A fluorescence micro-spectroscopy technique for the study of intracellular photobleaching of mTHPC

M. ATIF*
Physics and Astronomy Department, College of Science, King Saud University, Riyadh, Saudi Arabia
National Institute of Laser and Optronics, Nilore, Islamabad, Pakistan

A fluorescence micro-spectroscopic technique was applied to investigate intracellular photobleaching of mTHPC (Foscan) from micron-scale locations within individual formalin-fixed keratinocytes. The experimental results show that Foscan is highly photolabile in a cellular environment and the photobleaching phenomenon can be analysed *via* the modification of the fluorescence emission at 410 nm laser light. The progressive depletion of the 652 nm mTHPC fluorescence peak can be explained using bi-exponential decay kinetics which is consistent with singlet oxygen mediated process. The photobleaching plot against light dose shows an inverse-dose-rate dependence.

(Received June 02, 2014; accepted July 10, 2014)

Keywords: Fluorescence micro-spectroscopic technique, Photobleaching, mTHPC (Foscan), Keratinocytes

1. Introduction

Photodynamic therapy (PDT) is an emerging technique for different conditions like malignant and pre-malignant [1]. Weishaupt [2] *et al.* (1996) found that the target tissue destruction is a result of oxidative damage of cellular compartments that lie near to the site of $^1O_2$ production. The ALA (aminolevulinic acid) -PDT behaviour in photo-biochemical processes is complex and leads the treatment parameters difficult to be predicted for therapeutic efficacy. Wilson [3] *et al*., (1997) demonstrated that the indirect method used for predicting clinical result is to determine the rate of self–sensitised oxidation (photobleaching) *via* progressive decrease in Protoporphyrin IX (PpIX) fluorescence emission. It is assumed by this approach that the rate of photosensitiser oxidation is proportional to the cytotoxic activity that stimulates biological response.

A number of studies of *in vitro* systems containing Photofrin™ (Georgakoudi [4] *et al.,* 1997) and ALA induced PpIX (Moan [5] *et al.,* 1997; Georgakoudi and Foster [6], 1998) show that although bleaching is driven by $^1O_2$ interactions, the kinetics of fluorescence depletion cannot adequately be demonstrated by a fluence-dependent single exponential function. Robinson [7] *et al.,* (1998) describe that in a normal mouse skin, the PpIX bleaching rate is dependent on the fluence-rate and oxygen availability. It was observed that using reduced fluence-rate increased skin damage. Similar results were found by Finlay [8] *et al*., (2001)for normal rat skin.

The first generation photosensitisers have the following three major drawbacks, their selectivity is low and, hence skin photosensitivity is a major side effect; absorption in the red region is weak, thus the treatment of deep tumors is difficult; the photosensitisers possess complex mixtures of active oxygen.

The major drawbacks must be taken into consideration while designing improved photosensitisers. Extensive studies of chlorine led to the discovery of two promising second generation photosensitisers, (m-hydroxyphenyl) chlorin (m-THPC) and bacteriochlorin (m-THPBC) in PDT due to their strong absorption in the red region.

In this study, we used (m-hydroxyphenyl) chlorin (m-THPC, Foscan) one of the promising second-generation photosensitisers [9]. Foscan is a potent photosensitiser developed by Scotia Pharmaceuticals Ltd (European regulatory approved it in the treatment of head and neck cancer). It is easily localized within individual cell and seems that the lower light doses are necessary to cause necrosis *in vivo*. Furthermore, it is a single, pure compound with a high absorption in the red (ε_{652} = 22,000 M^{-1} cm^{-1}), selectivity for tumour tissue [10].

It causes considerable tumor necrosis in depth (> 5 mm) (11) in low concentrations (0.75 μmol kg^{-1}) combined with low flounce rate (10 J cm^{-2}). Hence, there is a low risk of skin damage due to high rate of photobleaching both *in vitro* [12] and *in vivo* [13].

Several studies show mTHPC to be the most potent of all the photosensitisers being so far used. In one of the clinical research, mTHPC at a dose of 0.3 mg kg^{-1} activated 48 h with a flounce of 10 J cm^{-2} at 652 nm causes 10 mm deep tumor necrosis in patients with malignant mesothelioma. Even at a lower dose of mTHPC (0.15 mg kg^{-1}) and flounce 10-20 J cm^{-2}, similar results of tumour necrosis were observed by Baas *et al.*, Savary *et al*.., and Mikvy *et al.*. [14-16].

