Organic Light Emitting Diode for *in vitro* Antimicrobial Photodynamic Therapy of *Candida* Strains

Uriel Melendez-Celis[a], Teresita Spezzia-Mazzocco[a*], Saydulla Persheyev[b], Cheng Lian[b], Ifor Samuel[b*], Julio C. Ramirez-San Juan[a], Ruben Ramos-Garcia[a]

[a] *National Institute of Astrophysics, Optics and Electronics. Luis Enrique Erro #1 Sta Maria Tonantzintla, 72840, Puebla, Mexico.*
[b] *Organic Semiconductor Centre, SUPA, School of Physics and Astronomy, University of St Andrews, St Andrews, Fife KY16 9SS, UK.*

[*]Corresponding authors:

terespezzia@inaoep.mx
idws@st-andrews.ac.uk

Highlights

- OLED devices are effective light sources for aPDT
- The present work expands the range of microbes that can be treated by OLED devices.
- Fractionating a given light dose gave more effective inhibition for aPDT

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[b] *Organic Semiconductor Centre, SUPA, School of Physics and Astronomy, University of St Andrews, St Andrews, Fife KY16 9SS, UK.*

Abstract

Organic light emitting diodes (OLEDs) are very attractive light sources because they are large area emitters and, can in principle, deposited on flexible substrates. These features make them suitable for ambulatory photodynamic therapy (PDT), in fact there are a few reports of *in vitro* or *in vivo* OLED based PDT studies for cancer or microbial inhibition but to our best knowledge, none against yeasts. Yeast infections are a significant health risk, especially in low income countries with limited medical facilities. In this work, OLED-based antimicrobial PDT (aPDT), using methylene blue (MB) as photosensitizer (PS), is studied to inactivate opportunistic yeast of four Candida strains of two species: *Candida albicans* and *Candida tropicalis*. Before aPDT experiments, fluconazole-resistance was evaluated for all strains, showing that both strains of *C. tropicalis* were resistant and both strains of *C. albicans* were sensitive to it. We found that is useful for aPDT and that 3 repetitive irradiations work better than a single dose while keeping the total fluence constant, and that this result applies whether or not the strains are resistant to fluconazole.

Key words: OLED, photodynamic therapy, Candida, methylene blue, in vitro

Introduction

For more than 3.5 billion years, microorganisms have populated our planet, currently accounting for about 90% of the mass of all living beings. Humans have learned to live with them in such a way that they are now indispensable for our survival. However, a small amount of these microorganisms are responsible for many diseases and deaths in humans. Pathogenic microorganisms are classified into four groups: bacteria, viruses, parasites and fungi. Fungi have the greatest worldwide distribution but are the least studied. Fungi are responsible for a large number of secondary infections. The most important etiological agent for opportunistic mycoses is the *C. albicans*. Candida infections can be significantly aggravated when they are systematically spread in the body, reaching mortality rates of 40% to 80% in immunocompromised hosts, 60% among non-immunocompromised patients and 67% in diabetic patients[1,2] leading to extended hospital stays and high mortality rate[3,4]. They are also responsible for prolonged stays in the hospital's intensive care areas and therefore[5,6], higher hospitalization costs. To make matters worse, in recent decades, a rise in the number of non albicans infections has been reported, with *C. tropicalis* the most prevalent.[7] In fact, *C. tropicalis* is one of the most virulent pathogens among Candida species and one of the most efficient biofilm-forming with adherence to epithelial cells and inert surfaces[5]. In addition, *C. tropicalis* is particularly a health threat given its resistance to azoles[8] and its ability to grow in highly salty environments. [6, 8]. *C. albicans* and *C. tropicalis* share many genome similarities and both produce true mycelia which help them invade the host. However, they also show unique characteristics that makes them special such as the amount and type of proteases, phospholipases and hemolysins they produce.

Antifungal treatments have evolved since early nonspecific antifungal treatments with low effectiveness. The introduction of amphotericin B in 1956 represented a cornerstone in the treatment of systemic antifungal infections[9]. However, the best results have been obtained with the advent of azoles, allylamine and morpholine derivatives.[10] Nowadays, one of the most commonly used antifungal drugs is fluconazole, for which *C. albicans* is normally sensitive. However, the excessive use of this drug has led to resistant species becoming a public health problem worldwide. That, added to the relatively short times that microorganisms and specifically Candida yeasts require to replicate and proliferate, its complete elimination is complicated. This is particularly true in low income countries where fungal infections show a high morbidity incidence.[2] The search for alternative treatments is essential, especially those that propose novel metabolic routes other than the classic ones.

