Photoacoustic Spectroscopy Applied to the Study of Protoporphyrin IX Induced in Mice


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We used Photoacoustic Spectroscopy (PAS) to study protoporphyrin IX (PpIX) in mice skin induced from δ-aminolevulinic acid (ALA) administrated by intraperitoneal route to CD 1 female mice. From obtained PAS spectra and by using the Phase-Resolved Method we estimated that PpIX in skin was preferentially concentrated near to the inner side of the basal membrane. It was also obtained the total attenuation coefficient ($\mu_t$) of the mouse skin as a function of wavelength. The obtained $\mu_t$ values, in particular at 630 nm agree with the literature values for similar samples. Also it was estimated in a first approximation the degradation time of PpIX when is irradiated at its maximum optical absorption wavelength.

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In the photodynamic therapy (PDT), porphyrins are currently used as photosensitizers of cancerous tumors, thus, is important to measure their distribution in tissues, mainly in relation to the possible side effects after their injection into patients. Among these porphyrins stands out the protoporphyrin IX (PpIX) which is induced by δ-aminolevulinic acid (ALA) being accumulated in high concentrations in cancerous cells and in low concentrations in normal cells. It is important to measure the distribution of PpIX in tissues and to study the products of its photobleaching in order to find possible collateral effects and optimize the PDT. Photothermal (PT) techniques could be useful for this kind of studies in tissues. Among the PT techniques, Photoacoustic Spectroscopy (PAS) is ideally suited for measuring the absorption spectrum of opaque materials, as it depends on thermal as well as optical properties of the sample, and provides different information from reflectance measurements.

It involves the measurements of heat produced as an excite species relaxes by a nonradiative path. The exciting light is chopped at a suitable frequency and the resulting modulated heat flow is detected as pressure fluctuations by using a microphone and a lock-in amplifier. As the exciting light is scanned in wavelength, a similar spectrum of the optical absorption spectrum is obtained in which the response is proportional to both the absorption cross section and the thermal diffusivity of the sample. PAS has been used in skin studies including stratum corneum maturation, sunscreen effectiveness, and in a study of both water content and tetracycline disappearance.

The purpose of this work is to determine, from mice exposed to ALA, the PpIX accumulation in skin also to calculate the degradation time of PpIX in vitro, when irradiated at its maximum optical absorption wavelength, and to obtain the total attenuation coefficient in mouse skin as a function of the wavelength radiation. In all these studies were used PAS.

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Experimental

Sample preparation

Two groups of CD1 female mice (n=7 per group) were used, one of them was treated by intraperitoneal injection of ALA to 160 mg/kg and sacrificed at 0.50 h after the injection. The other group was treated with physiological saline solution and sacrificed after one hour. ALA was obtained from Sigma Chemical Co. (St. Louis MO, USA) as a hydrochloric salts (98% pure) and this was dissolved in physiological saline solution (0.9 per cent NaCl) at concentration of 0.2 mg/ml before the experiment. To study the PpIX photodegradation in vitro we dissolved PpIX, from Sigma Chemical Co. as a disodium salt, in a HCl solution at 25%.

The mouse skin samples used for PAS analysis were obtained from skin biopsies (1cm²) of the abdominal zone, included in tissue-teck, and stored at −20 °C until their photoacoustic (PA) analysis. Minutes before use the samples were defrosted, shaved and dried.

Photoacoustic Spectroscopy

The optical absorption spectra in mice skin were obtained in the range of 300-700 nm by the use of a PA spectrometer. The experimental set-up consists of a 1000W Xenon lamp (Oriel), a variable frequency mechanical chopper set at 17 Hz, a monochromator, an air filled brass cell with a condenser microphone and a lock-in amplifier. The PA signal was normalized with respect to the signal obtained from charcoal powder in order to take into account the emission spectrum of our light source (Xe lamp).

In biological tissue, the radiance \( L(r, s) \) (W m⁻² sr⁻¹) of light at position \( r \) traveling in a direction of the unit vector \( s \) is decreased by absorption and scattering. The total attenuation coefficient is\(^{11}\)

\[
\mu_t = \mu_a + \mu_s
\]

(1)

where \( \mu_a \) (m⁻¹) is the absorption coefficient and \( \mu_s \) (m⁻¹) is the scattering coefficient.

