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Effects of photodynamic therapy on the absorption properties of disulphonated aluminum phthalocyanine in tumor-bearing mice

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Abstract

Time-resolved reflectance spectroscopy was performed on tumor-bearing mice, administered with disulphonated aluminum phthalocyanine (AlS_2Pc, 5 mg/kg body weight), before, during and after photodynamic therapy. This allowed us to evaluate the absorption spectrum of AlS_2Pc in vivo from 610 to 700 nm, and to investigate how the therapeutic irradiation affects it. Two tumor locations (intraderma on the back and intramuscular in the leg), and two uptake times (3 and 12 h) were considered. As already observed previously, the absorption spectrum of AlS_2Pc in vivo is centered at 680–685 nm. The irradiation causes a blue-shift of the measured line shape, more or less marked depending on the experimental conditions. A reduction in absorption is also often observed upon illumination with therapeutic light doses.

Keywords: Absorption; Photodynamic therapy; Photon migration; Phthalocyanine; Time-resolved reflectance

1. Introduction

Disulphonated aluminum phthalocyanine (AlS_2Pc) proved to be an effective photosensitizer for the photodynamic therapy (PDT) of tumors in animal models [1,2]. Strong absorption and fluorescence in the red region of the visible spectrum characterize it. Several authors have already reported on changes in the fluorescence detected during photodynamic therapy. In particular, two competing effects have been observed both in vitro and in vivo: an initial marked increase in fluorescence, followed by a progressive bleaching [3–5]. When incorporated into cells, AlS_2Pc localizes mainly in the lysosomes and in this environment is only weakly fluorescent. However, irradiation can photo-oxidize lysosomes, causing the release of AlS_2Pc into the cytosol and a consequent marked increase in the fluorescence. Prolonged irradiation then leads to a gradual decrease in the detected emission, attributed to the photodegradation of the drug.

The aim of the present work is to extend the study on the effects of PDT to the in vivo absorption properties of AlS_2Pc. To this purpose, time-resolved spectroscopy was applied, as it allows the non-invasive evaluation of absorption and transport scattering coefficients (μ_a and μ_s', respectively). Experiments were performed in vivo on mice bearing experimental tumors injected at two different locations, and two uptake times were tested. Moreover, data were acquired not only before and after the irradiation, but also during it, thus providing information also on the effect of different light doses.

2. Materials and methods

2.1. Chemicals

Aluminum phthalocyanine with average degree of sulphonation of 2.1 was kindly provided by Dr. A. McLennan (Paisley College of Technology, Paisley, UK) and diluted in saline at a concentration of 2.5 mg/ml. The mean degree of sulphonation was ascertained by means of chromatography. The compound consists of a mixture of isomers with sulphonic groups in both adjacent and opposite positions.

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2.2. Animals and tumor models

Experiments were performed on hybrid (Balb/c×DBA/2F_1) CDF_1 mice (Charles River, Calco, Italy). The MS-2 fibrosarcoma, originally induced by the Moloney murine sarcoma virus, was maintained by weekly intramuscular (i.m.) passage of tumor cell homogenate into the right hind leg. Five days before the experiments, tumor cells (10^6 cells/mouse) were injected intradermally (i.d.) into the back or i.m. into the right hind leg of mice. At the time of the measurements, the tumors on the back had a diameter of 6–8 mm and the tumor was encapsulated, while the neoplastic mass in the leg was deeply infiltrated into the muscle.

2.3. Photodynamic therapy (PDT)

A drug dose of 5 mg/kg body weight (b.w.) was administered intraperitoneally (i.p.) and two uptake times (3 and 12 h) were considered.

The irradiation was performed at 685 nm, using the beam of a continuous wave dye (DCM) laser (Coherent Mod. CR-599, Palo Alto, CA) coupled to a 200-μm optical fiber. A circular area (2 cm in diameter) was irradiated. The administered light dose (100 mW/cm^2 for 10 min) was divided into two subsequent sessions of equal duration (5 min+5 min), separated by a time interval shorter than 5 min, as necessary to perform reflectance spectroscopy.

