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Spectral properties of stilbazolium merocyanines – potential sensitizers in photodynamic therapy and diagnosis Part II. Merocyanines in resting and stimulated lymphocytes

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Abstract

Three stilbazolium merocyanines, selected on the basis of their investigations in model systems in part I of this work [J. Photochem. Photobiol., A: Chem. 163 (2004) 127], were introduced into resting and phytohemagglutinin-stimulated lymphocytes. All these merocyanines in solution exhibit high yield of generation of very photochemically active triplet states, but are characterized by different yields of fluorescence emission. The PHA-stimulated lymphocytes were the model of tumor cells. The fluorescence emission and fluorescence excitation spectra are more sensitive to small changes in the cell material than absorption spectra. The course of the photodynamic reactions in stained and unstained cells were established.

The efficiency of triplet states generation of the dyes incorporated into stimulated cells has been evaluated using time-resolved photothermal spectroscopy. As follows from the photographs of stained cancerous cells taken by means fluorescence microscope, merocyanines are located predominantly in cell membranes. The spectral properties of the dye-sensitizes in solutions and in the cells can differ therefore the selection of proper sensitizers for medical applications made on the ground of the investigation of dye molecules in simple artificial model systems is only preliminary. The selected dyes have to be further investigated after introducing them into resting and PHA-stimulated lymphocytes. It has been shown that the dyes exhibiting high yields of triplet state generation in the stimulated cells usually are also efficient sensitizers of photodynamic reactions. The exceptions are the dyes undergoing fast photobleaching in the cells.

Keywords: Fluorescence emission; Fluorescence excitation; Laser-induced optoacoustic spectroscopy; Resting lymphocytes; Stimulated lymphocytes; Photodynamic therapy; Stilbazolium merocyanines; Triplet state decay time; Triplet state generation

1. Introduction

Merocyanine (Mero) dyes are promising sensitizers for application in photodynamic therapy (PDT) [1,2]. The photosensitizer suitable for the medical application has to be incorporated selectively into cancerous and healthy cells and therefore, after illumination predominantly cancerous stained cells are destroyed. The efficiency of incorporation and the location of the dye in cells depend on the dye interactions with the surroundings. The efficiency of photodynamic reactions depends on the generation of long living, very photochemically active triplet states. On the basis of our earlier study [3] of seven stilbazolium Mero dyes, three of them labeled as Mero I, Mero B and Mero T* (Fig. 1) have been selected for investigations in cells. These dyes do not exhibit strong interactions with macromolecular polymer system therefore they are expected not to interact with lipid and proteins of cell membranes and hence to be easily introduced into cells. It is known [4,5] that the efficiency of various Meros incorporation strongly depends on their side groups attached. The dyes chosen for present study in solutions exhibit high efficiency of the triplet states generation, but they have very different yields of fluorescence emission [3]. Mero

Abbreviations: AV, annexin-V; BCP, bromocresol purple; BPB, bromophenol blue; FITC, fluorescein; ISC, intersystem crossing; LIOAS, laser-induced optoacoustic spectroscopy; Mero, merocyanine; PBS, phosphate buffered saline; PDD, photodynamic diagnosis; PDT, photodynamic therapy; PHA, phytohemagglutinin; PI, propidium iodide; PS, phosphatidiloserine; PVA, polyvinyl alcohol; TD, thermal deactivation; Trp, tryptophan; Tyr, thyrosine

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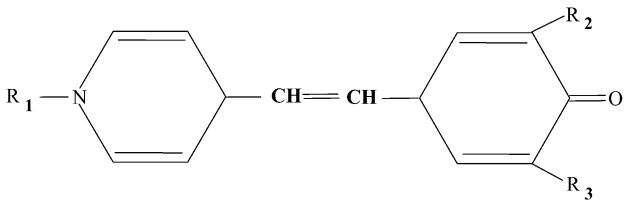


Mero B $R_1 = (CH_2)_{11}–OH$, $R_2 = H$, $R_3 = H$

Mero T* $R_1 = (CH_2)_{11}–OH$, $R_2 = OCH_3$, $R_3 = H$, salt HCl

Mero I $R_1 = (CH_2)_6–OH$, $R_2 = NO_2$, $R_3 = NO_2$

Fig. 1. Molecular structure of investigated merocyanine dyes.

I is practically nonfluorescent, but in simple model systems it shows very high efficiency of triplet state generation. It could be a result of a competition between the intersystem crossing (ISC) transition from excited singlet to triplet state and "prompt" fast thermal deactivation (TD). Mero B and Mero T* in model systems show lower, but still high, efficiency of triplet states generation and well measurable fluorescence emission [3]. The present results should show to a which extend the prediction made on the basis of the investigation of simple artificial models can be fulfilled in much more complex biological system, such as lymphocytes. It is known [6] that spectral properties of stilbazolium merocyanine dyes strongly depend on the medium, therefore they can be different in solutions and after incorporation into cells.

2. Materials and methods

The molecular structures of the stilbazolium merocyanines (Meros) investigated are presented in Fig. 1. The following Meros were investigated (Fig. 1): (i) Mero I (1-(6′-hydroxyhexyl)- 4 -[(3, 5-dinitro-4-oxocyklohexa-2, 5-dienylidene)etylidene]-1,4-dihydropyridyne), (ii) Mero B (1-(1,1′-hydroxyhexyl)-4-[(4-oxocyclohexa-2,5-dienylidene) etylidene]-1,4-dihydropyridyne) and (iii) Mero T* (1-(11′-hydroxyundecyl)-4-[(3-dimetoxy-4-oxocyklohexa-2,5-dienylidene)ethylidene]-1,4-dihydropyridyne salt HCl). Mero I differs from Mero B and Mero T* in the length of the chain (R_1) as well as the character of R_2 and R_3 groups. The Mero B and Mero T* differ in side groups at position R_2 and additionally Mero T* is salt form of the dye. These Meros were synthesized by Dr. I. Gruda (Université du Québec, Trois Rivières, Canada) according to the methods described in [6]. The Meros used in this study were dissolved at 10^{-3} M concentration in ethanol (Mero B and Mero T*) or in methanol (Mero I) and stored at 12 °C before use.

