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THE KINETICS OF PHOTOREACTIONS OCCURING IN RESTING AND STIMULATED LYMPHOCYTES STAINED BY TWO PHTHALOCYANINES EXHIBITING VARIOUS EFFICIENCIES OF TRIPLET STATES GENERATION

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Human leukocytes resting and stimulated by phytohemagglutinin (serving as model of cancerous cells) were stained by two phthalocyanines (complexed with Zn and with Al) exhibiting various efficiencies of triplet states generation. The stained resting and stimulated cells were illuminated by red laser light and kinetics of photoreactions were established by the measurements of relative change in fluorescence emission intensities excited in two spectral regions: at 296 nm wavelength predominantly absorbed by the cell material (fluorescence at 330nm) and at 610nm — light absorbed predominantly by the introduced dye (emission at 675nm). Both investigated dyes exhibit similar, rather low, yields of fluorescence. For the dye with a higher yield of triplet formation the cell material was very effectively changed as a result of illumination of stimulated cells, and almost unchanged in a case of resting cells. For the dye with a lower efficiency of triplet generation the cell material was much more photostable and the difference between the undergoing photoreactions in stimulated and resting cells was low. Both dye molecules were photodegraded in both types of cells but with various kinetics. Results show that choosing a proper sensitizer for the photodynamic therapy one should take into account the efficiency of the dye triplet state generation by the intersystem crossing.

INTRODUCTION

Phthalocyanines (Pcs) are promising candidates to the applications in photodynamic therapy (PDT) and/or photodynamic diagnosis (PDD) (Rosenthal, 1991; Feofanov, Grichine, Karmakova, Kazachkina, Pecherskih, Yakubovskaya, Luk'yanetes, Derkacheva, Egret-Charlier & Vigny, 2002; Langlois, Brasseur, Wagner & van Lier, 1986; Waszkowiak, Frąckowiak, Wiktorowicz & Miyake, 2002; Frąckowiak, Wiktorowicz, Planner, Waszkowiak & Ion, 2002; Frąckowiak, Planner & Wiktorowicz, 2001).

Pcs exhibit an absorption band in the region of 650nm–800nm in which the tissue is rather transparent (Rosenthal, 1991; Frąckowiak, Planner, Waszkowiak, Boguta, Ion & Wiktorowicz, 2001).

The complexation of Pcs with metal or attachment of the side groups to the Pc ring change their interactions with macromolecules and the orientation of the dye molecule with respect to the surroundings (Frąckowiak, Ion & Waszkowiak, 2002). Various Pcs exhibit different yields of fluorescence, as well as the different efficiencies of generation by intersystem crossing (ISC) of their triplet states (Frąckowiak *et al.,* 2001)

The long living triplet states are very active in destruction of the cancerous cell either directly or by generation of very active singlet oxygen ($^1O_2$) (Frąckowiak, Planner, Waszkowiak, Boguta, Ion & Wiktorowicz, 2001).

The efficiency of incorporation of the dye-sensitizer into cells can be established using several spectral methods: absorption, fluorescence, steady state photoacoustic (PAS) or cytometric fluorescence measurements (Frąckowiak, Wiktorowicz, Cofta, Niedbalska & Latosińska, 1995; Wiktorowicz, Niedbalska, Planner & Frąckowiak, 1995; Waszkowiak *et al.,* 2002; Frąckowiak, Waszkowiak, Manikowski, Ion, Cofta & Wiktorowicz, 2001; Frąckowiak *et al.,* 2002). Most practical is a combined application of several methods because each one has its good and bad sides: the yield of dye fluorescence is usually much lower in the cells than in the solution, absorption spectra are perturbed by light scattering,

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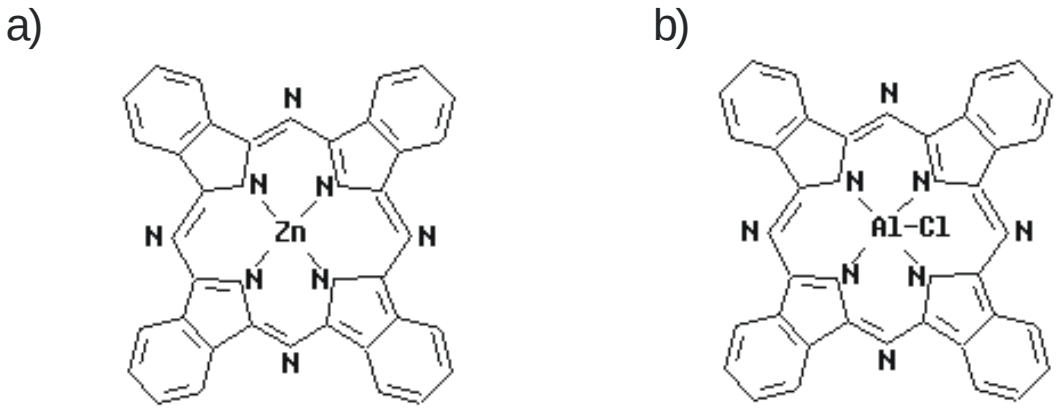

Fig. 1. Schemes and notations of investigated phthalocyanines. a) ZnPc; b)AlPc.

