Time-resolved *in-vivo* fluorescence of photosensitizing porphyrins†

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(Received March 16, 1993; accepted August 2, 1993)

Abstract

Various components of photosensitizing porphyrins (*e.g.* monomers, aggregates, ionic species) have been recently localized in single cells by time-resolved fluorescence microscopy. Novel time-resolving techniques, based on picosecond laser diodes, a frequency-doubled Nd:YAG laser and time-gated microscopic equipment, were used for *in-vivo* measurements of the chick chorioallantoic membrane (CAM) exhibiting a pronounced vasculature. Changes of the fluorescence decay kinetics after light exposure were correlated with the formation of a photoproduct (Photosan, aminolaevulinic acid) or changes of the intracellular binding sites (tetraphenyl-porphyrins). Fluorescent components with different decay times were shown to be distributed differently within the tissue.

Key words: Porphyrins; ALA; Photoproducts; Chick chorioallantoic membrane; Microscopy; Fluorescence lifetimes

1. Introduction

Time-resolved fluorescence spectroscopy has proved to be a valuable tool for differentiating between various components of complex photosensitizers. For example, monomers, ether- or ester-linked dimers and aggregates of Photofrin II^R and Photosan^R have been detected and localized in single cells [1–4] (decay times: 14 ns; 2.9 ns and 0.2 ns [4]). In addition, neutral and ionic species of tetraphenyl-porphyrins, as occurring in the cytoplasm and in lysosomes, respectively, were differentiated on the basis of their decay times of about 12 ns and 3.5 ns, and correlated with the photodynamic cell destruction [5, 6]. In the present article the experimental model of the chorioallantoic membrane (CAM) of chicken embryos was adopted [7], where the intact vascular system could be visualized by conventional and fluorescence microscopy. New experimental methods were applied, namely the measurement of sub-nanosecond fluorescence decay kinetics with a frequency-doubled laser diode, and time-gated (nanosecond) fluorescence imaging of microscopic samples.

2. Materials and methods

2.1. Samples

Photosan 3^R (PS3; Seehof Laboratorium, Germany), meso-tetraphenylporphyrin tetrasulfonate ($TPPS_4$; Porphyrin Products, Logan, UT, USA) and aminolaevulinic acid (ALA; Fluka, Neu-Ulm, Germany) were used as photosensitizers. PS3 is a purified and aggregate-enriched product of protoporphyrin, similar to Photofrin [8], whereas ALA is a precurser of protoporphyrin IX in the biosynthetic pathway of haem. Topical application of ALA seems to be promising for the photodynamic treatment of basal cell and squamous cell carcinoma [9].

Fertilized eggs were kept in an incubator at 37 °C up to the 6th–9th day prior to the removal of a small part of the shell [7] and incubation by the photosensitizer. All photosensitizers were applicated topically within a teflon ring of 5 mm diameter at a concentration of 2 μg in 20 μl of

†Paper presented at the First International Lithuanian–Italian Workshop on Photosensitized Tumour Therapy, Vilnius, May 25–31, 1992.

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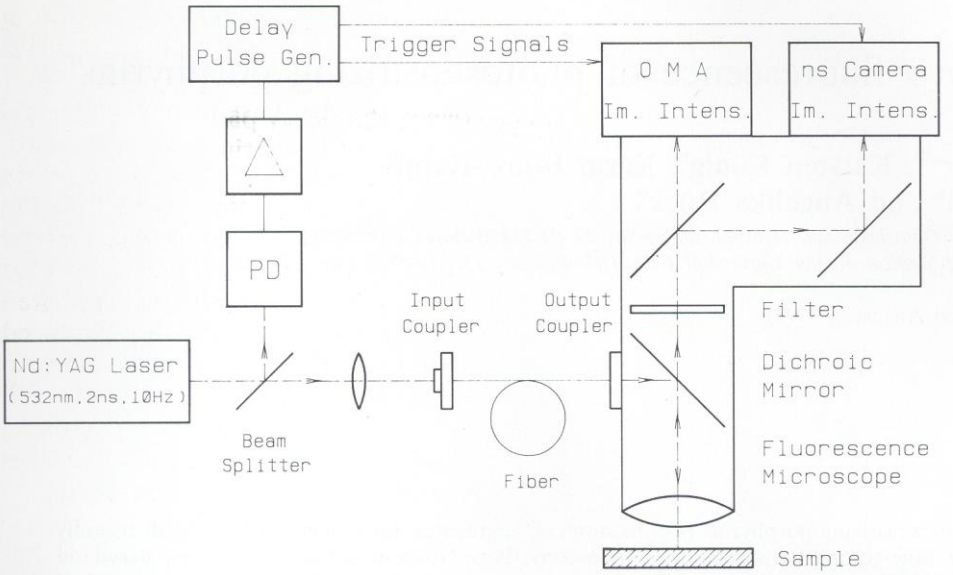


