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ORIGINAL ARTICLE

Silver colloidal nanoparticles: effect on matrix composition and structure of *Candida albicans* and *Candida glabrata* biofilms

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Abstract

Aim: The aim of this study was to assess the effect of different silver nanoparticles (SN) concentrations on the matrix composition and structure of *Candida albicans* and *Candida glabrata* biofilms.

Methods and Results: *Candida* biofilms were developed in 6-well microtiter plates during 48 h. After, these biofilms were exposed to 13·5 or 54 μg SN ml^{-1} for 24 h. Then, extracellular matrices were extracted from biofilms and analysed chemically in terms of proteins, carbohydrates and DNA. To investigate the biofilm structure, scanning electron microscopy (SEM) and epifluorescence microscopy were used. SN interfered with the matrix composition of *Candida* biofilms tested in terms of protein, carbohydrate and DNA, except for the protein content of *C. albicans* biofilm. By SEM, *Candida* biofilms treated with SN revealed structural differences, when compared with the control groups. Further, SN showed a trend of agglomeration within the biofilms. Epifluorescence microscopy images suggest that SN induced damage on cell walls of the *Candida* isolates tested.

Conclusions: In general, irrespective of concentration, SN affected the matrix composition and structure of *Candida* biofilms and these findings may be related to the mechanisms of biocide action of SN.

Significance and Impact of the Study: This study reveals new insights about the behaviour of SN when in contact with *Candida* biofilms. SN may contribute to the development of therapies to prevent or control *Candida* infections.

Introduction

Candida pathogenesis is associated with biofilm formation (Vediyappan *et al.* 2010), and *Candida* spp. can colonize tissues or inert surfaces, such as oral epithelia and dental prostheses, respectively (Kojic and Darouiche 2004; Ramage *et al.* 2006). An example of oral infection caused by *Candida* biofilms is the *Candida*-associated denture stomatitis, which is characterized by the presence of biofilm in the space between the denture base and the mucosa (Budtz-Jorgensen 2000). *Candida albicans* and *Candida glabrata* have been recognized as the most prevalent microorganisms found in denture stomatitis (Coco *et al.* 2008). Usually, *Candida* biofilms are resistant to host defence mechanisms and to antifungal drugs, representing a continuous source of infection (Baillie and Douglas 2000). One of the factors that may be associated with antifungal resistance of *Candida* biofilms is the presence of extracellular matrix (Mah and O'Toole 2001).

According to Flemming *et al.* (2007), the biofim matrices or extracellular polymeric substances (EPS) 'are biopolymers of microbial origin in which biofilm microorganisms are embedded'. This matrix is composed of a wide variety of proteins, carbohydrates and phosphate (Baillie and Douglas 2000; Blankenship and Mitchell 2006). In addition, extracellular DNA has also been mentioned as a component of the biofilm matrices (Martins *et al.* 2010).

The composition of extracellular material varies depending on the growth medium, environmental conditions and species (Conover *et al.* 2011). Al-Fattani and Douglas (2006) reported that *C. albicans* biofilm matrix consisted of carbohydrate (32·2% glucose), small amounts of proteins (5·0%), hexosamine (3·3%), phosphate (0·5%) and uronic acid (0·1%), while the matrix from *Candida tropicalis* biofilms consisted mainly of hexosamine (27·4%), carbohydrates (3·3%, including 0·5% glucose), proteins (3·3%), phosphate (0·2%) and uronic acid (1·6%). A study by Silva *et al.* (2009) demonstrated that the biofilm matrices of *C. glabrata* had higher concentrations of proteins and carbohydrates when compared with other species (*C. tropicalis* and *Candida parapsilosis*). On the other hand, antifungal drugs may bind to beta-glucans, which are constituents of the extracellular matrix of *Candida* biofilms, preventing the drug from reaching its target (Vediyappan *et al.* 2010). Thus, strategies that focus on eradication of matrix components may contribute to the control of infections related to biofilms.

Currently, nanotherapeutics have been used to control biofilm formation through the incorporation of nanoparticles into several materials (Monteiro *et al.* 2009, 2012a; Allaker 2010; Cheng *et al.* 2012; Wady *et al.* 2012). In this context, silver nanoparticles (SN) were reported as antimicrobial agents against bacteria (Sondi and Sondi 2004) and fungi (Kim *et al.* 2009; Panácek *et al.* 2009), and their mechanism of action is multifactorial. These nanoparticles preferably bind to sulphur-containing proteins, thereby forming pores in the cell wall and membranes and leading to loss of intracellular contents. Furthermore, nanoparticles attack respiratory chain enzymes, causing cell disintegration, and interact with phosphate in the DNA, preventing cell division (Monteiro *et al.* 2009; Rai *et al.* 2009).