2.1. Isolation of the mononuclear cells

Freshly, drawn samples of heparinized venous blood from normal donors were centrifuged at 400 × *g* for 10 min. The samples were diluted (1:4, v/v) with Hanks solution (Polfa, Poland) and centrifuged at 400 × *g* at 4 °C for 30 min in a Gradisol L (AQUA MEDICA, Poland) gradient (d = 1.077 g/cm^3) according to Bøyum [7]. The mononuclear cells were collected from the interphase, washed three times and resuspended to a concentration of 8×10^6 ml^{-1} with an 0.9% NaCl solution.

The purity and number of mononuclear cells (monocytes and lymphocytes) were established using flow cytometry. The contents of lymphocytes was about 75% of cells, henceforth the cells are referred as lymphocytes. The cells investigated have some admixture of erythrocytes attached to lymphocytes as it can be seen from cytometric and absorption measurements.

2.2. Cell stimulation and incubation with dyes

One part of lymphocytes (a concentration of 8×10^6 ml^{-1}) was stimulated with 10 μg/ml phytohemagglutinin (PHA, HA 17, Wellcome, UK) for 1 h at 37 °C. After incubation the cells were washed twice and resuspended with the 0.9% NaCl solution (concentration 40 × 10^6 cells/ml). The unstimulated (resting) cells were prepared in a similar manner but were not incubated with PHA. Next, 1 μl of ethanol or methanol Meros solution (10^{-3} M) per 1 ml cells was added to the samples of both stimulated and resting cells. The control samples, were cells with addition of the same volume of ethanol or methanol. The incubation of all sets of samples (1 h, 37 °C) was performed in the dark. During the incubation the concentration of Meros in the sample volume was 10^{-4} M (for spectral investigations) and 10^{-4} to 10^{-5} M (for estimation of cells apoptosis). After incubation the cells were washed three times with a 0.9% NaCl solution.

The cells used in present investigations were obtained from two donors. The cells stained by Mero B and Mero T* were from the same source, the cells stained by Mero I from the another one.

For all spectral and biochemical measurements the samples were resuspended in solutions of physiological salt, exception the samples used for the laser-induced optoacoustic spectroscopy (LIOAS) investigation which were resuspended in the mixture of the 15% fluid polyvinyl alcohol (PVA) water solution and physiological salt (2:1, v/v).

The samples of the lymphocytes were denoted as follows: resting (R) and stimulated (S) unstained cells; resting (RD) and stimulated (SD) – cells stained by dye. The type of the sensitizer is market in brackets, for example, RD(B) means resting cells stained by Mero B.

2.3. Estimation of cells apoptosis

For the detection of apoptosis the annexin-V (AV) staining kit (Annexin V/FITC Kit, Bender MedSystems) was used. The staining procedure for flow cytometry involved: (i) washing the cells with phosphate buffered saline (PBS) pH 7.4 and centrifugation at 200 × *g* for 5 min, (ii) incubation of the cells with 5 μl AV-fluorescein for 10 min, (iii) incubation of the cells with 10 μl propidium iodide (PI) for

Table 1
Effect of the Mero B and Mero T* on the structure of the resting cell membrane (measured by annexin-binding) and survival (PI-test: showed the part of necrotic cells) of the cells in light exposed and non-exposed samples (R: resting unstained; RD: resting stained by proper Mero dye)

Sample	Light non-exposed cells			Light exposed cells		
	Living cells (%)	Annexin-binding cells (%)	PI-test (%)	Living cells (%)	Annexin-binding cells (%)	PI-test (%)
R without methanol	99.70	0.10	<0.05	99.50	0.36	<0.05
R with methanol	99.45	0.26	<0.05	99.00	0.70	<0.05
RD(B)	97.15	0.74	2.00	97.55	0.66	1.80
RD(T*)	96.11	1.26	2.50	86.62	4.67	8.80

10–15 min and (iv) analysis of the samples on a flow cytometer using 488 nm excitation and 515 nm bandpass filter for fluorescein (FITC) fluorescence detection and a filter > 600 nm for PI fluorescence detection.

At the early stages of apoptosis which could be reversible, changes occur at the cell surface. One of them is a translocation of phosphatidiloserine (PS) from the inner side of the plasma membrane to the outer leaflet [8]. The analysis of PS availability in the apoptotic cell-membranes was performed by flow cytometry using fluorescein conjugated AV, which is a phospholipids-binding protein with high affinity to PS. The PI is commonly used for the differentiation of apopthotic from necrotic cells. Since, cells undergoing apoptosis are less permutable than dead cells, they show characteristic intermediate incorporation of PI and finally display a complete loss of membrane permeability and become PI high [9]. The effect of staining by Mero B and Mero T* on the cells survival is shown in Table 1. In our experiments, two sets of samples were used. The samples of the first set were non-exposed to light, while of the second one were exposed to the light through glass filter (cut off the light below 400 nm) for 15 min. The intensity of illumination was 2.11 mW/cm^2. From Table 1 it follows that the staining of the resting cells is, at used in spectral investigations dyes concentrations and illuminations, only weakly cytotoxic.

2.4. Measurements

The absorption, fluorescence emission and fluorescence excitation spectra were measured, using Specord M40 (Carl Zeiss, Jena, Germany) and fluorescence spectrophotometer F4500 (Hitachi, Tokyo, Japan), respectively. The measurements of time-resolved photothermal signals were carried by the LIOAS method developed by Braslavsky and Heibel [10] using experimental setup constructed in our laboratory [3,11]. The wavelengths of nitrogen-dye laser flash were: 384 nm (for Mero B), 414 nm (for Mero T*) and 412 nm (for Mero I). The analysis of LIOAS signals was made by two methods: that proposed by Marti et al. [12] and Rudzki-Small et al. [13] in the way described previously [3].

