Оптика и спектроскопия

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ЗАКОНОМЕРНОСТИ КОМПЛЕКСООБРАЗОВАНИЯ ИНДОТРИКАРБОЦИАНИНОВЫХ КРАСИТЕЛЕЙ С БЕЛКАМИ СЫВОРОТКИ КРОВИ ЧЕЛОВЕКА

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Изучено взаимодействие индотрикарбоцианиновых красителей и белков сыворотки крови человека. Определены спектрально-люминесцентные свойства красителей в бутаноле, натрий-калиевом фосфатном буфере Дюльбекко (0,14 моль/л) с pH 7,4 и растворах сыворотки крови человека. Показано, что спектральные свойства красителей в бутаноле значительно отличаются от спектральных свойств красителей в двух других растворах. Отмечено, что бутанол эффективно экстрагирует молекулы исследованных красителей, которые не образуют ковалентно связанные комплексы с компонентами сыворотки крови. Путем анализа продуктов экстракции определена доля ковалентно связанных комплексов красителей с белковыми молекулами. Установлено, что индотрикарбоцианиновые красители с хлорзамещенным ортофениленовым мостиком в цепи сопряжения переходят в бутанол частично и степень экстракции зависит от соотношения концентраций красителя и белков. Так, при концентрации

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10 мкмоль/л в 5 % сыворотке крови человека степень экстракции данных красителей составляет ~50–60 %. Соответственно, доля молекул красителей в прочных ковалентно связанных комплексах с компонентами сыворотки крови равняется ~40–50 %. Однако краситель со свободной полиметиновой цепью экстрагируется практически полностью (степень экстракции 91,4 %). Индотрикарбоцианиновые красители можно эффективно использовать в качестве фотосенсибилизаторов при проведении фотодинамической терапии.

Ключевые слова: индотрикарбоцианиновые красители; комплексообразование; флуоресцентная спектроскопия; белки сыворотки крови; экстракция; фотосенсибилизаторы; фотодинамическая терапия.

REGULARITIES OF COMPLEXATION OF INDOTRICARBOCYANINE DYES WITH HUMAN BLOOD SERUM PROTEINS

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The interaction of indotricarbocyanine dyes with human blood serum proteins was studied. The spectral and fluorescent properties of dyes in butanol, Dulbecco's sodium and potassium phosphate buffer (0.14 mol/L) with pH 7.4, and human blood serum solutions were determined. It was shown that the spectral properties of dyes in butanol differ significantly from the spectral properties in solutions of Dulbecco's sodium and potassium phosphate buffer and human blood serum, and that butanol effectively extracts the molecules of the studied dyes that do not form covalently bound complexes with blood serum components. By analysing the extraction products, the proportion of covalent complexes of dyes with protein molecules was determined. It has been established that indotricarbocyanine dyes with a chlorine-substituted orthophenylene bridge in the conjugation chain are partially passes into butanol, the degree of extraction depends on the ratio of dye and protein concentrations. Thus, at a concentration of 10 μmol/L in 5 % human serum, the degree of extraction of these dyes is ~50–60 % respectively, the proportion of dye molecules in strong covalently bound complexes with blood serum components is ~40–50 %. On the contrary, the dye with a free polymethine chain is extracted almost completely (91.4 % extraction rate). The indotricarbocyanine dyes are promising for use as a photosensitisers for photodynamic therapy.

Keywords: indotricarbocyanine dyes; complexation; fluorescence spectroscopy; blood serum proteins; extraction; photosensitisers; photodynamic therapy.

Introduction

Photodynamic therapy is one of the most minimally invasive methods of treating malignant neoplasms of various localisations [1–3]. Further development of the method continues by improving the parameters of the used photosensitisers (PS). According to modern concepts, an ideal PS should exhibit a pronounced photodynamic effect when activated by radiation in the transparency window of biological tissues, have a high selectivity of accumulation in target tissues, biocompatibility, an effective pharmacokinetic profile, be eliminated from the body relatively quickly, and fluoresce with sufficient efficiency for diagnostic purposes [3; 4]. In recent years, there has been an increase in research interest in polymethine dyes as promising photosensitisers for photodynamic therapy of malignant neoplasms [5].

