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Research Article

Organic and inorganic light-emitting diodes for photodynamic therapy of cutaneous leishmaniasis

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Abstract

For effectively fighting worldwide infectious diseases such as cutaneous leishmaniasis, novel approaches are required. Photodynamic Therapy (PDT) is one such possibility. PDT involves applying a light-sensitive chemical (photosensitizer), which should be highly efficient, non-toxic, and work at longer light wavelengths. This photosensitizer needs to be activated by a light source that provides uniform emission over a large area, high intensity, easy to fabricate, compact, and low cost. In this work, we designed and built light sources based upon commercially available Organic Light Emitting Diode (OLED) and LED parts to experimentally validate the combination with methylene blue photosensitizer to kill *Leishmania major* and *Crithidia fasciculata* cells *in vitro*. Our results showed that suitable-sized OLEDs, as compact and uniform light sources, are very good candidates for photodynamic therapy and can be used to efficiently kill such kinetoplastids *in vitro*. Therefore, it has real potential to be used in wearable devices for ambulatory treatment of patients.

Introduction

Leishmaniasis is an important parasitic disease that affects around two million people every year, mainly in developing countries [1-5]. Effective and affordable treatment is needed, and Photodynamic Therapy (PDT) is a promising approach. In photodynamic therapy, a light source in combination with a Photosensitizer (PS) is used to generate reactive oxygen species [6-10] which destroy parasites. Methylene blue is efficient and a well-known PS, licensed by the FDA as a topical agent, and has maximum absorption in the red-light spectra.

There are a small number of chemotherapeutic treatments for visceral and cutaneous leishmaniasis, with miltefosine being the most recent addition that can be used to treat both. Its mechanism of action is not well understood [11-13] and

given it has an increasing problem with resistance in the field [14] and common side effects such as vomiting, diarrhea, dizziness, skin reactions and is not recommended to people with kidney, liver diseases, or expectant mothers, there is an urgent need for alternative therapeutic approaches [1-3,15] such as photodynamic therapy.

Since the late 1970s, PDT has been under development and has been intensively used in oncology including dermatology, surgery, urology, and many more [16-19]. PDT experimental data on the treatment of leishmanial diseases is still limited and cannot so far be recommended in routine clinical practice, more laboratory-based research is required to validate the treatment [15,20]. In the case of cutaneous leishmaniasis, when parasites affect mainly the skin surface and dermis [21], the possibility of effective treatment of the infected area with



PDT is more viable [19,22-24]. *Crithidia fasciculata* is a free-swimming kinetoplastid and is not infective to vertebrates [25]. It is an example of a non-human infective trypanosomatid but is closely related to several human parasites including *Leishmania spp*, which cause leishmaniasis, and for that reason is often used to test new therapeutic strategies against parasitic infections [26].

The aim of this paper is to explore the potential of OLED and LED-based light sources for PDT to kill promastigotes of *Leishmania major* and *Crithidia fasciculata* cells, Light sources for PDT are usually large and bulky. Instead, we have designed and realized small, portable, efficient light sources and used them for *in-vitro* studies in 96 well plates with the photosensitizer (MB).

Experimental

Light sources

The requirements for light sources used for PDT could be described as follows - high intensity, high uniformity, and spectral conformity to the absorption spectra of the photosensitizer. High intensity of the light sources is usually required to reduce exposure times, it is particularly important when sources are used for light treatment of humans and on animal tests when exposure times cannot exceed reasonable times enabling animals to receive a treatment. In the meantime, intensities around 100 mW/cm² and higher are not advised due to the burning sensation, erythema [17] on patients, and PS bleaching effects [27]. High uniformity of the source is required for the homogeneous distribution of photon energy to photosensitizer used for higher quantitative estimation delivered optical power [19,22].

Table 1 shows some of the previously published data on light sources used for PDT on various *Leishmania* species in the last 20 years. Laser-based light sources have high output power parameters but are strictly limited.

The cost of laser systems also limits the applications for these types of devices and is a particular concern as *leishmaniasis* is prevalent in developing countries. Broadband light sources are bulky and expensive, generally for laboratory use only, and are often not efficient. The most commonly used devices are inorganic LEDs, due to their high-power output, high efficiency, and low cost compared to other easily available devices, but still large machines.

Organic light-emitting diode light sources (OLED) attracting more attention for using biomedical applications such as antimicrobial PDT due to high uniformity, large area, flexibility, and increased lifetime [32].

