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ORIGINAL ARTICLE

Antifungal susceptibility of *Candida albicans* biofilms on titanium discs with different surface roughness

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Abstract Although it is well known that fungal biofilms have increased resistance to antimicrobial agents, limited information is available on the formation of candidal biofilms on implant surfaces with different surface roughness and their resistance to conventional antifungal therapy. In the current study, the effect of increasing the surface roughness of titanium discs on the susceptibility of *Candida albicans* biofilms to amphotericin B was determined. Grade I commercially pure titanium discs were sandblasted with 99.6% aluminium oxide of different grit sizes, producing surface roughness of 0.90, 1.88 and 3.82 μm (Groups A, B and C), respectively ($P<0.001$). The antifungal susceptibility of *C. albicans* biofilm grown on different Ti discs was determined using XTT assay. The 50% reduction in metabolic activity (50% RMA) of planktonic *C. albicans* (0.5 μg/mL) was much lower than those from Groups A, B and C (2, 16, 2 μg/mL, respectively), while the 50% RMA from Group B was three-fold higher than those from Groups A and C. In conclusion, difference in titanium surface roughness was associated with variations in the antifungal resistance of the candidal biofilm. Group C appeared to have an optimum surface roughness for biofilm resistance.

Keywords Titanium · *C. albicans* · Biofilm · Surface roughness · Antifungal resistance

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Introduction

Microorganisms in their natural habitats are mostly found in biofilm ecosystems attached to surfaces rather than free-living organisms [12]. The formation of biofilm that leads to persistent human infection has long been appreciated and many of these infections are associated with medical devices [1, 10]. A substantial number are implant infections that may involve a single microbial species or a mixture of fungal and/or bacterial species [10, 15].

Candida albicans, which is a polymorphic fungus, is the major fungal pathogen in humans and candidiasis is the fourth most common nosocomial infection worldwide [4, 29]. The first step in the pathogenesis of candidal infection entails the adhesion of the yeast to a surface, which may be the host surface or a medical device such as a dental implant. The presence of such a surface is thought to modify the normal planktonic mode of growth of the yeasts to a sessile biofilm mode of growth, which is characterized by three phases of growth, namely early, intermediate and maturation development phases [7, 19].

Biofilms have been shown to be significantly less susceptible to antifungal agents [14, 16] and a number of resistance mechanisms have been proposed to explain this phenomenon. The mechanisms include the physiological state of the fungal cells, a barrier function of extracellular matrix, overexpression of drug efflux pumps, variations in fungal membrane sterol composition, and different development phases [14, 17]. A number of studies have also shown that architecture of *C. albicans* biofilms is affected by the nature of the substrate surface [7, 17].

Recently, titanium dental implants with various surface treatments such as plasma spraying, sandblasting and hydroxyapatite coating have been introduced because the increase in surface roughness has been shown to result in

firmer and faster bone integration [5, 6, 26]. However, it has been demonstrated that surface roughness is positively correlated with the rate of bacterial colonization of oral implants [21, 25]. If such rougher surfaces become exposed to the oral environment, they may be more susceptible to bacterial adhesion and biofilm formation and lead to implant-related infections.

Since little information is available on the formation of *C. albicans* biofilm on implant surfaces with different morphologies and the architecture of *C. albicans* biofilms is known to be affected by the topography of the substrate surface, the aim of the present study was to compare the amphotericin B susceptibility of *C. albicans* biofilms grown on titanium surfaces with different surface roughness in vitro.

Materials and methods

Preparation of the titanium discs

Grade I commercially pure titanium (CP-Ti) rods (Arkhe, Fukui, Japan) were cut into small discs (12 mm in diameter and 1 mm in thickness) in the University of Hong Kong Technology Support Centre using a wire cut electrical discharge machine (Agie Charmiles, Swiss). The discs were divided equally into three groups. Groups A, B and C were sandblasted with 99.6% aluminium oxide (Korox, Bego, Bremen, Germany) with mean grit size of 25 μm, 110 μm and 250 μm, respectively. Sandblasting was done with a pressure of 5 bar for 30 s at a distance of 1.5 cm from the disc's surface using a sandblasting machine (Dentastrah Combi, Krupp Medizinteehnik, Germany).

All Ti discs were cleaned by rinsing with sterile distilled water for 15 s, followed by 70% ethanol (ETOH) for 15 s and 99% ETOH for 15 s. The Ti discs were then immersed in 99% ETOH in a sterile plastic container with lid and sonicated in a sonicating bath (Branson Ultrasonics, Danbury, CT) for 15 min. The Ti discs were transferred to a sterile Petri dish with sterile artery forceps and dried under a safety cabinet for 15 min. The Ti discs were packed into autoclave bags (SteriCLIN, Germany) and autoclaved in an electronic steam autoclave (Eschmann SES-2000, UK) at 121°C for 15 min within 1 week before usage.

