Photodynamic Therapy at Low-Light Fluence Rate: *in vitro* Assays on Colon Cancer Cells

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***Abstract*—This paper presents the results of *in vitro* photodynamic therapy assays on RKO and HCT-15 cell lines. The envisaged implementation is in autonomous medical microdevices, such as endoscopic capsules for clinical treatment of several types of gastrointestinal tract tumors. Because of their very limited device volume, light fluence and fluence rate needed to destroy tumor cells should be minimized. Foscan or meta-tetra(hydroxyphenyl)chlorin (mTHPC) is used as a photosensitizer. The experimental results show that a small amount of mTHPC (0.15 mg/kg) and light fluence (5–20 J/cm^2) is sufficient to obtain significant photodynamic activity. An array of LEDs with peak transmittance at 652 nm is used as a portable light source for the maximum quantum efficiency in producing singlet oxygen. Irradiation to a light fluence between 2.5 and 10 J/cm^2 is achieved by an increased exposure time at an 11 mW/cm^2 light fluence rate, while mTHPC concentrations of 0.5, 1, 5, and 10 μg/mL are used. The experimental results show that decreased cell viability (down to 30%) can be obtained for 1–5 μg/mL of mTHPC concentrations and 2.5 J/cm^2 of light fluence. Such light fluence and light fluence rate are compatible with the endoscopic capsules batteries.**

***Index Terms*—Biophotonic therapeutic technique, light-tissue interaction, Photodynamic therapy.**

I. Introduction

CONVENTIONAL therapeutic modalities for the treatment of tumor lesions (such as surgery, chemotherapy and radiotherapy) have in common low selectivity and the consequent appearance of adverse reactions due to the high toxicity caused on healthy cells. When compared with conventional therapeutic modalities, photodynamic therapy (PDT) is distinguished by the high selective cytotoxic activity against tumor cells. Selectivity is due to ability of photosensitizers to localize in tumor lesions and the distribution of light throughout the tissue of interest. The photosensitizer is activated by exposure to light of a wavelength for which it has high absorption and sufficient photon energy is provided upon absorption to convert molecular oxygen into reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$) and free radicals [1]–[6]. The singlet oxygen enables the destruction of the target cells through direct cellular damage, promoting apoptosis, vascular destruction and activation of an immune response against the targeted cells [1], [2], [4], [6]–[9].

The incident light is usually monochromatic, red, and has a wavelength equal to the maximum absorption wavelength of the photosensitizer. The light wavelength must provide the energy required to photoactivate the molecules of the photosensitizer and produce ROS, as well as to ensure a good tissue penetration of light [2], [5]. The wavelength of the light generally used in PDT is in the range between 600-850 nm. Photons with a wavelength longer than 850 nm carry insufficient energy to activate the photosensitizer and produce singlet oxygen, while at short wavelengths the tissue penetration is very low [2], [4].

The PDT results depend on several factors. The first is the properties of the photosensitizer (the spectral characteristic of the absorption, as defined by the molar extinction coefficient, and the quantum yield of singlet oxygen). The second is the distribution of the photosensitizer within tumor cells (determining localization of the action). Other factors that contribute to the biological efficacy of PDT are the light fluence or light dose and light fluence rate or light intensity [2], [4], [7], [8]. The light fluence is the total energy of exposed light across a sectional area of irradiated spot (J/cm^2). The light fluence rate is the radiant energy incident per second across a sectional area of irradiated spot (W/cm^2) [1].

An inverse proportionally of the effectivity of PDT with light fluence rate (i.e., increased tumor destruction at reduced fluence rate) has been reported. Blant *et al.* (1996) concluded that, regardless of the light wavelength (652 nm or 514 nm) and drug-light intervals (4 days or 8 days) for a given light fluence (between 8 J/cm^2 and 20 J/cm^2), PDT of squamous cell carcinoma with mTHPC (meta-tetra(hydroxyphenyl)chlorin)) at lower fluence rate (15 mW/cm^2) caused more damage to both tumor and normal tissue than when higher fluence rate was used (150 mW/cm^2) [10]. When studying the parameters affecting photodynamic activity of mTHPC, Rezzoug *et al.* (1998) also concluded that the photodynamic activity is

