Disinfectants to Fight Oral *Candida* Biofilms

M. Elisa Rodrigues, Mariana Henriques, and Sónia Silva

Abstract

Oral biofilms, especially those caused by oral mycobiota, which include *Candida* species, are very difficult to eradicate, due to their complex structure and recalcitrance. Moreover, the mouth is prone to be colonized since it presents different types of surfaces, especially biomaterials and dental implants, often associated with a high rate of infections. Therefore, although disinfection of the oral cavity is of major importance, the number of commercially available disinfectants is not high. However, new solutions, as silver nanoparticles are being developed to help oral biofilms' eradication.

1 Introduction

The oral cavity is one of the most complex cavities of the human body, comprising hundreds of different bacterial, viral, and fungal species. The complexity of the oral cavity is in fact increased by the high number of different surfaces that can co-exist there, namely teeth, gingival sulcus, tongue, cheeks, hard and soft palates and medical devices (prosthetic devices or implants). In addition to the oral specific environment (variations on pH and sugar concentration, etc.), biomaterials are very prone to

infections, specially due to its heterogeneous composition (from ceramics to metals) and structure (from very smooth, as the crown surface, to very rough, as the implant). These infections can be less complex as caries, periodontitis and thrush or can evolve to severe diseases, as endocarditis or glomerulonephritis.

Several microorganisms can be found in the oral cavity, including the well-known bacteria, such as streptococci, lactobacilli, staphylococci and corynebacteria, as well as fungi. *Candida* species belong to the latter and are the most frequent (isolated from 75 % of participants), followed by *Cladosporium* (65 %), *Aureobasidium*, *Saccharomycetales* (50 % for both), *Aspergillus* (35 %), *Fusarium* (30 %) and *Cryptococcus* (20 %). Moreover, four of these predominant genera are known to be pathogenic in humans (Ghannoum et al. 2010). So, the

M.E. Rodrigues, M. Henriques (✉), and S. Silva
CEB, Centre of Biological Engineering, LIBRO –
Laboratório de Investigação em Biofilmes Rosário
Oliveira, University of Minho, 4710-057 Braga, Portugal
e-mail: mcrh@deb.uminho.pt

mycobiota is a medically important component of the oral microbiota, since opportunistic fungal infections commonly afflict the oral mucosa, being of special importance on immunocompromised hosts.

2 Oral *Candida* Species' Biofilms

The genus *Candida* contains over 200 different species that are ubiquitously distributed. However, only a few of these have been implicated in human oral infection (Calderone 2002). Oral candidoses have been recognized throughout human history and are often described as being 'the disease of the diseased', reflecting the opportunistic pathogenic nature of *Candida*. Whilst *Candida* species are generally regarded as harmless members of humans, infection can arise if a colonized individual becomes immunocompromised (Silva et al. 2012). The most prevalent *Candida* species recovered from the human mouth, in both commensal state and cases of oral candidoses, is *Candida albicans* (Pfaller and Diekema 2007; Samaranayake et al. 2009). However, the so-called non-*Candida albicans* *Candida* (NCAC) species are increasingly recognized as important agents of oral infections (Pfaller and Diekema 2007). In terms of oral prevalence, *C. albicans* is followed by *C. glabrata*, *C. krusei*, *C. tropicalis* and *C. parapsilosis* (Weems 1992; Williams et al. 2011). The apparently increased involvement of NCAC species in oral candidoses may partly be related to improvements in diagnostic methods, such as the use of primary agars with ability to differentiate species, and the introduction of molecular techniques in the routine diagnosis of fungaemia (Liguori et al. 2009). However, the increased prevalence of NCAC species in disease could also be a reflection of the inherently higher level of antifungal drug resistance in some NCAC species (González et al. 2008) compared to *C. albicans*, as this would promote their persistence in mixed-species infections treated with traditional antifungal agents. *Candida* species virulence has been attributed to their ability to form structured

aggregates of cells called biofilms (Silva et al. 2012). *Candida* biofilms can play a significant role in clinical oral infections because of their resilience and resistance to normal host removal mechanisms and also antifungal therapy. In general, for *Candida* species, biofilm formation occurs as a result of a sequence of events: (i) adherence/colonization to the surface; (ii) cell proliferation/invasion and well organized colony formation; (iii) matrix production with differentiation into a mature three-dimensional structure consisting of yeast, pseudohyphae, and/or hyphae embedded within extracellular matrix and finally (iv) detachment of biofilm cells to promote colonization and infection of distal sites (Seneviratne et al. 2008; Silva et al. 2012). Involvement of *Candida* biofilms in human oral infections is well recognized, particularly when occurring on biomaterials, such as acrylic and metals, used for implanted medical devices. Silva et al. (2009) confirmed that NCAC clinical isolates, as it is described for *C. albicans* (Chandra et al. 2001), are also able to produce biofilms, although these were less extensive for *C. parapsilosis* comparatively to *C. tropicalis* and *C. glabrata* (Fig. 1a) (Silva et al. 2009).

