ON THE DEPENDENCE OF FLUORESCENCE INTENSITY ON THE CONCENTRATION OF PHOTOSENSITIZER SOLUTIONS

Meerovich G.A.[1,2], Romanishkin I.D.[1,2], Akhlyustina E.V.[2], Loschenov V.B.[1,2]
[1]Prokhorov General Physics Institute of Russian Academy of Sciences, Moscow, Russia
[2]National Research Nuclear University MEPhI (Moscow Engineering Physics Institute), Moscow, Russia

Abstract

When studying the optical properties of photosensitizers, it is assumed that their fluorescence intensity depends linearly on concentration. However, there are many factors that need to be taken into account. At low photosensitizer concentrations, a part of the excitation radiation energy is lost beyond the volume of the excited solution, and due to local or one-directional registration, a large part of isotropically emitted fluorescence radiation is also not registered. At higher concentrations, the loss of fluorescence light increases due to its partial re-absorption by the photosensitizer molecules and subsequent isotropic re-emission with quantum yield much lower than 1, and further increase of concentration leads to partial aggregation of PS, and to the following decrease of effective fluorescence. At high absorption, fluorescence is excited only in a limited volume close to the excitation radiation source, leading to higher significance of light registration geometry. This should be taken into account in fluorescence diagnostics and navigation using this characteristic.

Keywords: photosensitizer, concentration, fluorescence, mathematical modeling.

Contacts: Romanishkin I.D., e-mail: igor.romanishkin@nsc.gpi.ru

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О ЗАВИСИМОСТИ ИНТЕНСИВНОСТИ ФЛУОРЕСЦЕНЦИИ ОТ КОНЦЕНТРАЦИИ РАСТВОРОВ ФОТОСЕНСИБИЛИЗАТОРОВ

Г.А. Меерович[1,2], И.Д. Романишкин[1,2], Е.В. Ахлюстина[2], В.Б. Лощенов[1,2]
[1]Институт общей физики им. А. М. Прохорова Российской академии наук, Москва, Россия
[2]Национальный исследовательский ядерный университет «МИФИ», Москва, Россия

Резюме

При исследовании оптических свойств фотосенсибилизаторов принято считать, что интенсивность их флуоресценции линейно зависит от концентрации. Однако, есть много факторов, которые необходимо учитывать. При низких концентрациях фотосенсибилизатора часть энергии возбуждающего излучения, выходящая за пределы объема возбуждаемого раствора, теряется, а из-за локальной или «односторонней» регистрации часть изотропно распространяющегося излучения флуоресценции, также не регистрируется. При более высоких концентрациях потери флуоресцентного света увеличиваются за счет перепоглощения его части молекулами фотосенсибилизатора и последующего изотропного переизлучения с квантовым выходом значительно ниже 1, а дальнейшее увеличение концентрации приводит к частичной агрегации ФС, и к следующему из этого снижению эффективной флуоресценции. При высоком поглощении, флуоресценция возбуждается только в ограниченном объеме вблизи источника возбуждающего излучения, из-за чего большое значение начинают иметь геометрические особенности регистрации света. Это необходимо учитывать при флуоресцентной диагностике и навигации с использованием данной характеристики.

Ключевые слова: фотосенсибилизатор, концентрация, флуоресценция, математическое моделирование