According to the results of complex studies of the photophysical properties of a number of indotricarbocyanine dyes in model media and tumors of experimental animals *in vivo*, a dye was chosen that largely satisfies the specified requirements [6]. It is based on an indotricarbocyanine dye with a chlorine-substituted orthophenylene bridge in the polymethine chain. Polyethylene glycols with a molecular weight of 300 g/mol (PEG300), covalently linked at the end groups, provided the new PS with high water solubility and biocompatibility.

The effectiveness of PS largely depends on the selectivity of its accumulation in tumor tissues. Therefore, studies aimed at developing new methods of targeted drug delivery are relevant. One way to solve this problem is to use complexes of PS molecules with carriers, which provide efficient accumulation in tumor cells. In this regard, blood plasma components, proteins and lipoproteins, have special attention as endogenous carriers [7; 8]. On the contrary, blood serum is an available model medium for studying the photophysical properties of a PS under the conditions in which it is found *in vivo*.

In [9], using the methods of fluorescence spectroscopy and size-exclusion chromatography, the formation of complexes of indotricarbocyanine dyes with blood serum proteins was shown. The formation of covalently bound complexes with albumin and high-density lipoproteins of indotricarbocyanine dyes with a chlorine-substituted orthophenylene bridge in the polymethine chain was established using gel electrophoresis [10].

In this work, extraction methods were used to quantify the proportion of indotricarbocyanine dye molecules that formed strong covalently bound complexes with the components of human blood serum (HBS).

Materials and methods of research

The objects of study were the symmetrical indotricarbocyanine dye PD1 (fig. 1), developed in the laboratory of spectroscopy of A. N. Sevchenko Institute of Applied Physical Problems of Belarusian State University, which is promising in many respects for use as a photosensitiser for photodynamic therapy [6], as well as two dyes with similar structure – PD2 and PD3. Compared to PD1, PD2 lacks polyethylene glycols at the end groups, and PD3 does not have a chlorine-substituted orthophenylene bridge in the polymethine chain.