The photobleaching mechanism of mTHPC has been a prime interesting area of research for several groups in recent years.

Ma *et al.* [17] compared the loss of mTHPC fluorescence with meso-tetrahydro phenyl porphyrin

(mTHPP) and photofrin during irradiation of sensitized cells with broad band light at 375-450 nm and found that the rate of mTHPC bleaching was much higher than the other two. Forrer *et al.* [8] studied the mTHPC bleaching kinetics *in vivo* and demonstrated that the decay of mTHPC fluorescence was consistent with the theory based on the assumption that $^1O_2$ was the agent responsible for bleaching. In the *in vitro* photodegradation of mTHPC in 10 % fetal calf serum, the evidence of involvement of $^1O_2$ was proved by Hadjur *et al.* [19].

Similar results were also observed by Kunz *et al.* [20] who studied the intracellular photobleaching of mTHPC in the murine macrophage cell line using 652 nm laser irradiation. Bonnett *et al.* [21] demonstrated the photobleaching of mTHPP, mTHPC, 5,10,15,20-tetrakis(m-hydroxyphenyl) bacteriochlorin (mTHPBC) in methanol and in methanol-water (3:2, v/v) using an argon laser with wavelength of 514 nm and found that mTHPC, mTHPBC photobleached rapidly whereas the mTHPP is resistant to photobleaching and undergoes photomodification to give a new chromophore.

The research carried out by Coutier *et al.* [22] revealed that fluence and oxygenation play a major role in the photobleaching of mTHPC sensitized tumour spheroids and their in PDT–induced cell toxicity. Their results verified the fact that at low fluences, a greater rate of photobleaching was found, besides a decrease in the fraction of cell surviving to treatment. These are observed in the presence of oxygen concentration gradients from a photosensitiser whose photodynamic damage and bleaching are both due to $^1O_2$. Their findings are in excellent agreement with the results of Ma *et al.*, Melnikova *et al.*, and Kunz *et al.* where $^1O_2$ was observed as the major toxic agent in mTHPC photosensitization and with the *in vivo* identifications of Blant *et al.* [23] who demonstrated more efficient treatment of Syrian hamster cheek pouch tumors at low fluences.

Finlay *et al.* [24] reported the measurements on the normal rat skin, which give information on the photobleaching kinetics and action mechanism of the photosensitiser mTHPC. The results show that at high fluences photochemical oxygen concentration will decrease the local oxygen concentration and as a result, the bleaching efficiency is decreased or *vice versa*.

Dysart *et al.* [25 observed the effect of varying sensitizer concentration in DP16 cells and studied fluorescence photobleaching versus biological damage. The results suggested that fluorescence bleaching could be also used to predict mTHPC PDT damage *in vitro*.

The previous studies involved the recording of fluorescence emission either from homogenized cell suspensions or used single-cell imaging techniques. No spectral data has been obtained from individual cells applying either of these techniques.

In our study, we have applied a micro-spectroscopic technique in order to record the laser induced fluorescence emission of the PDT photosensitiser, mTHPC, from micron-scale locations within individual keratinocytes. The nuclear region was found to be the most noticeable cellular component at the used magnification. A number of fluorescence spectra were studied to demonstrate mTHPC fluorescence photobleaching. No photoproduct emission is detected across the spectral region investigated. Specifically, the bleaching rate at the highest incident laser powers is limited by oxygen availability.

2. Materials and methods

In this study normal keratinocytes derived from human foreskin cells were used. To prevent laser-induced fluorescence emission of the glass, the cells were cultivated on sterile glass coverslips that were coated with gold having a density of 1×10^5 cells per dish. A medium containing 0.044 mM of Foscan (mTHPC) was then added. The culture conditions and Foscan administration are discussed in detail in the previous studies [26-29]. The experimental apparatus used for the current study is an adapted commercial Raman spectrometer (*System 1000*, Renishaw plc, Wotton-under-Edge, UK). Laser was focused into the nuclear region of individual cells, visually located through the microscope, using light of wavelength longer than 665 nm (to minimize mTHPC excitation). Spectral region was in the range, 635-675 nm, including the principal mTHPC emission peak. Each spectrum was recorded over 10 s; sequential spectra were recorded in order to investigate the dynamics of mTHPC photobleaching.

