

# Real time optical coherence tomography monitoring of *Candida albicans* biofilm *in vitro* during photodynamic treatment

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## ABSTRACT

The biofilm formed by *Candida albicans* is the mainly cause of infections associated to medical devices such as catheters. Studies have shown that photodynamic antimicrobial therapy (PAT) has lethal effect on *C. albicans*, and it is based on photosensitizer (PS) in the presence of low intensity light to generate reactive oxygen species in biological systems. The aim of this study was to analyze in real time, by Optical Coherence Tomography (OCT), the alterations in *C. albicans* biofilm *in vitro* during PAT using methylene blue (MB) as a PS and red light. An OCT system with working at 930nm was used, sequential images of 2000x512 pixels were generated at the frame rate of 2.5frames/sec. The dimension of the analyzed sample was 6000µm wide by 1170µm of depth corrected by refraction index of 1.35. We recorded 1min. before and after the irradiation with LED for PAT, generating 8min. of video. For biofilm formation, discs were made from elastomeric silicone catheters. The PS was dissolved in PBS solution, and a final concentration of 1mM MB was applied on biofilm, followed by a red LED irradiation ( $\lambda=630\text{nm}\pm 20\text{nm}$ ) during 6min. We performed a curve of survival fraction versus time of irradiation and it was reduced by 100% following 6min. of irradiation. OCT was performed for measurement of biofilm thickness of 110µm when biofilm was formed. During irradiation, the variation of biofilm thickness was ~70µm. We conclude that OCT system is able to show real time optical changes provided by PAT in yeasts organized in biofilm.

**Keywords:** Photosensitizer, Antimicrobial, Yeast photoinactivation, Methylene blue

## 1. INTRODUCTION

Photodynamic antimicrobial therapy (PAT) is a phototherapy based on the use of substances that can photosensitize biological tissues and are capable of being activated in the presence of light to kill microorganisms. The PAT target cells are stained by a photosensitizer and irradiated by resonant light [1-4]. The photochemical process rapidly generates reactive oxygen species (peroxides, hydroxyl radicals, superoxide ions and singlet oxygen). This technique has been reported as an effective method against a large number of different pathogens [1,3,4], including microorganisms that are resistant to antibiotics [5].

Biofilm is an aggregate of microorganism embedded in an organized polymeric extracellular matrix, which assume different forms such as micro-tubes adhered to surfaces [6]. The biofilm formed by fungi, especially by the pathogen *Candida albicans* is the cause of colonization of medical devices, for instance catheters [7,8]. This microorganism is the main pathogen found in those case, where a spread may occur in the bloodstream, leading to patient morbidity and mortality [7,8]. These infections are particularly serious because the cells in biofilms are resistant to common antifungal [9-12]. The resistance to antifungal is acquired at the beginning of the biofilm formation, and apparently is governed by different mechanisms in new and mature biofilm. Several models *in vitro* were used to show the stages of development and processes for the formation of biofilms of *C. albicans*. Today, it is clear that the interaction cell-substrate and cell-cell are key steps for the development of biofilm, as well as differentiation in hyphae and production of extracellular matrix. Thus, new therapies are needed to eliminate the biofilm on the surface of these catheters.

Recently, our group showed that the parameters of irradiation in photodynamic therapy on *C. albicans* play an important role, evaluating the susceptibility of this pathogen to PAT; it was achieved up to 6 logs of yeast reduction in suspension

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[1]. However, biofilms formed by *C. albicans* presents a bigger challenge to be tested and evaluated regarding treatment dynamic in real time.

This study aimed to develop a biofilm model and analyze it by Optical Coherence Tomography (OCT) in real time as well as evaluate the microbial reduction following PAT. OCT is non invasive technique capable to produce high resolution image of microstructured scattering media [13].

## 2. MATERIALS AND METHODS

### 2.1 Biofilm development

*Candida albicans* ATCC90028 was aerobically growth on Sabouraud dextrose (Sb) agar for 24h at 37°C. Then, it was harvest, placed in 5mL of Sb broth and incubated at the same conditions for more 24h. The cells were washed twice in phosphate buffered saline (PBS) pH 7.4 and the inoculum was prepared with  $\sim 1 \times 10^7$  colony form units (cfu)/mL. In parallel with cell growth in broth, silicon elastomeric (SE) disks were immersed on serum fetal bovine in a Petri dish and incubated at 37° C in orbital shaker (RPM 60) for 24h.