As it is well known, biofilms' formation on denture surfaces is promoted by poor oral hygiene and practices, such as failure to remove the denture whilst sleeping and reduced denture cleaning. In the event of host debilitation, which causes an ecological shift in favour of *Candida* growth, candidal biofilms may develop also on the mucosa itself, as it is possible to observe in Fig. 1b (Silva et al. 2011).

As described previously, the presence of candidal biofilms reduce the likelihood of removal of organisms by the host defense mechanism and antifungal agents. From the clinical perspective, the most important feature of *Candida* biofilms is, in fact, their role in resistance to conventional antifungal therapy (Hawser and Douglas 1995). Several groups have demonstrated that *Candida* cells present in biofilms show a dramatic increase in the levels of resistance to the most commonly used antifungal agents (Ramage et al. 2001). Although the mechanisms of biofilm resistance to antifungal agents are not fully understood, the

Fig. 1 (a) Scanning electron microscopy images of *Candida* species colonizing acrylic surfaces and (b) Confocal laser scanning microscopy images of *Candida* species colonizing and invading human oral

epithelium after 24 h. *Candida* cells are shown in red, and the nuclei of the epithelial cells appear in blue in confocal microscopy images; 3000 X corresponds to original magnification of scanning electron microscopy images

current consensus is that biofilm resistance to drugs is a complex multifactorial phenomenon involving different molecular mechanisms of resistance, compared to those displayed by planktonic cells (Silva et al. 2012). So, it is of major importance to know which oral disinfectants are available to fight these biofilms.

3 Oral Disinfectants

As described, *Candida* biofilms are very complex structures that can be formed on both biotic and abiotic surfaces, and are associated with important and recalcitrant infections. Therefore, disinfection of oral biomaterials is of major importance.

As well as the antifungal agents available, the number of disinfectants used for elimination of fungal colonization is very low. The most common are chlorhexidine, benzalkonium chloride and nystatin, among others. However, as their effect on biofilms is still reduced, other alternatives, as silver nanoparticles, are arising.

3.1 Chlorhexidine

Chlorhexidine is a highly cationic polybiguanide (bisbiguanide) with a broad spectrum of antimicrobial activity, including *C. albicans* and other common NCAC species (Suci and Tyler 2002; Ellepola and Samaranayake 2001). It is considered one of the most important medication in a basic health system (WHO 2013).

Additionally, chlorhexidine acts both as fungicide and fungistatic agent, but the specific mode of action is not yet fully understood. Nevertheless, several studies have assessed the antimicrobial effects of chlorhexidine identifying effects on *Candida* viability and structural integrity such as: (i) disruption of the attachment to the surface substrate; (ii) cell wall changes as retraction of the plasmalemma from the cell wall, and condensation and fragmentation of the cell wall (indicating possible lysis and escape of cytoplasmic components through the plasmalemma); (iii) increased vacuolation, aggregation and clumping of the cytoplasm contents; and (iv) inhibition of budding (MacNeill et al. 1997;

Bobichon and Bouchet 1987; Ellepola and Samaranayake 2001).

Chlorhexidine has also been shown to bind avidly to negatively charged surfaces such as epithelial cells (Audus et al. 1992), and to adsorb to enamel and salivary proteins (Hjeljord et al. 1973). Because of this, it has been suggested that a crucial feature of this compound is its high substantivity in the oral cavity, i.e. the capacity to be retained in the mouth (Bonesvoll et al. 1974a, b). The slow and continuous release of chlorhexidine from pellicle-covered oral surfaces seems to be a relevant pharmacodynamic feature, prolonging the therapeutic effect in the oral environment (Ellepola and Samaranayake 2001). However, this characteristic has also been associated with the destruction of the normal ultrastructure of epithelial cells and their receptors for microbes (Vaahtoniemi 1997), as well as with denaturation and precipitation of salivary mucinous proteins (Gjerme 1989). This may lead to side effects such as interference with taste sensations, soreness of the oral mucosa, and discoloration of the teeth (Gjerme 1989).