According to literature data, SN were able to prevent biofilm formation by *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* (Kalishwaralal *et al.* 2010). These nanoparticles were more effective in reducing biofilm biomass and number of colony forming units (CFU) when applied to adhered cells than to mature *Candida* biofilms (Monteiro *et al.* 2011). Moreover, the size of SN and the type of stabilizing agent were not crucial to their efficacy against *C. albicans* and *C. glabrata* biofilms (Monteiro *et al.* 2012b). Although the effect of SN on *Candida* biofilm biomass and on the number of CFUs has been reported, there are no studies on the effect of these nanoparticles in the matrix composition and structure of *Candida* biofilms. Therefore, the main aim of this study was to evaluate the effect of different SN concentrations on the matrix composition (protein, carbohydrate and DNA content) and the structure (using Scanning Electron Microscopy (SEM) and Epifluorescence Microscopy) of *C. albicans* and *C. glabrata* biofilms.

Materials and methods

Preparation of silver colloidal nanoparticles

For the preparation of silver colloidal nanoparticles, all chemicals were obtained from Merck KGaA, Darmstadt, Hesse, Germany. These nanoparticles were synthesized by a process that involves reduction of silver nitrate ($AgNO_3$) with sodium citrate ($Na_3C_6H_5O_7$) and their stabilization with ammonia (NH_3), as evidenced earlier (Gorup *et al.* 2011; Monteiro *et al.* 2011, 2012a,b). Briefly, a solution of $AgNO_3$ ($5{\cdot}0 \times 10^{-3}$ mol l^{-1}) in deionized water was brought to boiling and an aqueous solution of $Na_3C_6H_5O_7$ was added to the $AgNO_3$ solution. The solution turned yellow and a colloidal suspension was formed. Next, the silver colloids were stabilized by adding of 7·5 ml of an ammonia solution (1·4 mol l^{-1}). The silver colloidal nanoparticles suspensions were characterized as detailed in our previous studies (Gorup *et al.* 2011; Monteiro *et al.* 2011, 2012a,b), and SN having an average particle size of 5 nm were used.

Microorganisms and preparation of *Candida* cell suspension

The composition (per 1 l of deionized water) of artificial saliva medium (Lamfon *et al.* 2003) used in this study was as follows: 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, St Louis, MO, USA), 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 to 6·8.

An oral clinical isolate (324LA/94) of *C. albicans*, provided by the culture collection of Cardiff Dental School, Cardiff, UK, and an oral clinical isolate (D1) of *C. glabrata*, obtained from the biofilm group of the Centre of Biological Engineering, University of Minho, Braga, Portugal, were used in all of the experiments. All *Candida* strains were stored as frozen stocks with 20% glycerol at

−80°C until required. To prepare the *Candida* cell suspension for biofilm growth, clinical isolates were subcultured on Sabouraud dextrose agar (SDA, Liofilchem) plates for 24 h at 37°C and then, a loopful of the *Candida* colonies was transferred into 30 ml of Sabouraud dextrose broth (Liofilchem) and incubated overnight at 37°C in a rotary shaker (at 120 rev min^{-1}). Afterwards, *Candida* cells were harvested by centrifugation (6500 ***g*** for 5 min at 15°C), washed twice with 30 ml of phosphate buffered saline (PBS; pH 7), enumerated using a Neubauer chamber and then adjusted to a concentration of 10^7 cells ml^{-1} in artificial saliva medium. These *Candida* cell suspensions were used in the subsequent assays.

Biofilm formation and treatment with silver nanoparticles

Biofilm formation was performed as described by Silva *et al.* (2009). Briefly, 4 ml of standard cell suspensions of yeasts, prepared as above, were added into each well of a 6-well polystyrene microtitre plate (Orange Scientific, Braine-l'Alleud, Belgium), and the plate incubated with shaking (120 rev min^{-1}) for 48 h at 37°C. After 24 h, an aliquot of 2 ml of artificial saliva medium was removed and an equal volume of fresh artificial saliva was added. After the incubation period, non-adherent cells were removed by washing with 4 ml of PBS.