Fig. 1. Experimental setup for time-gated fluorescence imaging and spectroscopy (PD = photodiode, OMA = optical multichannel analyzer).

PBS solution for 6 h (ALA) or 24 h (other sensitizers), without any subsequent rinsing procedures. Intact embryos were investigated by time-gated fluorescence microscopy, whereas part of the CAM was cut off by scalpel and transferred into a perfusion chamber (POC; containing a 0.9% NaCl solution) immediately before measuring the fluorescence decay kinetics. This preparation kept the cells alive for up to 4 h [10].

2.2. *Apparatus*

A novel set-up for time-gated (nanosecond) fluorescence imaging and spectroscopy was developed, as shown in Fig. 1. The 2 ns Q-switch pulses of a frequency doubled Nd:YAG laser (532 nm, repetition rate 10 Hz) are coupled into a fluorescence microscope via a quartz fiber. The image intensifiers of an ultra-fast shutter camera (Proxitronic, NCA) and of an optical multichannel analyzer (Hamamatsu, IMD C4560, in combination with a self-developed monochromator) are triggered by the reference pulses from the laser, which are detected by a fast photodiode (PD), amplified and transformed into TTL pulses with variable delay times. Thus, time windows down to 5 ns can be selected. In addition, continuous spectra and images can be recorded using the different lines of a Hg high pressure lamp. In the following sections only fluorescence images are reported. Each image is stored during the time interval between two adjacent laser pulses prior to an integration over 80–100 images.

Fluorescence decay kinetics were measured from small parts of the CAM (about 2 mm^2) using a frequency doubled laser diode (Hamamatsu, LDH 038, wavelength 390 nm, pulse duration 40 ps, repetition rate adjusted to 1 MHz) and a single photon counting device as demonstrated in a previous article [6]. All decay curves were recorded in the spectral range of 590–800 nm with a time resolution of 50–100 ps, deconvoluted and fitted by a computer program for three exponentially decaying components (Edinburgh Instruments).

3. Results and discussion

Figure 2 shows typical decay curves of the CAM incubated with ALA or PS3 together with the time windows (1, 2) which were used for time-gated imaging (0–5 ns and 16–21 ns, respectively). The lifetimes obtained were about 0.3 ns, 2 ns and 14 ns for Photosan, and 18–20 ns as well as 5.2 ns for protoporphyrin IX produced endogeneously from ALA. The time constants obtained for Photosan are in excellent agreement with measurements of cultured cells, where these components were correlated with aggregated (or oligomeric), dimeric and monomeric porphyrin molecules, respectively [4]. In contrast to this, protoporphyrin IX as produced from ALA is mainly monomeric, as can be deduced from the almost mono-exponential decay pattern in Fig. 1 (with a time constant of about 19 ns). A rather weak component (10%) with a decay time of 5.2 ns is superimposed on