Profilometric and morphological analysis of titanium discs

Measurement of surface roughness of Ti discs

The Ra value, which is the arithmetic average of the absolute values of the profile height deviations from the mean line, was measured using a stylus profiler (Surtronic 3+, Taylor Hobson Precision, England). Five discs from each group were randomly selected for profilometric analysis. Five measurements were taken on each disc and Ra was then calculated.

Adhesion assay

C. albicans reference strain ATCC 90028 was subcultured and grown on Sabouraud dextrose agar plate at 37°C for 24 h. Cells were harvested, added into sterile Yeast Nitrogen Base (YNB, Difco, Maryland, USA) with 100 mM glucose and standardized to 1×10^7 cfu/mL by adjusting the optical density (O. D.) of the suspension to 0.385 at 520 nm. 250 μL of yeast suspension and 250 μL of YNB supplemented with 100 mM glucose w/v were added into each well of a pre-sterilized, 12 well polystyrene flat bottomed plate. The Ti discs were placed into the wells with sterile artery forceps. The cells were allowed to adhere on the disc surface for 90 min at 37°C in an orbital shake incubator at 75 rpm. Discs with no cells added served as negative controls.

Each Ti disc was picked up using sterile artery forceps and gently washed in 350 mL of PBS in a sterile container by back and forth movement for 2 s to remove non-adherent cells.

Biofilm formation

The adhesion phase of candidal biofilm formation was performed in the same fashion as the adhesion assays described above. The Ti discs were then placed in a new pre-sterilized, 12 well polystyrene flat bottomed plate with 750 μL of YNB supplemented with 100 mM glucose w/v for biofilm formation and growth at 37°C and 75 rpm for 96 h in an orbital shake incubator. The growth medium was replenished daily.

Antifungal susceptibility test for *C. albicans* biofilm

The 50% reduction in metabolic activity (50% RMA) of *C. albicans* biofilm grown on different Ti discs was determined using 2, 3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) assay, derived from a modification of the method by Chandra et al. [8].

Shortly before 48 h of biofilm growth, antifungal solutions with different concentrations were prepared. 0.0032 g of amphotericin B was dissolved in 3 mL of dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO) and 2 mL of ETOH. The amphotericin B solution was diluted by adding 45 mL of YNB with 100 mM glucose to obtain the concentration of 64 μg/mL. The solution was then serially diluted to obtain the following concentrations: 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, and 0.064 μg/mL. YNB with 100 mM glucose was used as a control with no drug added.

At 48 h, each disc was picked up by a pair of sterile artery forceps and gently washed for 2 s in 350 mL of PBS to remove non adherent cells. Each disc was placed into a well of a 12 well plate containing 1.8 mL of amphotericin B solution with different concentrations (0-64 μg/mL). The cells were placed into an orbital shake incubator and incubated for 45 h at 37°C and 75 rpm.

XTT reduction assay

Each Ti disc with the adherent yeasts (adhesion assay) or the biofilm (total biofilm formation and antifungal susceptibility test) was picked up using sterile artery forceps and gently washed in 350 mL of PBS for 2 s to remove non-adherent cells. The disc was then placed into a well of a 12 well plate containing 4 mL PBS per well. Fifty μL XTT (1 mg/mL in PBS) and 4 μL menadione solution (1 mM in acetone) were added to each well. A piece of aluminium foil was wrapped around the container and incubated in the dark for 3 h at 37°C.

The reagent containing dislodged yeast cells was pipetted into a sterile eppendorf tube and centrifuged (A 30, Beckman Coulter, CA) for 10 min at 13,200 rpm. 100 μL of the supernatant was pipetted into a pre-sterilized, flat bottomed 96 well polystyrene plate (Iwaki, Tokyo, Japan). The O.D. of the supernatant was measured with a microtiter plate reader (SpectraMAX 340 Tunable Microplate Reader, Molecular Devices Ltd., CA) at 492 nm.

A "blank" sample was also prepared following the same procedures except no disc was placed into the reagent. The O.D. for the blank sample was subtracted from the test wells to eliminate background interference. The antifungal concentration which resulted in 50% reduction in XTT metabolic activity compared with control was determined. The experiment was performed in triplicate on two separate occasions.

Antifungal susceptibility test for planktonic *C. albicans* cells

The antifungal susceptibility of planktonic cells against amphotericin B was determined by the XTT method [8]. An inoculum size of 1×10^7 cfu/mL was used and YNB was used instead of RPMI 1640 as the growth medium.