Контакты: Романишкин И.Д. e-mail: igor.romanishkin@nsc.gpi.ru

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Spectral-fluorescent methods for studying photosensitizers (PS) and sensitized biological tissues using local, 2D and 3D methods of fluorescence intensity registration are widely used for fluorescent diagnostics to determine the topology and boundaries of different pathological foci, fluorescent navigation, assessment of the kinetics and level of PS accumulation, what is important for optimizing of photodynamic therapy (PDT) [1–5]. The measurement of integral fluorescence intensity values at different time intervals after administration is used as a pharmacokinetic curve of PS, and the ratio of the values of this parameter in different organs and tissues is used for estimation of the selectivity of PS accumulation [6]. Typically, in such studies, excitation and registration of fluorescence are performed from one side of the examined sample (including a cuvette with a photosensitizer solution) using local (light-guide) or matrix photodetectors equipped with a focusing optics [7]. Fluorescence studies (spectral density distribution in the fluorescence and absorption bands of the working transition of the PS responsible for the photodynamic effect, the shape and features of these spectral characteristics, fluorescence lifetime and spatial distribution of all parameters in biological tissue, and also the integral fluorescence intensity are carried out, as a rule, in a wide range of PS concentrations [2]. However, most of these works do not take into account reabsorption in PS solutions with a significant overlap of absorption and fluorescence spectra in the working band [8], aggregation of PS molecules in solutions, as well as a number of geometric factors associated with the excitation of fluorescence in a volume of a PS solution and its registration at specific dimensions of this volume, that can significantly affect, for example, the dependence of the integral fluorescence intensity on the PS concentration.

The purpose of this work is to evaluate this dependence, taking into account these phenomena, which is important for assessing their influence on the integral fluorescence intensity. For such an assessment, we consider the simplest model (using photophysical characteristics of a tetracationic derivative of synthetic bacteriochlorin for quantitative assessments [9]) with the following assumptions:

- excitation is carried out in a shorter wavelength spectral range, far enough from the fluorescence band (in case of synthetic bacteriochlorin derivatives—in the Q2 band at 532 nm) to exclude the effect of scattered excitation light on the registered fluorescence spectra;
- the scattering in the sensitized liquid is negligible;
- PS is not aggregated;
- the distribution (spectral density) of intensity in the absorption and fluorescence bands is spectrally homogeneous within each of the bands

As a simplified calculation model, consider the scheme of registration of PS fluorescence in an optical cell with length *L* and a cell wall thickness of *l* (Fig. 2). Optical fiber for light irradiation and optical fiber for receiving fluorescence with a diameter D are in contact with this wall (this is a simplified model for studying the fluorescent properties of PS using a fiber-optic spectrometer[1]). The fluorescence of the solution is excited through a transparent wall by a parallel light flux with a power density $P_{0.}$

$$I = \frac{\varepsilon_B D^2 P_0 c}{16}\Gamma\int_l^{l+L}\frac{2.3\times\exp\left[-\varepsilon_2\left(x-l\right)c-\varepsilon_0 c\left(1-\gamma\right)\left(x-l\right)\right]}{x^2}dx, \quad (1)$$

where ε_B – PS extinction at a wavelength 532 nm, ε_0 – PS extinction in the spectral maximum of the absorption band of working transition, Γ – quantum yield of photoluminescence in the fluorescent band of working transition upon excitation at a wavelength 532 nm, γ – quantum yield of photoluminescence in fluorescent band upon excitation of absorption band of working

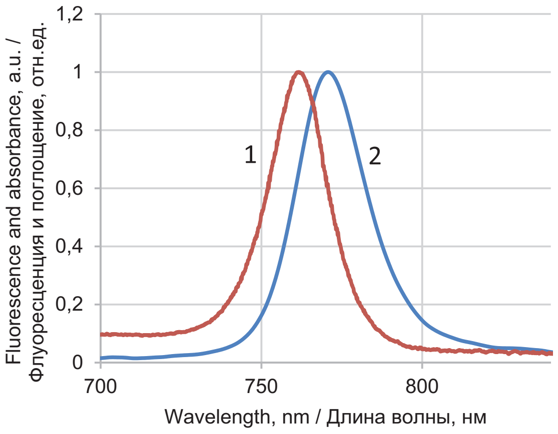

Рис. 1. Спектральные кривые поглощения (1) и флуоресценции (2) тетракатионного производного бактериохлорина при концентрации 5 мкМ.
Fig. 1. The spectral contours of absorption (1) and fluorescence (2) bands oftetracationic bacteriochlorin derivative at 5 µM concentration.