We report here the investigations carried out applying a laser power of 0.024 mW on individual cells. The period of light exposure is 50 s that paved the way for sequential accumulation of ten spectra (635-675 nm) of 5 s each exposure, from a single point within each cell. In order to study photobleaching mechanism, during each acquisition period, for the purposes of data acquisition, the first spectrum is recorded at the median exposure time of 2.5 s, with subsequent spectra collected as 7.5, 12.5 etc. up to 47.5 s. The values of intensity for a narrow wavelength band (50 detector elements = 1.8 nm) around the peak (652 nm) of each spectrum were then averaged and normalized using the magnitude of the first spectrum in the sequence. This method was applied for a set of ten individual cells and the mean of the 10 data points at each exposure time calculated. Plotting these values either as a function of time or light dose delivered (laser power × exposure time) induces a photobleaching profile. The method was followed again by applying different laser powers (0.049 mW, 0.074 mW and 0.099 mW) using a separate set of 10 cells each time for comprehensive study of photobleaching profiles. Using this experimental scheme, the photobleaching profiles generated at different laser powers, were compared.

3. Results and discussion

Cell cultures were studied at different laser powers. Population of each cell was heterogeneous (in different stages of the cell cycle) when mTHPC was added, and the cells formalin-fixed. It is important to mention that this

heterogeneity within the cell populations and the relatively small sample of cells observed from each culture, contributed to the large standard deviations. In order to identify individual components within the nucleus of the cells, the present arrangement is inadequate. Furthermore, the detection system may allow a contribution to the total fluorescence signal from the surrounding cytoplasm. As described previously, the analysis of live cells would be preferable, as it may allow a more representative comparison with data from previous *in vivo* reports. Using the present arrangement, this was not possible, whereas formalin fixing allowed the position and morphology of the cell to be maintained throughout the extended period of analysis. Despite this fact, these results are in qualitative agreement with those from previous studies, using different methods of fluorescence analysis, both *in vitro* and *in vivo*.

By irradiation of keratinocytes, mTHPC fluorescence was photobleached quickly by a singlet oxygen mediated phenomenon. A simple exponential function of delivered light dose is unable to fully describe the rate of mTHPC photobleaching, but is closely represented by a bi-exponential decay. However, other studies have described similar bleaching both *in vitro* and *in vivo*.

Georgakoudi *et al.* (1997) [4] presented a model for the dosimetry of sensitized multi cell tumour spheroids during photodynamic therapy and the effects mediated by singlet oxygen ($^1O_2$) and non-singlet ($^1O_2$) photobleaching on oxygen consumption. The expression of simple exponential decay used for sensitizer degradation depends upon fluence, which is not consistent with these mechanisms. Hence, we concluded that the description of $^1O_2$ mediated photobleaching was not understandable by a simple exponential with a constant photobleaching coefficient. We found for photofrin-sensitized spheroids that the $^1O_2$ mediated photobleaching model demonstrated accurately $^3O_2$ concentration changes, which is the outcome from sensitizer degradation. Their investigation illustrates non-$^1O_2$ mediated photobleaching mechanisms and observes their effects on dosimetry. They demonstrated $^3O_2$ concentration measurements and with Nile blue Selenium (EtNBSe) and ALA induced protoporphyrin IX sensitized spheriods cell survival assays. The results were analyzed by using a model of oxygen diffusion with consumption that includes the effects of various possible photobleaching mechanisms.

Niedre *et al.* (2002) studied the use of a novel photomultiplier tube in detecting singlet oxygen luminescence in cells *in vitro* and *in vivo* [30]. The photomultiplier tube is considered to be highly sensitive in the NIR region. Hence, due to their investigations, it was possible to detect the singlet oxygen luminescence in true biological media during PDT. These findings, previously explained, lead to the conclusion that mTHPC bleaching *in vivo* is dependent both upon singlet oxygen and irradiance.

Kunz *et al.* (2002) [20] reported the intracellular photobleaching of mTHPC in the murine macrophage cell line J744A.1. The investigations were carried out using quantitative fluorescence imaging microscopy, microspectrofluorometry, and microspectrophotometry. At 652 nm laser exposure , it was observed that mTHPC possess oxygen and fluence rate dependent intracellular photobleaching. The rate of photobleaching plotted against dose shows inverse dose-rate dependence.