Stock suspensions of silver colloidal nanoparticles were diluted in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich) to obtain two different concentrations of silver: 54 and 13·5 $\mu g\ ml^{-1}$. These SN concentrations were based on a previous study (Monteiro *et al.* 2011) in which it was verified some effect against *Candida* biofilms after exposure to silver concentrations at or higher than 13·5 $\mu g\ ml^{-1}$. A volume of 4 ml of each dilution was transferred to the wells of the 6-well polystyrene microtitre plate, containing pre-formed biofilms. In control experiments, RPMI 1640 medium without SN was added to the wells. The plates were incubated at 37°C for 24 h with shaking (120 rev min^{-1}). After treatment with SN, biofilms were washed once with 4 ml of PBS to remove loosely attached cells.

Biofilm matrix extraction

After treatment with SN, the biofilm matrix was extracted in accordance with a previously described procedure (Silva *et al.* 2009). Biofilms were scraped from the wells of 6-well microtitre plates using sterilized cell scrapers (Orange Scientific) and resuspended with PBS. Following, the biofilm samples were sonicated (Ultrasonic Processor, Cole-Parmer, IL, USA) for 30 s at 30 W, and the suspension was vortexed for 2 min. Finally, the suspension was centrifuged at 3000 ***g*** for 10 min at 4°C and the supernatant filtered through a 0·45 μm nitrocellulose filter (Orange Scientific).

Biofilm dry weight determination

For this assay, biofilms removed from the 6-well plates were filtered on 0·45 μm sterilized membrane (Pall Corporation, New York, MI, USA) and dried at 60°C until a constant dry weight. The membrane was also weighed before biofilm filtering. Thus, biofilm dry weight was obtained as the difference between the two measurements.

Protein, carbohydrate and DNA quantification

The protein content was determined using the BCA Kit (Bicinchoninic Acid; Sigma-Aldrich), using bovine serum albumin (BSA) as the standard (Silva *et al.* 2009). Briefly, 25 μl of the suspension obtained after biofilm matrix extraction was transferred into wells of 96-well microtitre plates containing 200 μl of the mixture of BCA kit reagents. The plates were incubated at 37°C for 30 min and the absorbance read in a microtitre plate reader (Bio-Tek Synergy HT, Izasa, Lisbon, Portugal) at 562 nm.

The total carbohydrate content was measured according to the procedure of Dubois *et al.* (1956), using glucose as a standard. Thus, into a glass tube, 500 μl of the suspension obtained after biofilm matrix extraction was mixed with 500 μl of 9% phenol (Panreac, Barcelona, Spain) and 2·5 ml of sulfuric acid (95–97%, Applichem). The solution was homogenized by vortexing, and after 15 min at room temperature, the absorbance was read at 490 nm.

To assess the DNA content in biofilm matrix, a small volume (1·5 μl) of the suspension obtained after biofilm matrix extraction was spectrophotometrically analysed (at 260 nm and 280 nm) using a Nanodrop Spectrophotometer (Nanodrop 1000; Thermo Scientific, Lisbon, Portugal).

Total proteins, carbohydrates and DNA contents were expressed as a function of the biofilm dry weight (mg g^{-1} of biofilm dry weight). Three to six separate experiments were carried out for each condition studied.

Biomass and cultivable cells quantification

Biofilm biomass, obtained after treatment with silver colloidal nanoparticles, was evaluated by the crystal violet (CV) staining method, as previously described (Silva *et al.* 2009; Monteiro *et al.* 2011). Briefly, biofilms were fixed with 4 ml of 99% methanol (Romil, Cambridge, UK). After 15 min, the methanol was removed and the microtitre plates were allowed to air-dry. *Candida* biofilms were stained with 4 ml

of CV stain (1% v/v) (Merck) for 5 min. The plates were washed with deionized water, allowed to dry at room temperature, and then the CV was removed by adding 4 ml of acetic acid (33% v/v) (Pronalab) into each well. Afterwards, 200 μl of the solution was transferred to a 96-well microtitre plate and the absorbance was measured at 570 nm. The experiments were carried out in triplicate and on three separate occasions.

In addition, biofilms exposed to SN were scraped from the microtitre plate wells and 100 μl samples were taken to culturability assessment. Appropriate dilutions of biofilm cell suspension were plated on SDA, in triplicate. The SDA plates were incubated at 37°C, and after 24 h, the number of CFUs per unit area of wells was enumerated. At least three independent assays were performed.

Biofilm structure

Candida biofilm structure was visualized by SEM. First, *Candida* biofilms were developed within the 24-well microtitre plates by dispensing standardized yeasts cell suspensions (1 ml of a suspension containing 10^7 cells ml^{-1} in artificial saliva) into each well and incubated for 48 h at 37°C in a shaker at 120 rev min^{-1}. After treatment with different SN concentrations (54 and 13·5 μg ml^{-1}) for 24 h, the RPMI 1640 medium was aspirated and each well was rinsed gently with PBS. Subsequently, samples were dehydrated by washing in a series of ethanol (70% for 10 min, 95% for 10 min and 100% for 20 min), and finally air-dried in a desiccator. Afterwards, samples were mounted onto aluminium stubs, sputter coated with gold and viewed under a S-360 scanning electron microscope (Leo, Cambridge, MA, USA).