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TABLE I
CHARACTERISTICS OF SOME PHOTOSENSITIZERS USED IN PDT

Photosensitizer	Photofrin®	Levulan®	Foscan®	Laserphyrin®	Tookad®
Maximum absorption (nm)	630	635	652	664	763
Molar extinction coefficient ($cm^{-1}M^{-1}$)	1170-3000	5000	22400-30000	40000	88000-108600
Singlet oxygen quantum yield	0.89	0.56	0.87	0.77	0.50
Photosensitizer dose (mg/kg)	2-5	30-60	0.1-0.15	0.5-3.5	2-4
Photosensitizer-light interval (h)	24-72	3-6	24-96	2-4	0.5
Skin photosensitivity	4-12 weeks	1-2 days	2-4 weeks	3-7 days	1.3 hours
Light fluence (J/cm^2)	100-200	100	5-20	100-150	360
Light fluence rate (mW/cm^2)	100	100-150	100	100	100
References	[14], [16], [20]	[14], [16], [20]	[14], [16], [20]	[16], [17], [19]	[16], [17], [20]

significantly higher when low-light fluence rate is used (32 mW/cm^2) rather than higher fluence rate (160 mW/cm^2) [7]. Coutier *et al.* (2001) studied the effect of fluence rate on cell survival and photo-bleaching in Colo 26 (non-metastasizing mouse colorectal tumor cell line) multi-cell spheroids photosensitized by mTHPC. Twenty four hours after administration of photosensitizer, cell spheroids were exposed to various light fluences administered at light fluence rates of 5, 30 and 90 mW/cm^2. The authors concluded that the therapeutic efficacy and photosensitizer degradation increased significantly and progressively as the light fluence rate decreased from 90 to 5 mW/cm^2. A high photo-bleaching rate of the photosensitizer results in an increased singlet oxygen production and associated biological effects [11]. A year later, Coutier *et al.* studied the influence of the light fluence rate in PDT of HT29 human colon adenocarcinoma xenografts in nude mice with mTHPC. The tumors were injected with 0.3 mg/kg of mTHPC and 72 h later were exposed to light fluence of 12 J/cm^2 delivered at fluence rates of 5, 30, 90 and 160 mW/cm^2. The low-light fluence rates (5 mW/cm^2 and 30 mW/cm^2) are associated with longer delays in tumor regrowth than light delivery at higher rates (90 mW/cm^2 and 160 mW/cm^2) [12]. The principal reason of less PDT efficiency at high fluence rate is the depletion of oxygen in tissues which leads to low photo-bleaching of the photosensitizer. The fluence rate have also impact on the dominant mechanism of cell death in PDT, shifting from necrosis to apoptosis when the fluence rate is lowered [13].

An ideal photosensitizer should have certain characteristics, such as high purity and chemical stability, accumulation and retention preferentially in tumor tissues, rapid excretion from the body (inducing a low-systemic toxicity), and strong absorption peak at light wavelengths above 630 nm [9], [14]. Another important requirement in choosing a photosensitizer is the high production of ROS, particularly singlet oxygen - in other words, the photosensitizer should have a high singlet oxygen quantum yield (ϕ_Δ). A strong absorption with a high molar extinction coefficient (ϵ) for higher light wavelengths is another important feature of an ideal photosensitizer. Finally, another important factor of an ideal photosensitizer is the price, which must be low enough to ensure widespread commercial availability for extensive utilization of PDT treatment [9], [14]–[18].

Photosensitizers are usually classified as porphyrins or non-porphyrins. Furthermore, porphyrin photosensitizers are classified as first, second or third-generation photosensitizers. First-generation photosensitizers include the hematoporphyrin derivative (HpD) and Photofrin (Porfimer Sodium). These photosensitizers have a reduced light tissue penetration due to its weak absorbance in the red region of the spectrum (light wavelength less than 630 nm), a low molar extinction coefficient (that leads to the necessity of a high dose of the photosensitizer and high light fluence) and prolonged skin photosensitization after treatment. Second-generation photosensitizers, as Levulan (ALA – 5-aminolevulinic acid), Foscan (mTHPC – meta-tetra(hydroxyphenyl)chlorin), Laserphyrin (Talaporfin sodium) and Tookad (Palladium bacteriopheophorbide) were developed to alleviate some problems associated with first-generation photosensitizers. These second-generation photosensitizers absorb light at a longer wavelength, are more effective in the production of ROS and cause less skin photosensitivity after treatment. Third-generation photosensitizers are formed by biological conjugates and are based on systems that carry the photosensitizer to the tumor tissue, with the aim of reducing the toxicity to normal tissue and provide a selective accumulation in tumor tissues [2], [16], [17], [19].