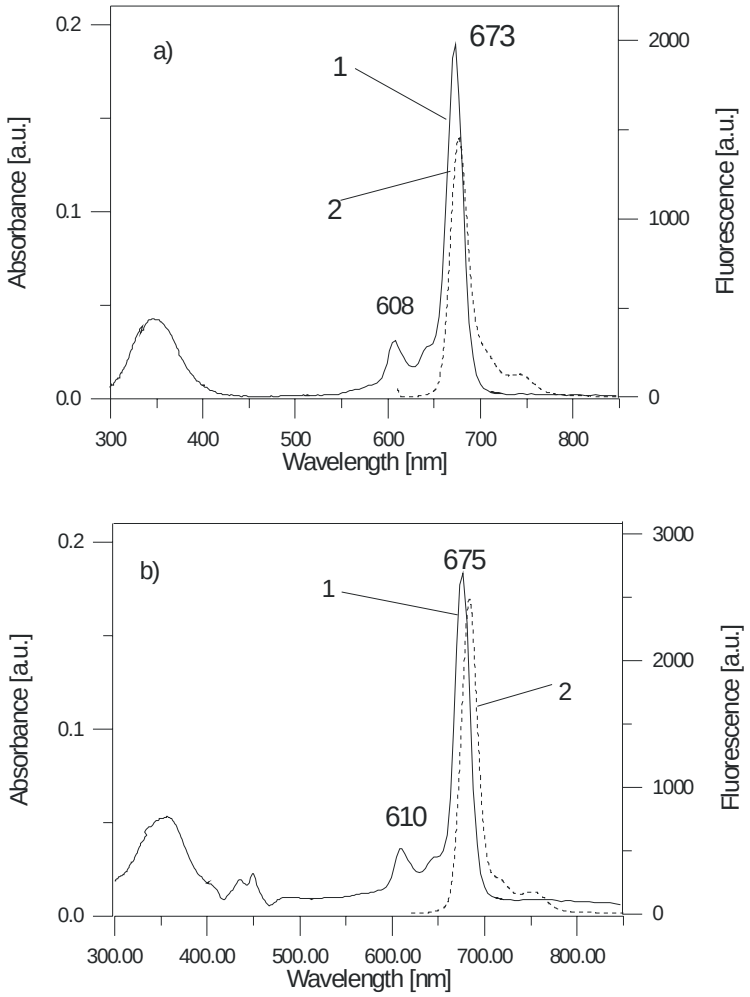

Fig. 2. Absorption (curves 1) and fluorescence emission (curves 2) spectra of ZnPc (a) and Al Pc (b) solutions in DMSO (concentration 10^{-6}M).

PAS amplitude depends not only on the amount of introduced dye, but also on the thermal deactivation (TD) of light absorbed by the dye molecule. TD depends again on the interaction of the dye with the cell material (Frąckowiak *et al.,* 2001; Frąckowiak *et al.,* 2002). Cytometry can be used only in a limited range of the dye concentration (Wiktorowicz *et al.,* 1995). Previously, we have established the yields of introducing various Pcs into cells (Waszkowiak *et al.,* 2002; Frąckowiak *et al.,* 2002). In this work we compare the kinetics of the photoreactions occurring in resting and stimulated cells stained by two Pcs exhibiting different yields of triplet state generation.

MATERIAL AND METHODS

The structures of investigated dyes are shown in Fig. 1. The investigated Pcs were purchased from Sigma-Aldrich and used without further purification.

Whole heparinized human blood cells remaining after routine analysis were used for experiments. The purity and numbers of mononuclear cells (lymphocytes and monocytes) were established using flow cytometry (Wiktorowicz *et al*., 1995). The analyzed cell population contained 95% of mononuclear cells, mainly lymphocytes (75%), therefore we called our sample "lymphocytes". In cytometric analysis the purity of lymphocytes gate was over 97%. The percentage of dead cells was established using propium iodide staining. Percentage of viable cells was about 90% before incubation with the dye and about 80% after incubation (Waszkowiak *et al.,* 2002). One part of the sample was stimulated using PHA (phytohemagglutinin PHA 17, Wellcome, England) at the concentration of 10μg/ml over a period of 1h at 37° C. The second part of the sample was not subjected to stimulation.