Bonnet *et al.* (1999) [21] described the comparative study across the compounds m-THPP, m-THPC, m-THPBC in methanolic solutions using 514 nm argon laser irradiation. The following results are observed: (a) m-THPP resists to photobleaching and undergoes photomodification to form a new chromospheres; (b) m-THPC photobleached immediately; (c) similar results are observed for m-THPBC and?? m-THPC. If one increases the polarity of the solvent, the photobleaching rates for these three substances are also increased. In conclusion, singlet oxygen was found to be the key reactive species for the bleaching of these compounds.

Coutier *et al.* (2001) [22] observed the impact of fluence rate on cell survival and photobleaching in mTHPC. Photosensitized multicell Colo 26 spheroid was irradiated with 650 nm light at 5, 30, 90 mW cm^{-2}. The experimental results showed more efficient bleaching at lower irradiance.

Finlay *et al.* (2002) [24] addressed the impact of mTHPC photobleaching in normal rat skin. Using 650 nm laser irradiation, loss of mTHPC fluorescence was observed in fluorescence spectroscopy during PDT. When the fluorescence is plotted against fluence, the rate of photobleaching decreases significantly with increasing fluence. This behaviour supports the view that higher irradiance cause more significant oxygen depletion which is consistent in singlet oxygen-mediated bleaching mechanism.

Dysart *et al.* (2002) [25] demonstrated the mTHPC photobleaching in DP16 cell using 514 nm laser irradiation. During the experiment, sensitizer concentration, fluence rate and oxygenation effects were studied. It was noticed that the fluorescence photobleaching obeyed second order bleaching kinetics. A dosimetric model that incorporates photosensitizer fluorescence photobleaching to biological damage during PDT was observed by varying sensitizer concentration, treatment fluence rate, and medium oxygenation. The prediction of the cell viability from mTHPC fluorescence photobleaching could be achieved by a single curve for a range of treatment parameters excepting higher concentrations.

The current investigation is based on the prior findings of Dysart *et al*., [25] to test larger incident powers, which was limited in his study. Efficiency of photobleaching and sensitiser photodynamic action in experimental systems will be based on the local oxygen concentration if these processes are mediated by singlet oxygen. Photochemical oxygen consumption refers to the reduction in the local oxygen concentration at higher

irradiance. Due to this, bleaching efficiency is also decreased. At lower irradiance, we found a more rapid loss of fluorescence against flounce in singlet oxygen mediated bleaching. Robinson [7] *et al.* (1998) also observed that, at low values of irradiance of normal mouse skin, the increased rate of photobleaching corresponded to enhanced, homogeneous, photodynamic damage across the treatment site. The rationale is that the reduced photochemical oxygen consumption places less demand upon the vascular system, allowing a critical supply of oxygen to be maintained throughout the illuminated area.

In order to deeply study the bleaching rates, different incident laser powers regime were selected and a group of experiments were performed.

In this experiment, mTHPC sensitized keratinocytes were irradiated at 410 nm with incident laser powers: 0.024 mW, 0.049 mW, 0.075 mW, and 0.099 mW.

One laser power (0.024 mW) is selected from four incident laser powers in order to explain the gradual degradation of fluorescence intensity. The progressive reduction in intensity of the 652 nm mTHPC fluorescence peak, detected from the peri-nuclear region of a fixed keratinocyte during exposure to a laser power of 0.024 mW for 50 s is demonstrated in Fig. 1

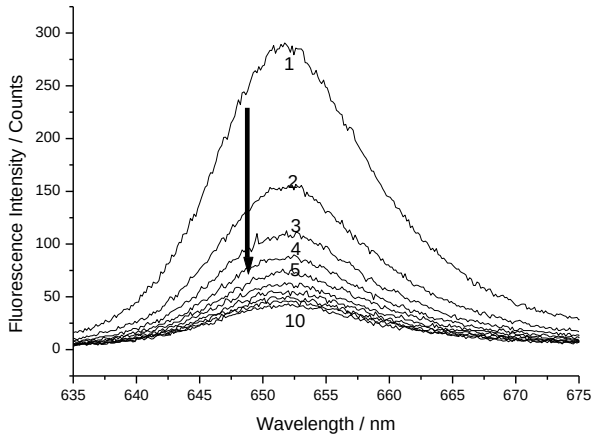

Fig. 1. Sequential fluorescence peaks of mTHPC from plateau phase kertinocytes incubated in 0.044 mM mTHPC for 3 hours, using a continuous laser power of 0.024 mW.

In Fig. 2a the peak (652 nm) fluorescence intensity from a complete set of 10 cells is plotted against time. The figure shows a large range in magnitude of the fluorescence intensity from different cells (23 % standard deviation around the mean for the first spectrum in each sequence).