This antimicrobial agent is widely prescribed as an adjunctive therapeutic supplement due to its antimicrobial activity against a broad spectrum of organisms that includes *Candida* (Epstein 1990; Zegarelli 1993; Suci and Tyler 2002; Ellepola and Samaranayake 2001; Torres et al. 2007). In particular, it is used in mouth rinse formulations to reduce microbial burden in the oral cavity, related to biofilms, having an improved action compared to washes with antifungal agents as nystatin and clotrimazole (Treister and Woo 2010). However, the effectiveness of chlorhexidine as an antimicrobial agent is influenced by its concentration and exposure time, and no firm conclusions have been taken regarding the best method to prevent infection in the oral cavity (Fathilah et al. 2012). Nevertheless, a 0.2 % chlorhexidine gluconate solution is typically used in clinical practice (Salem et al. 1987) against *Candida*-associated denture stomatitis and in acute pseudomembranous candidosis; and a 2 % chlorhexidine gluconate is used as an overnight denture disinfectant (Ellepola and Samaranayake

2000; Budtz-Jorgensen 1990). Chlorhexidine is also used as prophylaxis for both chemotherapy- and radiotherapy-induced mucositis (Kowanko et al. 1998). It has been suggested that chlorhexidine may be used as a pre-treatment on denture acrylic to reduce subsequent adherence of *C. albicans* (Spiechowicz et al. 1990; McCourtie et al. 1985; McCourtie et al. 1986).

However, there are some problems that lead to therapeutic failure of chlorhexidine: both the diluent effect of saliva and the cleansing action of the oral musculature reduce the availability of the compound below the effective therapeutic concentration (Ellepola and Samaranayake 2001); and the reduced susceptibility to antifungals of *Candida* biofilms (Suci and Tyler 2002; Baillie and Douglas 1999; Hawser and Douglas 1995).

3.2 Benzalkonium Chloride

Benzalkonium chloride is a quaternary ammonium compound consisting of a mixture of alkylbenzyltrimethylammonium chlorides whose alkyl group has different even-numbered alkyl chain lengths. This compound has been used as a biocide, a cationic surfactant and a phage transfer agent.

The amphiphilicity of benzalkonium chloride is essential for its antimicrobial activity. The action of this compound begins with the interaction of the hydrophilic cationic region with the negatively charged components of the surface of the pathogen, establishing electrostatic interactions. These outcompete the divalent cations that commonly stabilize the structure of the pathogen's surface. After this contact, the hydrophobic region of benzalkonium chloride penetrates the hydrophobic bilayer, compromising cellular permeability controls and causing cell leakage and lysis (Coughlin et al. 1983; Fazlara and Ekhtelat 2012; Mangalannalli-Illathu and Korber 2006; McDonnell and Russell 1999).

The main antimicrobial activity of benzalkonium chloride – membrane disruption, is most effective against Gram-positive bacteria,

having an effect also on Gram-negative bacteria, some enveloped virus, fungi, yeasts and protozoa (Fazlara and Ekhtelat 2012).

It is an active ingredient of biocides used in different applications, from clinical, food line to domestic household (Mangalannalli-Illathu and Korber 2006). Its main use is as a preservative in ophthalmic solutions (as eye drops), in concentrations ranging from 0.004 to 0.01 % since higher values can be caustic and cause irreversible damage to the cornea (Nelson and Goldfrank 2011; Baudouin et al. 2001). Additionally, it has also been tested against *Candida* in plastic surfaces of medical devices (e.g. denture materials), achieving a pathogen reduction when used at least at a concentration of 5000 mg/L. In opposition, benzalkonium chloride was shown to be unable to prevent fungal adherence to extracellular matrix proteins (Imbert et al. 2003b).

3.3 Nystatin

Nystatin belongs to the polyene class, fungicidal due to the ability to interact with the ergosterol component within the fungal cell membranes to generate pores causing cell leakage and loss of cytoplasmatic content. This agent is generally regarded to have the broadest spectrum of antifungal activity (Silva et al. 2001). Nystatin is frequently used topically and can be administrated in a variety of oral formulations in the treatment of oral candidoses including suspensions and pastilles (Williams et al. 2011). Silva et al. (2013) revealed that on single pre-formed biofilms, nystatin was able to significantly inhibit *C. glabrata* and *C. albicans* biofilms. Thus, one strategy that has been the focus of recent research is to actually modify the medical surfaces so they are less prone to biofilm formation by microorganisms, including *Candida* species (Price et al. 2002; Price et al. 2005). In addition, thin-film polymer formulations with incorporated antifungal agents, such as chlorhexidine and nystatin have also recently been shown to inhibit *C. albicans* biofilms growth on denture materials (Redding

et al. 2009). In fact, the incorporation of nystatin into the thin-film polymers was able to reduce biofilm formation between 70 % and 80 % (Redding et al. 2009). In studies in which the concentration of nystatin was evaluated, the higher concentration tested had the highest effect, being time dependent, and decreasing and/or disappearing after 1 week, 15 days or 1 month depending on the concentrations (Skupien et al. 2013).