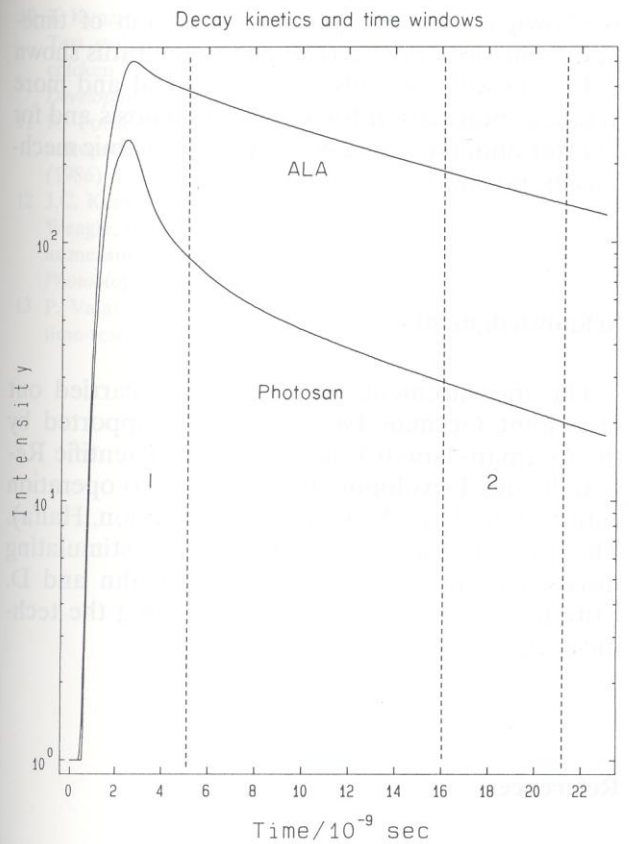


Fig. 2. Fluorescence decay kinetics of the CAM after incubation with ALA or Photosan measured at 590–800 nm. The two time windows used for gated imaging are indicated.

this long-lived component. It may be due to a photoproduct [4], whose intensity increases during irradiation (Table 1).

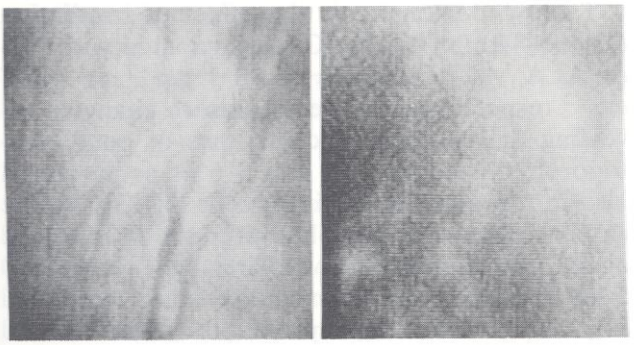
The fact that protoporphyrin IX is mainly monomeric, explains many advantages when using ALA for photodynamic therapy (PDT), in particular the high fluorescence yield, high photosensitizing efficiency (due to a high singlet oxygen quantum efficiency [11]), low clearance time [12] and rapid photobleaching (thus avoiding excessive photodamage).

Table 1 demonstrates the effect of irradiation (at 400–440 nm) of the CAM incubated with PS3 or ALA. At light doses of 90 J cm^{-2} or 180 J cm^{-2} respectively, the decay pattern changed, revealing the characteristic lifetimes of photo-protoporphyrin (about 1 ns and 5–6 ns as measured also in solution and in single cells [4, 13]). In the case of Photosan, this photoproduct disappeared again after application of higher light doses (180 J cm^{-2}), when decay times similar to non-irradiated PS3 were found. This proves the light-induced decomposition of the photoproduct itself and confirms previous measurements of cell cultures [4]. Those previous measurements, however, showed a significant decrease of the photoproduct at light doses of only 6–12 J cm^{-2}, *i.e.* more than one order of magnitude less than measured in the CAM. The role of the photoproduct for PDT has to be investigated further, but recent experiments carried out with erythrocytes revealed that its photodynamic activity was comparatively low, even after excitation in its absorption maximum of 670 nm [14].