Biofilm cells structure was also observed by Epifluorescence Microscopy. After treatment with SN, biofilm cells were fixed overnight at room temperature and stained with Calcofluor (Calcofluor White Stain; Sigma-Aldrich) (diluted 1 : 100 in ultra pure water) during 15 min. Prior to the microscopy observation, the cells were thoroughly washed with deionized water and observed using an Epifluorescence Microscope (BX51; Olympus, Tokyo, Japan) equipped with a CCD camera (DP71; Olympus) and filter capable of detecting the yeast cell wall (BP 365–370, FT 400, LP 421).

Statistical analysis

The results of triplicate experiments conducted for each of the above assays were analysed applying one-way ANOVA followed by Tukey multiple comparison *post hoc* test, using SPSS software (SPSS – Statistical Package for the Social Sciences, Inc., Chicago, IL, USA). $P < 0{\cdot}05$ was considered statistically significant.

Results

Protein, carbohydrate and DNA quantification

Table 1 summarizes the results of quantitative analysis of total proteins, carbohydrates and DNA extracted from *Candida* biofilm matrices after treatment with different SN concentrations. For *C. albicans* 324LA/94, the protein content of biofilm matrices was completely unaffected by SN treatment ($P = 0{\cdot}935$, ANOVA). On the other hand, the treatment with 13·5 and 54 μg SN ml^{-1} for 24 h increased the total carbohydrate content, but this increase, when compared with the control group, was only significant (approx. 71%, $P = 0{\cdot}017$) for the SN concentration of 54 μg ml^{-1}. There was a significant reduction in *C. albicans* 324LA/94 biofilm DNA content when treated with 13·5 μg SN ml^{-1} compared to the control group (approx. 36%, $P = 0{\cdot}002$). However, there was no significant difference in the DNA content between the control group and the biofilm treated with 54 μg SN ml^{-1}.

For *C. glabrata* D1, the matrix of biofilm treated with 13·5 μg SN ml^{-1} had a higher amount of proteins and DNA than the control group ($P = 0{\cdot}001$; $P = 0{\cdot}029$) and the group treated with 54 μg SN ml^{-1} ($P < 0{\cdot}001$; $P < 0{\cdot}001$). It was also observed that the matrix of biofilms treated with 54 μg SN ml^{-1} contained less total proteins (approx. 96%, $P = 0{\cdot}039$) and DNA (approx. 33%, $P = 0{\cdot}009$) compared to the control group. In addition, biofilm matrix of *C. glabrata* D1 treated with 13·5 μg SN ml^{-1} contained less total carbohydrates than the control group. However, interestingly, after treatment with 54 μg SN ml^{-1} occurred a significant increase regarding the total carbohydrates content compared to the control group (approx. 158%, $P < 0{\cdot}001$) and to the group treated with 13·5 μg SN ml^{-1} (approx. 270%, $P < 0{\cdot}001$).

Biomass and cultivable cells quantification

The efficacy of SN colloidal suspensions in reducing total biomass and the number of CFUs of *Candida* biofilms are shown in Fig. 1. According to Fig. 1(a), when compared with the control groups (biofilms without SN), *C. albicans* 324LA/94 and *C. glabrata* D1 achieved biofilm biomass reductions around 50% ($P < 0{\cdot}001$) and 47% ($P = 0{\cdot}025$) at a silver concentration of 13·5 μg ml^{-1} and around 54% ($P < 0{\cdot}001$) and 90% ($P = 0{\cdot}001$) at a concentration of 54 μg ml^{-1}, respectively.