The first method [12] is based on the following formula:

$$\phi_T E_T = (1 - \alpha) E_{las} - \phi_F E_F \quad (1)$$

where α is the part of absorbed energy of the laser light (E_{las}) converted into heat in a time shorter than 0.5 μs, ϕ_F and E_F the fluorescence yield and energy of singlet state, E_T and ϕ_T are the yield of triplet state generation and its energy.

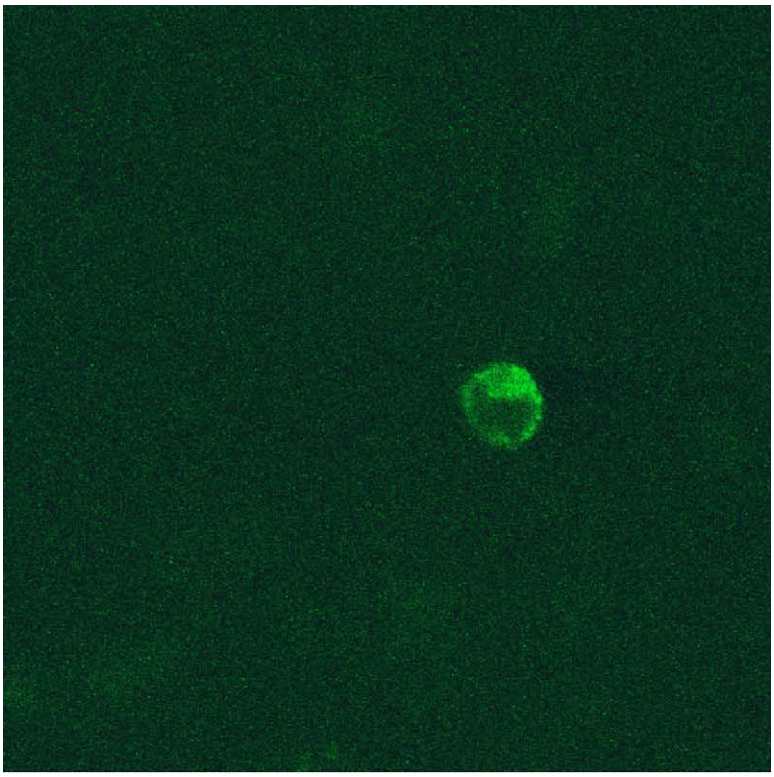
The second method [13] delivers by the deconvolution of LIOAS signals the decay times of triplet states (τ_i) and their contributions (k_i) to thermal deactivation, but reasonable results can be obtained only for decay times between 0.5 and 5 μs [3,11].

The reference samples were the unstained stimulated cells with addition of reference dyes (bromocresol purple (BCP) or bromophenyl blue (BPB) [14]). The concentration of these dyes were adjusted so that the absorptions of the sample studied and the reference one at the wavelength of the laser flash were the same. The reference dye can be attached to cell membrane or even in a small amount it can be introduced into cells. Therefore, the approach used in our study is a crude approximation because a reference dye in the presence of cells can have some component of slower deactivated TD. The cell material has also long living states [14,15], which can be changed as a result of sample degradation due to illumination, but in system of used samples this effect is at least partially compensated.

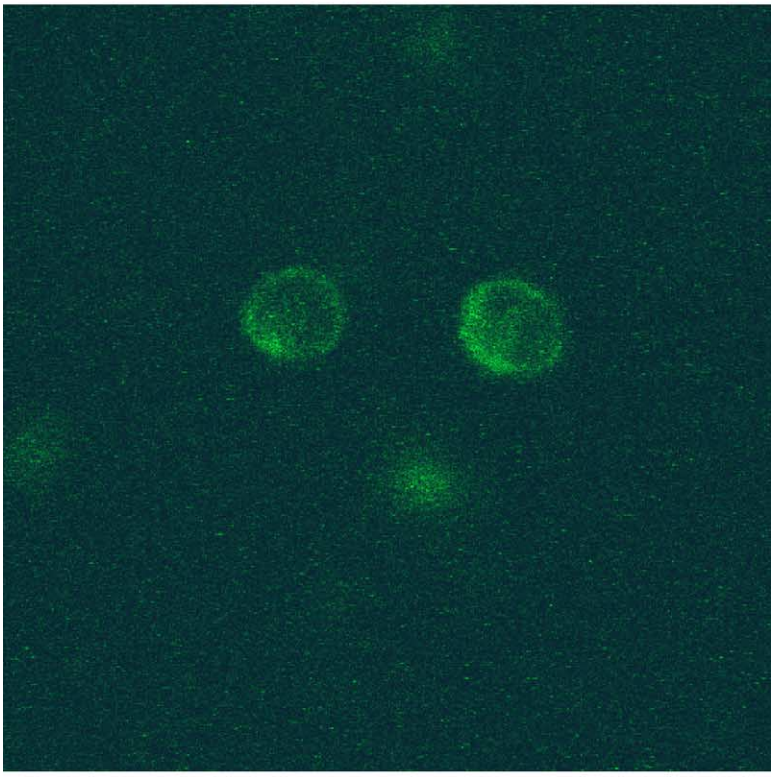
The photochemical reactions were induced by the illumination using xenon lamp (150 W) applied trough a glass filter (cut off the light below 400 nm). Intensity of illumination was 5.05 mW/cm^2. The dose of light energy supplied during 1 h was about 18.18 J/cm^2, which means it was almost one-and-half time greater than that used in [15] for killing 90% of cancer cells.

The photographs of stained cells were taken using a Bio-Rad MRC 1024 fluorescence microscope (at illumination by 488 nm). Some exemplary photographs of cancerous (JU-CAT) cells stained by incubation with Mero B for 3 and 24 h are shown in Fig. 2. The pictures show that this dye molecules are gathered predominantly in the cell membrane.

On the basis of our results it is possible to compare the yields of triplet state generation of the Mero dyes in model systems [3] and after introducing them into stimulated cells and to establish the efficiency of photodynamic reactions occurring in a case of stimulated cells. It help to establish if it is possible to predict the photodynamic action on the ground of spectral and photothermal investigations of sensitizers in model systems.



(a)



(b)

Fig. 2. Photographs of cancerous cells stained by Mero B by the incubation during 3 h (a) and 24 h (b) (taken under fluorescence microscope).