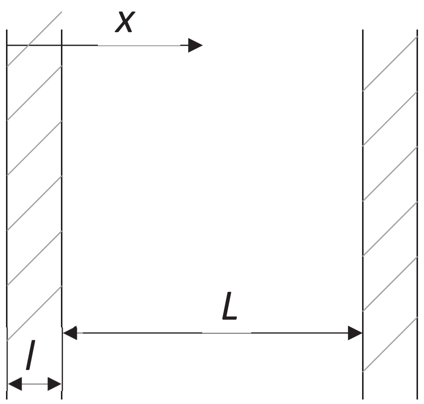

Рис. 2. Схема упрощённой модели.
Fig. 2. Scheme of the simple calculation model.
We take into account that the fluorescence emission from each point is distributed uniformly in all directions.

transition, Δ – "overlapping factor" of the spectral contours of fluorescence and absorption.

$$I = Bc\int_{l}^{l+L} \frac{2.3\times\exp\left[-(x-l)c\left(\varepsilon_2+\varepsilon_0\Delta(1-\gamma)\right)\right]}{x^2}dx$$

$$B = \frac{\varepsilon_2 D^2 P_0}{16}\Gamma,$$

$$a = -b = c\left[\varepsilon_2+\varepsilon_0\Delta(1-\gamma)\right]$$

$$\varepsilon_{\pounds=}\varepsilon_2+\varepsilon_0\Delta(1-\gamma)$$

$$b = -\varepsilon_\Sigma c$$

$$I = Bc\int_{l}^{l+L} \frac{\exp\left(-\varepsilon_\Sigma(x-l)c\right)}{x^2}dx$$

$$I = Bc\int_{l}^{l+L} \frac{e^{\varepsilon_\pounds cl}e^{-\varepsilon_\Sigma cx}}{x^2}dx$$

$$I = Bce^{-bl}\int_{l}^{l+L} \frac{e^{bx}}{x^2}dx$$

$$\int\frac{e^{bx}}{x^2}dx = -\frac{e^{bx}}{x}+b\ Ei(bx)\quad \int\frac{e^{bx}}{x^2}dx = -\frac{e^{bx}}{x}+b\ Ei(bx)$$

where $Ei(x)=\int\limits_{-\infty}^{x}\frac{e^t}{t}dt$ – exponential integral. Thus, the dependence of the integral fluorescence intensity the concentration of the photosensitizer for different optical cell lengths can be represented by the formula:

$$I = Bce^{-bl}\left(b\ Ei\left(b(l+L)\right)-b\ Ei(bl)-\frac{e^{b(l+L)}}{l+L}+\frac{e^{bl}}{l}\right). \tag{2}$$

The "overlapping factor" of the working absorption and fluorescence bands, according to [8], is about 0.4 for a lot of PS. As for the quantum yield of photoluminescence upon its excitation in the working band, its value for an effective PS should be significantly lower than the quantum yield of ROS generation (for example, for phthalocyanines, which have intense fluorescence, according to [10], the quantum yield of ROS generation is 0.60–0.65, and the quantum yield of their fluorescence is 0.03–0.11).

An analysis of ratio (2) shows that in the ranges of PS concentrations of 1–100 μM and cell length (sensitized layer thicknesses) of 1–10 mm, which are relevant for ongoing studies, the dependence of the integrated fluorescence intensity recorded from the excitation side on the PS concentration is sublinear. At low concentrations of PS, a part of the energy of the exciting light, which passes through the absorbing solution, is lost, and, due to local or "one-sided" registration, a part of the fluorescent light, which emits in all directions, is also not registered. Also (especially at high concentrations) the losses of fluorescent light are significantly higher due to the partial reabsorption by PS molecules and subsequent reemission with a quantum yield significantly less than 1 [11].

However, the results of calculations using the above formulas exceed the data obtained experimentally, especially at high concentration, due to a number of simplifying assumptions adopted for making these estimates.