2.4. Set-up for time-resolved reflectance spectroscopy

A cavity-dumped dye (DCM) laser (Coherent Mod. CR-599, California) pumped by a mode-locked Argon laser (Coherent Mod. CR-18, CA) was used as the light source, tunable between 610 and 700 nm. The pulse duration was $<$10 ps, with a repetition rate of 8 MHz.

One-millimeter plastic-glass optical fibers (Quartz & Silice Mod. PCS1000W, France) were used for both illumination and collection. The laser power at the distal end of the illumination fiber was $<$1 mW. The two fibers were set at a relative distance of 0.6 cm, parallel to each other, and in contact with the animal skin. The distal end of the collection fiber was positioned at the entrance slit of a scanning monochromator.

A double microchannel plate photomultiplier with extended red photocathode (Hamamatsu Mod. R1564U-11, Japan) and an electronic chain for time-correlated single-photon counting were used for detection. A small fraction of the incident beam was coupled to another 1-mm fiber and fed directly to the photomultiplier to account for any eventual time drift of the instrumentation.

Overall, the system transfer function was $<$70 ps (full width at half maximum) and the spectral resolution was $\cong$2 nm.

All the time-resolved reflectance curves were collected every 5 nm from 610 to 700 nm.

In order to allow measurements to be carried out in vivo, the system for the acquisition of time-resolved data was fully automated, and the analysis and display of the measured spectra were performed in real time. A PC controlled the laser tuning and power, and the monochromator scanning. The overall measurement time (for data acquisition and system adjustment) was of 5 s/wavelength.

2.5. Data analysis

The absorption and transport scattering spectra were constructed by plotting, versus wavelength, the values of μ_a and μ_s', as obtained from fitting the experimental data with a standard solution of the diffusion approximation to the transport equation for a semi-infinite homogeneous medium [6]. The diffusion coefficient D was taken to be independent of the absorption properties of the medium (i.e., $D=1/\mu_s'$), in agreement with Furutsu and Yamada [7], and the extrapolated boundary condition was used [8]. The theoretical curve was convoluted with the system transfer function and normalized to the area of the experimental curve. The fitting range included all points with a number of counts higher than 10% of the peak value on the rising edge of the curve and 1% on the tail. The best fit was reached with a Levenberg–Marquardt algorithm [9].

Due to the unfavorable experimental conditions (low scattering, high absorption, and small measurement volumes requiring small interfiber distances) the interpretation of time-resolved reflectance data using the diffusion theory is particularly critical [10,11]. No systematic changes in the scattering spectrum were observed upon therapeutic irradiation, although the scattering measurement is inaccurate in these experimental conditions. In order to increase the precision of the measured absorption values, and allow the observation of even small spectral differences and changes, we followed the approach labeled 'constrained diffusion' and described in detail in Ref. [11]. Briefly, the fitting procedure is performed with a free time-shift, while leaving only one optical coefficient (i.e., μ_a) free. The values of μ_s' at each wavelength to be input to the fitting procedure were preliminarily obtained with a best fit performed by varying both μ_a and μ_s'.

2.6. Experimental procedure

For the evaluation of the absorption spectra of AlS_2Pc in vivo, time-resolved reflectance spectroscopy was used, as it can provide the non-invasive estimate of the optical coefficients in turbid media, such as biological tissues. The possibility that the illumination light used for spectroscopic purposes could cause photobleaching, especially after the administration of the sensitizer, was investigated by performing the measurements twice without changing the fiber position. No remarkable differences were noted between the results of the former and the latter measure,

indicating that photobleaching did not affect the experiments significantly.

For the measurements, the mice were anesthetized with ketamine and xilazine. The fiber holder was placed in contact with the skin lining the tumor mass (either on the leg or on the back) and reflectance measurements were performed on untreated mice every 5 nm in the 610–700-nm range. For each mouse, after the first series of measurements, the fiber holder was moved and repositioned, and a second series was performed. Thereafter, the mice were injected i.p. with 5 mg/kg b.w. of AlS_2Pc, and either 3 or 12 h later the whole measurement procedure was repeated. Then the animals were administered a therapeutic light dose, dividing it into two sessions of 5 min, and reflectance measurements on the tumor were performed after each session.