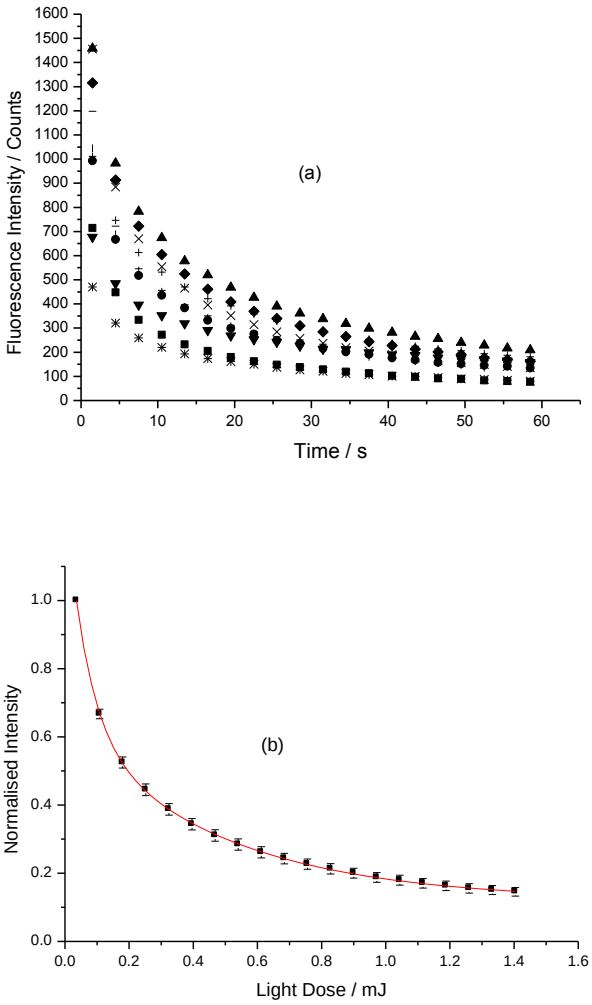

Fig. 2. mTHPC photobleaching recorded from a set of 10 cells (keratinocytes (in 0.044 mM mTHPC for 3 hours) in the form of (a) fluorescence intensity as a function of time and (b) normalised fluorescence intensity as a function of light dose. The solid line represents an empirical bi-exponential fit to the mean data points. Laser power is 0.024 mW.

The same data are shown after intensity normalization in Fig. 3b, this time plotted against the delivered energy dose. Included in this plot is the (normalized) mean of the 10 data points at each defined exposure time, and a bi-exponential fit (absolute chi-squared value < 10^{-6}) through this bleaching profile. The results are seen in Fig. 3 where bleaching is shown plotted versus irradiation time for the first set of laser powers: 0.024 mW, 0.049 mW, 0.075 mW, and 0.099 mW.

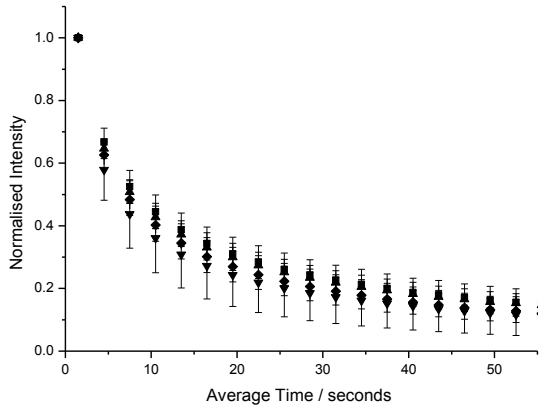

Fig. 3. Photobleaching plot of mTHPC (with respect to time) showing the normalised, averaged data points from sets of keratinocytes(in 0.044 mM mTHPC for 3 hours) exposed to different laser powers: 0.024 mW (■), 0.049 mW (▲), 0.074 mW (▼) and 0.099 mW (◆). Error bars are removed for clarity.