3. Results

3.1. Absorption spectra

Fig. 3 presents the absorption spectra of resting cells and those stimulated unstained (Fig. 3(a) and (b)) as well as stained by Mero B (Fig. 3(c) and (d)), Mero T* (Fig. 3(e) and (f)) and Mero I (Fig. 3(g) and (h)). All absorption spectra are shown in two regions: 240–310 nm – the absorption predominantly by the cell material and 350–600 nm – the absorption of heme and added Mero dyes. As follows from a comparison of the spectra in Fig. 3(a), (c), (e) and (g) the contributions of a cell material to absorption in the short wavelengths region is for all samples similar and does not changed dramatically as a result of cells stimulation and sensitization by dye addition. Stimulation and/or dye addition can cause small changes in absorption due to the degradation processes occurring in macromolecules. Such processes can also affect macromolecules conformations followed by the change in light scattering effect. The absorption modifications are different for various Meros (Fig. 2(a), (c), (e) and (g)). The 240–310 nm region corresponds to the absorption by aromatic amino acids: predominantly thyrosine (Tyr) and tryptophan (Trp) [14]. The long wavelengths range

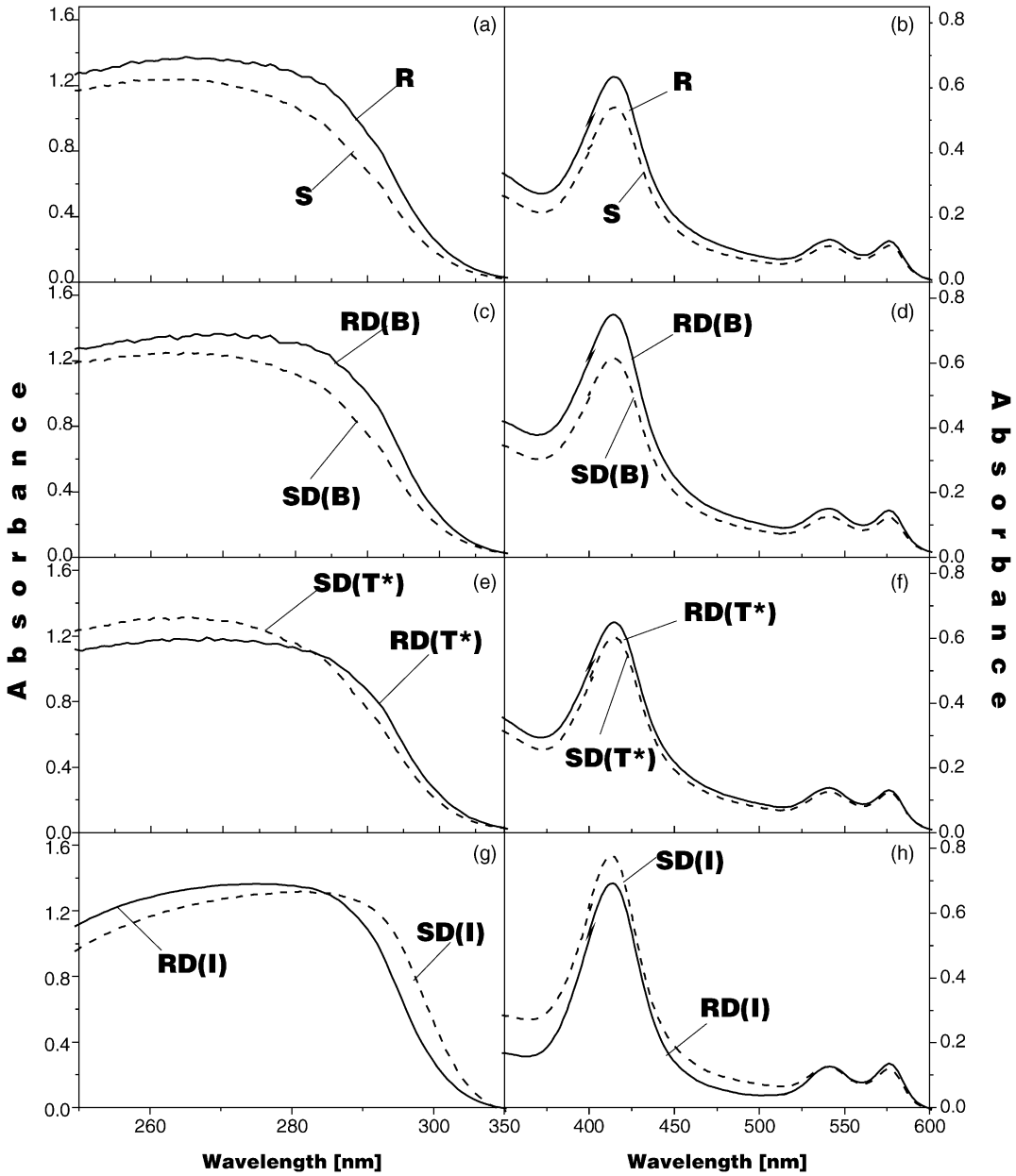


Fig. 3. Absorption spectra of resting and stimulated lymphocytes: unstained (a and b) and stained by Mero B (c and d), Mero T* (e and f), Mero I (g and h). Description of curves: resting (R) and stimulated (S) unstained cells; resting (RD) and stimulated (SD) stained by dye-sensitizer cells. Type of dye was marked in parenthesis, e.g. RD(B) means resting cells stained by Mero B.