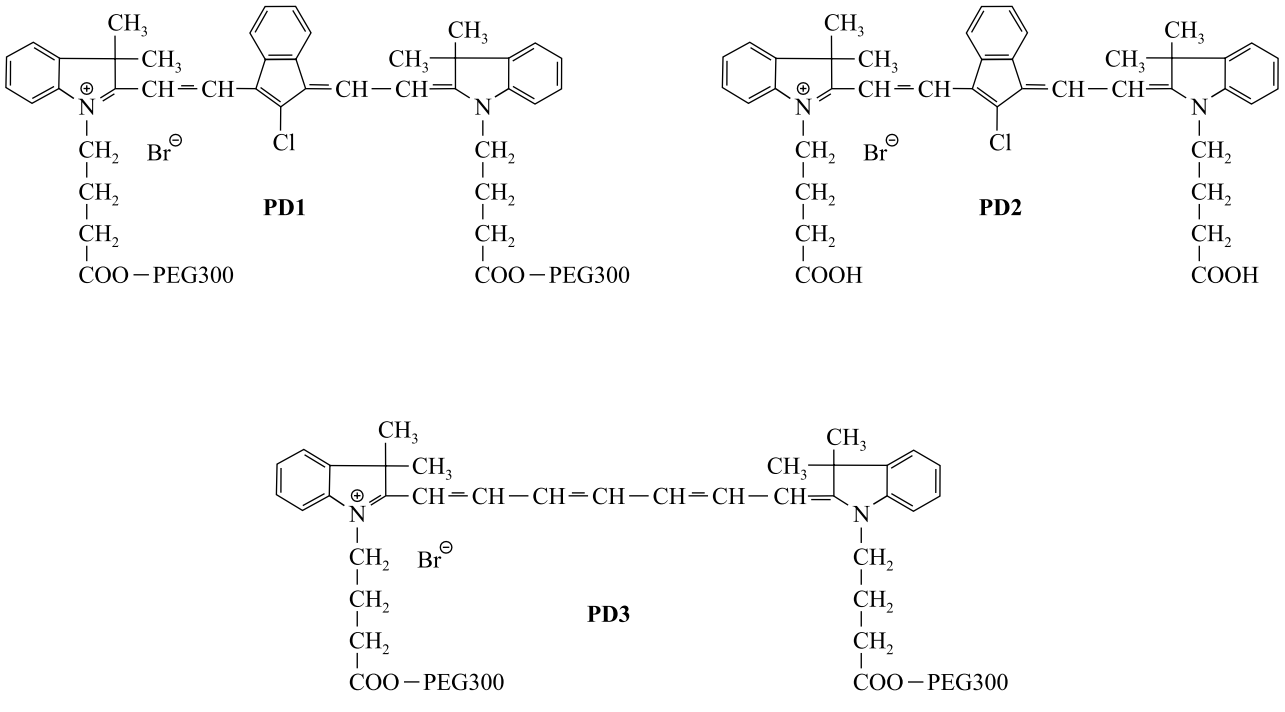

Fig. 1. Structure of the studied indotricarbocyanine dyes

Studies of the interaction of dyes with blood serum proteins were carried out in a 5 % solution of HBS in Dulbecco's sodium and potassium phosphate buffer (0.14 mol/L) with pH 7.4 (PBS), which corresponds to an albumin concentration of ~30 μmol/L. The concentration of dyes in the studied samples was 10 μmol/L (the ratio of dye to protein was 1 : 3). With this ratio of dye and protein concentrations, the analysis of spectral properties does not reveal dye molecules PD1 and PD2 localised in the aqueous environment. Dye-stained serum samples were prepared using stock solutions in PBS and incubated at 37 °C for 120 min. Dye PD2 has low solubility in water; therefore, the stock solution for it was prepared with 5 % ethanol content.

Electronic absorption spectra were recorded using a Solar PV 1251 spectrophotometer (Belarus). The fluorescent properties of the dyes were recorded using a Fluorolog spectrofluorometer (*HORIBA*, USA). The solvents used in the work were preliminarily purified by standard methods [11].

Results and discussion

In accordance with the methods of extraction [12; 13], a procedure for the extraction of the studied dyes from HBS solutions has been developed. The extractant should have a high extraction ability; in the case of biological samples, the organic solvent must good extract the test substance from the aqueous phase. Among the most common solvents (ethanol, methanol, acetonitrile, dimethyl sulfoxide, etc.), butanol was chosen due it efficiently extracts dyes PD1, PD2 and PD3 from water and PBS (table 1). The undoubted advantage of butanol is also that it is immiscible with water.

The photophysical properties of dyes in PBS, butanol, and HBS solution were determined (table 2, fig. 2). The spectral properties of dyes in PBS are significantly affected by the aggregation of their molecules in an aqueous solution. Dye PD2, which has no PEG in its structure, is hydrophobic and forms H- and J-type aggregates in water [14]. Dye PD1 is hydrophilic; its solutions in PBS are an equilibrium mixture of monomers and H-type dimers [15]. At the same concentration of dyes, their aggregation increases in the following sequence $3 \rightarrow 1 \rightarrow 2$, which manifests itself in an increase in the half-width of the long-wavelength absorption band. Wherein the fluorescence of the studied dyes in PBS is single-component: the shape of the fluorescence spectrum does not depend on the excitation wavelength when scanning within the long-wavelength absorption band, the decay kinetics is approximated by one exponent. The fluorescence spectrum of dyes upon excitation within the main absorption band is approximately mirror image of the absorption spectrum of their monomers.