After this period, the SE disks were carefully washed with PBS to remove the excess of calf serum and it was then incubated with *C. albicans* inoculum for 90min in orbital shaker at 37°C. This procedure was to provide adherence of yeast cells on SE disk surface.

Thereafter, SE disks were gently washed in PBS to remove non-adherent cells, and finally incubated in Sb broth for 48h at 37° C in orbital shaker [6-8,14].

### 2.2 PAT on biofilm

The biofilm on SE disks were immersed in Petri dish with 1mM methylene blue (MB) (Sigma Ltd, Poole, UK) in PBS solution and a pre-irradiation time of 10 min. was set in dark conditions. SE disks were carefully transferred to new Petri dish to perform irradiation.

A control group (L-MB-) without MB nor light, group with MB and without irradiation (L-MB+) and PAT groups (L+MB+) were performed to certificate PAT efficacy under 3, 6, 9 and 12 min. irradiation, that results in fluences of 35, 70, 106 e 141J/cm<sup>2</sup>. A LED device (MMOptics, São Carlos, São Paulo, Brazil) emitting wavelength  $\lambda=630\text{nm} \pm 20\text{nm}$  and 260mW output power was used to perform irradiation. A beam area of 1.33cm<sup>2</sup> was set to provide fluence-rate of 195mW/cm<sup>2</sup>, which covered all SE disk surface (Figure 1). Following irradiation, biofilm SE disks were transferred to a tube with 1mL PBS and it was agitated in vortex for 1min. After homogenizing, samples were removed from each tube and submitted to serial dilution in 96-well flat-bottomed microtiter plaque to provide 10<sup>-1</sup> to 10<sup>-4</sup> times the original concentration. Ten- $\mu\text{L}$  aliquots of each dilution were streaked onto a Sabouraud agar plate in triplicate and incubated to allow colony formation.

All samples group were counted and converted into cfu/mL for statistical analysis, which was performed using one-way analysis of variance (ANOVA). Mean comparisons were carried out with Tukey's test and the overall significance level was set at 5% (P<0.05) [15].

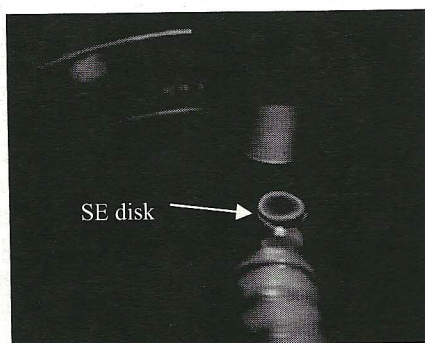


Figure 1. Biofilm in SE disks during PAT.

### 2.3 OCT monitoring PAT

An OCT system with super luminescent LED at 930nm with 2mW of power was used (OCP930SR-Thorlabs Inc.), sequential images of 2000x512 pixels were generated at the frame rate of 2.5 frames/sec. The dimension of the analyzed sample was 6000 $\mu$ m wide by 1170 $\mu$ m of depth corrected by refraction index of 1.35. The PAT group that we choose to OCT analysis was that during 6 min of PAT. We recorded 1min before and 1min after irradiation for PAT, generating 1,200 images and producing 8min of video.

## 3. RESULTS AND DISCUSSION

In all assays, control groups were set to observe the effects of irradiation, the toxicity of the dye in the dark and environmental control. In all experiments, we found no statistically significant differences between these groups. These results agree with findings in the literature, where there was no evidence of irradiation effects or toxicity of methylene blue in concentrations and pre-irradiation time studied [3,16-20].

The exposure time required to reduce 100% the number of viable *C. albicans* in biofilm was 6min. for 1mM MB (Figure 2). In fact, this concentration is ten times higher than that to kill cells in suspension [1,2]. Some authors observed that the extracellular polymeric matrix hinders the diffusion of antifungal agents, and when the drug penetrates the biofilm, its concentration is much lower than the concentration outside [9,11,21,22]. In addition, the biofilm, is rich in polysaccharides, which is able to significantly reduce, but not block, the penetration of antifungal agents [10,23]. The use of a higher concentration of MB improved its distribution in the biofilm. Using methylene blue 1.0mM, we obtained a reduction above 99% following 3min. of irradiation, and 100% after 6min (Figure 2). These findings suggest that MB at a concentration of 1.0mM, even with the protective factors of the biofilm, is widespread in the extracellular polymeric matrix in sufficient concentrations to PAT.