Scanning electron microscopy (SEM) study of *C. albicans* biofilm on Ti discs

C. albicans biofilm formed after incubation for 48 h on Ti discs from each group was observed under SEM. *C. albicans* biofilm subjected to amphotericin B for each group was also observed.

Ti discs were picked up by sterile artery forceps from the flat bottomed 12 well polystyrene plate and gently washed in 350 mL of PBS for 2 s to remove non adherent cells. The discs were then placed in a pre-sterilized, flat bottomed 12 well polystyrene plate. The biofilms were air fixed with 1% osmium tetroxide placed under the lid of the 12 well plate for 2 h. The discs were then coated with gold and then imaged using a SEM in a high vacuum mode at 10 kV. Scanning electron micrographs of the biofilm were taken at 1000-4000× magnification.

Statistical analysis

Calculation of Ra values of titanium discs

Calculation of the 95% confidence interval for Ra values of the four groups was made by descriptive analysis using SPSS software (version 12.0 for Windows). The difference between the Ra values produced by blasting with different sizes of alumina oxide and the untreated group were tested. Kolmogorov-Smirnov and Shapiro-Wilk Tests were performed to test for the normality of the data. The Levene Test was used to test for homogeneity of variances. When the data were parametric and homogenous, one way ANOVA was performed. Post Hoc tests including the Turkey HSD, Scheffe and Bonferroni tests were performed for multiple comparisons using SPSS software.

Results

Measurement of surface roughness of Ti discs

Sandblasting with 25 μm Al_2O_3 (group A), produced the smoothest Ti surface with a Ra of 0.90 μm (SD 0.06 μm). Sandblasting with 110 μm and 250 μm Al_2O_3 (Group B and C) produced rougher Ti surfaces with Ra of 1.88 μm (SD 0.09 μm) and 3.82 μm (SD 0.38 μm), respectively (Table 1). A significant difference in surface roughness was found among groups A, B and C ($P<0.001$).

Adhesion assay and biofilm formation

XTT analysis at $O.D._{492\ nm}$ for Groups A, B and C were 0.29±0.02, 0.28±0.03 and 0.26±0.02, respectively. No significant difference in adhesion was noted between the three groups. Figure 1 shows the biofilm formation of the three groups with different incubation time. Again, no significant difference was noted between the three groups.

Antifungal susceptibility of *C. albicans* biofilm grown on Ti surfaces

Six samples from each group were tested for each of the 12 amphotericin B concentrations (0-64 μg/mL). The 50% RMA for the biofilm grown in group B with moderately

t1.1 **Table 1** Surface roughness (Ra), the SD and the 95% CI for Ra of Ti discs in different groups A, B and C with surface blasted with 25, 110 and 250 μm Al_2O_3, respectively

t1.2 Group	Ra (μm)	SD (μm)	95% CI for Ra (μm)	
t1.3			Lower bound	Upper bound
t1.4 A	0.90	0.06	0.83	0.97
t1.5 B	1.88	0.09	1.78	1.99
t1.6 C	3.82	0.04	3.77	3.86

rough surfaces (Ra=1.88 μm) was the highest (16 μg/mL), being the least susceptible to amphotericin B. On the other hand, the biofilm grown in group A with minimally rough surfaces (Ra=0.90) and group C with rough surface (Ra=3.82) had the same 50% RMA (2 μg/mL). Figure 2 shows the activities of different concentrations of amphotericin B against biofilms from the three groups. Each result is representative of three separate experiments performed in duplicate.

Antifungal susceptability of planktonic *C. albicans* cells

The antifungal susceptibility of planktonic cells against amphotericin B, using 50% RMA was 0.5 μg/mL.

SEM study of *C. albicans* biofilm on Ti discs

Figures 3, 4 and 5 show titanium discs blasted with 25, 110 and 250 μm Al_2O_3 (Group A to C), respectively. *C. albicans* biofilms incubated for 48 h on the discs had similar appearance, consisting of densely packed yeast cells with scattered long hyphal cells among the yeast cells (Fig. 6).

Figure 7 shows *C. albicans* biofilms from Group B, incubated for 48 h and exposed to amphotericin B for 48 h at 32 μg/mL. The mature, dense layers of yeast cells were no longer present. A few yeast and hyphal cells were seen remaining in small pits. On the Ti surface, yeast and hyphal cells also remained in larger cavities in groups. Some of them showed the typical appearance of damaged yeast cells exposed to antifungal agent; yeast cells appeared shrunken, wrinkled, ruptured and ballooned while fused blastopores were also seen.