In summary, the absorption coefficient was evaluated four times as a function of wavelength: before drug administration, at the end of the uptake time, after the administration of half the therapeutic light dose, and at the end of the irradiation. The two spectra obtained in the same experimental conditions were not significantly different and were always averaged to improve the signal to noise ratio. In order to assess the average absorption line shape of AlS_2Pc in vivo, the spectrum taken from each untreated mouse was subtracted from each of the three spectra acquired from the same animal after drug administration. Then the resulting curves relative to measurements on four animals in the same experimental conditions (tumor location, uptake time, and irradiation time) were averaged, leading to the average absorption spectrum of the drug in vivo before, during and after PDT.

Control measurements were performed on tumor-bearing animals not administered with any drug, following the procedure described above for time-resolved reflectance spectroscopy before, during, and after irradiation.

For each tumor location and uptake time, the measurements were carried out on groups of four animals. All experiments were performed at least twice.

3. Results and discussion

The typical absorption spectrum of AlS_2Pc in solution (e.g., phosphate-buffered solution, methanol, aqueous solutions of human serum albumin) peaks between 672 and 676 nm, depending on the polarity of the microenvironment [4]. Conversely, the authors had already shown previously that the spectrum of the drug incorporated in vivo can be red-shifted to 680–685 nm [12,13]. The present study aims at investigating whether and how irradiation with therapeutic light doses can affect the absorption properties of AlS_2Pc. Thus, measurements were performed before, during, and after PDT.

In the spectral interval considered in the present study, the optical properties of the measured tissues are in the following ranges: $\mu_a \approx 0.2–1\ cm^{-1}$ and $\mu_s' \approx 5–10\ cm^{-1}$. Thus, when measurements are performed on the tumors injected i.d. on the back, due to their limited thickness (i.e., few mm), part of the collected photons are expected to propagate also in the underlying tissues. As a consequence, the measured optical properties may not refer strictly to the tumor volume, but they are more like to be an average over the neoplastic mass and some surrounding tissue.

Control measurements performed on animals not injected with AlS_2Pc showed that the therapeutic irradiation has no significant effect on the optical properties of untreated tissue (data not shown). Thus the results of subtraction between spectra collected after and before drug administration are expected to reflect the absorption properties of AlS_2Pc in vivo, and how they are modified by exposure to therapeutic light doses.

Fig. 1 shows the in vivo absorption spectra of AlS_2Pc, obtained from the average over the tumors injected i.m. in the leg of four animals treated with 5 mg/kg b.w. of drug for 3 h. Before irradiation, the absorption maximum peaks at 680 nm. This red-shift of the absorption spectrum with respect to what measured in solution in various solvents is typical of in vivo situations. As noted above, the absorption peak of AlS_2Pc in solution never reaches wavelengths longer than 676 nm [4], while the red-shifted line shape has been detected in different tumor models (leukemia and fibrosarcoma), and does not depend on the type of tissue involved [12–15]. Thus, the spectral behavior observed in vivo has been attributed to the presence of strong interactions with the biological matrix, particularly the extracellular component. In fact, it has been previously detected not only in vivo in solid tumors, but also in the supernatant

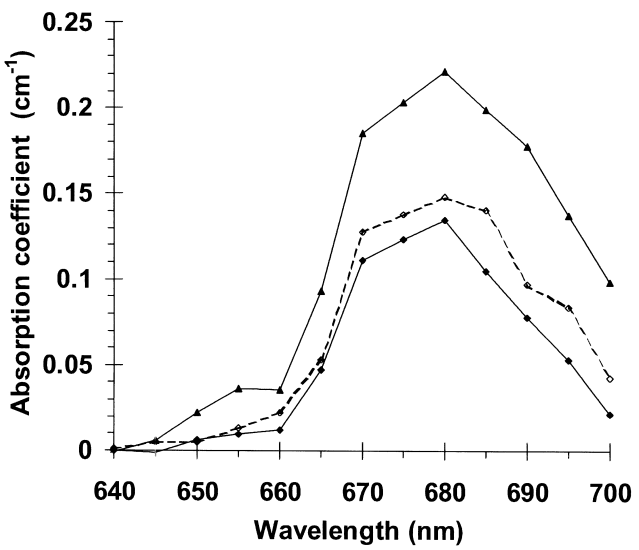

Fig. 1. Average absorption spectra of AlS_2Pc (5 mg/kg b.w.) in vivo in the tumor injected i.m. in the leg: 3 h after drug administration (closed triangles, solid), after 5 min of irradiation (open diamonds, dashed), and at the end of the irradiation (10 min, closed diamonds, solid).