Staining of the cells was done in darkness by the dye solution in DMSO (dimethylsulfoxide) at the dye concentration of 10^{-5} M for 1h at 37ºC. After incubation the cells were washed and resuspended in 0. 9% water solution of NaCl. The final suspension contained 10^6 cells in 1ml.

The fluorescence emission and fluorescence excitation spectra were recorded using a Hitachi F 4500 (Japan) spectrofluorimeter.

Fluorescence spectra were excited in two spectral regions: in a region of absorption of the dye incorporated into lymphocytes at 610 nm (maximum of emission at about 675 nm) and at about 296 nm in a region, in which the absorption of the cell material dominates (observation of fluorescence at about 330 nm).

The photochemical reaction in investigated cells was carried out by illumination of the sample with the He-Ne laser (Amepol, Poland) light (632. 8nm, 3mW/cm^2). Duration of illumination is given in Figures 2-5.

RESULTS AND DISCUSSION

Fig. 2 presents the absorption and fluorescence spectra of investigated Pcs (ZnPc and AlPc Fig. 1) measured in the incubation solvent DMSO.

The yields of both dyes fluorescence measured in DMSO are similar (0. 18 for ZnPc and 0. 17 for AlPc (Frąckowiak *et al.,* 2001). The yield of generation of triplet states by intersystem crossing is higher for ZnPc (close to unit) than that for AlPc (0. 88). These results are not very precise, because the sum of both yields cannot be of course higher than unity, and because the part of excitation energy is converted into heat in a fast process by internal conversion from $S_1 \rightarrow S_0$ (from the excited singlet to the ground state singlet). However the sequence of the yields of the triplet state generation is usually, for a given set of dyes, measured under the same conditions using laser induced optoacoustic spectroscopy (LIOAS), reasonable (Frąckowiak *et al.,* 2001, Frąckowiak *et al.,* 2002).

Fig 3 shows the set of fluorescence emission and excitation spectra of illuminated resting and stimulated samples stained by ZnPc, Fig. 4 the same sets

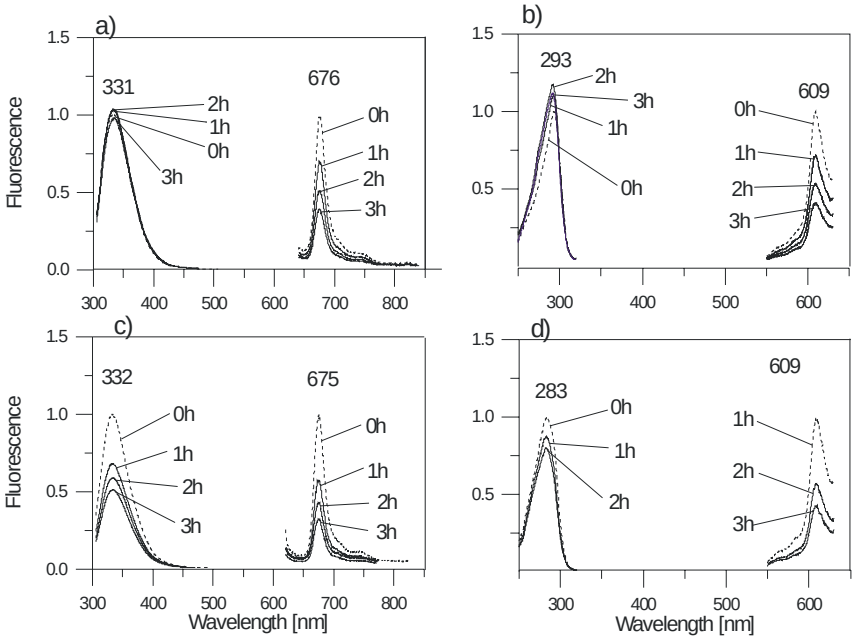

Fig. 3. Fluorescence emission spectra (a and c) and fluorescence excitation spectra (b and d) for cells stained by ZnPc.Figs a) and b)-resting cells; c)and d) –stimulated cells. Short wave emission excited at 296 nm,observed at 330 nm, long wavelength emission excited at 610 nm-observed at 675 nm Maxima of bands (in nm) and duration of illumination are shown in figures.