With regard to biofilm cultivable cells (Fig. 1b), it was possible to observe that the treatment (during 24 h) with SN colloidal suspensions decreased the number of CFUs for *C. albicans* 324LA/94; however, only the concentration of 54 μg ml^{-1} showed a significant statistical

Table 1 Mean results and standard deviation for the amounts of each matrix component of *Candida* biofilms obtained after treatment with two different silver nanoparticles concentrations

Matrix composition (mg g^{-1} of biofilm dry weight)	*Candida albicans 324LA/94*			*Candida glabrata D1*		
	Silver concentration (μg ml^{-1})			Silver concentration (μg ml^{-1})		
	0	13·5	54	0	13·5	54
Proteins	27·28 ± 6·34	27·37 ± 3·25	28·56 ± 4·09	8·67 ± 0·40	27·93 ± 5·41*	0·34 ± 0·02*
Carbohydrates	202·33 ± 52·05	222·64 ± 30·24	346·77 ± 47·50*	238·94 ± 73·72	166·84 ± 42·36	617·88 ± 37·86*
DNA	15·02 ± 1·38	9·66 ± 0·38*	14·20 ± 1·01	10·07 ± 1·27	12·60 ± 0·66*	6·73 ± 0·54*

*$P < 0·05$, as compared to the control group, using one-way ANOVA with Tukey *post hoc* test.

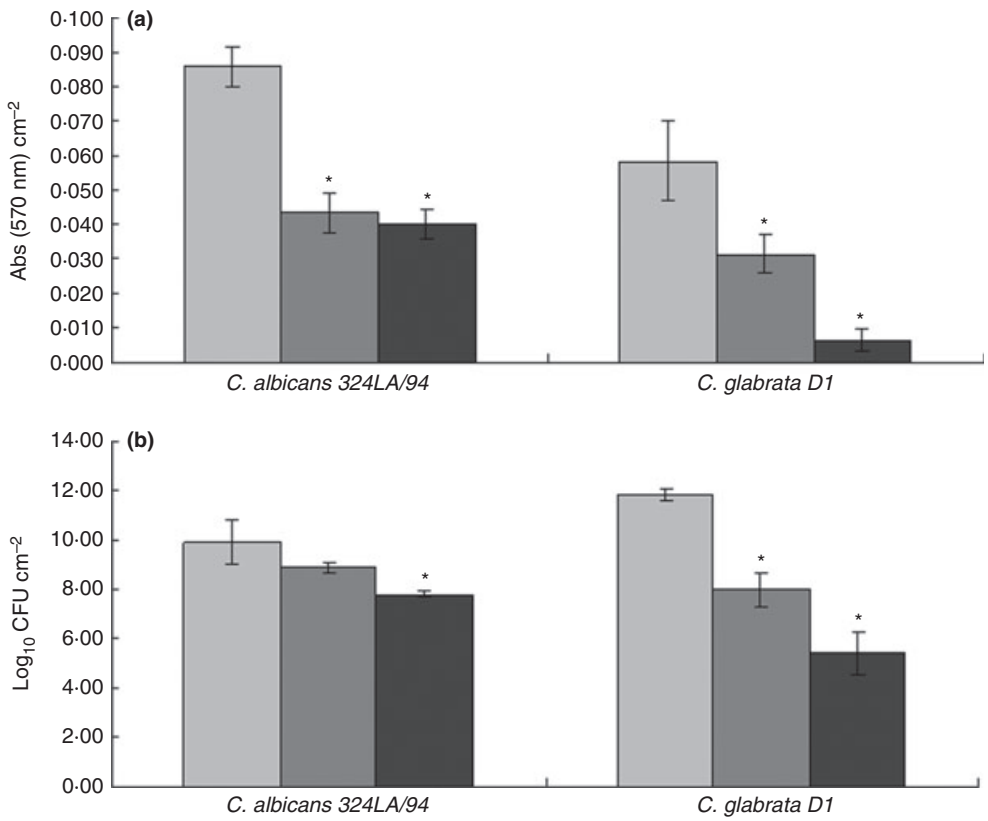

Figure 1 Absorbance values per cm^2 (a), obtained with crystal violet staining assay, and logarithm of colony forming units per cm^2 (b) for *Candida albicans* 324LA/94 and *Candida glabrata* D1 biofilms treated with two different silver nanoparticles concentrations. Error bars denote the standard deviations of the means. *$P < 0·05$, as compared to the control groups by using one-way ANOVA with Tukey *post hoc* test. Silver concentration (μg ml^{-1}): (□) 0; (▩) 13·5 and (■) 54.

decrease (reduction of 2·12−$\log_{10}$; $P = 0·008$) when compared with the control group. For *C. glabrata* D1 biofilms, the effect of SN was more pronounced, with decreases of 3·86−$\log_{10}$ ($P = 0·001$) and 6·46−$\log_{10}$ ($P < 0·001$) at silver concentrations of 13·5 and 54 μg ml^{-1}, respectively. Furthermore, for *C. glabrata* D1 biofilms, the reduction in total biomass (Fig. 1a) and in the number of CFUs (Fig. 1b) were dependent on silver concentration and there were statistically significant differences ($P = 0·020$ and $P = 0·007$, respectively) between the silver concentrations tested.