Among these alternative treatments, the aPDT is a promising option since it activates processes of cellular self-destruction through excitation of photosensitizing molecules. When a photosensitizer (PS) is in contact with the cell wall or within subcellular components and is photoactivated, it leads to the generation of reactive oxidizing species (ROS).[11] These species cause localized damage, promoting the activation of intrinsic processes of cell death, such as apoptosis or necrosis.[12] In addition, aPDT is a technique that is minimally invasive and maintains several advantages over traditional processes, such as low probability of drug resistance[10], acting on more than one cell target at the same time, being useful to treat multiple infections, and low cost.

In fact, PDT has emerged as an alternative treatment for many diseases (cancer, psoriasis, microbial infections, among others),[11–13]for which different light sources have been used. It is common to use LED-based or laser-based devices, however, an emerging and promising new light source has been recently demonstrated: organic light emitting diodes (OLEDs). In OLEDs, the light-emitting layer is a nanometric

thin layer of an organic semiconductor. This results in a light source that emits over a large area and can be flexible. OLEDs are lightweight, compact and wearable, making them suitable for patients to undergo ambulatory treatment. For example, Attili et al.[14] reported an open pilot study of ambulatory PDT using a wearable low-irradiance OLED source for treatment of nonmelanoma skin cancer. Likewise, Guo et al.[15] presented a pilot study of PDT using OLED to treat brain tumors.

aPDT has proven to be quite efficient in the inactivation of antimicrobial resistant microorganisms, particularly in bacteria[16–18] and yeast[19,20] using LED or laser-based devices, however, the use of OLEDs devices in aPDT is still very scarce. As far as we know, there is only one report using OLED in aPDT for bacteria inhibition (*Staphylococcus aureus*) using a large-area flexible OLED.[21] Finally, there are no reports for the inhibition of yeasts using OLEDs as light source for aPDT. In this work, we present a novel application of OLED light source to study the *in vitro* aPDT effect on opportunistic yeast, and additionally we show that aPDT efficiency can be improved by light fluence dose fractionation.

Materials and Methods

Biological material

We tested four yeast strains of *Candida* genera (two of *C. albicans* and two of *C. tropicalis*); all strains were donated by the Mycology Department of the Benemerita Universidad Autonoma de Puebla (BUAP), Mexico. For each species of yeast there was a laboratory reference strain and a patient-isolated strain from the collection of the Mycology Department BUAP. *Candida* strains were grown in Sabouraud dextrose agar (SDA) (Omnichem, Mexico) media for 24 hours and suspended in phosphate buffered saline solution (PBS): 287.5 mg sodium dibasic phosphate, 55 mg potassium chloride, 2015 mg sodium chloride, 50 mg monobasic potassium phosphate (Omnichem, Mexico) pH 7.4, before experiments.

Photsensitizer solution

MB (Omnichem, Mexico) was used as PS. A 200 μM concentration stock solution was prepared in PBS. The solution was sterilized with a syringe filter (cellulose acetate; pore size, 0.20 μm; diameter, 25 mm; GVS Life Sciences, USA), and stored at 4°C in the dark before used. The MB stock solution was diluted to 20 μM concentration with sterile PBS before aPDT application.

OLED fabrication

OLEDs were deposited on a glass substrate with thermal evaporation under 3×10^{-7} mbar (Angstrom EvoVac). The device structure of OLEDs is shown in **Figure 1a**. Materials used in the OLED fabrication: 80 nm aluminum as anode, 50 nm 2,2′,7,7′-tetrakis(N,N′-di-p-methylphenylamino)-9,9′- spirobifluorene (Spiro-TTB) doped with 2,2′-(perfluoronaphthalene-2,6-diylidene)dimalononitrile (F6-TCNNQ) (4 wt%) as hole-transport layer, 10 nm N,N′-di(naphtalene-1-yl)-N,N′-diphenylbenzidine (NPB) as electron-blocking layer. 40 nm NPB doped with Bis(2-methyldibenzo [f,h]quinoxaline)(acetylacetonate) iridium(III) [$Ir(MDQ)_2(acac)$] (10 wt%) as emission layer, 10 nm bis-(2-methyl-8-chinolinolato)-(4-phenyl-phenolato)-aluminium(III) (BAlq) as hole-blocking layer, 40 nm 4,7-diphenyl-1,10-phenanthroline (BPhen) doped with cesium as electron-transport layer, 20 nm silver as semi-transparent cathode, 80 nm NPB as capping layer. The OLEDs were encapsulated at the nitrogen atmosphere with glass lids and UV-curable epoxy glue (Norland NOA68). The active area of OLEDs is 2 cm by 2 cm.