Discussion

A number of previous studies have shown that *Candida* biofilms exhibit increased resistance to antifungals. Although a number of hypotheses have been proposed, none of them seem to explain clearly the phenomenon of increased resistance. And although surface roughness has been shown to influence the adhesion of microorganisms to medical devices such as catheters and dental implants [13], no studies have been done to ascertain the relationship between surface roughness and antifungal resistance in biofilms. It is notable that our results showed that there was no significant difference in adhesion amongst the three groups. Although biofilm biomass showed no significant difference, whether any other structural or biochemical difference in the result biofilm may have any effect on increased antifungal resistance warrants further investigations.

In the current study, only three groups with different surface roughness were included because pilot studies using Al_2O_3 of other grit sizes, e.g. 50 or 220 μm, showed little difference in terms of profilometric and morphological analysis (data not shown). A number of studies have shown that rougher implant surfaces promote better and faster bone formation [6, 24, 32]. As a consequence, a range of dental implants with roughened surfaces created by coat-

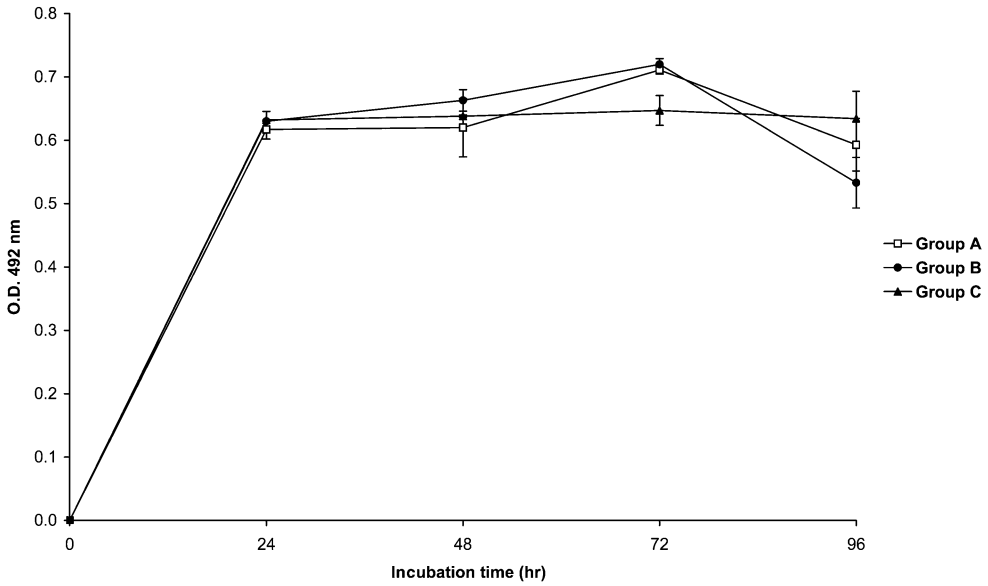

Fig. 1 Effect of incubation time on biofilm formation by *C. albicans* ATCC 90028 as determined by means of the XTT assay. Data are means± standard error of three independent experiments performed in duplicate

