in the effect of SN on both species could be due to their biofilm architectures, which present several dissimilarities. *Candida glabrata* biofilms are exclusively composed of blastospores and devoid of hyphal cells, while *C. albicans* is a polymorphic organism, able to grow as hyphae, pseudohyphae and blastospores (Silva *et al.* 2011). Accordingly, *C. albicans* biofilms are more complex and difficult to eradicate. Furthermore, the biofilm formation and matrix production processes are dependent upon species, strains and environmental conditions (Silva *et al.* 2011). When biofilms are exposed to stress conditions, physiological changes can occur to protect the cells (Mah and O'Toole

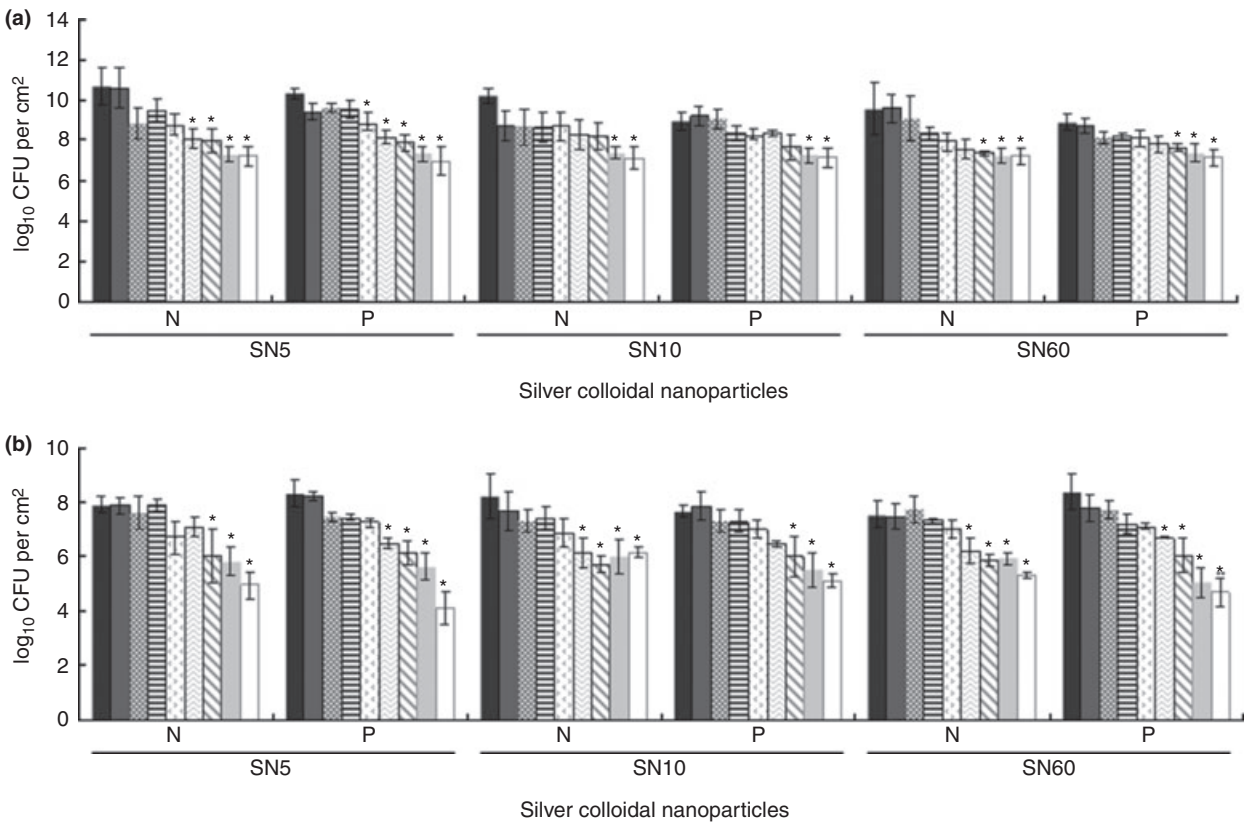

Figure 4 Logarithm of colony forming units per cm^2 obtained for *Candida albicans* 324LA/94 (a) and *Candida glabrata* D1 (b) mature biofilms after 24 h of treatment with silver nanoparticles (SN) with 5 (SN5), 10 (SN10) and 60 nm (SN60), which were stabilized using ammonia (N) and plyvinylpyrrolidone (P). Error bars indicate the standard deviations of the means. *$P < 0{\cdot}05$, when each SN colloidal suspension was evaluated separately and compared to the control groups by using a one-way anova followed by Bonferroni test. (a) Silver concentration ($\mu g\ ml^{-1}$): (■) 0; (■) 0·4; (▨) 1·6; (▤) 6·7; (▨) 13·5; (▨) 27; (▧) 54; (▩) 108 and (□) 216. (b) Silver concentration ($\mu g\ ml^{-1}$): (■) 0; (■) 0·4; (▨) 1·6; (▤) 6·7; (▨) 13·5; (▨) 27; (▧) 54; (▩) 108 and (□) 216.

2001). This may suggest an increased production of exopolymeric substances by *C. albicans* 324LA/94 biofilms in the presence of SN, which could explain the increase in total biomass observed in Fig. 3a, accompanied by a slight decrease in the number of viable biofilm cells.