OLED characterization

The electrical characteristics of the OLEDs were measured with a source meter. (Keithley 2400, Keithley). Electroluminescence spectra were measured using a spectrograph (MS125, Oriel) coupled to a charge coupled device (CCD) camera (DV420-BU, Andor). The light output and operational lifetime of OLEDs were measured with an irradiance meter (Gigahertz Optik P9710 with RW3703 detector head).

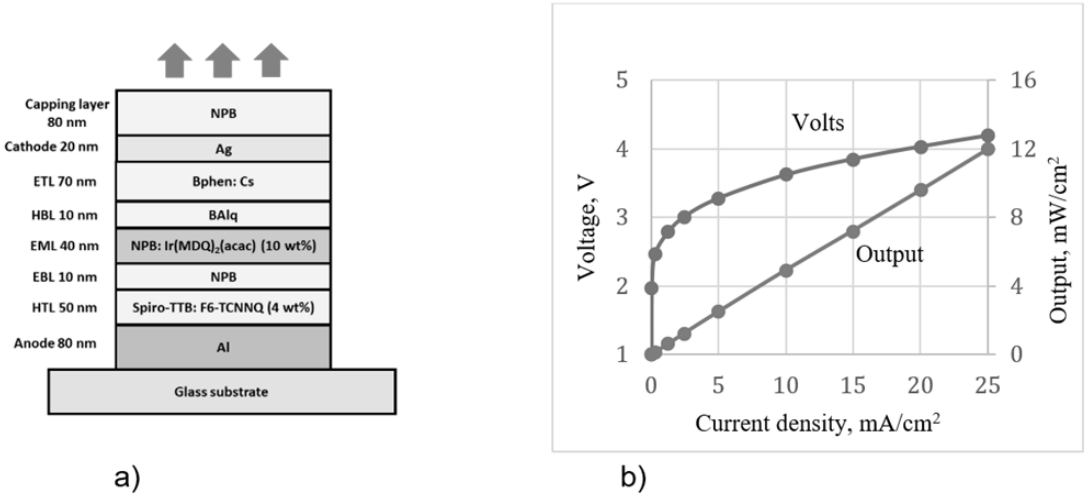

Figure 1. (a) OLED device structure. (b) Current-voltage-light output characteristics of the OLED

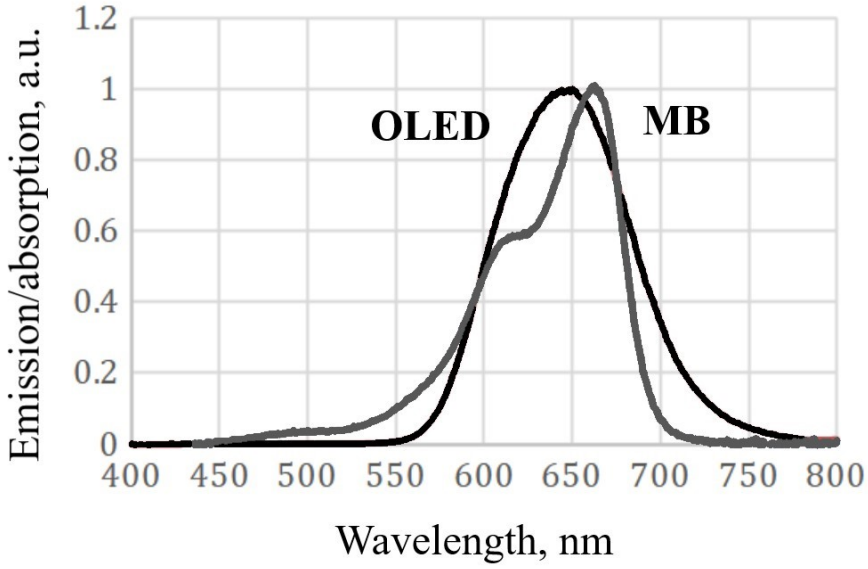

Figure 2. MB absorbance at 20 µM and OLED device emission spectrums.