We have designed and built our light emitting devices for PDT using OLED and LED (Lighting panel, Red, KANEKA Corp., and Red LED, CREE) based upon devices used for *in vitro* experiments that utilize common and useful 96-well plates for culturing and testing of cells including single cells such as *Leishmania major* and *Crithidia fasciculata*. We have analyzed different lighting approaches and came to the conclusion that underneath or bottom-up exposure of cells in the well plates is preferable because it allows to delivery of higher illumination intensities to the media containing the PS and cells, due to the shorter distance from the source (Supplementary Data Figure 1), thus avoiding use of high currents in our devices. High currents are undesirable because they shorten device lifetime and cause device heating, initiate aging and decrease the OLED lifetime of organic devices, and can affect the ambient temperature inside well plates, both of which we wish to minimize.

For easy and practical illumination of the live cells *in vitro* we have built the light sources based upon high performance, large area, high uniformity OLEDs, and using off-the-shelf high power LEDs. The wavelength of the sources was selected to match the spectral adsorption property of the chosen PS, i.e. methylene blue (662 nm) used in our experiments (Supplementary Data Figure 2). As can be seen from the figure, the methylene blue maximum absorbance overlaps with a local maximum of both the LED and OLED sources. This wavelength overlap of the methylene blue provides very good penetration through the human skin as well. The experimental LED setup accommodates 96-well plates and illuminates simultaneously 12 wells (Figure 1a,b) uniformly from underneath, with variable power ranging from 12.5, 25 up to 50 mW/cm².

The optimal distance from LED to the 96-well plate was chosen to be 4 mm, it permits emission intensity tolerance to be around $\pm 5\%$ when the incident angle does not exceed 40 degrees (Supplementary Data Figure 3). It is a very acceptable deviation of the optical power delivered to the cells in such experiments.

For large-area OLED devices, we used "Kaneka Corp." (part number 159002443A32, Red) high stability and uniform emitting devices with an emitting area of 80 mm x 80 mm,

Table 1: Light sources used for PDT to kill *Leishmania sp in vivo* and *in vitro*

Authors	LED Light source	Wavelength (nm)	PS	Method	Intensity, (mW/cm ²)	Fluence (J/cm ²)	Exp Time (min)
Song, et al. 2011 [24]	LED	650	MB	<i>In vivo</i>	5	9	30
Enk, et al. 2003 [21]	Broadband	570-670	ALA	<i>In vivo</i>	150	100	11
Peloi, et al. 2011 [28]	LED	665	MB	<i>In vivo</i>	5	12	60
Aureliano, et al. 2018 [20]	LED	645	MB	<i>In vitro</i>	100-250	20-260	1-5
Pinto, et al. 2017 [29]	LED	660	MB	<i>In vitro</i>	2.77	10	60
Pinto, et al. 2017 [30]	Laser	659	AIPcS4	<i>In vitro</i>	40	10	4
Nesi-Reisa, et al. 2018 [31]	LED	630	AIPcCl AIPcOH	<i>In vitro</i>	0.0025	0.0045	30

allowing simultaneous illumination of 64 out of 96 wells in the plate (Figure 1c,d) with fixed output intensity of around 2 mW/cm². During the exposure process, the temperature was monitored and did not go higher than 28 °C.

Other advantages include being an area light source instead of a point source for LEDs, being thin, and lightweight, and having the potential to be flexible or conformable to wear directly on the skin area being treated.

Photosensitizer

In photodynamic therapy, photosensitizer absorbs the energy of the photon and through the chain of energy, transfers cause the production of cytotoxic radicals such as singlet oxygen and oxygen reactive species. The more radicals created in the process of light absorption, the more effective the PS. Nontoxicity to cells, and biocompatibility of the PS when it is not illuminated with light are also of great importance for developing new therapies against leishmaniasis. Methylene blue (0–200 nm, pre-incubated for 30 minutes) was chosen as the best candidate in our case for the effective killing of *L. major* and *C. fasciculata* *in vitro* with a maximum of absorption around 662 nm wavelength.

Cells and culture

Crithidia fasciculata clone HS6 was cultured at 27 °C with gentle agitation in a liquid medium defined below in non-vented flasks. Cells were passaged to sustain mid-log phase growth by transferring cells into fresh media before the density reached $\sim 5 \times 10^7$ cells/mL [26]. Promastigotes from *Leishmania major* Freidlin strain were grown at 28 °C in M199 medium pH 7.4, supplemented with 40 mM HEPES pH 7.4, 100 μM adenosine, 5 μg/mL haemin, and 10% heat-inactivated fetal bovine serum. Cells were passaged every 2–3 days to maintain mid-log growth.

Cell viability

Alamar Blue cell viability reagent was used for quantitative fluorescence live/dead assays to measure the toxicity of methylene blue photosensitizer as part of PDT, against both *L. major* and *C. fasciculata* [26].

The cell viability was assessed with Alamar blue 24 h after control or PDT treatment. All assays were conducted in replicates of three or four.