obtained by centrifuging an ascitic tumor and removing the cells [13]. However, it should be taken into account that the localization of AlS_2Pc in the lysosomes has been reported in the literature [3] and at present a red-shift induced by that specific biological environment cannot be excluded. Five minutes of irradiation with a power density of 100 mW/cm^2 cause a significant reduction in the absorption (from 0.22 to 0.14 cm^{-1} at 680 nm). This effect is more marked on the trailing edge of the spectrum, leading to some spectral shift, even though the peak position of the average spectrum does not change significantly. The administration of the second half of the light dose further enhances the observed changes of the absorption spectrum, albeit the effect is weaker.

The average absorption spectra of AlS_2Pc incorporated in tumors injected i.d. on the back of mice are reported in Fig. 2. After an uptake time of 3 h, the peak absorption is detected at 685 nm and reaches 0.22–0.23 cm^{-1}. Even though the irradiation does not cause any significant reduction in absorption, the spectral change is still present, shifting the peak to 680 nm already after the first 5-min session. In this case, both raising and trailing edge of the line shape shift to shorter wavelengths upon irradiation.

The results of the measurements performed on the leg of animals treated for 12 h with 5 mg/kg b.w. AlS_2Pc are displayed in Fig. 3. Before irradiation, the maximum absorption is lower than after an uptake time of 3 h (approximately 0.18 cm^{-1} instead of 0.22 cm^{-1}). Such a decrease is in agreement with published data on the pharmacokinetics of sulphonated aluminum phthalocyanines in both normal and tumor-bearing animals [16–18]. Moreover, the line shape is sharper and peaks between 670 and 680 nm, thus being closer to what obtained for the drug in solution. This seems to suggest that a longer uptake time leads to weaker interactions between the sensitizer and the biological matrix. The appearance of the phenomenon is similar to what observed previously upon increasing the drug dose [13], even though its nature is reasonably different, as the drug concentration reduces going from 3 to 12 h of uptake time. The therapeutic irradiation causes no significant reduction in the measured values of μ_a, and only a small blue shift is observed on the long wavelength side of the spectrum.

For what concerns the tumors injected i.d. on the back (Fig. 4), the drug release occurring between 3 and 12 h of

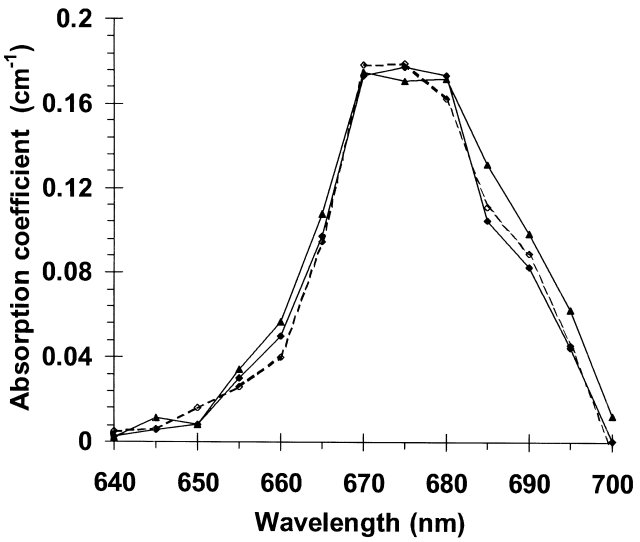

Fig. 3. Average absorption spectra of AlS_2Pc in vivo in the tumor injected i.m. in the leg: 12 h after drug administration (closed triangles, solid), after 5 min of irradiation (open diamonds, dashed), and at the end of the irradiation (10 min, closed diamonds, solid).