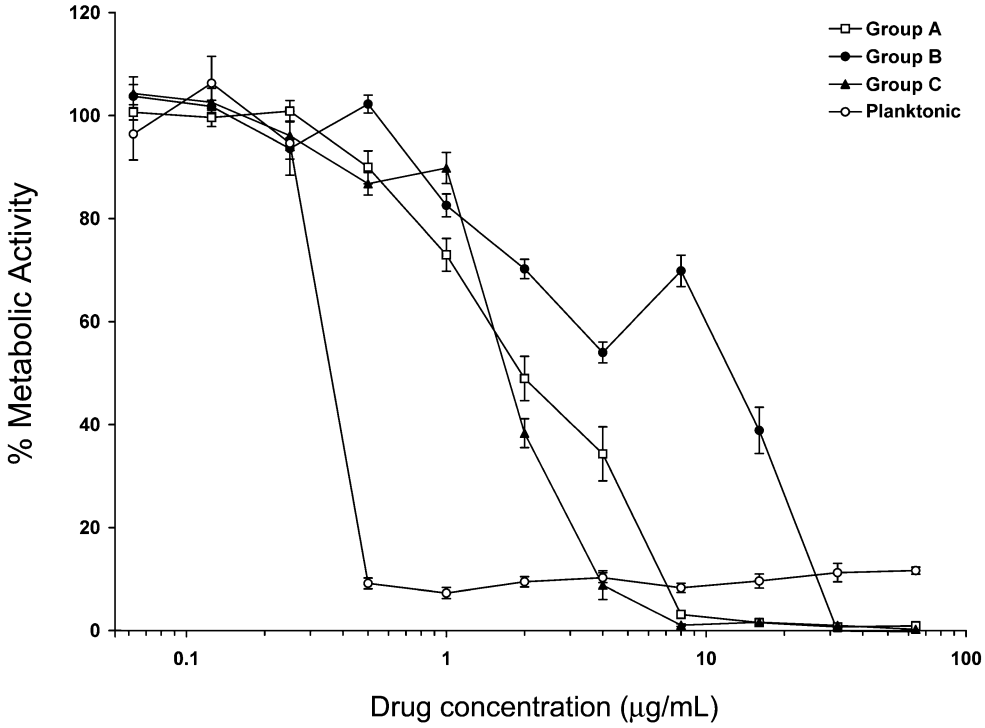

Fig. 2 Effect of antifungal susceptibility on *C. albicans* ATCC 90028. Biofilms or planktonic cells were exposed to different concentrations of amphotericin B and their metabolic activities were determined by means of the XTT assay. Data are means± standard error of three independent experiments performed in duplicate

ings, blasting, acid etching or a combinations of these treatments have been introduced commercially [9]. However, Mustafa et al. [20] showed that by further increasing the size of the blasting particles did not further increase the initial attachment of the cells.

In the current study, different Ti surface roughness was produced by sandblasting in order to study the effect of implant surface roughness on fungal biofilm formation and the susceptibility to antifungal agent. A potential drawback of sandblasting with Al_2O_3 is the possible embedding of alumina particles on Ti surfaces [11]. The presence of Al_2O_3 particles may modify the surface property, potentially affecting cell adhesion and growth [2]. In addition, since commercial dental implants are pre-sterilized, the Ti discs were also sterilized by steam autoclaving after cleaning. However, Vezeau et al. [28] have shown that steam autoclaved CP-Ti surfaces showed discoloration indicating possible surface oxide changes and particulate contamination, resulting in lower fibroblast cell attachment level; the use of ultraviolet sterilization technique may be a better alternative.

In the current study, the 50% RMA of *Candida* biofilm formed on titanium disc was found to be much higher than their planktonic counterpart, which is in agreement with previous studies [8, 22]. A notable finding was that the *Candida* biofilms formed on titanium surfaces with

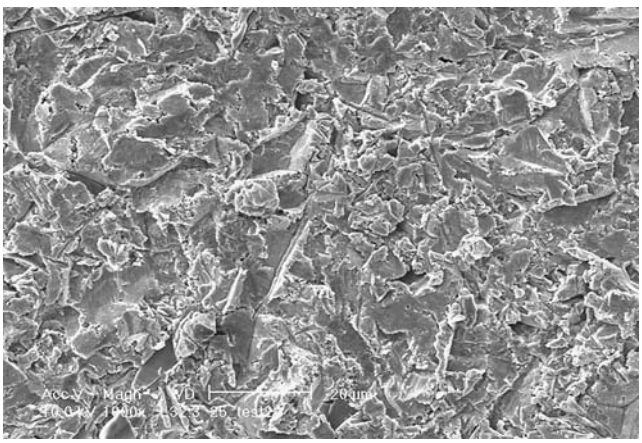

Fig. 3 Titanium surface blasted with 25 μm Al_2O_3 and viewed under 1000× magnification, showing a homogenous surfaces composing of numerous small pits and some larger cavities

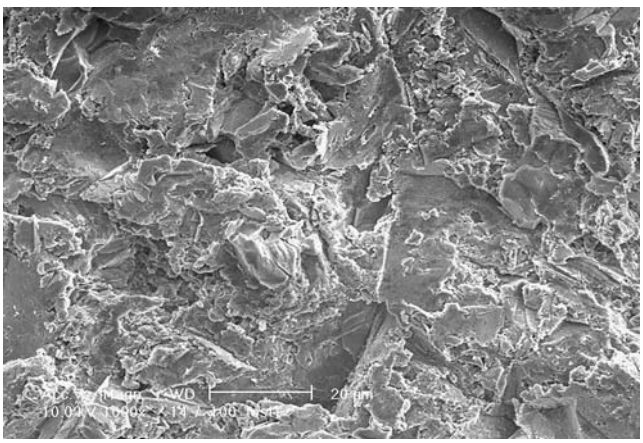

Fig. 4 Titanium surfaces blasted with 110 μm Al_2O_3 and viewed under 1000× magnifications, showing a surface with multiple small pits and grooves superimposed on larger cavities