The Alamar Blue assay allows a simple, reproducible method for PDT experiments [33]. When added to cells, Alamar Blue is metabolized by the reducing environment of viable cells turns red in color, and becomes highly fluorescent. Fluorescence measurements were performed with the Spectra Max Gemini XPS Microplate reader using an excitation between 530–560 nm and an emission at 590 nm.

Results

Initially, in our experiments, the *L. major* and *C. fasciculata* were illuminated at the highest intensity of LED light source 50 mW/cm² and the exposure time was 30 minutes with varying

concentrations of the MB in the solution; 3.15, 6.25, 12.5, 25 and 50 μM. Two control groups were done in parallel: the first one was cells that did not have any MB introduced, just exposed to light, and the second with no light, only with MB. Cells in control groups were not affected after 30 minutes. As we can see from our results (Figure 2), *C. fasciculata* demonstrated a more resistive nature to PDT at concentrations of MB up to 25 μM.

Next, the exposure of *C. fasciculata* cells to different fluences ranging from 22.5 to 45 and 90 J/cm² (accordingly exposure times 7.5, 15, and 30 minutes) while the light intensity was kept constant at 50 mW/cm² (Figure 3) was investigated.

Figure 3 shows how the higher doses increase the killing rate of *C. fasciculata*, such that at concentrations 50 μM and above, PDT with MB generates enough cytotoxic radicals to kill around 100% of cells under these *in vitro* conditions. We continued our investigations by using OLED-based light sources (Figure 4), to compare outcomes of LED and OLED light sources. The optimal output for OLED devices was 2 mW/cm² and exposure time to kill cells was 45 and 60 minutes, which is equal to fluences of 5.4 and 7.2 J/cm² respectively.

The direct comparison of OLED and LED exposures and their effect on *C. fasciculata* is shown in Figure 5. The effect of high-intensity light is more profound when similar doses

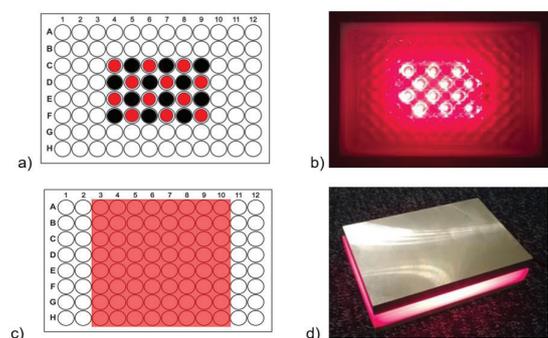


Figure 1: LED-based light source fitted with 96 well plates: a) LEDs positions with 96 well plates; b) 96 well plate image with LEDs on, top view; c) OLED underneath (bottom) illumination; d) complete working setup, side view.

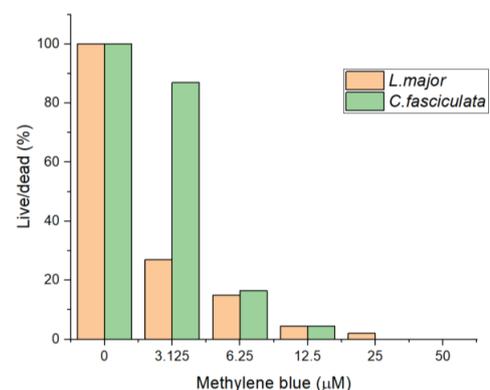


Figure 2: Cell viability of *L. major* and *C. fasciculata* after exposure 30 min to LED light source. A Fluence of 90 J/cm² was received at light intensity 50 mW/cm².

are delivered to parasites. Evidently, it is due to the higher efficiency of the high density of light (50 mW/cm^2), which generates a high concentration of radicals facilitating the killing and overcoming growth rate (doubled every 5–7 hours) of parasites [34].

In Table 2, we summarize our results with typical parameters, which have been used by authors in the current work for comparison when $25 \mu\text{M}$ MB is utilized.

Exposing *Leishmania* to MB photosensitizer at $50 \mu\text{M}$ or below only in the dark / in the absence of red light shows it has a detrimental effect on the cells in accordance with several other studies [20,22,24,28]. Our investigations showed no significant noticeable change in the viability of either *L. major* or *C. fasciculata*, which is consistent with the results from other groups as recently reviewed [35,36] and (Supplementary Data Table 1).

Discussion

The increasing prevalence of drug resistance in *Leishmania* over the past decades has become one of the primary causes of leishmaniasis treatment failure, Horácio, et al. 2021 [37]. Thus, it is unsurprising that the use of PDT as a potential alternative to treat CL is gaining traction to prevent the widespread number of recurrence and unresponsive cases to drug therapies Cabral, et al. 2021 [9].