Table 1

Degree of extraction of dyes from distilled water and 5 % HBS solution

Dye	Distilled water (C_{dye} = 3.5 μmol/L), %	Distilled water (C_{dye} = 35 μmol/L), %	5 % HBS solution (C_{dye} = 10 μmol/L, 2 h incubation at 37 °C), %
PD1	99.4	94.7	49.7
PD2	99.7	96.4	60.1
PD3	99.3	95.0	91.4

Table 2

Photophysical properties of dyes (10 μmol/L) in PBS, butanol and 5 % HBS solution

Solution		λ_{abs}^{max}, nm	$\Delta\lambda_{abs}^{max}$, nm	λ_{f}^{max}, nm	$\Delta\lambda_{f}^{max}$, nm	$\varepsilon \cdot 10^5$, L/mol · cm^{-1}	τ (20 °C), ns	*P* (20 °C)
PBS	PD1	707	134	738.0	64	–	0.4 ± 0.1	0.28 ± 0.02
	PD2	706	170	737.0	77	–	0.3 ± 0.1	0.31 ± 0.02
	PD3	746	78	772.0	49	–	0.3 ± 0.1	0.30 ± 0.02
HBS	PD1	729	64	757.0	43	1.88	1.4 ± 0.1	0.42 ± 0.02
	PD2	731	65	756.5	46	1.96	1.4 ± 0.1	0.39 ± 0.02
	PD3	748	68	776.0	55	1.89	0.4 ± 0.1	0.40 ± 0.02
Butanol	PD1	728	47	753.0	49	2.27	1.1 ± 0.1	0.28 ± 0.02
	PD2	732	53	756.0	53	2.37	1.0 ± 0.1	0.30 ± 0.02
	PD3	755	58	785.0	51	2.16	1.0 ± 0.1	0.30 ± 0.02

Molecules of polymethine dyes in polar organic solvents are in the form of monomers [16; 17], the predominant ionic form of the studied dyes in such media is free ions [18]. This is confirmed by the stability of the absorption, fluorescence excitation and emission spectra of the studied dyes in butanol over time, with changes in concentration and heating. Compared with the localisation of molecules of the dyes in an aqueous environment, in butanol there is a bathochromic shift of the long-wavelength absorption bands and fluorescence spectra, and an increase in the duration of fluorescence decay. It is important to note that the spectral properties of the molecules of the studied dyes in this solvent differ significantly from those in PBS and HBS solution; therefore, it is possible to determine with certainty in which medium the studied compounds are located during the extraction process.

The spectral properties of PD1 and PD2 in HBS solutions differ strongly from those in PBS (see table 1): a shift of absorption and fluorescence maxima to the long wavelength region is observed, absorption in the band of aggregates decreases, the fluorescence lifetime increases (from 0.3–0.4 to 1.4 ns), and the fluorescence polarisation increases (from 0.28–0.31 to 0.39–0.42). The change in the spectral properties of the dye molecules in the presence of proteins indicates the formation of a complex with them [19]. A bathochromic shift in the position of the maximum of the absorption and fluorescence spectra for these dyes is usually observed upon passing from polar solvents to low-polarity ones [6; 20; 21], which is achieved when localised near protein molecules.