The optical power of the OLED measured at a distance of 4 mm and considering an area equal to the one of the well´s plate (A=0.352 cm^2) the intensity was calculated as ~9.44 mW/cm^2. The fluences employed were 5.6 J/cm^2, 11.3 J/cm^2, 17 J/cm^2 and 34 J/cm^2. **Figure 2** shows that the emission spectrum of the OLED

device (570- 750 nm) coincides with the absorption spectrum of the MB (500 to 700 nm, with a peak at 662 nm).

Fluconazole resistance

Fluconazole resistance test was performed adding fluconazole (Afungil, Altia, Mexico) at different concentrations (10, 20, 40 and 60 µg/ml) in SDA medium before pouring in petri dish at 30-32°C. When solidified, 100 µl of yeast solution at 5×10^3 CFU/ml in PBS was inoculated in each petri dish containing the fluconazole. Antifungal effect was evaluated by CFU counting after 24 hours at 30ºC incubation temperature.

aPDT procedure

The cells concentration in PBS suspension was calculated with a hemocytometer (Neubauer improved cell counting chamber 0.1 mm depth; Marienfeld, Germany). The initial concentrations were adjusted by diluting the yeast suspension with PBS solution to reach 2-4×10^4 cell/ml. From previous aPDT studies[22] we found that 20 µM concentration of MB ensures cell deaths at the right energy density. Of course, better results are obtained at higher concentrations, but we want to reduce the MB concentration and energy density as much as possible. An initial 40 µM concentration of MB solution was prepared. Then 50 µL of MB solution and 50 µL yeast were placed in the central wells of a 96 well microplate to give a 100 µL solution with a final PS concentration of 20 µM and 1-2×10^4 cell/ml. For light controls, 50 µL of PBS was added instead of the MB solution. The yeast in the microplate was incubated for 30 minutes at 30°C in the dark. Subsequently, activation of the PS was performed in two ways: 1) applying a single irradiation (1R) with the total light fluence of 17 and 34 J/cm^2 and, 2) fractioning these light fluences into 3 irradiations (3R) (5.6 and 11.3 J/cm^2 per irradiation respectively) with 30 minutes dark incubation time intervals (DIT) between each irradiation as seen on **Figure 3.**

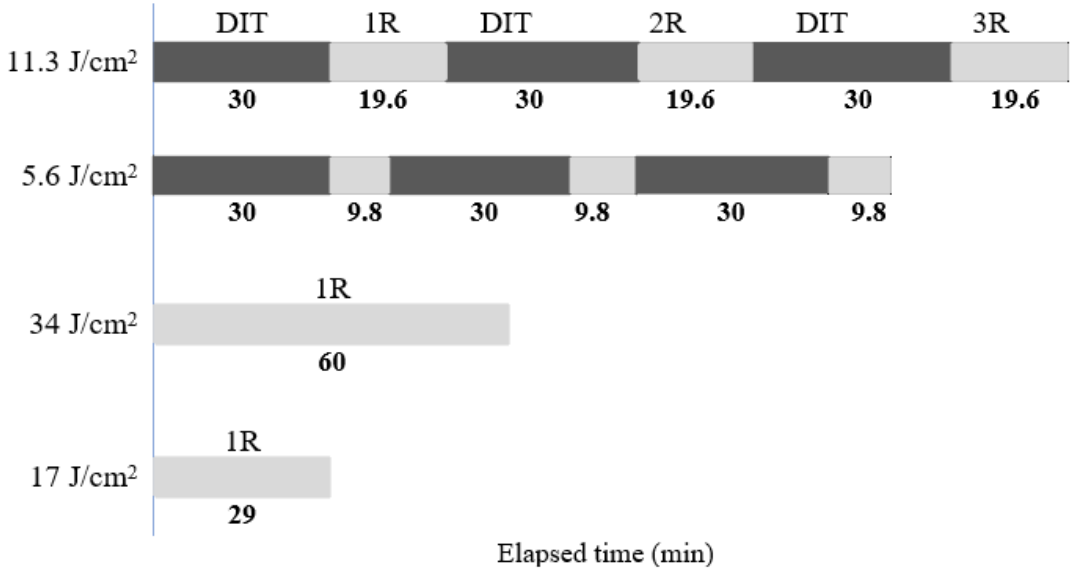

Figure 3. Irradiation sequences employed for the *in vitro* aPDT treatments. The dark incubation time (DIT) was set to 30 min between each radiation (1R, 2R and 3R) of 11.3 and 5.6 J/cm^2 to give a total fluence of ~34 J/cm^2 and ~17 J/cm^2 respectively. In comparison a single radiation of 17 and 34 J/cm^2 was also tested.