Table I shows the characteristics of some photosensitizers used in PDT.

In this paper the emphasis is put on low-light fluence activation. PDT has so far been used mainly on lesions that in principle easily accessible (skin, retina, lung etc. [1], [3]) without penetration through a layer of tissue. Endoscopic capsules have become available for imaging the all gastrointestinal tract (especially small bowel) as a diagnostic tool [21]. However, this concept can be extended to also include treatment. The integration of the PDT technique in medical instruments, such as endoscopic capsules for clinical treatment of several types of gastrointestinal tract tumors, will be a breakthrough as these medical devices are designed for diagnostic and not as therapeutic tools. The endoscopic capsule is the only minimally invasive medical device that is able to diagnose and provide light to perform the PDT throughout the gastrointestinal tract. Fig. 1 envisages the integration of PDT in the endoscopic capsule. Within this concept, we envisage implementation of PDT for

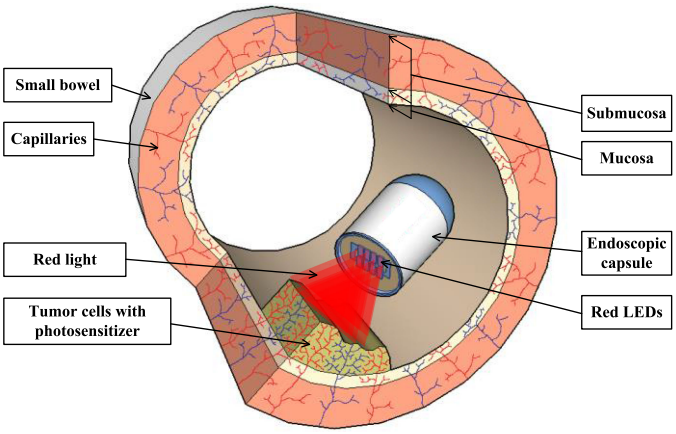

Fig. 1. Illustration of PDT integrated in the endoscopic capsule.

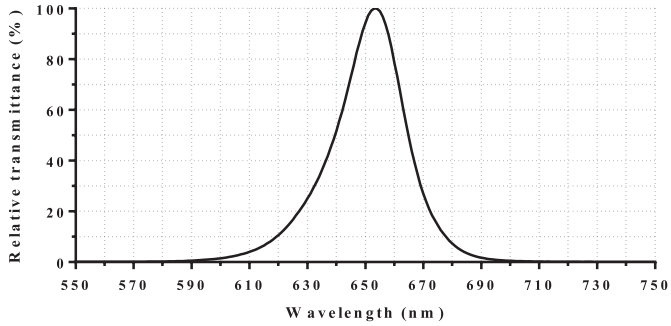

Fig. 2. Relative emission spectrum of LED WP7113SURC/E.

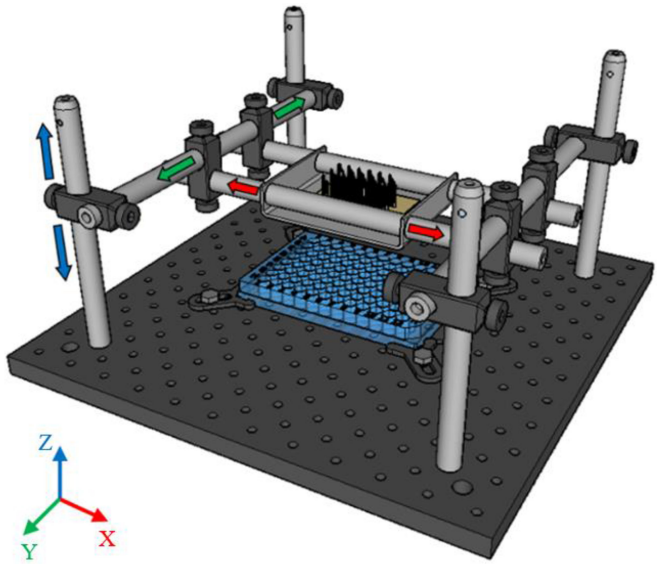

Fig. 3. Structure for supporting and placing the light source above of culture cell plate.