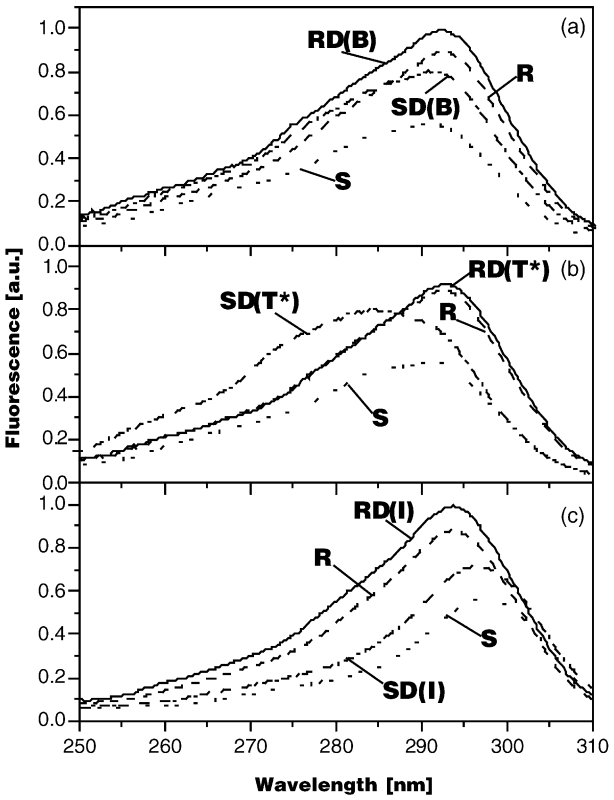


Fig. 4. Fluorescence excitation spectra of lymphocytes stained by (a) Mero B, (b) Mero T* and (c) Mero I. Observation at 330 nm. Description of curves as in Fig. 3.

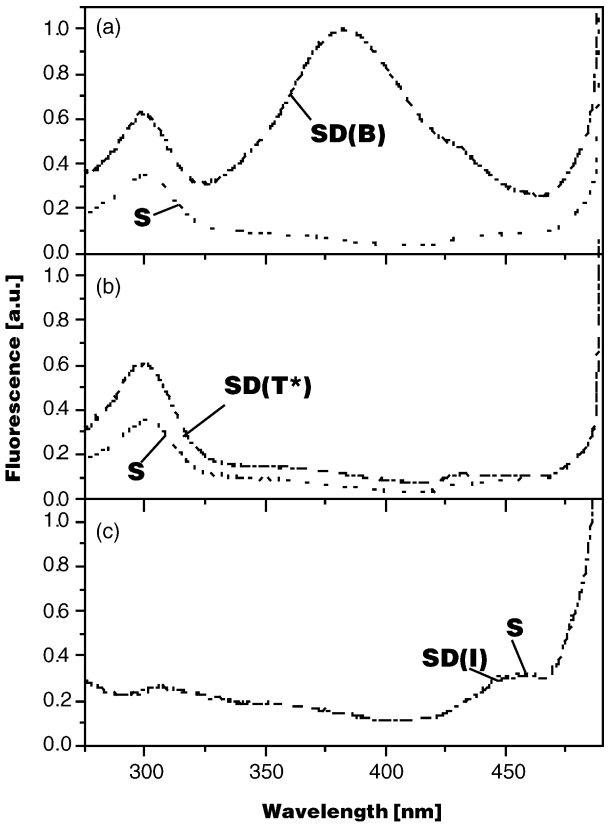


Fig. 5. Fluorescence excitation spectra (observed at 500 nm) of stimulated lymphocytes stained by (a) Mero B, (b) Mero T* and (c) Mero I. Spectra are normalized with respect to highest value of SD(B). Description of curves as in Fig. 3.

(350–600 nm) (Fig. 3(b), (d), (f) and (h)) corresponds to the absorption of heme (400–420 nm, 543 and 578 nm) and that of introduced photosensitizers. Even at the presence of low concentration of erythrocytes, the heme absorption strongly predominants, therefore from the absorption spectrum it is not easy to establish the amount of dye-sensitizer introduced into cells. Hence much more useful for evaluation of the efficiency of dye incorporation are fluorescence excitation (Figs. 4 and 5) as well as the fluorescence emission spectra (Figs. 6 and 7). The shapes of absorption spectra of resting and stimulated cells obtained from different donors were similar.

3.2. Emission spectra

Emission of proteins is dominated by Trp because of the efficient excitation energy transfer from Tyr to Trp [14–17]. Due to the efficient excitation energy transfer from heme to Trp in the heme protein intrinsic fluorescence is very low [14]. For monitoring the changes occurring in the cell material several authors [9–11] have applied the measurements of the cell intrinsic fluorescence. Such measurements are used also for monitoring the degree of cell differentiation as a result of photodynamic treatment [15,16].

Each set of samples (i.e. R, RD, S and SD) was prepared from the same suspension of cells therefore, the numbers and properties of cells in each sample were similar. In a such conditions, the changes in the intensity of emission in fluorescence excitation and emission spectra (Figs. 4–7) due to the cell staining are a function of the efficiency of the dye incorporation. The yield of the fluorescence of various dyes introduced into even similar cells can be different because of the effect of the dye surroundings [18].

Fig. 4 shows the normalized fluorescence excitation spectra at excitations in the region of the absorption of the cell material (250–310 nm) and at the observation of the fluorescence predominantly related to the cell material (at 330 nm). The staining of the cells by different Mero dyes and/or cell stimulation have different effects on the shape and intensities of the fluorescence excitation spectra of the cells. It means that samples characterized by similar absorptions (Fig. 3) can emit fluorescence with various yields. The reason is because the emission is much more sensitive than the absorption to small changes in the cell properties, such as the modification of macromolecules conformation, because the excitation energy transfer between various cell components depends on the mutual orientations of their transition moments.

