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Photoacoustic Spectroscopy of *Candida albicans* Treated with Methylene Blue

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Abstract In the present work the phototoxic effect of methylene blue (MB) on *Candida albicans* cultures was studied using the photoacoustic spectroscopy (PAS) technique. An aliquot of 50 μL of *C. albicans* suspension—strain ATCC 10-231—was incubated with 50 μL of MB solution (0.5 mg/mL), at room temperature. After the proper incubation time, a colony forming unit (CFU) with approximately 3 mm diameter was chosen in each plate. The CFU selected was irradiated using an InGaAlP laser during 20 s. After irradiation and new incubation, the CFUs were collected and stored at −70 °C, until spectroscopy analysis. The spectroscopy analysis was performed using an open PAS setup. The study was conducted in different groups: (1) control (non-treated); (2) irradiated with laser light; (3) treated with MB (non-irradiated); and (4) treated with MB and irradiated with laser light. The PAS measurements were performed on *C. albicans* in a sterile physiological solution. The measurements indicate that the presence of MB and irradiation promotes a change in the redox state of the cells to the reduced state. The absorption spectrum after photodynamic therapy (PDT) was observed 12 h and 36 h later. It was inferred that PDT can be related to structural changes in cytochrome molecules after 36 h. It is concluded that MB can be an efficient photosensitizer in *C. albicans* through modification of the cytochrome molecule affecting the cell metabolism.

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1 Introduction

Photoacoustic spectroscopy (PAS) is an experimental technique very useful in the study of the protozoan parasite. PAS is a nondestructive spectroscopy technique based on the photoacoustic effect, that consists of the conversion of light (electromagnetic energy) into sound (mechanical energy) [1]. PAS is a very versatile technique, because the studied materials do not need any special preparation. PAS has been used to study the malaria parasite (*Plasmodium chabaudi*) [2], the leishmaniasis parasite (*Leishmania mexicana*) [3], and the amoebiasis parasite (*Entamoeba histolytica*) [4]. Photodynamic therapy (PDT) is a process which combines light and a photosensitizing drug, promoting a phototoxic response on the treated cells, in general via oxidative damage [5,6]. The potential of photodynamic antimicrobial chemotherapy (PACT) to promote microbial eradication is progressively more accepted. This technique, such as PDT, involves the production of highly cytotoxic singlet oxygen and other reactive oxygen species, promoting photodynamic microbial damage. The photodynamic effects of different photosensitizing drugs on the pathogen *Candida albicans* were demonstrated by distinct authors [7–11]. However, in spite of the increasing interest by PACT, the specific cellular mechanisms that participate in this event are not clear. In this work we studied the phototoxic effects of methylene blue (MB), a Phenothiazinium photosensitizer, on *C. albicans* cultures. To study the absorption spectrum of *C. albicans*, the best technique is PAS because it does not need previous sample preparation, and samples can be analyzed as solid, liquid, paste, powder, or in another state. The amount of sample necessary for spectroscopy is also different when compared with other techniques [1,12].

2 Experimental Apparatus and Procedures

2.1 Organisms and Growth Conditions

Cultures of *C. albicans*—strain ATCC 10-231—were cultivated on a Sabouraud dextrose agar medium (Merck) and incubated in atmospheric air at 37 °C. After 48 h of incubation, a sample of colonies was removed from the surface of the agar plate and suspended in sterile normal saline and adjusted, by transmittance at 530 nm, to a 1 McFarland standard. An aliquot of 50 μL of *C. albicans* suspension was incubated with 50 μL of MB solution (0.5 mg/mL), at room temperature. After 30 min of incubation, an aliquot of 10 μL was evenly spread on a Petri dish with a Sabouraud dextrose agar medium and incubated for 48 h at 37 °C. After the incubation time, a colony forming unit (CFU) with approximately 3 mm diameter was chosen in each plate. The CFU selected was irradiated using an InGaAlP laser (Thera Lase®—DMC) during 20 s. The laser parameters, as informed by the manufacturer, were $\lambda = 685$ nm, power of 35 mW, and energy density of $10\,\mathrm{J \cdot cm^{-2}}$. In accordance to manufacturer parameters, the calculated energy density in our 3 mm diameter CFU is about $4.4\ \mathrm{J \cdot cm^{-2}}$ (in the literature was observed the use of different energy densities as $40\,\mathrm{J \cdot cm^{-2}}$ to

$80\,\mathrm{J \cdot cm^{-2}}$ [9], $0\,\mathrm{J \cdot cm^{-2}}$ to $12.6\,\mathrm{J \cdot cm^{-2}}$ [10], or $28\,\mathrm{J \cdot cm^{-2}}$ [11]). After irradiation, the selected colony was removed from the surface of the agar plate and suspended in sterile normal saline and adjusted by transmittance at 530 nm to a 1 McFarland standard. An aliquot of 10 μL was evenly spread on a Petri dish containing Sabouraud dextrose agar medium, and incubated for 72 h at 37 °C. After that, the CFUs were collected and stored at −70 °C, until spectroscopy analysis. The *C. albicans* groups studied were: non-treated group (control), irradiated with laser light, treated with MB (non-irradiated), and treated with MB and irradiated with laser light.