effective clinical treatment of several types of gastrointestinal tract tumors. Because of the endoscopic capsule dimensions and battery life, light fluence and fluence rate of the red light must be minimized to reduce the PDT treatment time. The light fluence and light fluence rate should be compatible with respectively the charge capacity and the discharge current of the type of batteries with dimensions that are suitable for integration within an optical microsystem. The mTHPC photosensitizer has potential to meet these requirements.

II. Materials and Methods

A. Photosensitizer

One of the most effective photosensitizers available is mTHPC. A relatively small dose (0.15 mg/kg) and small light fluence (5–20 J/cm^2) are sufficient for a very strong response [16], [17]. Its operating mechanism is based on direct tumor cell toxicity and vascular damage [16]. It was approved in the European Union for the palliative treatment of head and neck cancers in 2001 [17], [22]. It has a well-defined chemical structure and is available with 98% purity [10]. Besides, it also presents a large molar extinction coefficient in the red region at 652 nm, ensuring a good tissue penetration, and a high singlet oxygen quantum yield [10].

For experimental testing mTHPC was kindly provided by Biolitec research GmbH (Jena, Germany). Four stock solutions were made by dissolving mTHPC powder in methanol, resulting in concentrations of 0.5, 1, 5 and 10 mg/ml. A subsequent single 1:1000 dilution in complete medium of each stock solution yielded the final mTHPC concentrations of 0.5, 1, 5 and 10 μg/ml.

B. Light Source

The light source for illuminating the photosensitizer at the wavelength of maximum absorption of mTHPC is composed of a custom-made planar array of LEDs (30 hyper red LEDs reference WP7113SURC/E from Kingbright Electronic Co, Ltd) to best fit the 96-wells plate. The emission spectrum of the red LEDs was validated using an optical setup for the characterization of LEDs. This optical setup comprises: a monochromator (Oriel Cornerstone 260 ¼ m, Newport), a fiber optic (Model: 77533, Newport), a photodiode detector (Model: 71675, Newport), a power meter (Model: 1918-R, Newport) and a data acquisition system. The light source is placed at the entrance of the monochromator. The fiber optic guides the light beam from the monochromator output to the photodiode detector, which is connected to the power meter. The monochromator and power meter are connected to a computer to enable the data collection through the acquisition program.

Fig. 2 shows the relative emission spectrum of LED WP7113SURC/E. The central wavelength is 653 nm with a FWHM (full width at half maximum) of 25 nm. The central wavelength of light emitted by the LED WP7113SURC is very close to 652 nm, which is the wavelength of maximum absorption of mTHPC. Therefore, the conditions for optimum excitation are fulfilled. The light power per area (light fluence rate or light intensity) of this light source was measured with an optical power meter at approximately 15 mm from de detector (which is the minimum distance between the cells and the light source in the experimental assays). The light fluence rate at 653 nm is 11 mW/cm^2. Significant temperature raise was not detected.

An aluminum supporting structure (Fig. 3) was built for positioning the light source above the clear 96-wells plate. This structure is composed by screws and holders that allow the displacement and positioning of the light source over the three

coordinate axes and four position retainers that clamp the 96-wells plate to the structure base. A black breadboard acts as a structure base and prevents the reflection of light back into the wells.

The light source was positioned above the central 5 × 3 wells (column × line) to assure a homogeneity of light fluence rate. The light fluence rate of each illuminated well was measured with an optical power meter. The central 5 × 3 wells considered in the experimental assays showed almost the same values of light fluence rate.

C. Cell Culture

Two different human colon cancer-derived cell lines were used in order to study the influence of light fluence and mTHPC concentration on cell survival of mTHPC-mediated PDT treatment at low-light fluence rate.