Biofilm structure

Biofilm structure was monitored by SEM (Fig. 2). In the control (Fig. 2ia), *C. albicans* 324LA/94 biofilm consisted of a mixture of yeasts and hyphae surrounded by a thick extracellular matrix. The treatment with 13·5 μg SN ml^{-1}

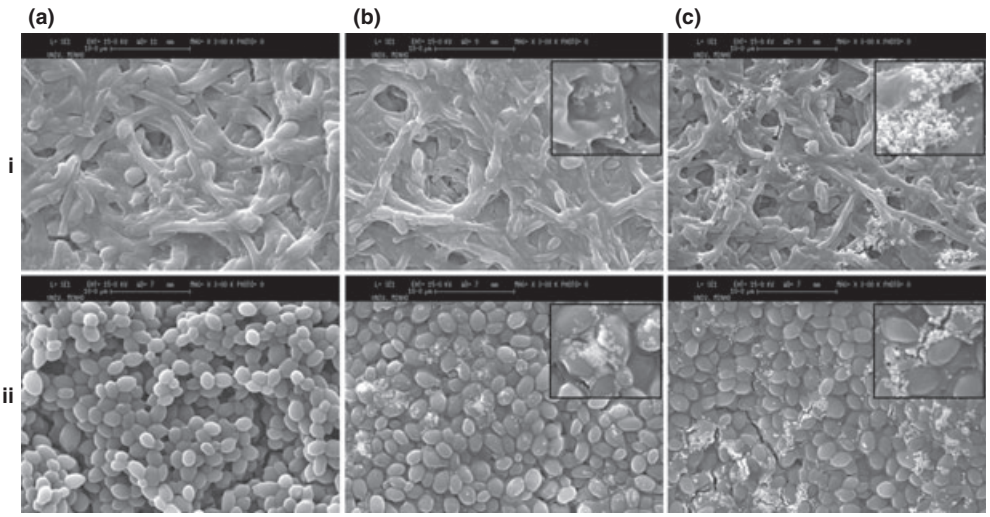

Figure 2 Scanning electron microscopy images showing the structure of *Candida albicans* 324LA/94 (i) and *Candida glabrata* D1 (ii) mature biofilms (48 h) under different experimental conditions: (a) control group, (b) experimental group (mature biofilms treated during 24 h with silver nanoparticles at 13·5 μg ml^{-1}) and (c) experimental group (mature biofilms treated with silver nanoparticles at 54 μg ml^{-1}). Note agglomerated silver nanoparticles in an enlarged view of part of biofilms (images ib, ic, iib and iic).

(Fig. 2ib) did not have any visible effect on the structure of *C. albicans* 324LA/94 biofilm, and similarly to that observed in Fig. 2(ia), this biofilm synthesized large amounts of extracellular matrix material and the fungal cells were almost hidden by the matrix. By contrast, the treatment of *C. albicans* 324LA/94 biofilm with 54 μg SN ml^{-1} (Fig. 2ic) resulted in a much less compact biofilm and lower amounts of extracellular polymeric material, when compared with the control group.

Interestingly, the structure of *C. glabrata* D1 biofilms was affected by SN. In the control group (Fig. 2iia), this strain revealed a multilayer biofilm composed entirely of yeasts. However, after treatment with SN in both concentrations of 13·5 (Fig. 2iib) and 54 μg ml^{-1} (Fig. 2iic), the biofilms displayed a more compact structure than the control group and having a thinner layer of yeasts covering the surface. Additionally, it was possible to observe some clusters of SN (Fig. 2ib,ic,iib,iic) attached to the *Candida* biofilms matrices and to the fungal cells.

Epifluorescence microscopy was also used to screen for structural differences between biofilm cells in the absence or in the presence of SN. The fluorescent dye Calcofluor white used in this assay binds to the fungal cell wall allowing its visualization. As observed by SEM, mature *Candida* biofilms, under different experimental conditions (without SN and after treatment with SN) consisted of a network of cells of all morphologies: yeasts and filamentous forms (pseudohyphae and hyphae) for *C. albicans* 324LA/94 biofilms and only yeasts for *C. glabrata* D1 biofilms, as shown in Fig. 3. Moreover, in general, increasing the silver concentration, the amount of fluorescence and cell walls stained with Calcofluor white decreased, for both biofilms (Fig. 3), suggesting that SN induced damage to cell walls of *Candida* biofilms.