For a more correct assessment of the dependence of the integral fluorescence intensity on PS concentration and its comparison with the experimentally obtained dependence, we will carry out estimates in a modified model with assumptions closer to the experimental conditions. We utilize the real shape of the absorption and fluorescence spectral contours from the experimental data obtained at low concentrations, when PS in solution is not aggregated. We also consider that the delivery of laser light and the reception of a fluorescent signal are carried out via two parallel optical fibers.

An offset of the receiving fiber axis reduces the value of the solid angle at which fluorescence must be emitted to be registered through the receiving fiber, and complicates the calculation form. In the simple case of coaxial transmitter and receiver, the solid angle is defined as

$$\begin{gathered}\Omega = 2\pi(1-\cos\theta)\\ \theta = \operatorname{atan}(r/d)\end{gathered}, \tag{3}$$

where θ is the cone angle at the fluorescent light emission point with the base representing the optical fiber core; r – the radius of the base of the cone, d is the height of the cone. Equations (3) is reduced to

$$\Omega = 2\pi\left(1-\frac{1}{\sqrt{\left(\frac{r}{d}\right)^2+1}}\right). \tag{4}$$

However, in the general case, the calculation of the solid angle is more complicated [12]. It is also necessary to take into account the angle at which the optical fiber is able to receive light, which is determined by its numerical aperture.

Laser light is delivered through an optical fiber directed along the x axis and the center of theend diskof which is located at the point of origin, and its radius is r_{fib}. The laser radiation emerging from the optical fiber propagates at an angle θ not exceeding the critical angle θ_{crit} determined from the numerical aperture of the optical fiber (NA = 0.22). It was determined that in our experiments the sine of the divergence angle of the exciting laser light from the optical fiber was $sin(\theta) = 0.062$, and this value was used in the calculations.

The following model for calculating the fluorescence signal obtained in reverse geometry with offset fiber optic source and receiver has been implemented:

Laser radiation is represented by a set of beams emerging from the point $O = (x_0, 0, 0)$, where $x_0 = -r_{fib}\,ctg\,(\theta)$, at an angle to the x-axis, less than θ. For each ray r_0, we simulate the absorbed light intensity along its path, calculate the effective intensity of the excited fluorescence at different points in the volume, and calculate the fluorescent signal transmitted through the absorbing photosensitizer medium. The resulting intensity of the fluorescent signal from the beam was calculated using the formula

$$F = \int_{\vec{r}_1}^{\vec{r}_2} A_{exc}\left(\vec{r}\right)\cdot f_{eff}\left(\vec{r}\right)\cdot T_{fluo}\left(\vec{r}\right)d\vec{r}.$$

Here $\vec{r}_1$ and $\vec{r}_2$ are the radius vectors of the intersection points of the beam r_0 with thefrontandrear innerwalls of the optical cell.

The absorption of exciting light A_{exc} is calculated using the Bouguer-Lambert-Beer law in differential form:

$$A_{exc}\left(\vec{r}\right) = -\frac{dT_{exc}\left(\vec{r}\right)}{d\left|\vec{r}\right|}$$

$$T_{exc}\left(\vec{r}\right) = e^{-\varepsilon c\left|\vec{r}-\vec{r}_1\right|}$$

where ε is the value of the molar absorption of the PS at the wavelength of the exciting light, c is the molar concentration of the PS.

The value of the effective emitted fluorescent signal is determined by the formula

$$f_{eff}\left(\vec{r}\right) = \frac{\Omega\left(\vec{r}_f\right)}{4\pi}\Phi I_f\left(\lambda\right). \qquad (5)$$

Here Φ is the fluorescence quantum yield, $I_f(\lambda)$ is the value of the fluorescence spectrum curveof the photosensitizer, normalized on maximum, measured at a low PS concentration (when aggregation can be neglected), at wavelength λ, $\vec{r}_f$ is the vector between the fluorescence emission point and the center of the receiving fiber face. $\Omega\left(\vec{r}_f\right)$ determines the value of the solid angle within which all emitted fluorescence enters the receiving optical fiber.