Despite polyenes being very poorly absorbed through the gut and their use being relatively limited, in view of their efficacy on the control of *Candida* growth and biofilm development it is possible to consider that nystatin can be used as an alternative disinfectant to fight *Candida* species.

3.4 Silver Nanoparticles

Silver nanoparticles (SN), due to their unique properties, are starting to be used in many day-to-day applications, including medical applications. Although there are various theories about the microbicidal action of SN, the exact mechanism is not clearly known and is yet a debate topic. SN has the ability to anchor to the microorganism's cell wall and subsequently penetrate it, thereby causing structural changes in the cell membrane integrity. It has also been proposed that the microbicidal activity can be due to the release of silver ions by nanoparticles, which can interact with important enzymes and inactivate them. It has also been postulated that nanoparticles can even modulate the signal transduction in microorganisms (Prabhu and Poulouse 2012).

The effect of SN against *Candida* biofilms was first reported by Monteiro et al. (2011). In this study, SNs were synthesized by silver nitrate reduction with sodium citrate and stabilized with ammonia. Then, these nanoparticles were applied (during 24 h) to adherent cells (2 h) or biofilms (48 h) of *C. albicans* and *C. glabrata* previously developed (Fig. 2).

Fig. 2 Scanning electron microscopy images showing the structure of *Candida albicans* (i) and *Candida glabrata* (ii) mature biofilms under different experimental

conditions: (a) control and (b) biofilms treated with SN (Note agglomerated SN in an enlarged view of part of biofilms (Monteiro et al. 2013))

The results for adhered cells showed a significant reduction in the total biomass and in the number of cultivable cells for SN concentration at/or higher than 3.3 $\mu\text{g/mL}$ for both species. So, SNs showed the ability to inhibit biofilm formation when applied to adherent cells. Importantly, SNs were more effective in the reduction of the number of cells and total biomass when applied on adhered cells than pre-formed biofilms (Monteiro et al. 2011). Regarding pre-formed *Candida* biofilms, the highest SN concentration tested (54 $\mu\text{g/mL}$) significantly reduced biofilms of *C. glabrata*. In general, *C. glabrata* biofilms have reduced thickness and are devoid of hyphae compared with *C. albicans* biofilms (Silva et al. 2009; Samaranayake et al. 2005). Consequently, all these features can help explain the better effect of SN against *C. glabrata* biofilms, which is still of major clinical importance since

this species is known to be resistant to conventional antifungal drugs, making it very difficult to eliminate. Furthermore, the effective SN concentration against *C. glabrata* biofilms can be considered low when compared with the concentrations of conventional antifungal used. For instance, fluconazole at concentrations ranging from 50 to 1250 $\mu\text{g/mL}$ was ineffective against *C. glabrata* biofilms (Fonseca et al. 2014).

In the view of the SN application as disinfectant of medical devices against *Candida* biofilms proliferation, and considering that the loss of the chemical stability might reduce their effectiveness, a study was conducted to verify whether heating or changing pH of a SN stock solution, as well as the treatment period, would affect the anti-biofilm activity (Monteiro et al. 2014). Several parameters were evaluated regarding SN

(54 µg/mL) stability, namely temperature (50 °C, 70 °C, and 100 °C) and pH (5.0 and 9.0) in mature biofilms grown on acrylic resin specimens. Surprisingly, SN colloidal suspensions heated at 50 °C and 70 °C for 30 min did not display modifications in their absorption spectra as compared with SN suspension that was not heated. In addition, heating SN at 50 °C and 70 °C did not compromise the effectiveness of SN against *Candida* biofilms (Monteiro et al. 2014). Regarding pH variations and despite the appearance of dark aggregates, evidencing instability of SN at both acid and basic pH, the results also showed that the variations in pH did not impair the effectiveness of SN in reducing *Candida* species biofilms. One of the factors that may contribute to *Candida* biofilms' antifungal resistance is the presence of extracellular matrix (Silva et al. 2001). The matrix of *Candida* biofilms is constituted by several proteins, carbohydrates (Silva et al. 2009) and DNA (Martins et al. 2010). Antifungal drugs may bind to constituents of the extracellular matrix, preventing the drugs from reaching the cells in the deeper layers of the biofilm (Vediyappan et al. 2010). Therefore, strategies focusing on eradication of extracellular matrix may collaborate to fight *Candida* infections associated with biofilms. Monteiro *et al.* (2013) applied SNs on *C. glabrata* mature biofilms and it was possible to observe a significant reduction in matrix protein and DNA contents (Monteiro et al. 2013). Moreover, an *in vitro* study compared the antifungal effects of nystatin and SN on pre-formed single and dual species (*C. albicans* and *C. glabrata*) biofilms on acrylic resin surfaces under conditions that attempted to mimic the oral environment (Silva et al. 2013). This work revealed that *C. albicans* and *C. glabrata* colonized acrylic surfaces in the presence of artificial saliva and co-existed in dual species biofilms without antagonism and that both nystatin and SN were able to inhibit single and dual species biofilms. SNs were also tested in combination with chlorhexidine and a synergetic activity was observed for *C. glabrata* biofilms, achieving a reduction in biofilm biomass of more than 84 % (Monteiro et al. 2013).

