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ABSTRACT

Measurements of concentration of sensitizers for photodynamic therapy can provide important information in the dosimetry planning and can also give input to the optimal time for treatment. There has been skepticism towards fluorescence techniques for this purpose, as the signal depends on the fluorescence yield and optical properties of the tissue. Absorption based techniques, lack on the other hand, often the sensitivity required for many sensitizers with relative weak absorption in a wavelength region where hemoglobin absorption is dominant. A direct comparison between absorption and fluorescence techniques for measuring mTHPC concentration after topical application on hairless SKH-1 mice bearing skin carcinomas has been performed. 20 μl/cm^2 of m-THPC thermogel (0.5 mg m-THPC/ml) were applied on normal and tumor area and the concentration of mTHPC was measured at 4 and 6 hours after drug application by two methods: 1. A fluorescence imaging system capturing images at two wavelengths (500 and 650 nm) following 405 nm excitation. Signals from different regions of interest were averaged and the intensity ratio at 650 to 500 was calculated. 2. A diffuse reflectance spectroscopy system with a fiber separation of 2 mm, providing the absorbance at 652 nm. Both systems provided consistent results related to the photosensitizer concentration. The methods show a remarkable difference in the concentration of photosensitizer in normal skin and tumor. No significant difference in mTHPC concentration in tumor could be observed between the 4 and 6h groups after drug application.

Keywords: mTHPC, pharmacokinetics, fluorescence spectroscopy, absorption spectroscopy, fluorescence imaging, photodynamic therapy

1. Introduction

Photodynamic therapy (PDT) as a cancer treatment modality has shown promising treatment results in terms of efficacy and selectivity(1). PDT relies on the excitation of a photosensitizing agent by light of an appropriate wavelength. The PDT effect is caused by a combination of treatment induced apoptosis, direct necrosis and vascular damage(2,3), where the extent of tissue damage depends on the total light dose, the sensitizer concentration and the tissue oxygenation(4,5).

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Pharmacokinetic studies are performed in order to follow sensitizer concentration build-up and match the time point of light delivery to the maximum sensitizer concentration in the target tissue. The measurements often rely on invasive methods such
as chemical extraction or taking biopsies since these methods give accurate results, although making it difficult to follow the time-history of sensitizer build-up. Optical methods, such as reflectance, fluorescence and absorption spectroscopy, have attracted more attention lately and the advantage of being less invasive. In addition, optical methods suit many sensitizing agents as they often display high extinction coefficients and characteristic fluorescence. These methods, however, require more extensive theoretical modeling to give accurate results. The special case of superficial tumors and topical sensitizer administration constitutes a highly complex situation where one needs to take into account the inhomogeneous drug distribution throughout the highly layered structure that human skin represents.

One of the major drawbacks when using systemic administration of many sensitizers is the extended photosensitivity remaining after the treatment procedure(6). In the case of easily accessible and shallow lesions, e.g. superficial skin malignancies, topical sensitizer application would be highly desirable from a clinical point of view. ALA-induced protoporphyrin IX is a photosensitizer that has been successfully used for various superficial skin tumors(7). The relatively small light penetration of the activating light and the lower triplet state yield of the protoporphyrin IX molecule are some of the factors that limit the treatment volume(8). In contrast to protoporphyrin IX, mTHPC has been reported as one of the most efficient sensitizers, as relatively small drug and light doses are required in order to achieve treatment response(9). For shallow skin lesions, the optical methods for investigating sensitizer concentration are to prefer.

In this paper we compare two different methods for assessing sensitizer concentration following topical application of mTHPC in an animal skin tumor model. The first method consists of fluorescence imaging where the fluorescence is induced by near-ultraviolet light. Because of the limited light penetration of the excitation light, this method mostly probes the very superficial tissue layers. The second method utilizes one single fiber optical source-detector pair to probe the overall tissue absorption within the spectral interval 400 to 850 nm(10,11). The source-detector separation has been chosen to make the method insensitive to variations in scattering parameters for the range of typical scattering values found in biological tissue. This method investigates the average sensitizer concentration slightly deeper than the fluorescence imaging method and Monte Carlo simulations have shown that more than 75% of the detected photons have probed tissue layers 2 mm or less(11).