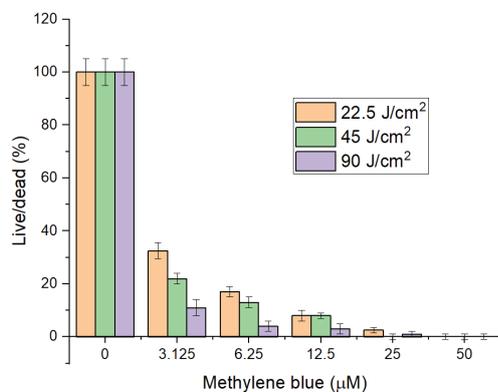


Figure 3: *C. fasciculata* exposed (LED) for 7.5, 15, and 30 minutes in the presence of different concentrations of MB.

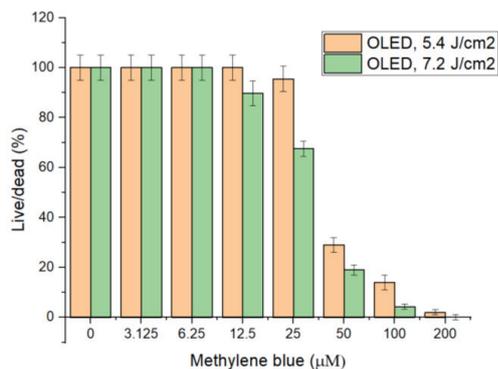


Figure 4: *C. fasciculata* was exposed for 45, and 60 minutes to OLED light in the presence of different concentrations of methylene blue photosensitizer.

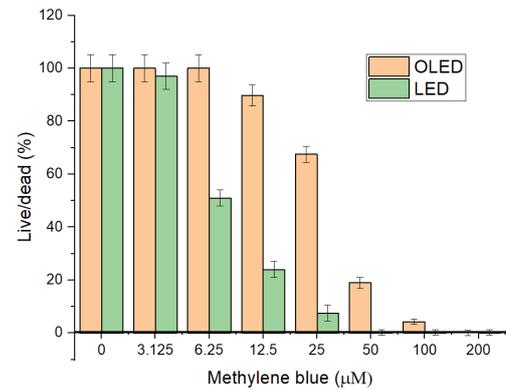


Figure 5: Live *Crithidia* cells after PDT (fluence 7.2 J/cm^2) using OLED and LED light sources at their basic intensities of 2 mW/cm^2 and 50 mW/cm^2 . The exposure times were for OLED 60 min and for LEDs 2.5 min.

Table 2: Comparison of obtained results on *L. major* and *C. fasciculata* species, photosensitizers, and light sources.

Cells	fluence (J/cm^2)	MB (μM)	LED, (mW/cm^2)	OLED (mW/cm^2)	Live cells (%)	Time (Sec)
<i>C. fasciculata</i>	5.2	25	-	2	96	2600
<i>C. fasciculata</i>	7.2	25	-	2	63	3600
<i>L. major</i>	7.2	25	50	-	8	144
<i>C. fasciculata</i>	22.5	25	50	-	3	450
<i>C. fasciculata</i>	90	25	50	-	1	1800
<i>L. major</i>	90	25	50	-	3	1800

We have demonstrated that OLED and LED PDT can be effectively employed to kill *C. fasciculata* (a suitable non-infective to vertebrate model) and *L. major in vitro* even at low light intensities, i.e. at around 2 mW/cm^2 .

To improve the killing rates, thus the effectiveness of PDT for *L. major*, i.e. CL there is a need for higher output intensities for OLEDs, i.e. $\sim 10 \text{ mW/cm}^2$. In other words, the higher efficiency of high density of light generates a high concentration of radicals facilitating the killing of the parasites. In the future, it will be important to understand the mode of killing of these parasites caused by the PDT and if other photosensitizers provide a more efficient killing profile. Organic light-emitting diodes would be more efficient if it is brighter, they are already highly attractive for this practical application, where the flexibility of the material lends itself well to direct contact with the skin in any body location.

Conclusion

In this work, we have demonstrated that OLED and LED PDT can be effectively employed to kill *C. fasciculata* (a suitable non-infective to vertebrate model) and *L. major in vitro* even at low light intensities, i.e. at around 2 mW/cm^2 . Maybe unsurprisingly, due to their lifestyle *C. fasciculata* cells are more resistive than *L. major*. This may well be due to their lifestyle, as *C. fasciculata* are more likely to encounter harsher environmental conditions and therefore have mechanisms to cope with such stress. With further development, we foresee our light sources as wearable devices for the treatment of cutaneous leishmaniasis, when either small or large skin areas are infected.

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(SUPPLEMENTARY-DATA)

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