The factor $T_{fluo}\left(\vec{r}\right)$ takes into account the absorption of fluorescent light on the way from point $\vec{r}$ to the receiving fiber.

$$T_{fluo}\left(\vec{r}\right) = e^{-2.3\times\varepsilon(\lambda)\, c\left|\vec{r}_f\right|\times\left(1-\frac{l}{\left|\vec{r}_f\right|\cos\left(\theta_f\right)}\right)} \qquad (6)$$

Here $\varepsilon(\lambda) = \varepsilon A(\lambda)$ is the value of the molar absorption of the photosensitizer at the fluorescence wavelength, θ_f is the angle between the x-axis and the vector $\vec{r}_f$ (Fig. 3).

The dependence of the integral fluorescence intensity of the PS (obtained numerically using the proposed model with the parameters of tetra-cationic derivative of bacteriochlorin as an example) agrees better with the experimental data (at least in the concentration range under 30 μM, wch is important from a practical point of view) than the dependence obtained in the simplified model (Fig. 4).

This calculation was also carried out in the approximation of a non-aggregated solution corresponding to the Bouguer-Lambert-Beer law. The observed difference between the results of calculations and experiments in the range of concentrations above 30 μM seems to be due to a number of phenomena associated with aggregation. Although the dependence of the PS solution absorption is linear with respect to its concentration, the fluorescence kinetics of its solutions upon excitation by a picosecond laser had features that indicate partial aggregation of solutions with higher concentrations. The values of the fluorescent lifetime of PS molecule aggregates are much shorter than those of non-aggregated molecules [13,14]. If the fluorescence decay kinetics of

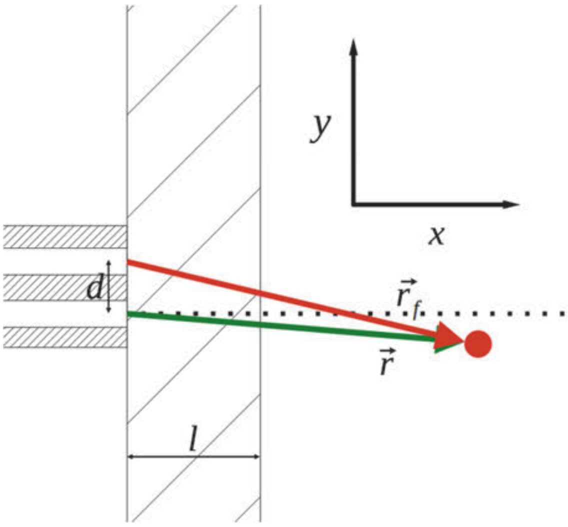

Рис. 3. Диаграмма расширенной модели для расчёта интенсивности флуоресценции раствора ФС в оптической кювете, учитывающая смещение принимающего оптического волокна.

Fig. 3. Diagram of a modified model for calculating the fluorescence intensity of a photosensitizer solution in optical cell, taking into account the shift in the position of the receiving fiber.

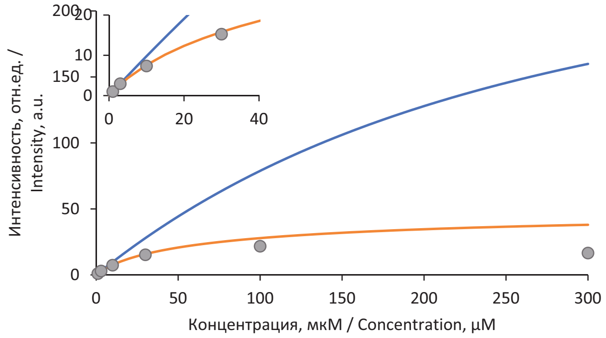

Рис. 4. Зависимость интенсивности интегральной флуоресценции тетракатионного производного бактериохлорина, полученная расчётом с помощью упрощённой модели (синий) и расширенной модели (оранжевый), а также значения (серый), полученные в эксперименте с оптической кюветой длиной 10 мм. Значения нормированы на интенсивность флуоресценции при концентрации 1 мкМ.