All experiments were done in triplicate with the corresponding controls: cell control without any treatment, light control (OLED irradiation without PS), and dark toxicity control (only MB without light). After aPDT experiments, 100 µl of *Candida* solution from each well were massively inoculated onto a petri dish with SDA media and incubated at 30°C for 48 h. CFU/ml were counted after 48 h and the efficiency of the treatments was assessed. The results were analyzed by analyses of variance (ANOVA) and the Tukey test. A significance level of 5% ($p < 0.05$) was considered to indicate a statistically significant difference.

Results

Fluconazole resistance

Table 1, shows Candida antifungal response to fluconazole, measured as the percent of CFU inhibition compared with the control. These results showed that the CFU inhibition of the two strains of *C. albicans* is higher than 90% at all doses of fluconazole studied. On the other hand, the two strains of *C. tropicalis* showed an CFU inhibition smaller than 5% at all the fluconazole doses. Therefore, *C. albicans* strains were considered sensitive and *C. tropicalis* strains resistant to fluconazole, no matter whether they are laboratory or patient-isolated strains.

Table 1. Candida response to fluconazole resistance test

Percent of CFU inhibition / Yeast strain	Fluconazole (µg/ml)			
	10	**20**	**40**	**60**
Laboratory reference *C. albicans*	91.4	97.2	97.6	99.5
Patient *C. albicans*	96.4	97.5	96.4	96.7
Laboratory reference *C. tropicalis*	1.3	1.4	3.6	4.4
Patient *C. tropicalis*	5.0	2.7	5.1	0.6

aPDT controls: 1R vs 3R fractionated light doses

Figure 4 shows the dark toxicity controls (only MB) for all the tested strains. In all cases, low inhibition (less than 10 %) of CFU was observed.

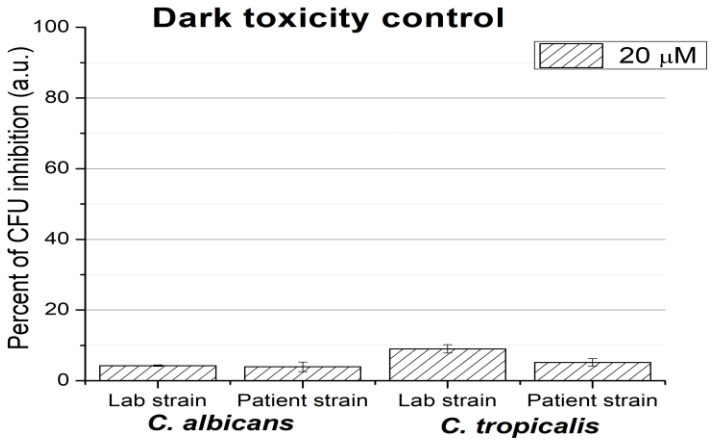

Figure 4. Dark toxicity controls of MB for the four strains tested. No statistically significant difference was observed compared with the control without any treatment.

On the other hand, all light controls (1R) with the highest light fluence (34 J/cm^2) showed inhibition rate between 20 and 40% (**Figure 5**). The light control (1R) of *C. tropicalis* strains presents a significant statistics difference (marked with "*") compared with the control without treatment. Contrary, fractional light controls (3R) cause less than 20% inhibition in all strains, showing statistically significant difference (**) between light controls 1R and 3R in 3 of the 4 strains evaluated (**Figure 5**).