2.2 Spectroscopy

The spectroscopy analysis was performed using an open photoacoustic setup as described by Marquezini et al. [13]. Basically, a white light beam supplied by a 1000 W xenon lamp (Model 66071, Oriel) was modulated at 17 Hz by a mechanical chopper (Model 197, EG&G) and passed through a monochromator (Model 77250, Oriel). The monochromatic beam was focused onto the sample using quartz lenses and mirrors. The sample was placed over an open photoacoustic cell. The open photoacoustic cell consisted of a cylindrical electret microphone closed in its top with a quartz window, attached to the microphone with vacuum grease. A two-phase lock-in amplifier (SR850, Stanford Research Systems) was used to record simultaneously the amplitude and phase of the signal. The lock-in amplifier was interfaced to a microcomputer to record the signal. A drop of 10 μL of the respective analyzed sample was placed over the quartz window attached to the microphone, and after that, the optical spectrum was obtained from 360 nm to 700 nm. As the obtained spectrum corresponds to the transmittance, to get the absorption spectrum, the natural logarithm of the normalized spectrum was calculated. To normalize the spectra, they were divided by the corresponding one without sample over the window, as described by Marquezini et al. [13]. The obtained data were analyzed using the software Microcal™ Origin®

3 Results and Discussion

The absorption spectra obtained for the *C. albicans* control and with MB are shown in Fig. 1. An adjustment of each spectrum by Gaussian curves was done to provide us a better idea of the contribution from different absorption peaks, as was done by Karu et al. [14]. The observed absorption bands in the control spectrum (Fig. 1A) correspond with the sum of the absorption spectra of cytochrome *b* or *c* (peaks at 413 nm and 549 nm) and ferredoxin (peaks at 413 nm and 481 nm) extracted from protist organisms [15–17]. Figure 1B shows the absorption spectrum of *C. albicans* treated with MB. Figure 2 shows the absorption spectra of *C. albicans* treated with MB and irradiated, respectively, 12 h and 36 h after treatment. In the control spectrum (Fig. 1A) and *C. albicans* irradiated spectrum (figure not shown), only three Gaussian curves were necessary to fit the whole absorption spectrum. The number of Gaussian curves necessary for the adjustment of the absorption spectrum in the presence of MB non-irradiated (Fig. 1B) and 12 h after irradiation (Fig. 2A) increased to four. The fourth band must be related with the presence of the MB pigment in the yeast. When

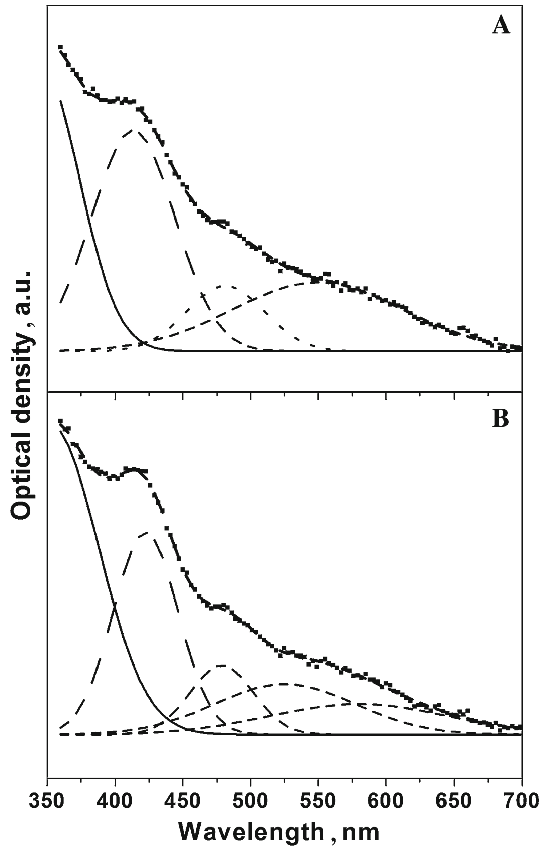

Fig. 1 (A) Absorption spectrum of *C. albicans* control. (B) Absorption spectrum of *C. albicans* plus methylene blue. In both cases, *dotted lines* represent the Gaussian curves used to adjust the experimental spectrum. Observe that in (B) is necessary one Gaussian curve more in comparison to (A)