Despite probing different tissue volumes, we report on a good correlation between the sensitizer concentration variations as determined by the two methods. The non-invasive nature of the methods make them suited for assessing sensitizer build-up in superficially situated lesions and will also be used in future studies trying to optimize treatment conditions for the new mTHPC formulation.

2. Materials and Methods

2.1 Fosgel® preparation

Fosgel® (Biolitec, Jena, Germany) is comprised of a liposomal formulation of mTHPC and a thermogel matrix (Lutrol F127). The gel was prepared according to vendor's instructions to a concentration of 0.5 mg mTHPC/ml gel. The gel, consisting of 19% (w/v) Lutrol® F127, 6.0 mg/ml of dipalmitoylphosphatidylcholine (DPPC), 0.70 mg/ml of DPPG, 17.0 mg/ml of glucose and water, is a heat-setting thermo-reversible gel. When heated by the skin to a temperature above 26°C a highly viscous gel is formed, whereas the compound is liquid at the storage temperature of 4°C.

2.2 Animal procedures

Seven male SKH-HR1 albino hairless mice, 8-10 weeks old and weighing 30-35 g, were used for studying the pharmacokinetics of Fosgel®. Malignant skin tumors were induced by repeated application of DMBA for 25 days in total followed by ultraviolet irradiation for 5 weeks. Mice with tumor diameter greater than 10 mm were euthanatized for ethical reasons.

Six animals were group into two groups with three animals in each for study of the mTHPC concentration before drug administration and at 4 and 6 hours after application. 20 μl of Fosgel® containing 0.5 mg/ml mTHPC was applied on each of the areas investigated, which included normal skin, tumor and skin surrounding the tumor. When present, several tumor regions within the same animal were studied. Application of the sensitizer and fluorescence and absorption measurements were performed under general anesthesia (ip injection 20 μl of γ-hydroxybutiric lactone solution in 0.9% sodium chloride (50:50)). Animals were killed by cervical dislocation and the investigated tissue regions were excised for extraction measurements. The mTHPC concentration in blood, liver, spleen and muscle was investigated for some animals. The study was carried out according to performed according to the guidelines established by European Council Directive 86/909/EC and the Greek Committee for experimental animals.

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2.3 Fluorescence imaging

A 405-nm continuous-wave diode laser (Power Technology Inc., Little Rock, Arkansas) emitting 2.1 mW was used to induce fluorescence from an area 27 mm in diameter. The fluorescence at 500 and 654 nm was filtered out using bandpass filters (Oriel, Stratford, Connecticut) and imaged using a cooled, intensified CCD (iStar, Andor Technology, Belfast, Northern Ireland). Two cut-off filters, GG475 and GG455 (Schott, Stafford, UK) were used to attenuate the reflected excitation light. The optical setup, shown in Figure 1.a, was calibrated for differences in spectral response using a NIST-traceable light source.

Drug concentration was quantified by forming a spectral ratio between the fluorescence intensities at 654 and 500 nm;