Figure 3 shows the time-integrated (a) and time-gated (b; time window 0–5 ns) images of PS3. The time-integrated image was almost identical with the time-gated image obtained after a delay time of 16–21 ns. This image shows dark blood vessels and rather homogeneous fluorescence from outside the vessels, mainly arising from the long-lived monomers. In contrast to this, no blood vessels, but a rather diffuse pattern of fluorescent "smears" was found at a gating range of 0–5 ns, probably due to the short-lived aggregates. This demonstrates the different localization of porphyrin components within the tissue and allows one to carry out more detailed studies of their vascular and cellular uptake and retention. After incubation of the CAM with ALA, the time-integrated image and both time-gated images (obtained in the ranges of 0–5 ns and 16–21 ns after the laser pulse) were almost identical, as is expected for an almost mono-

TABLE 1. Fluorescence lifetimes and relative intensities (CAM)

Photosensitizer	T_1 (ns)	I_1 (%)	T_2 (ns)	I_2 (%)	T_3 (%)	I_3 (%)	Comment
Photosan (0 J cm^{-2})	0.2–0.35	15	1.6–2.3	20	11–14	65	Monomers + Aggregates
Photosan (90 J cm^{-2})	0.7–1.0	20	5.2–6.0	25	12–17	55	Photoproduct
Photosan (180 J cm^{-2})	0.3	15	2.0–2.5	25	11–14	60	Disappearance of Photoproducts
ALA (0 J cm^{-2})	–	–	5.2	10	18–20	90	Monomers
ALA (180 J cm^{-2})	0.6	5	6.0	30	18–20	65	Increase of Photoproducts
$TPPS_4$ (0 J cm^{-2})	0.5	20	2.3–3.5	15	12–14	65	Cytoplasm + Lysosomes
$TPPS_4$ (90 J cm^{-2})	0.05	50	1.0–1.2	35	8–10	15	Cell Nucleus (?)



(a) (b)

Fig. 3. Fluorescence images of the CAM incubated for 24 h with Photosan (image size 1.0×0.8 mm^2; emission measured at 590–800 nm); (a) time-integrated, excitation wavelength 546 nm; (b) time-gated (0–5 ns), excitation wavelength 532 nm.

exponential decay. The only difference consisted of the higher background luminescence in the time-integrated image, which was due to the microscope optics, and which could be discriminated against by time-gated detection.

Before light exposure, the fluorescence decay kinetics of $TPPS_4$ showed the typical decay times of the unprotonated (12.6 ns) and the dicationic species (2.9 ns), together with a rather short-lived component (Table 1). As shown in previous papers [5, 6] the neutral species is expected to be localized in the cytoplasm (pH 7), whereas both (neutral and diprotonated) species co-exist within the lysosomes (pH 5). The lifetimes after irradiation (90 J cm^{-2}) are considerably shorter and may result from porphyrins localized in the cell nucleus [15], where they are possibly bound to proteins or nucleic acids.

The fluorescence images of $TPPS_4$ (before irradiation; figures not shown) showed almost non-fluorescent blood vessels with brightly fluorescent edges and some preferential accumulation of the dye in endothelial cells. However, the fluorescence within the time window of 16–21 ns was distributed more uniformly over the tissue than the fluorescence detected at 0–5 ns. This is another indication that the various components of a complex photosensitizer are localized differently within the tissue.

4. Conclusion

The measurements of the decay kinetics and time-gated images as reported above, are a first step of an *in-vivo* quantification of individual fluorescent components. Variations of the excitation wavelength (*e.g.* by using a tunable dye laser, as shown in ref. 16) and the detection of time-gated emission spectra (using the apparatus shown in Fig. 1) will certainly give additional and more detailed information for tumour diagnosis and for a better understanding of the photodynamic mechanisms involved.

Acknowledgments

The measurements of CAM were carried out in a joint German–Israeli project, supported by the German–Israeli Foundation for Scientific Research and Development (GIF), in co-operation with S. Kimel and V. Gottfried (Technion, Haifa). The authors thank W. Strauss for stimulating discussions and W. Nothdurft, R. Hahn and D. Fritz for their co-operation in installing the technical equipment.

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