C. albicans was treated with MB, it can be observed that the bands at 413 nm and 549 nm dislocated to 422 nm and 525 nm, respectively; however, the band at 481 nm in the control spectrum remains the same in the presence of MB (at 478 nm). So, it could be inferred that MB changes the redox state of the cytochrome molecule and does not affect the ferredoxin molecule. Also, the translation of the peak position indicates that the cytochrome molecule changed its redox state from oxidized to reduced [15]. Twelve hours after irradiation, the cytochrome molecule and ferredoxin absorption peaks maintain its central position as in Fig. 1B; however, the peak associated to MB (579 nm at Fig. 1B) apparently changes its center position to lower wavelengths (509 nm at Fig. 2A). This Gaussian curve after 12 h may be related not to the MB pigment but to photoproducts originated from MB light absorption. After 36 h (Fig. 2B), just three Gaussian curves are necessary to adjust the absorption curve. The absorption peaks associated with ferredoxin in Fig. 2B are in similar positions to the *C. albicans* control spectrum (Fig. 1A), showing that the ferredoxin molecule is not affected in its spectral properties, but the absorption curve associated with the cytochrome molecule disappears and a Gaussian curve at 455 nm composes the whole spectrum at Fig. 2B, which could be associated with modified byproducts from the irradiated MB.

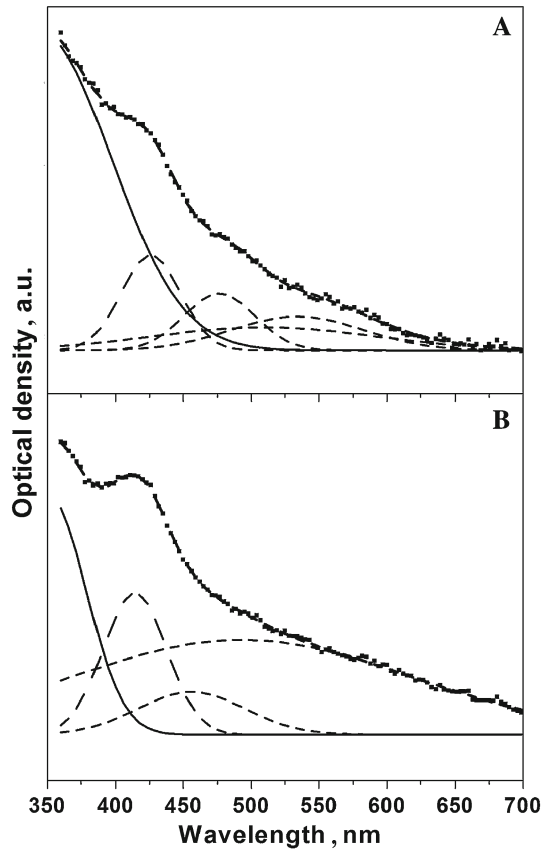

Fig. 2 (A) Absorption spectrum of *C. albicans* plus methylene blue 12 h after irradiation. (B) Absorption spectrum of *C. albicans* plus methylene blue 36 h after irradiation. In both cases *dotted lines* represent the Gaussian curves used to adjust the experimental spectrum

MB has been used as an efficient photosensitizer in PDT [18–20]. The absorption spectra obtained from *C. albicans* shows differences according to the treatment employed. The Gaussian curves associated with cytochrome *b* or *c* show that this chromophore is in a reduced state in the presence of MB and after 12 h of irradiation; however, after 36 h, its characteristic peak at 525 nm disappears. These data indicate that the presence of MB promotes a change in the redox state of the *C. albicans* cells, and the irradiation provokes the modification of the molecule after 36 h, changing its radiation absorption properties. It is clear that the event of PDT can be related to the modification of the cytochrome molecule. Thus, MB can be an efficient photosensitizer in *C. albicans* through the modification of the cytochrome molecule affecting the cell metabolism.

4 Conclusion

For the first time the absorption spectra of the *C. albicans* yeast were obtained. The absorption peaks have been associated to cytochrome and ferredoxin molecules. Our results indicate that PDT using MB on *C. albicans* must be associated to structural

modifications of cytochrome molecules, affecting the cell metabolism and, consequently, killing the cells.

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