Discussion

In the study described here, *C. glabrata* D1 biofilm matrix treated with SN showed significant modifications in the protein and DNA contents compared to untreated biofilm. At a silver concentration of 13·5 μg ml^{-1}, it was possible to observe statistically significant increases in the protein and DNA contents of *C. glabrata* D1 biofilm matrix, when compared with the control group (Table 1), probably due to fungal cell lysis and consequent release of intracellular contents. However, the treatment with the highest SN concentration (54 μg ml^{-1}) showed significant reductions in the matrix protein and DNA contents compared with the control group (Table 1). As silver has affinity for proteins (Furno *et al.* 2004) and for phosphate groups present in the DNA molecule (Rai *et al.* 2009), one reason for the results obtained may be the precipitation of the protein and DNA contents of extracellular matrix during its exposure to SN at high concentration. Thus, SN at 54 μg ml^{-1} were able to promote cell lysis, release of intracellular constituents, and precipitation of a part of released protein and DNA.

Surprisingly, the phenomena described above were not observed for *C. albicans* 324LA/94. The total protein content of this biofilm matrix was not affected by treatment with SN. In addition, *C. albicans* 324LA/94 biofilm exposed to 13·5 μg SN ml^{-1} demonstrated a significant

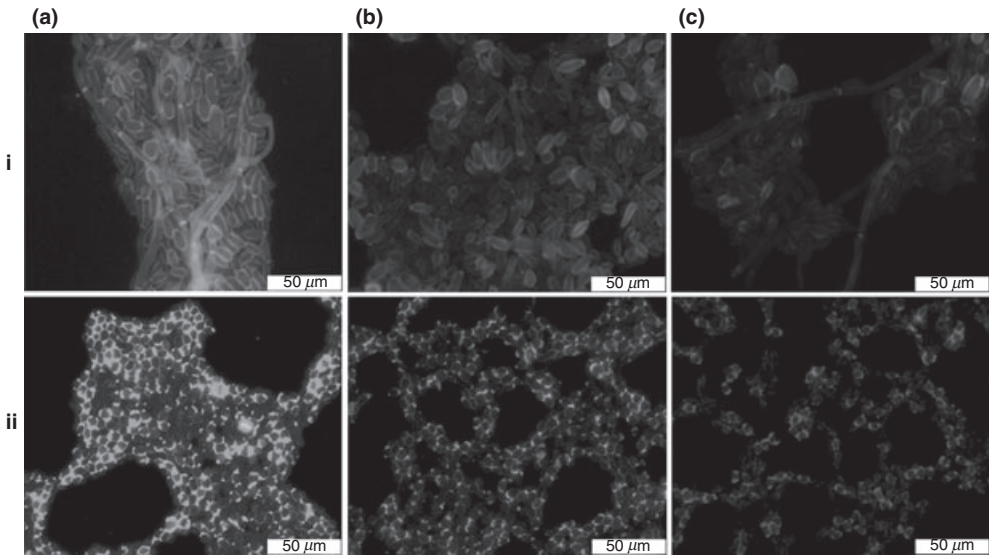

Figure 3 Epifluorescence microscopy images showing the structure of *Candida albicans* 324LA/94 (i) and *Candida glabrata* D1 (ii) mature biofilms (48 h) under different experimental conditions: (a) control group, (b) experimental group (mature biofilms treated during 24 h with silver nanoparticles at 13·5 μg ml^{-1}) and (c) experimental group (mature biofilms treated with silver nanoparticles at 54 μg ml^{-1}).

decrease in DNA content compared to the control, while the group treated with SN at 54 μg ml^{-1} did not differ from the control. DNA can be released into extracellular matrix by dead cells (Mulcahy *et al.* 2008), from DNA containing outer membrane vesicles (Schooling and Beveridge 2006), or can be regulated by quorum sensing (Allesen-Holm *et al.* 2006). As *C. albicans* biofilms are more complex than those of *C. glabrata*, the results obtained for protein and DNA contents of *C. albicans* 324LA/94 biofilm might result from the increased or decreased expression of genes involved, for example, in amino acid and phosphate biosyntheses, in response to SN exposure. In this regard, Vediyappan *et al.* (2010) found that *C. albicans* biofilms exposed to caspofungin exhibited marked changes in the expression of hypha-specific genes (such as ALS3 and HWP1), irrespective of the antifungal concentration used.

The major components of EPS are carbohydrates (Al-Fattani and Douglas 2006; Paramonova *et al.* 2009). Moreover, some studies have shown that the cell wall of *C. albicans* is composed by 80 to 90% of carbohydrates, and the most frequently found are β-glucans (Nett *et al.* 2007; Paramonova *et al.* 2009). The fungal cell wall provides physical strength, limits permeability, and their components may contribute for resistance to antifungal agents (de Groot *et al.* 2008).