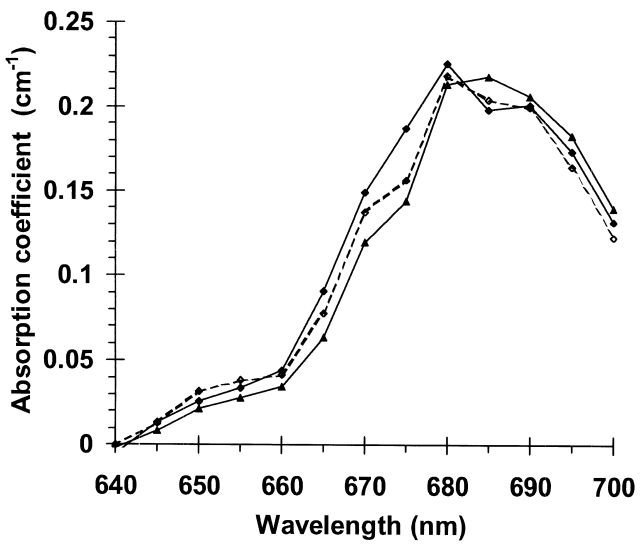

Fig. 2. Average absorption spectra of AlS_2Pc in vivo in the tumor injected i.d. in the back: 3 h after drug administration (closed triangles, solid), after 5 min of irradiation (open diamonds, dashed), and at the end of the irradiation (10 min, closed diamonds, solid).

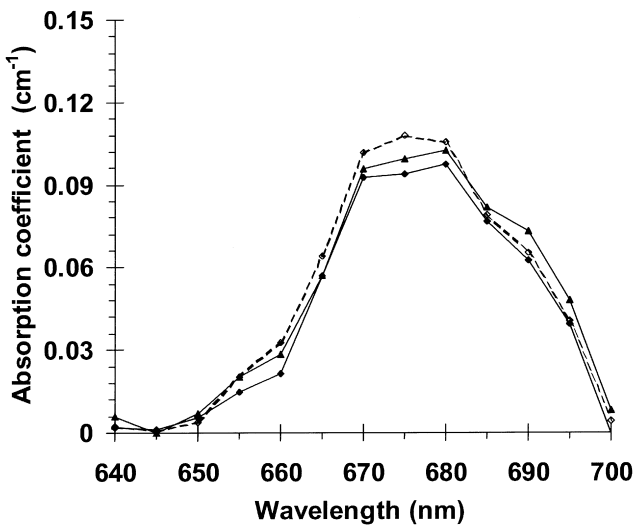

Fig. 4. Average absorption spectra of AlS_2Pc in vivo in the tumor injected i.d. in the back: 12 h after drug administration (closed triangles, solid), after 5 min of irradiation (open diamonds, dashed), and at the end of the irradiation (10 min, closed diamonds, solid).

uptake time reduces the measured absorption by a factor of 2 (from 0.22 to 0.1 cm^{-1} on the peak) and shifts the spectrum to the blue. The maximum absorption is detected at 680 nm, and comparable values are measured at 670 nm, while only a shoulder remains at 690 nm. Conversely, with the shorter uptake time, the spectrum peaked at 685 nm and similar values were obtained at 690 nm, whereas only a shoulder was present at 670 nm. The peculiar features of these line shapes seem to indicate quite clearly the simultaneous presence of two spectral shapes. The former peaks around 670–675 nm, close to the typical position of the free chromophore, and is attributed to a drug moiety not significantly influenced by the environment, while the latter is centered at 685–690 nm, as expected from the phthalocyanine molecules strongly interacting with the extracellular matrix. Thus, confirming that reported above for the tumor injected i.m. in the leg, longer uptake times seem to involve not only a partial drug release, but also weaker interactions with the tissue. The therapeutic irradiation has no effect on the absolute values of the absorption and causes only a very small blue shift.

In general, in all the conditions considered, two spectral forms seem to contribute to the absorption line shape of AlS_2Pc in vivo: a 'blue' component peaks at 670–675 nm and is attributed to the 'free' chromophore, while the other one is red-shifted, due to the interactions of the drug with the environment. This hypothesis is supported by the spectral differences observed when the uptake time is increased from 3 to 12 h. In the latter case, a sharper peak, centered at shorter wavelengths, suggests that the contribution from the 'free' AlS_2Pc is dominant, in agreement with the expected pharmacokinetics. Actually, 12 h after administration the drug release is occurring, as reported in the literature [16–18] and confirmed by the lower absorption values measured in the present study.