The position and shape of the fluorescence excitation band (Fig. 4) differ for the samples of different sets. Therefore,

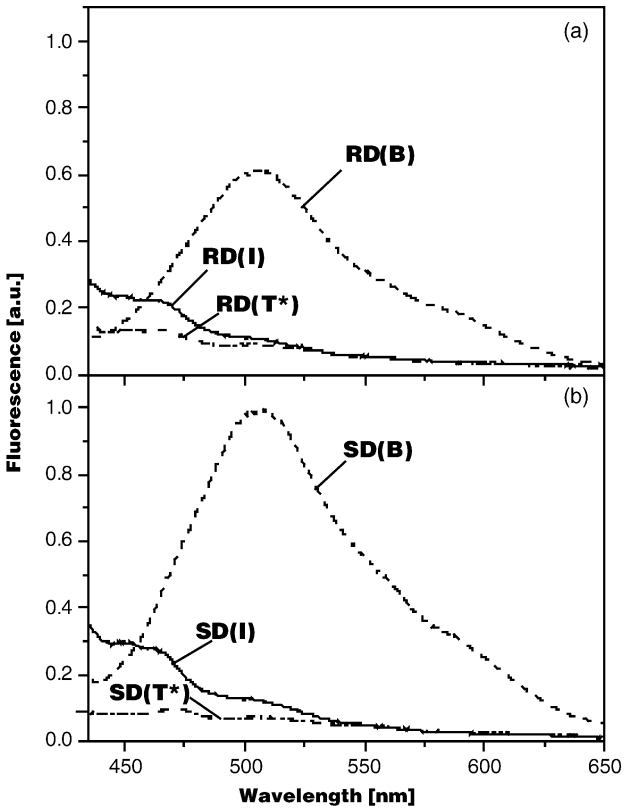


Fig. 6. Fluorescence emission spectra (excited at 410 nm) of (a) resting and (b) stimulated lymphocytes stained by Meros. Description of curves as in Fig. 3.

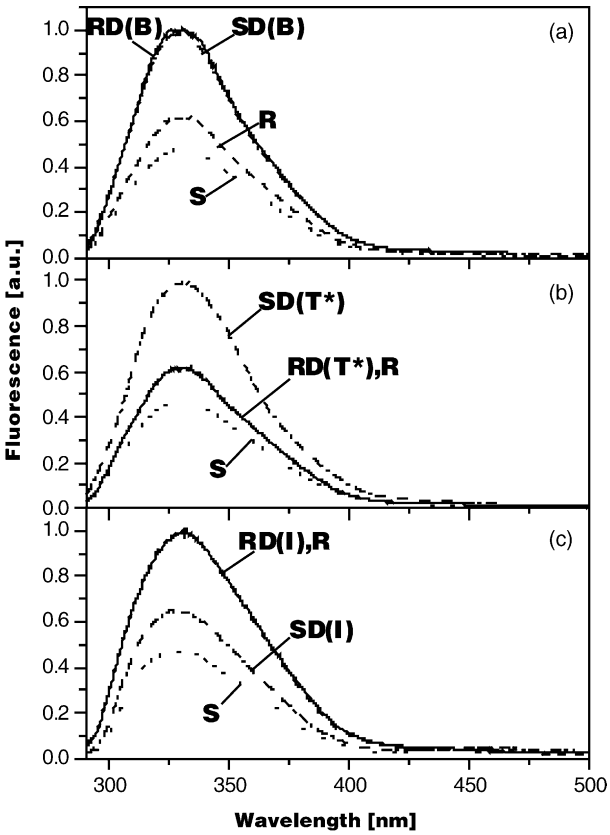


Fig. 7. Fluorescence emission spectra (excited at 275 nm) of resting and stimulated lymphocytes stained by (a) Mero B, (b) Mero T* and (c) Mero I. Description of curves as in Fig. 3.

the spectra of stained cells have to be compared with those of the unstained sample from the same set.

The excitation efficiencies of resting cells with and without dye are not very different (Fig. 4), but staining causes usually a small increase in the emission intensity of the excitation band. The stimulation of cells causes a strong decrease in the emission of the cell material and a change in the shape of the fluorescence excitation band (Fig. 4). The staining of the stimulated cells causes a stronger increase in the emission intensity than that observed for the resting cells. It could suggest more efficient incorporation of dyes into the stimulated than resting cells. The spectra shown in Fig. 4 strongly suggest the conformation changes occurring in the cell material due to the cells stimulation and staining. Such changes seems to be similar for the resting and stimulated cells stained by Mero B and Mero I but are different for the stimulated cells stained by Mero T*.

Fluorescence excitation spectra (Fig. 5) at observation in a region of the dyes emission shows that a very high fluorescence maximum at 384 nm, related to sensitizer absorption [3], appears only in the spectra of the stimulated cells stained by Mero B (Fig. 5(a)). Such a maximum is absent for unstained samples from this set.

The yields of fluorescence of Meros in the cells (Table 2) are different than those previously [3] observed in solutions. For Mero I incorporated into the cells the fluorescence yield value is very low similarly as it was found in solution, for Mero B this yield in the cells is much lower than in solution. The strongest decrease in the fluorescence yield occurs for Mero T* exhibiting a yield about 20 times lower in cells than in the solution [3]. In a case of very low yield of fluorescence of Mero in the cells it is not easy to establish the amount of incorporated dye on the basis of fluorescence spectra.

Fluorescence spectra in Fig. 6 show that Mero B is incorporated, in higher degree, into stimulated than into resting cells. The intensity of maximum at about 500 nm due to dye

Table 2
Results of LIOAS signals analysis of the stimulated lymphocytes (SD) stained by Mero dyes in mixture of 15% PVA water solution and physiological salt (2:1, v/v) (temperature 20 °C); α and ϕ_T on the basis of formula (1), k_1 is pre-exponential factor for $\tau_1 \leq 0.5\,\mu s$, k_2 and τ_2 – pre-exponential factor and decay time, respectively, of the slow component obtained from deconvolution, accuracy of calculated values α, $k_{1,2}$, τ and ϕ_T – about 5%, λ_{exc} – excitation wavelength used in LIOAS measurements

SD stained	λ_{exc} (nm)	ϕ_F	α	k_1	k_2	τ_2 (μs)	ϕ_T
Mero B	384	0.11	0.53	0.53	0.37	3.3	0.79
Mero T*	414	0.02	0.76	0.76	0.30	1.15	0.44
Mero I	412	<0.01	0.92	0.94	0.11	1.02	0.16

emission (Fig. 6) is at 410 nm excitation almost two-fold higher for Mero B in stained stimulated samples (SD(B)) than in the resting cells (RD(B)).