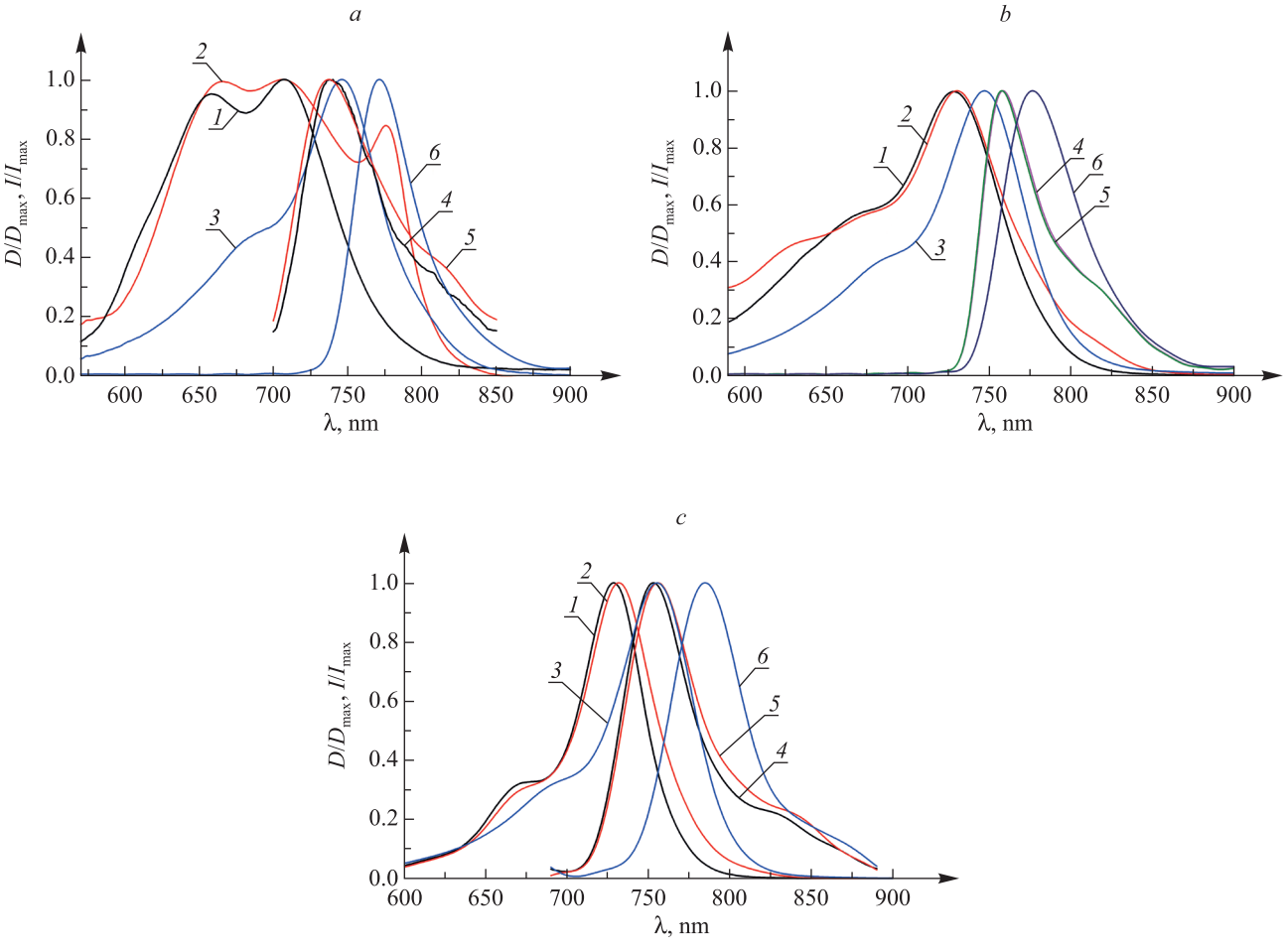

Fig. 2. Absorption spectra (*1–3*), and fluorescence spectra upon excitation at 684 nm (*4–6*) of indotricarbocyanine dyes (μmol/L) in PBS (*a*), 5 % HBS solution (*b*) and butanol (*c*): *1*, *4* – PD1; *2*, *5* – PD2; *3*, *6* – PD3