The RKO (human colon carcinoma) and HCT-15 (human colon adenocarcinoma) cell lines were cultured in Dulbecco's MEM (Gibco, Invitrogen, USA) and RPMI-1640 medium (Roswell Park Memorial Institute), respectively, with phenol red indicator and supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco, Introgen, USA) and 1% (v/v) penicillin/streptomycin (Introgen, USA) at 37 °C in a 5% CO_2 atmosphere to reach exponential growth

D. In vitro Light Treatment

RKO (6000/100 μl/well) and HCT-15 (7500/100 μl/well) cells were seeded in 96-well plates and incubated at 37 °C in a 5% CO_2 atmosphere during 72 h. The culture medium was aspirated and the cells were incubated with mTHPC (0.5, 1, 5 and 10 μg/ml) during 24 h. Then, the cells were washed twice with phosphate-buffered saline (PBS) and 100 μl of fresh medium was added. The cell culture plates were irradiated, in the dark, with red light at 653 nm, light fluence of 2.5, 5 or 10 J/cm^2 and light fluence rate of 11 mW/cm^2. The irradiation time of each culture cell plate is given by the division of light fluence by light fluence rate. So, the irradiation times are approximately 3 min 47 s, 7 min 35 s and 15 min 9 s for light fluences of 2.5, 5 and 10 J/cm^2, respectively.

Fig. 4 shows the PDT treatment of one 96-well plate with the light source and structure developed for *in vitro* assays.

Three culture cell plates were used for different light fluences (2.5, 5 and 10 J/cm^2) and one without illumination (0 J/cm^2, to be used as control). Each culture cell plate has a control without mTHPC (0 μg/ml concentration) and the mTHPC concentrations of 0.5, 1, 5 and 10 μg/ml, in triplicate.

E. Cell Viability Assays

Cell viability was assessed by MTS colorimetric assay. MTS is reduced to colored compound, the formazan, by the action of dehydrogenase enzyme of the viable cells. The amount of formazan product (reflected in color change) is directly proportional to the number of live cells, which can be measured in absorbance terms around of 490 nm.

The amount of cells added to each well was based on previews absorbance calibration curves of the two cell lines. Immediately after PDT treatment, 10 μl of MTS were added to each well and the cell culture plates were incubated for 1 h at 37 °C to allow MTS metabolization. After incubation, the absorbance of the resulting solution at 490 nm was measured using a Varioskan Flash Multimode Reader (Thermo Scientific). The background absorbance was also measured and subtracted to the absorbance values of MTS assay.

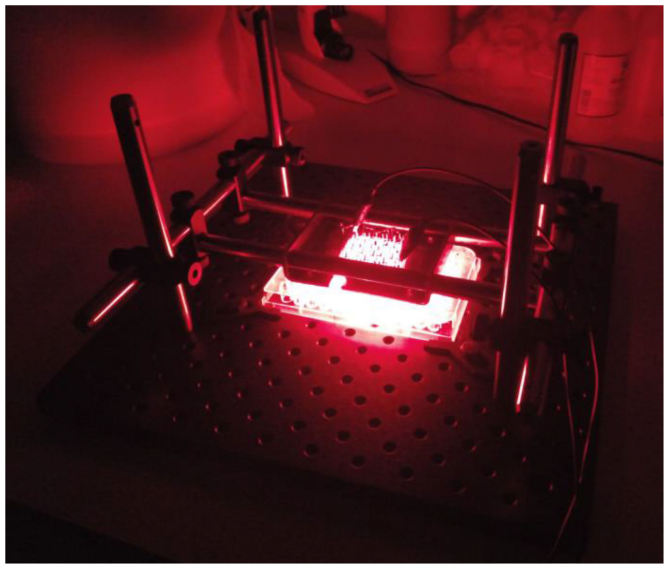

Fig. 4. *In vitro* light treatment of one 96-well plate, in the dark, with the light source and structure presented in Fig. 3.

III. Results and Discussion

The experimental results are presented as average ± standard deviation for three independents assays. Cell viability was normalized to the control.

Fig. 5 and Fig. 6 show the cell viability of RKO and HCT-15 cell lines, respectively.

As the data of Fig. 5 (a) and Fig. 6(a) were only normalized to the mTHPC concentration control, these graphs allowed to assess the influence of the mTHPC concentration on the cell viability values. On the other hand, when the data were only normalized to the light fluence control (Fig. 5 (b) and Fig. 6(b)), the graphs allowed to assess the influence of the light fluence on the cell viability values.