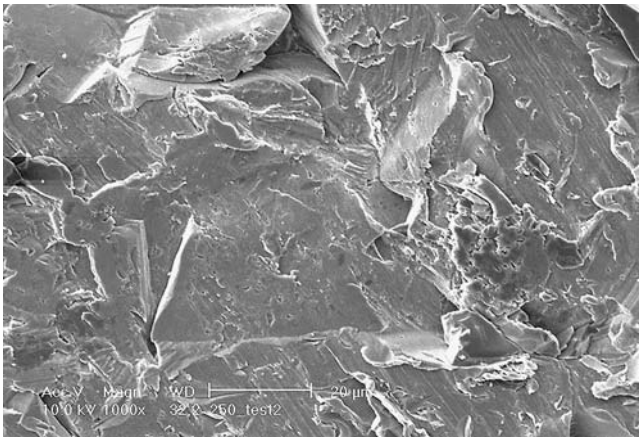

Fig. 5 Titanium surfaces blasted with 250 μm Al_2O_3 and viewed under 1000× magnifications, showing a surface with a few small pits and grooves superimposed on very large cavities. Large and rather smooth areas could also be observed on the same surface

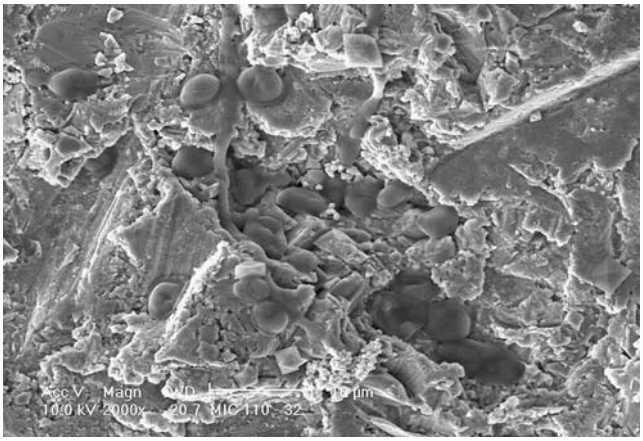

Fig. 7 Group B after 48 h exposure to amphotericin B at a concentration of 32 μg/mL shown under 2000× magnification. A few yeast and hyphal cells are seen remaining in small pits and group of yeast and hyphal cells are also seen remaining in larger cavities

different surface roughness also exhibited different degrees of resistance to antifungal agent. It appeared that an increase in surface roughness was associated with an increase in biofilm resistance but beyond a certain degree further increasing the surface roughness would decrease the biofilm resistance again.

In the current study, biofilm growth from the three groups showed little extracellular polymer matrix (ECM) formation after 48-72 h. This is probably due to dehydration during SEM procedures. Previous studies have shown that ECM might not play a major role in biofilm resistance [3]. ECM does not hinder penetration of antifungal drugs [3, 23] and biofilms grown with and without shaking, which exhibit different extent of EMC formation, showed no difference in biofilm resistance. Moreover, ECM-deficient biofilms formed by filamentation defective mutants of *C. albicans* retained high level of antifungal resistance. Therefore, it appeared that ECM did not contribute to the difference in biofilm resistance between the three groups.

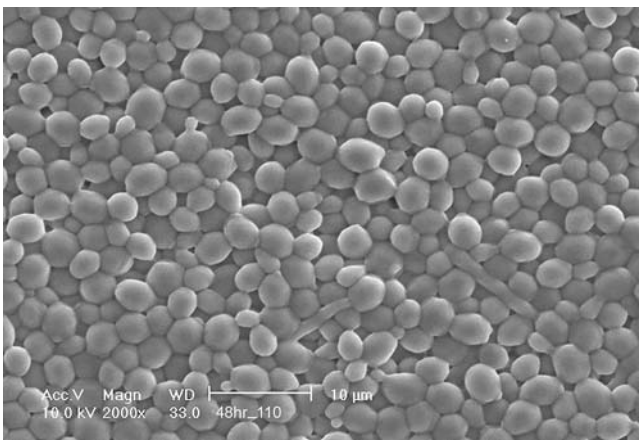

Fig. 6 Biofilm formed on Ti surface blasted with 110 μm Al_2O_3 shown under 2000× magnification. Matured biofilm with a few scattered long hyphal cells can be observed among densely packed yeast cells

Another hypothesis that explains antifungal resistance of biofilm is that the genes encoding multidrug resistance (MDR) transporters, are upregulated upon attachment of *C. albicans* to a surface, and may account for the resistance of young biofilms to azole. However, it should be noted that amphotericin B is not a substrate of Cdr1p, Cdr2p, Mdr1p, or any known drug efflux pump. Minimum inhibitory concentration (MIC) of the wild type was found to be the same as that of a strain with multiple deletions in the MDRs [18]. Therefore, surface-induced upregulation of drug efflux pumps also may not contribute to the difference observed in the present study.