Moreover, the silver concentrations above which significant reductions were detected in the number of biofilm cells of *C. albicans* 324LA/94 (Fig. 4a) and *C. glabrata* D1 (Fig. 4b), were about 17–135 and 16·5 to 34-fold higher, respectively, than the corresponding MICs (Table 1), confirming the lower susceptibility of biofilm cells. These MIC results revealed that all SN colloidal suspensions presented similar behaviours, all being fungicidal (in concentrations ranging from 0·4 to 3·3 $\mu g\ ml^{-1}$) against the two *Candida* isolates when in the planktonic growth mode (Table 1). Additionally, these fungicidal activities were confirmed by plating the content of each well on SDA. The data obtained are in agreement with the previously published results by Hawser and Douglas (1995). These authors found that all of the antifungal agents tested (amphotericin B, flucytosine, fluconazole, itraconazole and ketoconazole) showed much less activity against 48-h *C. albicans* biofilms than against planktonic cells. The drug concentrations that caused 50% inhibition in biofilms were 30–2000 times higher than the relevant MICs (Hawser and Douglas 1995). In fact, *C. albicans* and *C. glabrata* form recalcitrant biofilms which are difficult to eradicate (Lewis 2001), because of increasing tolerance to conventional antifungal therapy (Silva *et al.* 2012). According to Silva *et al.* (2012), the mechanisms of biofilm drug resistance/tolerance are not fully understood, but can be considered a multifactorial phenomenon. In combination or alone, these mechanisms include decreased growth rate (Lewis 2001), heterogeneity, expression of resistance genes and presence of 'persister' cells within the biofilm (Lewis 2001; Mah and O'Toole 2001). In addition, although some SN may diffuse inside the matrix through the water channels present in the biofilm

architecture, the micro-organisms present on the deeper layers may escape the treatment as the matrix hampers the drug diffusion (Kalishwaralal *et al.* 2010).

Regarding cell viability (Fig. 4), although all SN presented a similar behaviour, they were more effective on *C. glabrata* D1 than against *C. albicans* 324LA/94 biofilms. However, for both species in general, a significant reduction in the number of biofilm viable cells for silver concentrations at or higher than 13·5 μg ml^{-1} was observed. Interestingly, the highest silver concentration (216 μg ml^{-1}) did not have a significantly greater antibiofilm effect than the lowest concentrations (54 and 108 μg ml^{-1}), characterizing a relatively weak dose–response, except for SN5-P against *C. glabrata* D1, highlighting the similarities between the particles. Accordingly, these findings also suggest that it may not be necessary to use the highest silver concentrations.

SN colloidal suspensions were prepared with NH_3 and PVP as stabilizers. NH_3 stabilizes metallic SN by the formation of soluble diammine silver (I) complexes, which trap free silver ions responsible for particle growth (Gorup *et al.* 2011). The polymers of the PVP group bond on the SN surfaces through the nitrogen atom in their molecule (Kvítek *et al.* 2008), resulting in flocculation. Kvítek *et al.* (2008) reported that despite the flocculation process, the SN stabilized by PVP are separated from each other through the chain of the polymer molecule, and these SN can interact with the cell wall of a micro-organism because of their high surface energy and mobility. Furthermore, according to the literature (Baker *et al.* 2005), smaller SN are more effective than larger particles, because of a larger surface area available for interaction with cells. However, as referred, CV staining and CFU analysis (Figs 3 and 4) showed that the particle size range assayed and the type of stabilizing agent did not interfere with the antifungal activity of SN against *Candida* biofilms. Choi *et al.* (2010) demonstrated, through laser-scanning confocal microscopic observations, that the interactions of SN with *Escherichia coli* biofilm cells resulted in particle aggregation with a final average aggregate size of about 800 nm (an increase by a factor of 40). Moreover, Stewart and Franklin (2008) reported that biofilm features, such as oxygen availability, substrate, pH range and extracellular polymeric substances composition, may influence aggregation, dissolution and diffusive transport of SN. Taken together, all these observations reinforce the idea that SN aggregation may have occurred and, consequently, causing an increase in particle size. Probably due to this fact, it was not possible to observe any significant difference among SN colloidal suspensions and an inverse relationship between SN size and antifungal activity.

Furthermore, it would be reasonable to hypothesize that the aggregation of SN within the biofilms led to a particle size which conflicted extensively from that originally synthesized. This fact disallowed discriminating the effect of particle size and stabilizing agent on *Candida* biofilms. These observations emphasize that the original particle size may be a poor display of true nanoparticle size in biofilms and therefore also of antifungal activity. In other words, the results of the present study imply that biofilms are influencing by the aggregation of SN and that the size of nanoparticles and the type of stabilizing agent were not crucial to their positive effect on *Candida* biofilms. Possibly, only the well dispersed SN may act against *Candida* biofilms effectively.

In summary, the particle size and the type of stabilizing agent do not interfere with the *in vitro* antimicrobial efficacy of SN. On the basis of these findings, additional investigations with silver colloidal nanoparticles will be needed regarding their therapeutic potential, namely to assess formulation and delivery means.

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