For Mero T* and Mero I (Fig. 5(b) and (c)) the fluorescence of dyes in the staining cells is low. It is due to the low yield of fluorescence of these dyes in the cells. It can suggest that the excitation of Mero T* and Mero I could be, as a result of interactions with cell material, deactivated rather nonradiative, not by fluorescence emission (Fig. 6, Table 2) and/or that amounts of these dyes introduced into cells was low.

Fig. 7 shows the fluorescence emission of the cell material excited at 275 nm. For the cells stained by Mero B the intensity of the cell emission for resting and stimulated cells is almost the same, but for the stimulated sample the increase due to the staining is higher than for the resting lymphocytes. More interesting are observation made for cells stained by Mero T* (Fig. 7(b)): staining causes strong increase of the fluorescence of stimulated cells, whereas has no influence on the emission of the resting sample. Much lower is the increase in the fluorescence due to the staining by Mero I of the stimulated cells (Fig. 7(c)). For this dye the staining has no influence on the fluorescence intensity of resting cells, but resting cell fluorescence is higher than that of stimulated cells. The cells used for this set came from a different donor than those cells used for staining by Mero B and Mero T* (Fig. 7(a) and (b)). It was shown previously [19] that leukocytes obtained from different donors exhibit different fluorescence. The low intensities in the 500 nm region of dye emission (Fig. 7) suggests that for all types of cells and dyes the efficient excitation energy transfer from the cell material to dyes introduced is not observed.

The results presented in Figs. 3–7 show that all Mero dyes used, even before illumination, interact differently with the cell material. The fluorescence spectra suggest that Mero B is to a higher degree accumulated into the stimulated than into the resting cells where it is inserted inside the cell membrane and does not change the cell structure (Fig. 2, Table 1). The fluorescence results also suggest that Mero T* (Table 1) and Mero I to some extent perturb the cell membrane. This perturbation is better seen from fluorescence (Figs. 4(b) and (c) and 7(b) and (c)) than from the absorption data (Fig. 3(e)–(h)), because the fluorescence of complex systems depends on the mutual excitation energy transfer between various component and this transfer is strongly dependent on macromolecules conformation and aromatic amino acid surroundings.

The low influence of dye on the resting cells properties (Table 1 and Fig. 7) is, from the point of view of PDT and photodynamic diagnosis (PDD) applications, very satisfactory, because such dyes cannot be harmful to healthy cells. As follows from a comparison of the shapes of the fluorescence excitation spectra of cells taken from two different donors (Figs. 4(a)–(c) and 5(a)–(c)) the individual characteristics of the cells affect their fluorescence properties, especially strongly for the stimulated cells. This observation is in agreement also with our previous results [19]. To be able to conclude on properties of different sensitizers on the basis of fluorescence results it is necessary to introduce them to the cells obtained from the same donor. Fluorescence is very sensitive method, because even at similar low percent of AV-binding stained by various dyes resting cells (Table 1) the changes in fluorescence spectra can be observed.

3.3. Time-resolved optoacoustic data

Table 2 shows the results of LIOAS signals analysis performed for stimulated lymphocytes stained by the Mero dyes studied. The yield of fluorescence of Mero T* and Mero I (exception Mero B) in cells is very low (Table 2) and because of light scattering cannot be precisely established. This yield, because of its low value, has inconsiderable influence on the calculated values of the yield of the triplet state generation (formula (1)). As it follows from Table 2 almost all energy absorbed by Mero I stained cells is thermally deactivated in a very short time (high values of α and k_1). Efficiency of the triplet states generation (ϕ_T) is for this dye very low relative to that of the other dyes. The situation is different for the stimulated cells stained by Mero B and Mero T* in which the yield of dye triplet state generation is still relatively high. The values of the efficiency of fast thermal deactivation (α) and ϕ_T are the same for Mero B in cells and in the solution [3] and only the ϕ_F value is in cells slightly lower. Whereas in a case of Mero T*, the strong decrease in ϕ_F value, increase of α and decrease of ϕ_T values are observed in the cells. In all measured lymphocytes samples the triplet state decay times are shorter and the contribution of the slow thermal decay (k_2) are larger than were observed in solutions (Table 2, [3]).

The efficiencies of the triplet states generation of all three dyes located in solution without contact with oxygen are high [3] whereas in the cells these yields are different. It shows that it is not possible to predict the yield of dye triplet state generation in cells only on the ground of simple model system investigation. Nevertheless, such studies are useful because their results can help exclude from further examination of the dyes exhibiting low efficiency of triplet state generation. The efficiency of triplet state generation is usually lower in the cell than in model systems. It is known [20–22] that the oxygen presence and the dye close surroundings play important role in photodynamic reactions. These factors are probably responsible for different yields of Mero T* and Mero I triplet states generation in the model systems [3] and in the cells.

3.4. Photochemical reactions

In investigation of the possibility of the dyes application in PDT the influence of stimulation and staining procedures on the cell photosensitivity is crucial, therefore, all emission