No significant effect of blood serum components on the spectral and fluorescent properties of PD3, which lacks a chlorine-substituted orthophenylene bridge in the polymethine conjugation chain, was found. A significant difference was recorded only in the fluorescence polarisation, the value of which in HBS solutions increases in the same way as for the dyes PD1 and PD2. This is possible provided that dye PD3 molecules bind to proteins, but the dye chromophore remains localised in the aqueous environment. It is reasonable to assume that the binding of PD3 to proteins occurs with the participation of PEG chains. Polyethylene glycol is an amphiphilic molecule; it has hydrophilic and hydrophobic properties [22]. The literature shows its extremely low ability to interact with proteins [23], the protein – PEG affinity constant for low molecular weight polyethylene glycols (1000–8000 Da) is about 10^1–10^2 L/mol. Within the model of steric exclusion of preferential hydration [24; 25], it was shown that the interaction of various PEGs with proteins is thermodynamically unfavourable. A positive change in enthalpy is associated with an unfavourable interaction of PEG molecules with charges on the protein surface [26], as well as with the breaking of hydrogen bonds of structured water near the PEG molecule and the hydrophobic protein surface [26; 27]. This effect is enhanced with an increase in the molecular weight of polyethylene glycols. The presence of a significant interaction of low molecular weight PEGs with the surface of albumin (especially PEG600) was shown in [27], it was suggested that such an interaction is carried out by van der Waals forces between the hydrophobic surfaces of PEG molecules and bovine serum albumin. Apparently, the weak binding of dye PD3 molecules to proteins is realised through this mechanism.

The extraction was carried out by mixing the investigated dye solution with butanol in a ratio of volume fractions of 1 : 1. The resulting mixture was sonicated for 60 min. The layers were then separated by centrifugation for 15 min (8000 rpm). The content of dyes in the supernatant and precipitate was determined by the spectrophotometric method. The state of the dye molecules was analysed by comparing the spectral and fluorescent properties of the dyes in the supernatant and precipitate with those in the initial samples (PBS and HBS solutions) and in pure butanol. The spectral properties of the dye molecules that passed into the butanol extract match with the

values in pure butanol. The coincidence was established based on the analysis of the shape of the absorption and fluorescence spectra, as well as the decay time and the fluorescence lifetime and polarisation. It can be argued that dyes are in the free state in the butanol extract.

The measurements have shown that under such conditions dyes PD1 and PD2 are extracted only by 50–60 %. Re-extraction from the aqueous precipitate fails to extract the remaining dye. This indicates that their molecules in solution are distributed over several types of complexes with blood serum components, which differ in their bond strength. It was shown in [10] that these dyes are able to form covalent complexes with albumin and high density lipoproteins. With the help of extraction it is possible to determine the proportion of these complexes in solution. Extraction of dyes PD1 and PD2 from solutions with a higher protein concentration leads to a decrease in the degree of extraction. Thus, dye PD1 is not extracted at all from undiluted human blood serum (4 μmol/L, 2 h incubation at 37 °C).

On the contrary, the degree of extraction for dye 3, which lacks an orthophenylene bridge in the conjugation chain, from the HBS solution is close to the value for the dye solution in PBS. Taking into account the error in determining the molar absorption coefficient, we can say that this dye is completely extracted. Consequently, when interacting with blood serum components, it does not form strong complexes with them, which is consistent with the results of a study on gel electrophoresis [10].

Conclusion

Thus, we have developed a method for the extraction of indotricarbocyanine dyes from model media based on blood serum using butanol. Based on the analysis of the spectral and fluorescent properties of the studied dyes in PBS, butanol and HBS solutions, as well as in the precipitate and supernatant during the extraction process, it was shown that butanol effectively extracts the molecules of the studied dyes that do not form covalently bound complexes with blood serum components. This makes it possible to determine the proportion of strong covalent complexes with protein molecules by extraction. It was found that indotricarbocyanine dyes with a chlorine-substituted orthophenylene bridge are extracted only partially, while the dye with a free polymethine chain is extracted completely, which is consistent with the results of gel electrophoresis studies [10].

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