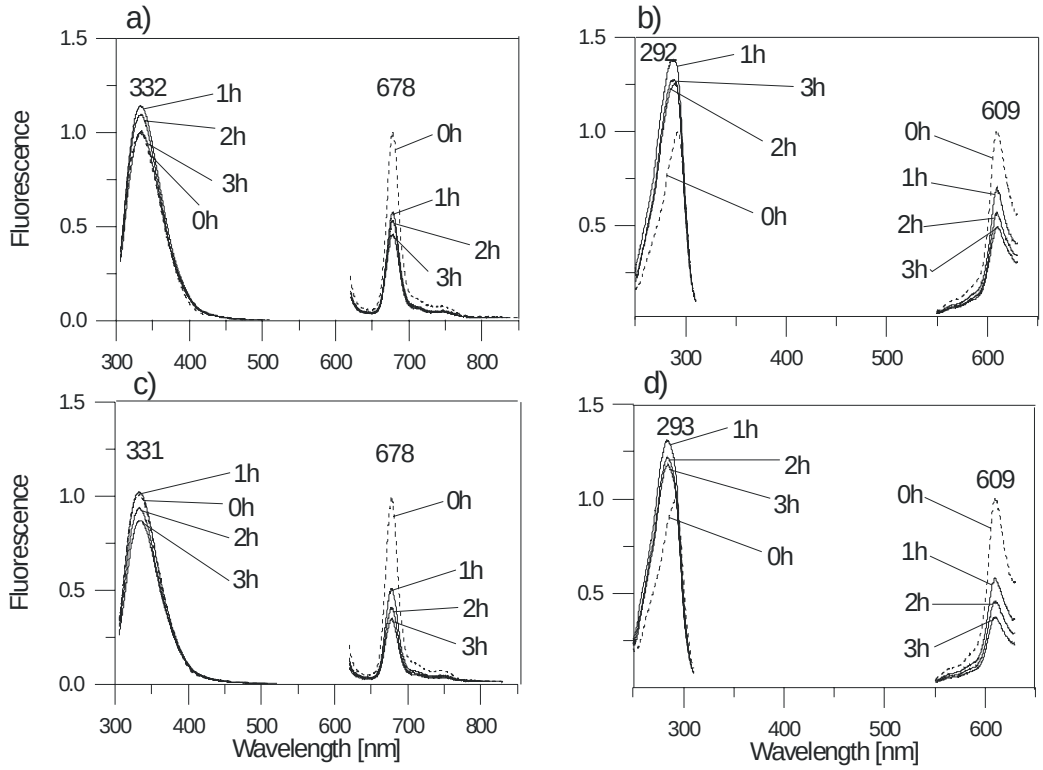

Fig.4. Fluorescence emission spectra (a and c) and fluorescence excitation spectra (b and d)of cells stained by AlPc. Wavelengths of excitation and observation as in Fig.3.

of spectra for cells stained by AlPc. The intrinsic fluorescence of cells observed with the maximum at 331nm-332nm is due to the superposition of tyrosine (maximum of emission at 304nm) and tryptophan (emission with maximum at 353nm). The used wavelength of excitation (296nm) is more efficiently absorbed by tryptophan. Additionally the tyrosine excitation is transferred to tryptophan (Lakowicz, 1999, pp. 63-65). In a result the tryptophan emission is predominant, but the position and bandwidth of observed fluorescence show that contributions of tyrosine are also present.

In order to avoid errors connected with unequal numbers of cells in exciting beam and various yields of fluorescence of dyes in the cells (which can be much more variable than in DMSO) all spectra of non illuminated samples (Fig. 3) are normalized to unity. The shapes of fluorescence emission spectra, obtained after various times of illumination, are similar. Illumination causes some shift towards longer wavelengths of the auto fluorescence maximum in fluorescence excitation spectra (Fig. 3b, d). It suggests that contributions from tryptophan, comparing with those from thyrosine, are slower decreasing during illumination. Due to sample illumination changes in intensities of emission observed in the short wave region are for ZnPc and AlPc different showing different, types and degrees of cell material degradation.

The ratios of amplitudes of fluorescence emission and fluorescence excitation measured at wavelength used for fluorescence excitation for all illuminated samples are similar, but for whole excitation spectra are not identical to those obtained for the set of emission spectra. These differences can be due to additional degradation of cells caused by excitation light. It is important that the tendency of changes is in both sets of spectra similar.