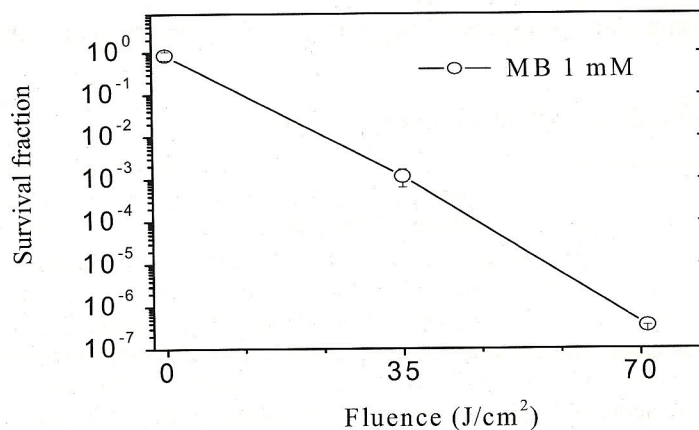


Figure 2. Mean values  $\pm$  SD of the survival fraction of *C. albicans* biofilm following PAT.

Figure 3 shows the architecture of the biofilm using the OCT technique. It is observed that the biofilm surface presents a cellular layer, characterized by high scattering of light (brightest region) followed by a thicker layer (dark region), which is the disc SE. The biofilm thickness was 110 $\mu$ m.

Destruction of biofilm structure mediated by PAT has been evaluated by electronic and confocal microscopy; however, OCT method was able to monitor changes in biofilm structure in real time. The biofilm surface is rich in water, retained by the hyphae formed by presenting a greater light transmission (Figure 3). During photodynamic therapy, this layer has increased, suggesting the destruction of the architecture of the biofilm probably caused by ROS destruction of these yeast cells and biofilm structure. The major mechanism of action of PAT is due to high levels of ROS production following photosensitizer irradiation [1,3]. Thus, it is a non selective mode of interaction, which may affect microbial cells, as well as non-cellular biomolecules such as polysaccharides involved in extra cellular polymeric substances [24,25].

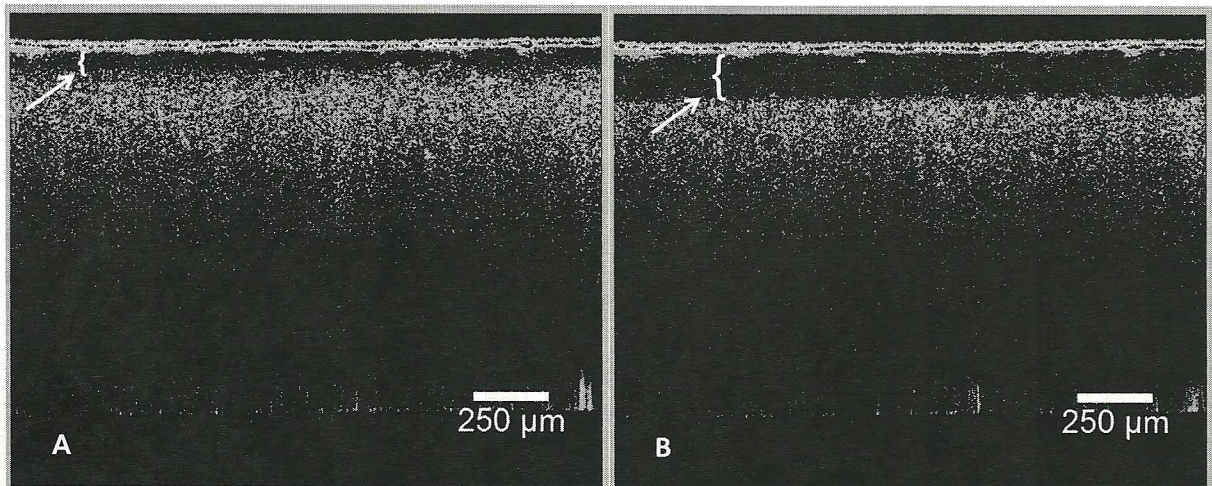


Figure 3. Video of OCT monitoring of *Candida albicans* biofilm in vitro during photodynamic treatment. A) before and B) after PAT with 1mM MB and red laser. Note the increased thickness of the layer indicated by arrow after PAT (A and B).  
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#### 4. CONCLUSION

MB at 1mM was efficient to reduce 100% of *C. albicans* after 6min. irradiation. The OCT showed to be a powerful tool to be used to monitor biofilm changes following photodynamic antimicrobial therapy in real time.

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