Moreover, distinct tumor locations are characterized by a different relative weight of the two spectral components, leading to a more red-shifted line shape for the tumors injected i.d. on the back, especially 3 h after drug administration. However, illumination with therapeutic light doses causes general changes in the absorption spectrum, independent of the tumor location and uptake time. In particular, the irradiation seems to affect both the spectral shape and the absolute absorption values. For the specific set of experiments described above, a marked reduction in the measured μ_a was observed only in leg tumors treated 3 h after the drug injection. However, even though this situation (i.e., tumors i.m. in the leg and uptake time of 3 h) seems particularly sensitive to the irradiation, an appreciable decrease in absorption was observed some times also in other experimental conditions (different tumor location and/or uptake time). When the absorption values are not significantly affected by the therapeutic irradiation, a blue-shift of the whole line shape (both raising and trailing edges) is usually observed, especially after the shorter uptake time. This seems to suggest that the irradiation alters the drug–tissue interactions, reducing their strength, at least for part of the drug molecules. When the absolute μ_a values are markedly reduced by the irradiation, the decrease in absorption is definitely more evident on the trailing side of the spectrum, thus hinting that the administration of therapeutic light doses favors the release of the 'red-absorbing' moiety. The higher sensitivity of the 'bound' molecules to the irradiation light may depend on their different state and microenvironment or merely on the irradiation wavelength (i.e., 685 nm), which is expected to be more effective for the 'red-absorbing' components.

The biological substrate is very likely involved in most features of the observed behavior and in the differences between back and leg tumors. The murine tumor that was used is a fibrosarcoma that was originated and transplanted in a leg muscle. When the tumor grows in the muscle, it is able to infiltrate very rapidly the normal tissue, and to partially substitute the normal muscle organization with a less homogeneous mass. In contrast, when the tumor is transplanted intradermally in the skin, its mass remains encapsulated and does not spread into the normal skin structure. This means that the tumor mass is more homogeneous, and it is not so much infiltrated with normal cells from the dermis layer. The different biological environment, which causes such a distinct behavior, is likely responsible also for the stronger interactions leading to a broader and more red-shifted spectrum in the back. Similarly, the same environment seems to maintain a high degree of interaction with the drug molecules even upon irradiation, and thus limit the blue-shift associated with PDT.

It should be noted that the presence of just two spectral shapes is the most elementary hypothesis to account for the experimental findings, but is likely to be an oversimplified assumption. Actually, in a complex environment like the intra- and extracellular one a full range of intermediate situations are probably present.

An uptake time of 24 h is often chosen for therapy with AlS_2Pc. The action spectrum for PDT showed that the therapeutic efficacy remains high even for wavelengths longer than 680 nm [19]. This suggests that, from a therapeutic point of view, the 'red-absorbing' component might be more effective. If this were the case, a shorter uptake time might be preferred, as the concentration of the drug incorporated into tissues would be higher, and its absorption spectrum more shifted to the red. Of course, also the concentration contrast between tumor and surrounding tissue should be taken into account, and it can reach its maximum at significantly different times depending on the nature of the tumor/tissue involved [20]. Consequently, the optimal uptake time for PDT should probably try to meet those two — sometimes opposing — requirements for the specific situation of interest. The experiments reported above cannot give indications on that optimal value, as they were performed at therapeutic drug

doses. This led to the cure of all back tumors, while comparable therapeutic effects were obtained at both 3 and 12 h for the leg tumors. Similar to what generally used for the measurement of the action spectrum for PDT, lower doses are needed to effectively investigate the dependence of the therapeutic efficacy on the uptake time.

On the other hand, the present work identified different contributions to the complex in vivo situation, possibly providing elements to better target further studies on the optimization of PDT with AlS_2Pc.

4. Abbreviations

AlS_2Pc	disulphonated aluminum phthalocyanine
b.w.	body weight
i.d.	intradermal
i.m.	intramuscular
i.p.	intraperitoneal
λ	wavelength
μ_a	absorption coefficient
μ_s'	transport scattering coefficient
PDT	photodynamic therapy

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