The relative changes in fluorescence emission intensities due to samples illumination are shown in Fig. 5

The intensity of cell autofluorescence is lately very often used for monitoring cell variability due to photodynamic therapy (Agati, Barnabei, Fusi, Pratesi, Paglierani, Santini & Bernabe, 2003; Pogue, Pitts, Mycek, Sloboda, Wilmot, Brandsema & O'Hara, 2001; Dysart, Petterson, Farrell & Singh, 2002). In most cases the cell degradation is followed by the decrease in tissue auto fluorescence intensities. For various samples proper spectral region of cells autofluorescence for evaluation of cell damage should be established. Such investigations are for our samples are actually carried out in our laboratory and will be presented in our

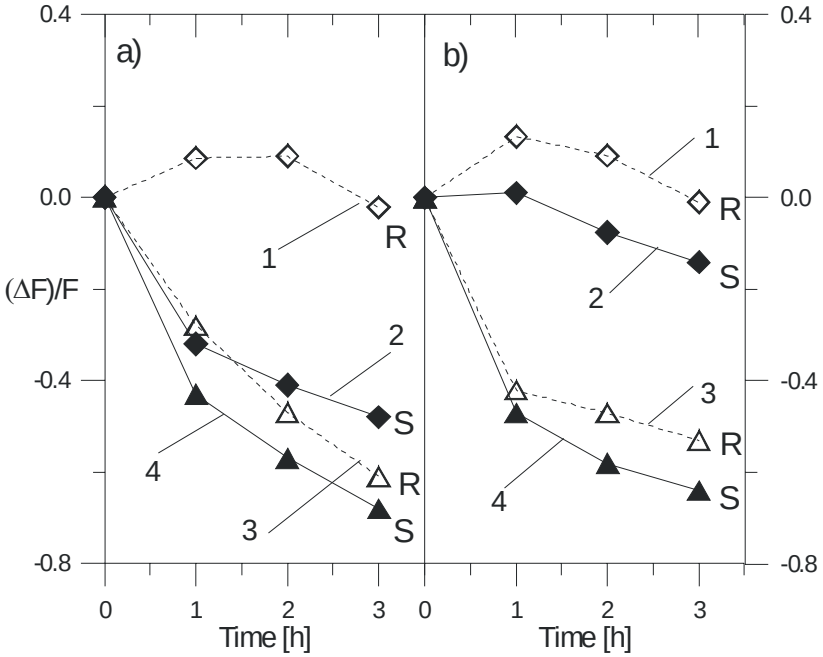

Fig. 5. The relative changes of emission maxima intensities as a result of illumination (calculated with respect to emission of non-illuminated samples). Curves 1 and 2 —emission at 330 nm; curves 3 and 4 at 675 nm. R-resting cells; S-stimulated cells. (a) Cells stained by ZnPc; (b)by AlPc.

further study. On this stage of our investigations is sure that the decrease in cell autofluorescence is due to cell degradation. Intrinsic fluorescence emitted from tissue is not completely understood at this time (Lakowicz, 1999), therefore the further studies are necessary.

As it follows from Fig. 5 a the lymphocytes stained by ZnPc, the dye having higher efficiency of triplet state generation, the illumination causes a much more effective destruction of cell material in a case of sensitized cells than for resting cells. This shows that ZnPc is not toxic for healthy cells. The dye molecules are photodegraded in similar ways in both types of cells, but slightly stronger in sensitized cells. Fast degradation of the dye in sensitized cells can stop the photodynamic action, but in this case the kinetics of cell material and dye degradation for sensitized cells are similar.

The kinetics of photodegradation of cell material for a case of staining by AlPc (Fig. 5b) are quite different than those observed for ZnPc (Fig. 5a). For both types of cells stained by AlPc the cell material is not strongly degraded. The fluorescence of resting cells even increases showing some conformational changes influencing the intensity of emission. The degradation (not very strong) is observed only after prolonged illumination. The AlPc in both type of lymphocytes samples is degraded much faster than the cell material. In the resting sample AlPc is slightly more resistant to photodegradation than in the sensitized sample.

The different changes generated by illumination in short wavelengths fluorescence region (Fig. 5) are due to tryptophan high sensitivity on its local environments (Lakowicz, 1999). These changes show that the cell sensitization strongly influences on the cell material properties.

Results from Fig. 5b show that AlPc is not a suitable photosensitizer for PDT. Both dyes, on the grounds of usually applied spectral parameters (absorption, fluorescence emission in the cells) seem almost equally proper for PDT applications. The presented results show that additionally, the efficiency of triplet state generation should be checked and that from LIOAS measurements in an incubation solvent one can predict the dye interaction with the cell material.

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