In general, after treatment with 54 μg SN ml^{-1}, both *C. albicans* 324LA/94 and *C. glabrata* D1 demonstrated significant increases in total carbohydrate content compared to the untreated biofilm groups (Table 1). The increase in carbohydrate content was higher for *C. glabrata* D1 biofilm, on which SN were more effective in reducing total biomass and number of CFUs (Fig. 1). These results should be seen in conjunction with the epifluorescence microscopy assay, which showed that SN induced damage to cell walls of both *Candida* biofilms, mainly at silver concentration of 54 μg ml^{-1} (Fig. 3ic, iic). Thus, taken together, these results suggest that the increases in carbohydrate content can be associated with changes in cell walls and secretion of their carbohydrates.

Furthermore, it is perhaps not surprising the apparent increase in the amount of extracellular matrix of *C. glabrata* D1 biofilm, compared to the control group, seen in Fig. 2(iic). During treatment with SN, in addition to changes in cell walls, the surviving cells could increase the production of exopolymeric substances in an attempt to protect the biofilm, and this fact may have contributed to the increase in carbohydrate content observed. However, as the Fig. 2 (ic) shows a apparent decrease in the amount of EPS, the increase in carbohydrate content for *C. albicans* 324LA/94 biofilm treated with SN at 54 μg ml^{-1} (Table 1) may represent only the carbohydrates that were released from cell walls affected by SN.

Parallel experiments carried out to examine the effects of SN on total biomass and number of CFUs (Fig. 1a and 1b) showed that SN were more effective in eradicating *C. glabrata* D1 biofilms, achieving a total biomass reduction around 90% at a silver concentration of 54 μg ml^{-1}, and reductions in the number of biofilm cells of 3·86$-\log_{10}$ and 6·46$-\log_{10}$ at silver concentrations of 13·5 and 54 μg ml^{-1}, respectively. These findings confirm those reported in a previous study (Monteiro

et al. 2011), which also revealed the greater susceptibility of *C. glabrata* biofilms to SN compared to *C. albicans* biofilms. However, the same SN concentrations tested in the present study (13·5 and 54 μg ml^{-1}) were less effective against *C. albicans* and *C. glabrata* biofilms developed in the 96-well microtitre plates (Monteiro *et al.* 2011), especially regarding to total biomass of *C. albicans* 324LA/94. Probably, the higher shear force acting on the biofilms formed in 6-well plates, compared with the force on the biofilms in 96-well plates, may have facilitated the diffusive transport of SN into and out of the biofilms (Stewart 2012), contributing to a better antifungal action of these nanoparticles.

On the other hand, SEM observations described here (Fig. 2) revealed a trend of SN aggregation, which was most obvious at the highest silver concentration (54 μg ml^{-1}). These findings are in agreement with those of Choi *et al.* (2010), who observed SN aggregation in the presence of *Escherichia coli* biofilms, resulting in an increase of total particle size. It seems reasonable to speculate that SN might bind to EPS, which have polyanionic nature (Hoyle *et al.* 1990), thus accumulating in the biofilm and retarding their diffusion (Hoyle *et al.* 1990). Additionally, these observations support the conclusions that the original particle size may be a poor exhibit of true nanoparticle size in biofilms (Monteiro *et al.* 2012b) and that the clusters of SN in some regions of *Candida* biofilms (Fig. 2) can restrict the antifungal effect of SN front of an extensive field of action. Possibly, SN agglomerated were unable to kill biofilm cells completely because these particles did not reach their targets.

In summary, it may be concluded that irrespective of concentration, SN affected the matrix composition of *Candida* biofilms assessed in terms of proteins, carbohydrates and DNA, except for the protein content of *C. albicans* 324LA/94 biofilm, which remained unchanged. After treatment with SN, SEM observations revealed structural differences for both biofilms with regard to spatial arrangement, and it was possible to observe clusters of SN on the biofilms. Moreover, epifluorescence microscopy images show that SN induced damage to the cell walls of the *Candida* oral isolates tested, especially at the highest silver concentration assayed (54 μg ml^{-1}). Finally, a limitation of this study is that only one strain of each *Candida* species was evaluated. Furthermore, future works are needed to fully elucidate how SN can act within *Candida* biofilms. Studies on the cytotoxicity of SN are also necessary for *in vivo* testing with such nanoparticles. Researches focusing on these issues are crucial for the development of alternative therapies that may prevent or control *Candida*-associated denture stomatitis and other *Candida* infections.

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