Fig. 4 shows photobleaching plots as a function of light dose delivered for different incident laser powers:L 0.024 mW, 0.049 mW, 0.075 mW, and 0.099 mW. The rate of photobleaching (as a function of dose) increases significantly when the incident power is reduced. This is the inverse dose rate effect. The results are in excellent agreement with the results of Kunz *et al*., *[20]*and Finlay *et al [24]*. The analysis of experimental results supports the idea of faster bleaching at lower powers which leads to the conclusion that the bleaching kinetics is identical for data obtained with different incident laser powers. Furthermore, our results are in agreement with a number of *in vivo* studies. We believe that this is the first comparative study of bleaching reported so far using different laser powers. This is limited in the work of Kunz *et al.*, and Finley *et al.*, and also suggested by Dysart *et al.*

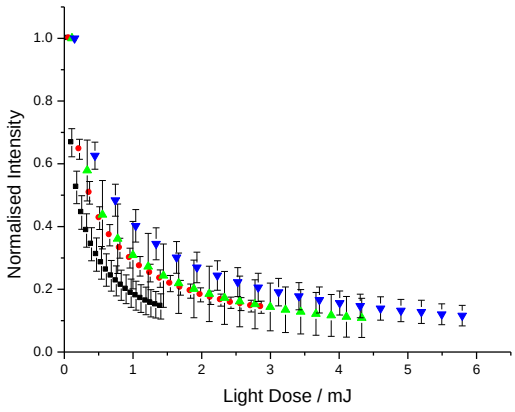

Fig. 4. Photobleaching plot of mTHPC showing the normalised averaged data points from sets of keratinocytes (in 0.044 mM mTHPC for 3 hours) exposed to different laser powers: 0.024 mW (■), 0.049 mW (▲), 0.074 mW (▼) and 0.099 mW (◆). Error bars are removed for clarity.

The inverse of the fluorescence signal amplitude will show a linear dependence on fluence / energy dose as shown by the experimental results in Fig. 5. However, the obtained broad agreement gives support to photobleaching as a result of singlet oxygen attack of the photosensitiser.

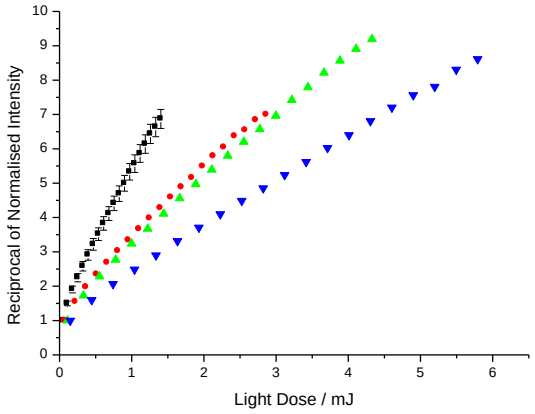

Fig. 5. Reciprocal of normalised intensity versus dose for cells following 3 hours of mTHPC incubation of keratinocytes at incident laser powers of 0.024 (■), 0.049 (●), 0.074(▲) and 0.099 (▼) mW.

The results show that the availability of free oxygen may also be limited, even within formalin-fixed cells. It is clear from the results presented, and the results of others, that a fluence-dependent single exponential decay is inadequate to describe the rate of mTHPC photobleaching both *in vitro* and *in vivo*.

4. Conclusion

In this study the laser micro-spectroscopic apparatus was used to study the fluorescence of individual keratinocyte cells. This permitted the fluorescence detection from micron-scale size regions of optical excitation in fixed cells. The results obtained on the intracellular photobleaching dynamics of mTHPC shows that a dose dependent reduction was seen in the 652 nm fluorescence characteristic peak for mTHPC. This is consistent with degradation occurring via a Type II singlet oxygen mediated process.

Acknowledgements

This project was supported by King Saud University, Deanship of Scientific Research, College of Science Research Centre.

References

[1] J. J. Schuitmaker, P. Baas, H. L. Van Leengoed, F.W. Van der Meulen, W. M. Star, N. Van Zandwijk, J. Photochemistry and Photobiology, **B 34**, 3 (1996).

[2] K. R Weishaupt, J. Gomer, T. J. Dougherty, Cancer Research, **36**, 2326 (1976).

[3] B. C. Wilson, M. S. Patterson, L. Lilge, Lasers in Medical Science, **12**, 182 (1997).

[4] I. Georgakoudi, M. G. Nichols, T. H. Foster, Photochemistry and Photobiology, **65**, 135 (1997).

[5] J. Moan, G. Streckte, S. Bagdonas, O. Bech, K. Berg, International Journal of Cancer, **70**, 90 (1997).

[6] I. Georgakoudi, T. H. Foster, Photochemistry and Photobiology, **67**, 612 (1998).