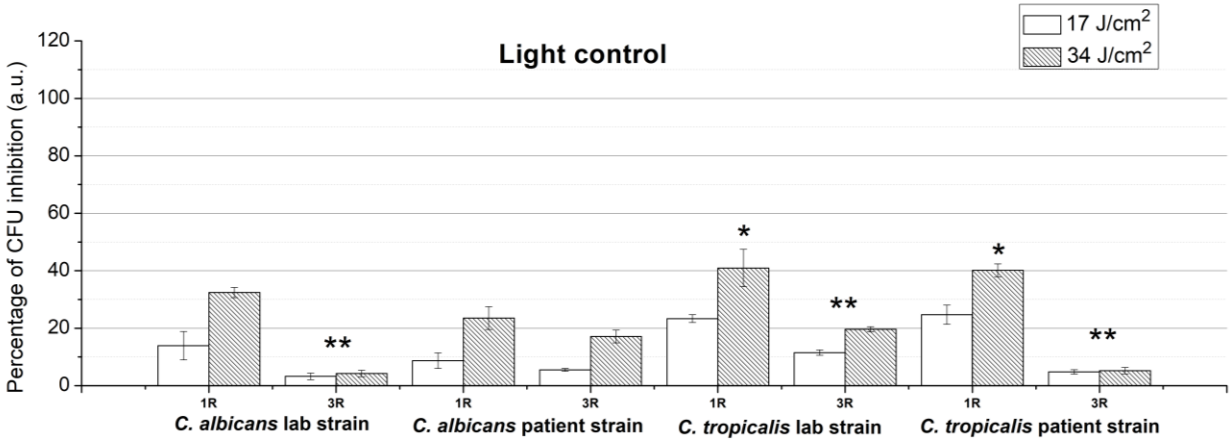

Figure 5. Light controls using one single (1R) and three fractioned irradiations (3R) with a total fluence light of 17 and 34 J/cm^2 for the four strains evaluated. There is a statistically significant difference (*) ($p < 0.05$) with respect to the control without treatment in 34 J/cm^2 for both strains of *C. tropicalis*. A statistically significant difference (**) is also observed when comparing the inhibition produced by applying the total light dose in a single irradiation (1R) and when applied in a fractional manner with 3 irradiations (3R), in 3 of the 4 strains evaluated.

aPDT treatment: 1R vs 3R fractionated light doses

Recently, we demonstrated[22] that it is possible to significantly reduce the amount of PS and light energy density requirements by using fractionated light doses using a LED-based device MB-aPDT, against *C.*

albicans. In the present work we extended and demonstrated similar results in different species of Candida: resistant strains versus sensitive strains and laboratory strains versus strains isolated from patients.

For aPDT evaluation, the yeast inhibition was tested by counting CFU 48 hours after treatment. **Figure 6** shows the performance of aPDT for 1R of 5.6, 11.3, 17.0 and 34 J/cm^2, as well as 2R and 3R (5.6 and 11.3 J/cm^2 each irradiations) for each of the four strains of *Candida*. Comparing the total fluence of light administrated in 1R vs 2R and 3R, it was found that the inhibition increased when the total fluence was fractionated.

As shown in **Figure 5**, the greater light fluence the higher inhibition. On the other hand, when the accumulated amount of light fluence increases (i.e. the number of irradiations increases), the inhibition increases too, that is, 2R is more efficient than 1R and 3R is more efficient than 2R, this behavior is observed for the four yeasts under study (**Figure 7**). When the amount of light fluence remains constant but is fractioned into 3 irradiations, the following is observed: *C. albicans* (sensitive to fluconazole) showed to be more sensitive than *C. tropicalis* (resistant to fluconazole) to aPDT since even for 2R (fluence of 11.3 J/cm^2) the percentage of inhibition for *C. albicans* was greater than 90%, while for *C. tropicalis* 3R were required to achieve a similar inhibition rate. OLED-aPDT can indiscriminately inactivate resistant and sensitive strains when an adequate fluence is used, especially when the application of light to the sample is fractionated.

The most notorious cases were: *C. tropicalis* laboratory strain, when it was subjected to aPDT with 17 J/cm^2 which, as can be seen in Figure 6, the inhibition was 46% applying 1R and 73% fractionating the light in 3R, that is, an increase in inhibition of 27%. Similarly, the aPDT of *C. albicans* patient strain using 34 J/cm^2, 73% inhibition was observed with 1R and 99% with 3R, that is, an increase in inhibition of 26%. For *C. albicans* lab strain, given their high sensitivity to aPDT, we obtained an inhibition of 95.3% even with 2R.

For *C. albicans* and *C. tropicalis* lab strains, 3R (accumulated fluence of 17 J/cm^2) are as efficient as twice the dose (34 J/cm^2) applied in 1R. For *C. albicans* patient isolated strain, it was even more efficient 3R (accumulated fluence of 17 J/cm^2) than twice the dose (34 J/cm^2) applied in 1R (**Figure 6**).