The key factors which have been proposed to explain the increased resistance of *Candida* biofilm to antifungal agents, however, appear to be directly or indirectly affected by the surface properties on which the biofilm is formed [20]. This trend is similar to that found between surface roughness and host bone response [30, 31]. In these studies, the authors found that surface roughness of Sa 1 to 1.5 μm appeared to be the optimal roughness with regard to retention in bone and bone-to implant contact. Further increase in surface roughness to Ra=2.11 did not result in an improved bone response. The authors surmised that the rugofile bone cells may recognize the very rough surface as a smooth surface and the medium rough surface as a trough rough surface. Whether the *Candida* cells responded to the surface topography the same way is unclear and warrants further investigations.

In the current study, *C. albicans* from group B blasted with 110 μm Al_2O_3 was least susceptible to amphotericin B. The yeast cells were found to remain in groups in larger cavities. It has been shown by Verran and Maryan [27] that yeast cells tend to be retained on roughened surfaces in higher numbers

than on smooth surfaces, and cells were observed within surface irregularities after the washing process.

A possible explanation for biofilm grown on group B discs showing the highest resistance to amphotericin B could be that the size of cavities produced by 110 μm Al_2O_3 is optimal for adhesion and provides shelter from the drug and from biofilm dislodgement. It has been shown previously that surface features smaller than cells or greatly exceeding the microorganisms will have little effect on retention [33]. The reason could be that when the surface features are small relative to the size of the cells, the small pits cannot act as a shelter to protect the cells; whereas if the surface features are too large relative to the size of the cells, they act more or less similar to a flat surface. Therefore, surfaces created by blasting with 25 μm Al_2O_3 may be too small while those blasted with 250 μm Al_2O_3 created cavities too large to provide a sheltering effect.

In conclusion, although biofilm resistance to amphotericin B formed on titanium discs with different surface roughness differed significantly, the precise resistance mechanisms are still unknown. It would appear that a balance has to be found so as to allow optimal implant osseointegration and yet minimize antimicrobial resistance should biofilm form on the implant surface. Further studies which include analyses of the yeast cell attachment, biofilm structure and gene expressions of the yeast cells at different development stages would provide further insight into the surface roughness/antimicrobial resistance relationship.