[7] D. J. Robinson, H. S. de Bruijn, N. van der Veen, M. R. Stringer, S. B. Brown, W. M. Star, Photochemistry and Photobiology, **67**, 140 (1998).

[8] J. C. Finlay, D. L. Conover, E. L. Hull, T. H. Foster, Photochemistry and Photobiology, **73**, 54 (2001).

[9] R. Bonnet, M. Berenbaum, Porphyrins as photosensitizers, in : Photosensitizing Compounds: their Chemistry, Biology and Clinical Use, Ciba Foundation Symposium, 146, Wiley Chichester, UK, 40 (1989)

[10] M. C. Berenbaum, R. Bonnet, E. B. Cheveretton, S.L. Akande-Adebakin, M. Ruston, Lasers in Medical Science, **8**, 235 (1993).

[11] R. Bonnet, R. D. White, U. J. Winfield, M. C. Berenbaum, Biochemistry Journal, **J 261**, 277 (1989).

[12] L.W. Ma, J. Moan, K. Berg, Lasers in Medical Science, **9**,127 (1994).

[13] D. Braichotte, G. Wagnieres, J.M. Philippoz, R. Bays, H. B. Ris, H. Van den Bergh, Preliminary clinical results on a second generation photosensitizer: mTHPC, in : Spineeli, P., Dal Fante, M. and Marchesini, R. (Eds.), Photodynamic therapy and Biomedical lasers, Elsevier, Amsterdam, 461 (1992).

[14] P. Baas, A.E. Saarnak H., Oppelaar, H. Neering, F. A. Stewart , British Journal of Dermatology, **145**, 75 (2001).

[15] J. F. Savary, Ph. Monnier, C. Fontolliet, J. Mizeret, G. Wagnieres, D. Braichotte, H. Van den Bergh, Archives Otolaryngol. Head neck Surgery, **123**, 162 (1997).

[16] P. Mikvy, J. Messmann, J. Regula, M. Conio, C. E. Millson A. J., MacRobert, S. G. Brown, Neo-plasma **45**, 157 (1998).

[17] L. W. Ma, S. Bagdonas, J. Moan, Journal of Photochemistry and Photobiology B: Biology, **60**, 108 (2001).

[18] M. Forrer, T. Glanzmann, D. Braichotte, G. Wagnieres, H. Van den Bergh, J. F. Savary, P. Monnier, Proceedings SPIE **2627**, 33 (1995).

[19] C. Hadjur, N. Lange, J. Rebstein, P. Monnier, H. Van den Bergh, G. Wagnieres, Journal of Photochemistry and Photobiology, **45,** 170 (1998).

[20] L. Kunz, A. J. MacRobert, Photochemistry and Photobiology, **75**(1), 28 (2002).

[21] R. Bonnet, B. D. Djelal, P. A. Hamilton, G. Martinez, F. Wierrani, Journal of Photochemistry and Photobiology, **53**, 136 (1999).

[22] S. Coutier, S. Mitra, L. N. Bezdetnaya, R.M. Parache, I. Georgakoudi, T. H. Foster, F. Guillemin, Photochemistry and Photobiology, **73**, 297 (2001).

[23] S. A. Blant, A. Woodtli, G. Wagnieres, C. Fontolliet, H. Van den Bergh, P. Monnier, Photochemistry and Photobiology, **64**, 963 (1996).

[24] J. C. Finlay, S. Mitra, T. H. Foster, Photochemistry and Photobiology, **75**, 282 (2002).

[25] J. S. Dysart, M. S. Patterson, T. J. Farrell, G. Singh, Photochemistry and Photobiology, **75**, 289 (2002).

[26] M. Atif, Laser Physics, **23**, 055603 (2013).

[27] M. Atif, Laser Physics Letters, **9**, 387 (2012).

[28] M Atif, M. R. Stringer, J. E. Cruse-Sawyer, P.E. Dyer, S. B. Brown, Photodiagnosis and Photodynamic Therapy Amsterdam: Elsevier. **2**(3), 235 (2005).

[29] M. Atif, M. R. Stringer, J. E. Cruse-Sawyer, S. B. Brown, Photodiagnosis and Photodynamic Therapy Amsterdam: Elsevier. **1**(2), 173 (2004).

[30] M. Niedre, M. S. Patterson, B. C. Wilson, Photochemistry and Photobiology, **75**, 382 (2002).

*Corresponding author: atifhull@gmail.com