Fig. 4. The dependence of the integral fluorescence intensity of tetracationic bacteriochlorin derivative, obtained by calculation using the proposed simplified (blue) and modified (orange) models, and the values (gray markers) obtained in the experiment with the optical cell length of 10 mm. Numerical values are normalized to the fluorescence intensity at a concentration of 1 μM.

a partially aggregated PS is described by the sum of two exponential components with different weight coefficients [13]:

$$I(t) = A_1 \exp\left(-\frac{t}{\tau_1}\right) + A_2 \exp\left(-\frac{t}{\tau_2}\right), \qquad (7)$$

then the ratio between the weight coefficients $A_1\tau_1$ for non-aggregated molecules and $A_2\tau_2$ for aggregates can characterize the degree of PS aggregation. Studies using the approach [13] showed that in solutions of the studied PS with a concentration in the range of interest, there are two fluorescent molecular groups: a monomeric PS with a relatively long lifetime (in the range of 1.5–3 ns), and the second group with a significantly shorter time lifetime (< 1 ns), the fluorescence of which is associated with self-aggregated complexes of PS molecules. The ratio of monomeric PS, estimated from the number of photons with such a lifetime among the total number of fluorescence photons, was more than 74% in an aqueous solution, that is, the degree of aggregation is low and does not noticeably affect the efficiency of this PS. but it can lead to a decrease in fluorescence intensity, which and is observed when the experimental data on the integral intensity of the solution deviate from the calculated values. Moreover, at very high concentrations (>100 μM), which go beyond the values observed in biotissues during PDT, the dependence of the integrated fluorescence intensity due to aggregation can saturate or even start decreasing.

The assumption about the partial aggregation of this PS at high concentrations, which affects its fluorescent characteristics, is additionally confirmed by the fact that when studying its fluorescence kinetics of its solution in blood plasma, the fraction of photons emitted by the monomeric component of the PS increases to 85%, and the second component, presumably associated with self-aggregated complexes of PS molecules, correspondingly decreases compared to a solution in water (Table 1). This is typical of tetrapyrroles due to the disaggregating effect of plasma proteins [15,16]but is only now becoming widely used. Originally developed as cancer therapy, some of its most successful applications are for non-malignant disease. The majority of mechanistic research into PDT, however, is still directed towards anti-cancer applications. In the final part of series of three reviews, we will cover the possible reasons for the well-known tumor localizing properties of photosensitizers (PS. These results confirm the assumption that the deviation of the experimentally obtained dependence of the integral fluorescence intensity from the theoretical (calculated) curve may be associated with PS aggregation at an increased concentration of its solution.

Таблица 1.
Время разрешенные компоненты флуоресценции тетракатионного производного бактериохлорина в воде и плазме крови

Table 1.
Lifetime components of tetracationic bacteriochlorin derivative fluorescence in water and in blood plasma

Раствор Solution	$A_1\tau_1$, %	τ_1, ns	$A_2\tau_2$, %	τ_2, ns
Вода Water	74	1.9–2.9	26	<1
Плазма крови Blood plasma	85	2.4	15	<1

Conclusion

At low concentrations of PS a part of the energy of the exciting light, which goes beyond the volume of the excited solution, is lost, and due to local or "one-sided" registration, a part of the fluorescence light, which propagates in all directions, is also not registered. At higher concentrations, the losses of fluorescence light significantly increase due to the reabsorption of part of fluorescent light by PS molecules and subsequent re-emission with a quantum yield significantly below 1. With a further increase in concentration, partial aggregation of PS also begins to reduce the efficiency of fluorescence. At very high concentrations, the influence of aggregation begins to dominate, and therefore the dependence of the integrated fluorescence intensity on concentration saturates and even starts to decrease. Thus, the dependence of the integral fluorescence intensity on PS concentration is sublinear, and this should be taken into account in fluorescence diagnostics and navigation using this parameter.

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