In the absence of light, i.e., no light activation (0 J/cm^2), it was found that mTHPC exposure led to approximately 30% toxicity in RKO cells incubated with 10 μg/ml of mTHPC concentration (Fig. 5(a)). The other mTHPC concentrations revealed no dark toxicity in RKO cells. At light exposure, the different mTHPC concentrations led to similar values of cell viability. However the mTHPC concentration of 1 μg/ml led to the lowest average values of cell viability.

Fig. 5(b) revealed that the light by itself has no cytotoxic activity on RKO cells, since the cell viability values for the condition without mTHPC remain around 100%. For the other mTHPC concentration, the different light fluences led to similar values of cell viability. However the light fluence of 2.5 J/cm^2 led to the lowest average values of cell viability for the mTHPC concentration of 1, 5 and 10 μg/ml.

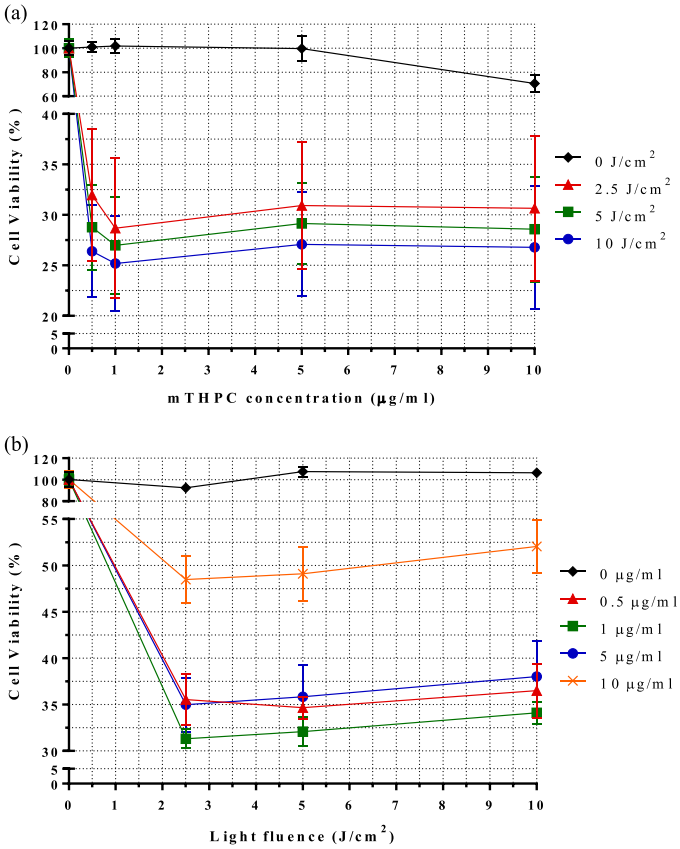

Fig. 5. Cell viability of RKO cell line: (a) normalized to the control without mTHPC (0 μg/ml) for each light fluence; (b) normalized to the control without illumination (0 J/cm^2) for each mTHPC concentration.