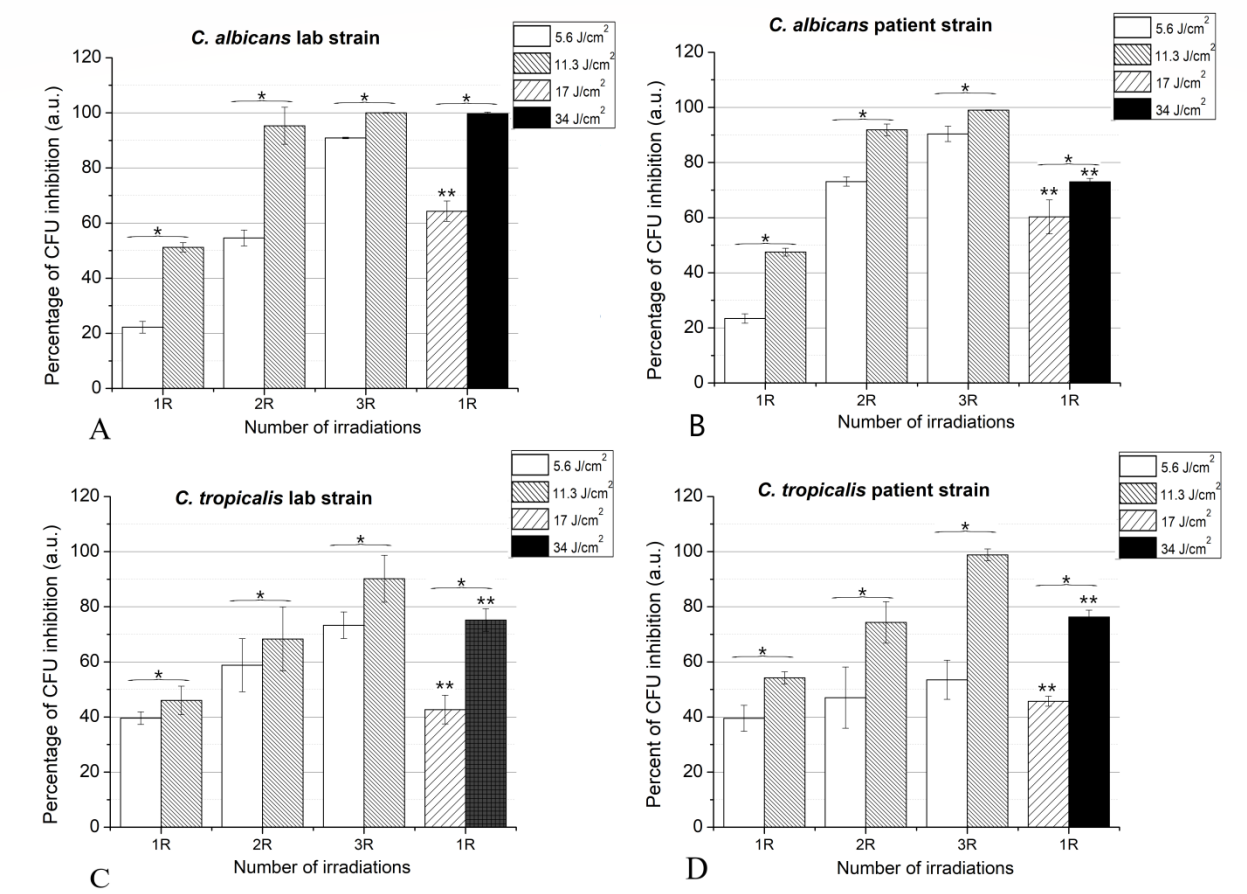

Figure 6. Inhibition obtained by activating 20 μM MB with three irradiations of 5.6 and 11.3 J/cm^2 each, and the effect of single and fractioned irradiation in MB-aPDT on: A) *C. albicans* lab strain, B) *C. albicans* patient isolated strain, C) *C. tropicalis* lab strain and D) *C. tropicalis* patient isolated strain. Statistically significant difference was obtained ($p < 0.05$) for all the treatments compared with the control group (*) and between a single irradiation and three irradiations (**) with the same total fluence.