In our case all the samples are thermally thick (which means \( \alpha_1 l_1 >> 1 \)), where \( \alpha_1 = 10^7 \mu \text{m}^2/\text{s} \) is the thermal diffusion coefficient with \( \mu \) the light modulation frequency, \( \chi \) and \( l_1 \) are the thermal diffusivity and thickness of the sample respectively) by considering \( \chi \equiv 10^7 \mu \text{m}^2/\text{s} \), then the total attenuation coefficient for thermally thick samples was obtained from the normalized PA signal by using the relation\(^{12}\)

\[
\mu_t = \left( a_s \right) \left[ q^2 + q(2-q^2) \frac{1}{\chi} \left[ 1 - q^2 \right] \right] (2)
\]

where \( q \) is the normalized PA intensity.

The method used to separate the two levels of chromophore in the mice skin is the named Phased Resolved Method used in PAS technique.\(^{6,8}\) In heterogeneous samples it is possible to generate separate spectra from different compounds by observing spectra in a phase-delay mode; this effect can be used at advantage in the examination of multilayered samples. In our case we can consider the skin sample as a two layer sample, which are mainly compound by epidermis and dermis. The PA measurement to obtain the photodegradation time in vitro was performed by using the PA spectrometer to irradiate the PpIX sample, contained in the PA cell, at fixed wavelength (\( \lambda = 413 \) nm) and the signal was monitored as a function of time. The light modulating frequency was fixed at 17 Hz and its intensity at 10 mW/cm².

Results and Discussion

The PA optical absorption spectrum of the mice skin were taken in such a way that the epidermis in the PA cell was illuminated by the monochromatic light. In this case we have in all samples the first layer (epidermis) with an absorption spectrum (corresponding to melanin) different to the absorption corresponding to the pigments in the dermis (mainly blood and PpIX). By using the Phase Resolved Method it was possible to separate the two components (melanin and heme group) obtained from this method, with an average value can be obtained from Eq. (3), ±5% for all samples.

The phase shift \( \Phi \) in the epidermis is given by \( a_s X_A \) where \( X_A \) is the mean epidermis thickness. Assuming that both layers (epidermis and dermis) have approximately the same thermal diffusivity and denoting by \( X_B \) the mean thickness of the dermis layer; the phase difference can be written as:\(^{9}\)

\[
\Delta \Phi = a_s (X_B - X_A)
\]

(3)

The average value can be obtained from Eq. (3), which gives us a value of 86 \( \mu \text{m} \). From the \( X_A, X_B \) value we propose that the PpIX would be localized in the dermis and some muscle, then we expect that this value must be close to the reported values in the literature for some of other
this layers but we don’t have $\mu_t$ values for this kind of samples (three layers). We found in the literature values for muscle of rabbit (2.7-12.5 cm$^{-1}$) and skin Caucasian human dermis (7.08 cm$^{-1}$) at 630 nm, which are close to the value obtained from our layered samples (4.577 cm$^{-1}$) at the same wavelength.

In the case of the PpIX in vitro photodegradation experiment, we can see from the time evolution of the photoacoustic signal, that the kinetic behaviour of the observed degradation is approximately an exponential decrease with time (see fig. 3). A first order constant of 400 sec was obtained from the fitting of experimental data to a exponential decrease equation. We observed that the PA signal decrease around 10% which is directly correlated to loss in absorption of the same magnitude if we considered that the light intensity is fixed during this time. This result agree with some results reported in the literature where at similar irradiated intensities, in the same wavelength, and by using a spectrophotometer it was observed a similar percentage decrease in the PpIX absorbance.

From PAS it was obtained the total attenuation coefficient ($\mu_t$) of mouse skin as function of the light wavelength, particularly at 630 nm the effective $\mu_t$ values agree with the reported values in the literature for the components (epidermis, dermis and muscle) of our multilayer samples. The determination of this optical coefficient is very important in PDT in order to determine the light penetration in the skin or another tissue. From the Phase-Resolved Method we estimated that the induced PpIX is mainly accumulated in the dermis near to the basal membrane. Also we obtain 400 s as characteristic time of photodegradation of PpIX in HCl.

**Fig. 1** Phase Resolved method applied to the skin PAS spectrum corresponding to 160 mg/kg of ALA administered. The inner spectrum (continuous line) corresponds to heme group molecules (peak at 400 nm) and the surface spectrum (dashed line) is due to melanine.

**Fig. 2** Total attenuation coefficient $\mu_t$ (cm$^{-1}$), calculated from Eq. (2).

**Fig. 3** Photoacoustic signal vs. time of irradiation of PpIX (as a disodium salt) dissolved in 25% HCl. The used $\lambda$ during irradiation was 413 nm.

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