$$F = \frac{I(654\,nm)}{I(500\,nm)} \qquad (1)$$

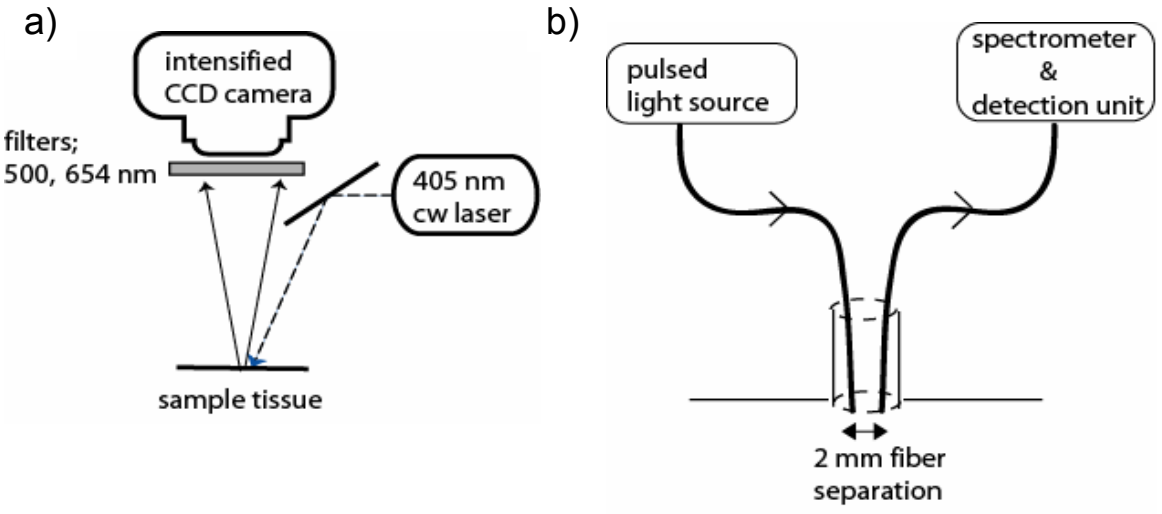

Figure 1a) Fluorescence imaging setup. b) Optical absorption setup.

2.4 Absorption spectroscopy

The optical absorption setup is illustrated in Figure 1.b and has been described in greater detail elsewhere(10,11). The output from a pulsed xenon short-arc lamp is delivered by a 400 μm diameter optical fiber and after interacting with the tissue the transmitted light is collected by a 200 μm fiber. The center-to-center distance between delivery and collection fibers is 2.0 mm. An S2000 miniature spectrometer (Ocean Optics Inc., Dunedin, Florida) is used to disperse and detect the collected light. Wavelength dependent fluctuations in source output and detector response were accounted for by taking a reference measurement from a spectrally flat diffuse reflector based on Spectralon material (Lab Sphere Inc., Cranfield, UK) in connection to each measurement.

When the pathlength of the detected photons is known, Beer's law can be used to determine the absorption coefficient of the tissue under investigation. It has been shown previously that for fiber separations in the range 15 to 26 mm between source and detector fibers, the pathlength of the collected photons is relatively insensitive to variations in tissue scattering properties. Using Beer's law in order to compare a measurement before sensitizer administration ($I(t_0)$) to that after a certain time interval ($I(t)$), one can relate the variation in elastic scatter signal to changes in tissue absorption coefficient due to the sensitizer;

$$-\ln\left(\frac{I(t)}{I(t_0)}\right) = B + \Delta\mu_a \cdot L(\mu_a), \qquad (2)$$

where B denotes a wavelength dependent background signal and $L(\mu_a)$ indicates that the effective pathlength depends on the total absorption coefficient. The reason for introducing B is that even though the effective pathlength does not change with tissue scattering properties, the amount of collected light will. Assuming the change in tissue absorption between measurements is caused by variations in mTHPC concentration, the negative logarithm of the detected light intensity can be expressed as;

$$-\ln\left(\frac{I(t)}{I(t_0)}\right) = d_0 + d_1\lambda + d_2\lambda^2 + \Delta C_{mTHPC}\varepsilon_{mTHPC} \cdot \left(x_0 + x_1 \exp\left(-x_2 \cdot \Delta C_{mTHPC}\varepsilon_{mTHPC}\right)\right) \qquad (3)$$

where d_i are coefficients determined by fitting the experimental data to a second order polynomial in wavelength within the spectral regions 450-460 nm and 750-800 nm. Monte Carlo simulations combined with experimental work has been used to determine the absorption dependence of the effective pathlength, x_i(11). In Eq. (3), ΔC_{mTHPC} and ε_{mTHPC} represent the concentration change and the extinction coefficient of mTHPC, respectively. The concentration change of mTHPC was found by non-linear fitting of the extinction coefficient, shown in Figure 2, to the background corrected experimental data in the wavelength interval 630-680 nm. By performing the fit only within this spectral interval, changes in blood content cold be disregarded due to the low extinction coefficients of oxy- and deoxy-hemoglobin.

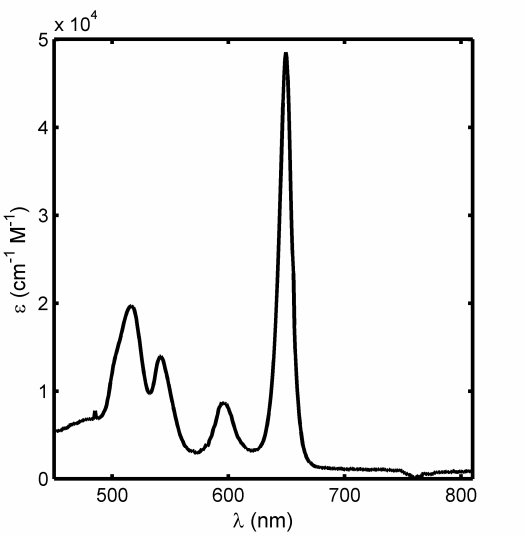

Figure 2 Extinction coefficient of mTHPC(12).