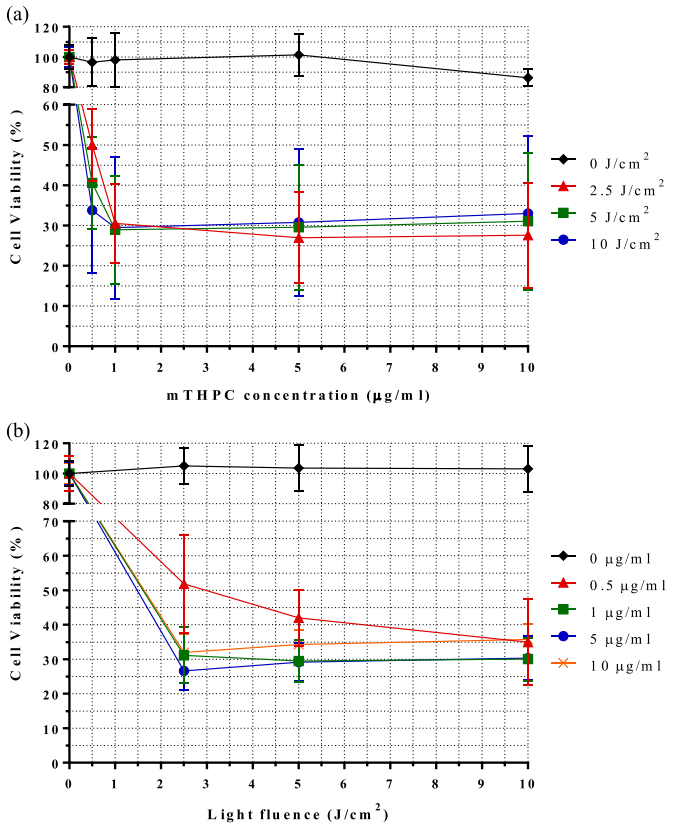

Fig. 6. Cell viability of HCT-15 cell line: (a) normalized to the control without mTHPC (0 μg/ml) for each light fluence; (b) normalized to the control without illumination (0 J/cm2) for each mTHPC concentration.

Fig. 6(a) showed the dark toxicity of the highest mTHPC concentration (about 15%) in the HCT-15 cells. The other mTHPC concentrations showed a cell viability very close to 100% in the absence of light, revealing that the inactivated mTHPC at low concentrations is not cytotoxic. At other light fluences, the lowest mTHPC concentration (0.5 μg/ml) presented the highest cell viability values, while the other mTHPC concentrations showed similar values of cell viability.

Similarly to what happened for RKO cells, the HCT-15 cell viability in the absence of mTHPC (0 μg/ml) is about 100% (Fig. 6 (b)). For the lower mTHPC concentration (0.5 μg/ml), an increasing of light fluence resulted in a decreasing of HCT-15 cell viability. For the other mTHPC concentration, the different light fluences led to similar values of cell viability.

Giuseppe Tortora *et al.* [23] presented a device for the treatment of Helicobacter pylori bacteria. This device consists of an ingestible capsule with a light source to perform photodynamic therapy by the excitation of the natural photosensitizers that Helicobacter pylori naturally owns. In this study, the authors performed the photodynamic therapy with a light fluence (irradiation density) of 9–16 J/cm^2 and light fluence rate (power density) of 5–8.9 mW/cm^2, corresponding a treatment time of 30 minutes. The target light fluence (16 J/cm^2) is only reached using 3-17 research capsules [23]. mTHPC-mediated PDT using light fluence of 2.5 J/cm^2 and light fluence rate of 11 mW/cm^2, reduces the PDT treatment time to approximately 4 minutes. A quick treatment requires less battery capacity and consequently a small number of capsules. The estimated charge capacity needed to perform the mTHPC-mediated PDT with small red SMD LEDs at 11 mW/cm^2 of light fluence rate and 2.5 J/cm^2 of light fluence is approximately 10 mAh with current discharge of 120 mA.

IV. Conclusion

This paper presented light fluence and mTHPC concentration response curves for *in vitro* PDT assays of RKO and HCT-15 cell lines. The 2.5, 5 and 10 J/cm^2 light fluences at 11 mW/cm^2 of light fluence rate and 0.5, 1, 5 and 10 μg/ml mTHPC concentrations were used in this study. A custom-made red light source and a structure for supporting and positioning of the light source on the culture cell plates were used.

In both cell lines, mTHPC showed some dark toxicity at a concentration of 10 μg/ml, however low concentrations of mTHPC are not cytotoxic in the absence of light. The studied mTHPC concentrations led to a similar values of RKO cell viability. In HCT-15 cells, the mTHPC concentration of 0.5 μg/ml led to the highest cell viability values.

The red light used in the activation of the mTHPC introduces no cytotoxic activity for either RKO or HCT-15 cells. The studied light fluences led to similar values of RKO cell viability. In the HCT-15 cells, an increasing of light fluence results in a decreasing of cell viability for the mTHPC concentration of 0.5 μg/ml. For the other mTHPC concentration, the different light fluences led to similar values of cell viability.

For 11 mW/cm^2 of light fluence rate, the optimum studied mTHPC concentrations and optimum studied light fluence are 1–5 μg/ml and 2.5 J/cm^2, respectively.

This paper demonstrates that the illumination at low fluence and low fluence rate lead to a good photodynamic response. Therefore, PDT using minimum light intensity and minimum illumination time can be integrated in autonomous medical devices, such as endoscopic capsules of very small dimensions, for providing them with an advanced therapeutic function.

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