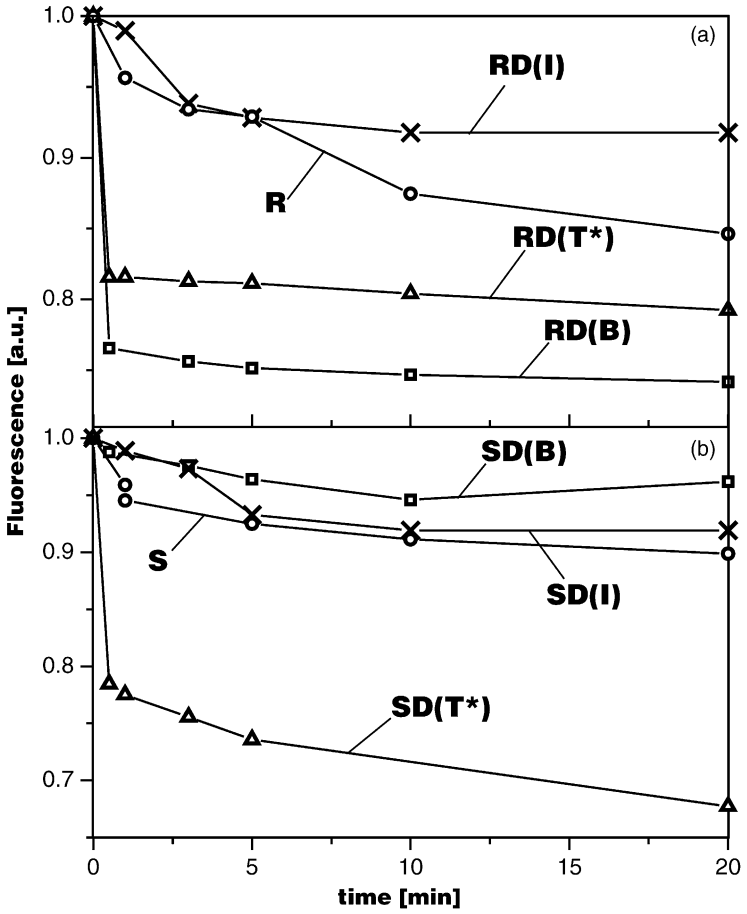


Fig. 8. Intensities at main fluorescence band of cell material emission (at 330 nm) as a function of illumination time: (a) resting and (b) stimulated cells. Wavelengths of fluorescence excitation 275 nm (normalized for intensity at time 0 min). Description of curves as in Fig. 3.

and fluorescence excitation spectra in every one set are normalized supposing that the intensity of the highest emission maximum is equal to unit. Fig. 8 shows the change in such normalized fluorescence excited at 275 nm and observed at 330 nm. The course of the change in the fluorescence intensity shows the occurrence of some photochemical processes in the cell material. For most dyes the kinetics of photoreactions in resting and stimulated cells are similar. Taking into account the numbers of dye molecules incorporated into cells evaluate on the grounds of the fluorescence result one can predict that the amount of destroyed material should be larger at higher efficiency of dye incorporation. The Mero B, which is probably efficiently incorporated into cells and has high yield of fluorescence, is not such effective in the changing of the emission for stimulated cell material as one could expect from (Fig. 8). Fig. 8 shows that the most effective in photodynamic reaction is Mero T*. The change in fluorescence excited at 410 nm and observed at 500 nm (Fig. 9) evidences the photobleaching of the sensitizers. For Mero B the intensity of emission in the short wavelength region (250–310 nm) is much higher than that in long wavelength range (400–700 nm) (not shown). The effects of the illumination observed in these two ranges are different as it follows from the comparison of Figs. 8 and 9. For Mero B the decrease in the fluorescence intensity at 500 nm due to illumination for both resting and stimulated cells occur predominantly during first 5 min of cells illumination. This shows that prolongated illumination of the sample is not effective because the photobleaching of dye interrupt the PDT treatment. The dose of light energy received by samples during 5 min is 0.6 J/cm^2, which means is rather low in comparison with illuminations applied usually in PDT treatment [16]. Hence, it seems that Mero B dye because of its strong fluorescence observed in stimulated cells and different in stimulated and resting cells (Fig. 6) could be a rather good candidate for PDD. The strong photodestruction of stimulated cells stained by Mero T* (Fig. 8(b)) suggests that this dye would be the best sensitizer, from the group of investigated Meros, for the application in PDT because it shows: (i) low yield of fluorescence in the cells, (ii) relative high yield of triplet state generation, (iii) high stability against photobleaching and (iv) high phototoxicity with respect to stimulated cells. It is interesting to note, that on basis of the present and earlier [3] results, it seems that the OCH_3 group

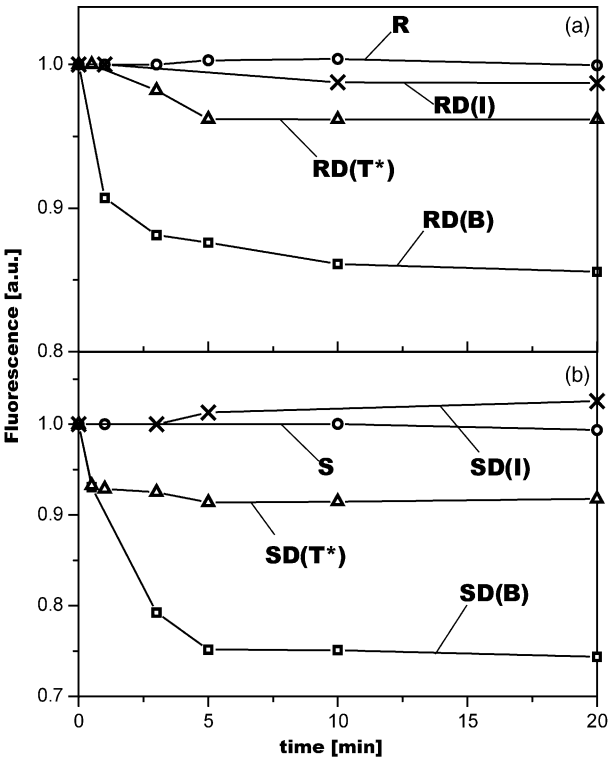


Fig. 9. Intensities at main fluorescence band of dye emission (at 500 nm) as a function of illumination time: (a) resting and (b) stimulated cells. Wavelengths of fluorescence excitation 410 nm (normalized for intensity at time 0 min). Description of curves as in Fig. 3.

in position R_2 in the Mero chain has strong influence on the Mero T* behavior in stimulated lymphocytes.

4. Conclusions

The above discussed results have allowed us to draw the following conclusions:

(i) different dyes, even chemically very similar, in human cells exhibit different spectral properties, such as yields of triplet states generation and ability to trigger the photodynamic reaction;
(ii) properties of dyes in simple model systems and in the resting and stimulated cells can be different, but some conclusion concerning possibility of given dye application in PDT or PDD can be drawn;
(iii) to be able to compare the properties of various sensitizes, they should be studied in the cells obtained from the same donor;
(iv) the dyes exhibiting in the cells, not only efficient triplet states generation, but also high photostability are promising sensitizers for the application in PDT, therefore on the basis of the optical spectroscopy and LIOAS result analysis it is possible to select from group of dyes the sensitizers suitable for medical purposes.

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