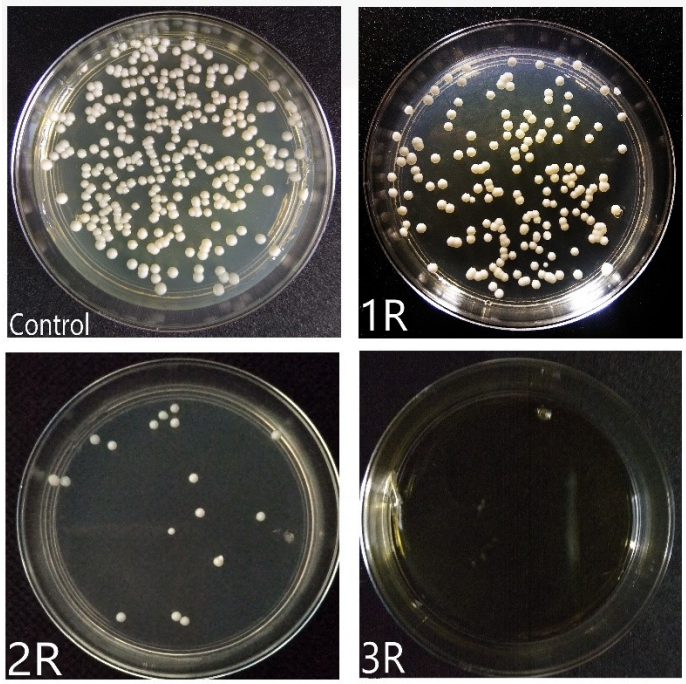

Figure 7. CFU inhibition after each irradiation compared with the control plate in *C. albicans* lab strain after 48 hours aPDT, notice that 100% inhibition was obtained for the third irradiation with 11.3 J/cm^2 (total fluence of 34 J/cm^2).

Discussion

The use of OLEDs in aPDT is quite attractive given its obvious advantages like lightweight, large area and low power consumption. However, OLEDs have lower optical power output than other PDT light sources. This is a potential limitation, though low intensity PDT allows more time for oxygen to diffuse to the site to be treated, and the wearable nature of OLEDs means that longer treatment times are likely to be acceptable. Some microorganisms replicate so fast that long exposure time to lower light intensity might not be effective since the microbial growth is commonly asynchronous i.e. every microorganism may be in different stages of the cellular cycle, which in turn may produce differences on the susceptibility of the treatment resulting in reduced efficacy. Despite these inconveniences, it was recently demonstrated that bacterial (*Staphylococcus aureus*) inhibition using a large-area flexible OLED-based aPDT.[21] Our study extends the use of OLEDs to different species of fungus.

The results show that aPDT results in higher CFU inhibition of *C. albicans* and *C. tropicalis* no matter their origin or its resistance to fluconazole. The metabolic diversity between *Candida* species may explain their different response to light exposure. It is known that different fungal species use light as a signal to regulate developmental transitions such as the germination of spores or conidia, the growth of vegetative hyphae, and the development of sexual or vegetative reproductive structures.[23] In fact, the presence of blue and red receptors in the secondary metabolic paths have been reported in fungi.[24] Also, it has been reported that visible light absorbed by cytochromes in mitochondria can have a negative effect on yeast cell respiration.[25] Thus, it is safe to assume a higher presence of light activated metabolites in *C. tropicalis* than in *C. albicans* that makes them more susceptible to light induced inhibition.

Regarding the higher efficiency of CFU inhibition by fractionated light doses may be associated to the rate of oxygen depletion. This problem was identified by Dougherty et al who decreased the light dose in order to diminish the oxygen depletion to keep the tissue oxygenation during PDT.[26] In addition, they showed that PDT treatments based on continuous low light or intermittent light dose delay the tumor recurrence.The dark illumination time (DIT) is critical to an efficient PDT based on light fractionation. In fact, it was speculated that oxygen diffusion beyond the illuminated region determines the optimal DIT (30 min). As second possible reason for the enhanced efficiency CFU inhibition is the incremental damage produced in the cells with each irradiation. During the first irradiation, the cell may suffer little damage, so a second or third exposure to light further damage the cell beyond any possibility of self-repair mechanism.

Finally, OLED devices having a lower intensity than traditional light sources are especially suited for fractional light fluence, reducing the risk of pain and sensitivity of the patient to the treatment, as some patients reported pain in treatment after irradiation.[27,28]

Conclusions

We have performed *in vitro* measurements that show OLEDs are effective light sources for aPDT of opportunistic yeasts. Our results expand the range of microbes that can be treated by OLEDs. In this work we demonstrated that both fluconazole-sensitive and fluconazole-resistant strains were inhibited by OLED aPDT. We found that fractionating a given light dose into three parts gave much more effective inhibition for all the strains studied. For many light sources, implementing such a regime in practice would be very inconvenient because the patient would need to stay close to large, fixed light sources. The effectiveness of fractionating the light dose is particularly relevant to OLEDs for aPDT because they are wearable devices. Overall our results show that OLEDs are attractive light sources for aPDT, and that fractionating light doses is likely to enhance their effectiveness.

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Declarations of interest: none

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