Recently, Longhi et al. (2015) revealed that the combination of SN and fluconazole reduced the concentration of the latter around 16–64 times against planktonic cells of *C. albicans* and a significant dose-dependent decrease in the viability of both initial and mature *C. albicans* biofilm (Longhi et al. 2015). In a clinical context, these combinations are very important to decrease the concentrations used on traditional antifungal therapy and reduce the probability of their increased resistance.

In view of all these attributes it is possible to consider that SN can be used as an alternative disinfectant to fight *Candida* biofilms; however, further research is needed to elucidate the mode of action, cytotoxicity and the longevity of SN formulations and microbial properties of SN generated by the incorporation of nanoparticles for example into denture acrylic.

3.5 Other Alternative Disinfectants

Besides the previously described disinfectants, the literature suggests other alternatives, as the use of 0.5 % sodium hypochlorite concentration that can help disinfect tissue conditioners and denture lines (Skupien et al. 2013).

In addition, povidone iodine is recognized as an effective broad spectrum biocidal agent, whose *in vitro* biocidal activity has been studied for years against bacteria, yeast, moulds, viruses, fungi, protozoa, actinomycetes and rickettsia (Duarte and Hamdan 2006; Zamora 1986; Amend and Pietsch 1972). Typical iodine solutions present significant oral toxicity, but this complex exhibits markedly lower toxicity, being less hazardous in case of accidental ingestion (Kumar et al. 2009). Povidone iodine has been mostly used for surgical scrubbing and as a prophylactic irrigation solution against surgical site infection (Chundamala and Wright 2007). A study has also demonstrated that when used at 2.5 %, povidone iodine is able to completely inhibit yeast adherence, suggesting that it could be a good candidate for prevention of candidoses related to medical devices (Imbert *et al* 2003a). However, it has only been applied: in

gynaecology for vaginitis associated with *Candida* (Dugal and Chaudhary 2013); as an antiseptic for infected inflammatory conditions of the mouth and pharynx caused by *C. albicans* (Silva et al. 2009); in reducing odor causing bacteria in mouth wash products at a small concentration (0.5 %) (Addy et al. 1977; Becker and Nel 1976).

Moreover, microwave irradiation has also been described as an alternative method of disinfection (Skupien et al. 2013).

Other option of disinfection is the low-level laser therapy (LLLT) and photodynamic therapy (PDT), which are also emerging as promising alternatives to conventional treatments for *C. albicans* infections (Dougherty 2002; Dougherty et al. 1998). LLLT uses low doses of visible light of appropriate wavelength to activate existing biological chromophores in cells, leading to the generation of reactive oxygen species (Souza et al. 2010). For its turn, PDT uses photosensitizing agents that are also activated by an appropriate wavelength of light in the presence of oxygen, promoting a phototoxic response of the cells, usually via oxidative damage (Dougherty et al. 1998; Mima et al. 2010). In consequence of the use of nonspecific oxidizing agents, organisms resistant to conventional antifungals may be successfully killed by PDT and the development of resistance to this therapy seems unlikely (Mima et al. 2010). A few *in vitro* studies have shown that *C. albicans* is susceptible to photoinactivation, but the effects on its growth, adherence and virulence factors are far from being defined (Mima et al. 2010; Sennhenn-Kirchner et al. 2009). Evidently, the knowledge about the application of LLLT and PDT applied to NCAC species is even scarcer.

4 Conclusions

Although there are some alternatives to combat oral infections, they are not always efficient on biofilms, especially those from *Candida* species. So, more research should be developed in this field to better treat oral *Candida* infections associated to biofilms, which can lead to serious

infections, with special relevance in immunocompromised patients.

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