3. Results

Fluorescence imaging, absorption spectroscopy and extraction were compared as three different methods for assessing the time course of mTHPC concentration following topical mTHPC application. Regions of interest included lesion and normal skin not in the surrounding area of any tumor. Using the two former methods, intact skin in close proximity to the lesion was also investigated.

Since the fluorescence imaging setup was not calibrated against any phantom with known fluorophore concentration, this method did not yield any absolute data and instead the sensitizer concentration was quantified by the contrast function value according to Eq. (1). In order to compare the imaging data to the extraction result, which determines the mTHPC concentration over the entire tissue volume, the contrast function value was averaged within each region of interest.

Using the optical absorption setup, two to five measurements from each tissue area were acquired. These were averaged in order to give $I(t_0)$ and $I(t)$ and Eq. (3) was used to determine the mTHPC concentration.

In order to compare the three methods, the covariance of the results from each technique was studied:

$$\mathrm{cov}(x, y) = E\left[\left(x - m_x\right) \cdot \left(y - m_y\right)\right] \qquad (4)$$

E denotes the mathematical expectation, x and y represent the mTHPC quantity as determined by each method and m_i is the respective expectation value.

The *in vivo* measurements reported on high selectivity in mTHPC accumulation between lesion and surrounding skin characterized by an intact epidermis. This is illustrated in Figure 3 for the imaging fluorescence study where the two tumor regions show a much stronger fluorescence signal at 652 nm than the surrounding areas. The selective accumulation of mTHPC was confirmed by the extraction results, which also showed no traces of mTHPC in any of the internal organs investigated. Both fluorescence imaging and absorption spectroscopy revealed heterogeneous sensitizer distribution within lesions where necrotic areas displayed significantly lower mTHPC accumulation.

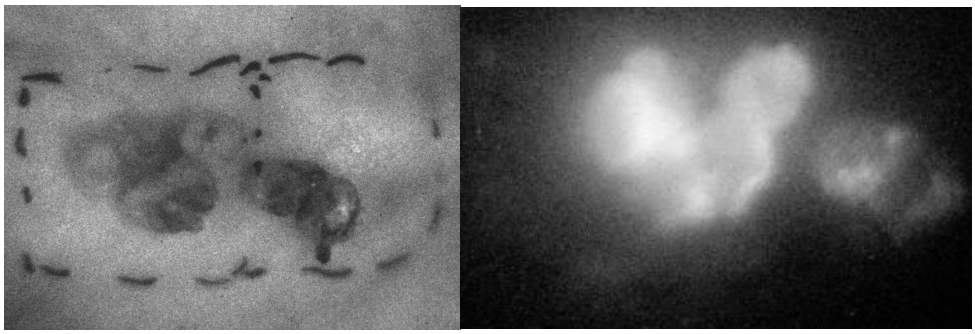

Figure 3 a) Room-light image showing lesions and surrounding skin. b) Fluorescence signal at 652 nm showing selective accumulation of mTHPC.

The temporal profile of the mTHPC build-up as assessed by the fluorescence technique is shown in Figure 4.a. Each marker represents the average within the region of interest and error bars denote the standard deviation. At 4 and 6 hours after mTHPC administration, lesions exhibited significantly higher sensitizer concentration than normal tissue with intact skin layer. No significant difference could be observed between the 4 and 6 hour accumulation times for the malignant tissue, whereas normal skin and skin surrounding lesions displayed slightly higher contrast function values at 6 hours.

The sensitizer concentration, determined by the absorption spectroscopy technique, is plotted as a function of time in Figure 4.b. The absorption technique yielded results in agreement with the fluorescence spectroscopy studies, where the lesions displayed a significantly higher sensitizer accumulation than tissue having an intact skin layer. The two different mTHPC accumulation times did not result in any significant variations in sensitizer build-up. As is evident from Figure 4, large variations in sensitizer concentration were found between animals, individual lesions and within each lesion. The covariance between the absorption spectroscopy and fluorescence imaging results yielded a correlation coefficient of 0.61. The correlation is based on the data at four and six hours from normal skin, lesion and intact skin immediately surrounding the lesion.