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References

1. Abi-Said D, Anaissie E, Uzun O, Raad I, Pinzcowski H, Vartivarian S (1997) The epidemiology of hematogenous candidiasis caused by different *Candida* species. Clin Infect Dis 24:1122–1128
2. Bagno A, Di Bello C (2004) Surface treatments and roughness properties of Ti-based biomaterials. J Mater Sci, Mater Med 15:935–949
3. Baillie GS, Douglas LJ (2000) Matrix polymers of *Candida* biofilms and their possible role in biofilm resistance to antifungal agents. J Antimicrob Chemother 46:397–403
4. Banerjee RP, Emori TG, Culver DH, Gaynes RP, Jarvis WR, Horan T, Edwards JR, Tolson J, Henderson T, Martone WJ (1991) Secular trends in nosocomial primary bloodstream infections in the United States, 1980-1989. National Nosocomial Infections Surveillance System. Am J Med 91:86S–89S
5. Becker J, Kirsch A, Schwarz F, Chatzinikolaidou M, Rothamel D, Lekovic V, Laub M, Jennissen HP (2006) Bone apposition to titanium implants biocoated with recombinant human bone morphogenetic protein-2 (rhBMP-2). A pilot study in dogs. Clin Oral Investig 10:217–224 Erratum in: Clin Oral Investig 2006, 10:225. Laub, Markus (added)
6. Buser D, Schenk RK, Steinemann S, Fiorellini JP, Fox CH, Stich H (1991) Influence of surface characteristics on bone integration of titanium implants. A histomorphometric study in miniature pigs. J Biomed Mater Res 25:889–902
7. Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormick T, Ghannoum MA (2001) Biofilm formation by the fungal pathogen *Candida albicans*-development, architecture and drug resistance. J Bacteriol 183:5385–5394
8. Chandra J, Mukherjee PK, Leidich SD, Faddoul FF, Hoyer LL, Douglas LJ, Ghannoum MA (2001) Antifungal resistance of candidal biofilms formed on denture acrylic in vitro. J Dent Res 80:903–908
9. Cochran DL (1999) A comparison of endosseous dental implant surfaces. J Periodontol 70:1523–1539
10. Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. Science 284:1318–1322
11. Darvell BW, Samman N, Luk WK, Clark RKF, Tideman H (1995) Contamination of titanium castings by aluminium oxide blasting. J Dent 23:319–322
12. Donlan RM (2002) Biofilms: microbial life on surfaces. Emerg Infect Dis 8:881–890
13. Douglas LJ (2002) Medical importance of biofilms in *Candida* infections. Rev Iberoam Micol 19:139–143
14. Douglas LJ (2003) *Candida* biofilms and their role in infection. Trends Microbiol 11:30–36
15. Jenkinson HF, Douglas LJ (2002) In: Brogden KA, Guthmiller JM (eds) Interactions between *Candida* species and bacteria in mixed infections Polymicrobial diseases. ASM Press, Washington, DC, pp 357–373
16. Kuhn DM, George T, Chandra J, Mukherjee PK, Ghannoum MA (2002) Antifungal susceptibility of *Candida* biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. Antimicrob Agents Chemother 46:1773–1780
17. Kumamoto CA (2002) *Candida* biofilms. Curr Opinion Microbiol 5:608–611
18. LaFleur MD, Kumamoto CA, Lewis K (2006) *Candida albicans* biofilms produce antifungal-tolerant persister cells. J Antimicrob Chemother 50:3839–3846
19. Mukherjee PK, Chandra J (2004) *Candida* biofilm resistance. Drug Resist Updat 7:301–309
20. Mustafa K, Wennerberg A, Wroblewski J, Hultenby K, Lopez BS, Arvidson K (2001) Determining optimal surface roughness of TiO (2) blasted titanium implant material for attachment, proliferation and differentiation of cells derived from human mandibular alveolar bone. Clin Oral Implants Res 12:515–525
21. Quirynen M, Mei HC, Bollen CML, Schotte A, Marechal M, Doornbusch GI, Naert I, Busscher HJ, Steenberghe D (1993) An in vivo study of the influence of the surface roughness of implants on the microbiology of supra-and subgingival plaque. J Dent Res 72:1304–1309
22. Ramage G, Vande Walle K, Wickes BL, Lopez-Ribot JL (2001) Standardized method for *in vitro* antifungal susceptibility testing of *Candida albicans* biofilms. Antimicrob Agents Chemother 45:2475–2479
23. Samaranayake YH, Jin Y, Yau JY, Cheung BP, Samaranayake LP (2005) *In vitro* method to study antifungal perfusion in *Candida* biofilms. J Clin Microbiol 43:818–825
24. Schwarz F, Bieling K, Bonsmann M, Latz T, Becker J (2006) Nonsurgical treatment of moderate and advanced periimplantitis lesions: a controlled clinical study. Clin Oral Investig 10:279–288
25. Schwarz F, Sculean A, Romanos G, Herten M, Horn N, Scherbaum W, Becker J (2005) Influence of different treatment approaches on the removal of early plaque biofilms and the viability of SAOS2 osteoblasts grown on titanium implants. Clin Oral Investig 9:111–117
26. Thomas KA, Cook SD (1985) An evaluation of variables influencing implant fixation by direct bone apposition. J Biomed Mater Res 19:875–901

27. Verran J, Maryan C (1997) Retention of *Candida albicans* on acrylic resin and silicone of different surface topography. J Prosthet Dent 77:535–539
28. Vezeau PJ, Koorbusch GF, Draughn RA, Keller JC (1996) Effects of multiple sterilization on surface characteristics and in vitro biologic responses to titanium. J Oral Maxillofac Surg 54:738–746
29. Viudes A, Peman J, Canton E, Ubeda P, Lopez-Robot JL, Gobernado M (2002) Candidemia at a tertiary-care hospital: epidemiology, treatment, clinical outcome and risk factors for death. Eur J Clin Microbiol Infect Dis 21:767–774
30. Wennerberg A, Albrektsson T, Andersson B (1996) Bone tissue response to commercially pure titanium implants blasted with fine and coarse particles of aluminum oxide. Int J Oral Maxillofac Implants 11:38–45
31. Wennerberg A, Albrektsson T, Johansson C, Andersson B (1996) Experimental study of turned and grit-blasted screw-shaped implants with special emphasis on effects of blasting materials and surface topography. Biomaterials 17:15–22
32. Wennerberg A, Hallgren C, Johansson C, Danelli S (1998) A histomorphometric evaluation of screw-shaped implants each prepared with two surface roughnesses. Clin Oral Implants Res 9:11–19
33. Whitehead KA, Colligon J, Verran J (2005) Retention of microbial cells in substratum surface features of micrometer and sub-micrometer dimensions. Colloids Surf, B Biointerfaces 41:129–138