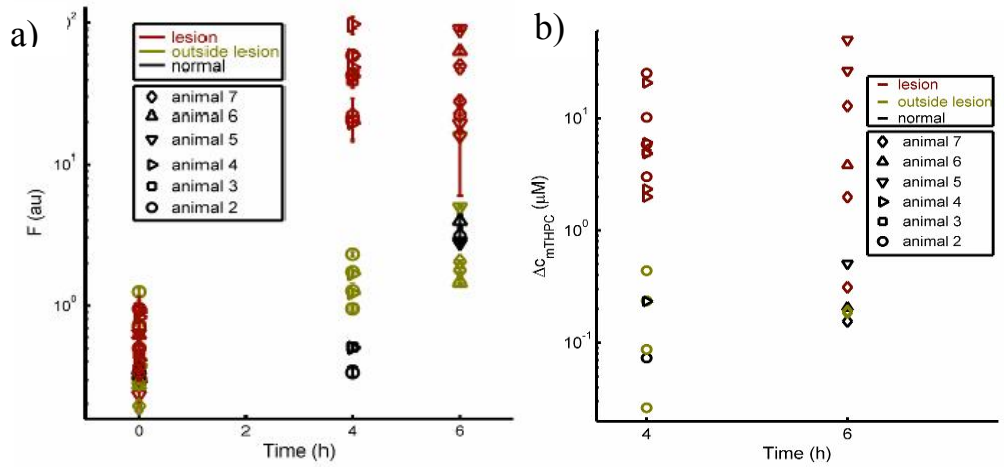

Figure 4 a) Contrast function value and b) mTHPC concentration as a function of time for six animals and all tissue types.

4. Discussion

Optical methods for quantifying sensitizer concentration *in vivo* have become increasingly popular over the years. Theoretical models exist that relate fluorescence signal to absolute fluorophore concentration in simple geometries. Here we report on experimental work that confirms the correlation between two different methods for assessing mTHPC concentration following topical sensitizer administration in a mouse skin tumor model. The near-ultra violet light used for exciting the mTHPC fluorescence has very limited tissue penetration, making this method applicable only when studying very superficial tissue regions. The covariance between fluorescence and absorption spectroscopy was, despite differences in probing volume, rather pronounced, possibly reflecting a certain overlap of probing volumes.

The method that relies on the differences in tissue absorption spectra has several advantages. The skin tumors likely to be treated by PDT using topical irradiation have depths that match the probing depth of this method. The absorption spectroscopy method also has the advantage of being applicable in the case where the compound does not exhibit any fluorescence. Further, when treating massive tumors embedded deep within normal tissue, the absorption spectroscopy method might be used by having two side-looking optical fibers aligned but having approximately two millimeters in between fiber tips. This approach would still allow for the use of a thin probe.

All methods indicated a highly selective mTHPC accumulation in lesions as compared to normal skin and skin surrounding the visible lesion. No significant difference in sensitizer concentration was found between the four and six hour application times. We believe the reason behind the selective sensitizer accumulation is due to differences in mTHPC penetration. The damaged epidermis covering the lesion makes the sensitizer penetrate more easily, whereas intact skin prevents the lower tissue layers from accumulating any substantial amount of mTHPC.

The absolute mTHPC concentrations found within lesions are in the range shown to induce significant PDT effects once irradiated at an appropriate wavelength(13).

Future work includes studying the PDT effect as a function of the sensitizer application time. In the case for systemic mTHPC administration, the treatment efficacy has been shown not to correspond to maximum sensitizer concentration within the target tissue(14). The largest amount of tissue damage is achieved when using rather short drug-light intervals in order to match the time of light delivery to when the mTHPC concentration within blood plasma is the highest. In the case of topical mTHPC administration, we anticipate PDT effect to more strongly correlate to maximum sensitizer concentration since the mTHPC levels found in blood are very low for all time points investigated. As a direct consequence, the treatment result is expected to be less influenced by vascular effects.

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