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SPECTRAL-FLUORESCENT RESPONSE OF DIOXABORINE DYES WITH VARYING CHROMOPHORE CHARGE IN THE PRESENCE OF SERUM ALBUMINS

Aim. *The aim of the work is the study of a spectral-fluorescent response of a series of dioxaborine dyes in the presence of serum albumins.* ***Methods.*** *UV-Vis absorption and fluorescence spectroscopy.* ***Results.*** *The fluorescent response of eight polymethine dyes (six of them being dioxaborine-based) in the presence of human, bovine, and equine serum albumins was studied and compared to the response in the presence of DNA and ovalbumin. It was shown that di-anionic dioxaborine* **A74** *and thiobarbituric-based merocyanine* **In4tb** *demonstrated the most promising properties as fluorescent probes for human serum albumin detection, with fluorescence intensity increasing by 62- and 56-fold, respectively. Meanwhile,* **A74** *shows the highest selectivity to HSA among other serum albumins: in the presence of ESA and BSA, the signal increased by only 28-fold and 7.9-fold, respectively. Thus, one can suppose that* **A74** *may be sensitive to slight variations in the globular conformation of serum albumins. Besides, dioxaborine merocyanines* **B33** *and* **B36** *demonstrated nearly 10-fold fluorescence intensity increase in the presence of serum albumins.* ***Conclusions.*** *The most promising dyes* **A74** *and* **In4tb** *can be further studied for various applications that require fluorescent detection or imaging of HSA, and, in the case of* **A74**, *as a probe sensitive to minor changes in the HSA globular structure.*

Keywords: *dioxaborines, polymethine dyes, fluorescent probes, serum albumins.*

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Introduction

Fluorescence quantum yield (FQY) and its dependence on the molecular environment (solvation) are arguably the most important characteristics of fluorescent probes and labels. Among the various factors influencing FQY, internal molecular motions, particularly torsional vibrations and rotations around chromophore bonds that distort planarity, are the most widely recognized cause of non-radiative excited-state decay [1]. This decay pathway also presents the most direct strategy for its mitigation, which is chromophore rigidization. Introducing internal cycles or bridging groups into the chromophore is a typical approach to restrain such motions and thereby increase FQY.

In dioxaborine-based fluorophores, introducing the 1,3,2-dioxaborine ring either into the central part of the π-chromophore or as an electron-acceptor end group increases molecular rigidity and, accordingly, enhances the fluorescence characteristics of the resulting dyes, endowing them with both theoretically interesting and practically appealing properties [2]. Hence, dioxaborines have been shown to be promising in a wide range of applications [2, 3], particularly as molecular rotors for solvent viscosity measurements [4], sensors for amines and carbon nanotubes [5—7], and probes or labels for proteins [8, 9], among others. Far-red and NIR dyes with high molar absorption and FQY values have also been developed [10—12], as well as dyes with promising nonlinear optical properties [13].

Serum albumins are the primary transport proteins in blood. To bind small molecules for transport, serum albumins possess multiple binding sites; in human serum albumin (HSA), the most important are the so-called Sudlow sites I and II [14, 15]. Detecting these proteins in biological fluids is clinically important, as their level can be indicative of various diseases; for example, an elevated level in urine may suggest diabetic nephropathy, while decreased level in blood plasma may be associated with the liver failure, cirrhosis, or chronic hepatitis [16]. Moreover, certain diseases lead to structural changes in the serum albumin molecule, such as glycation (in diabetes) [17, 18], oxidation (in liver disease) [17], allosteric modifications by tumor metabolites (in cancer) [19], or *N*-terminal alterations (in ischemia/reperfusion) [17]. Therefore, detecting not only the presence and concentration of serum albumin, but also changes in its globular structure, could be valuable for the diagnosis of various diseases.

In this study, we investigated the spectral-fluorescent response of a series of dioxaborine dyes in the presence of several serum albumins — human, bovine, and equine. Ovalbumin and DNA were also used to assess the selectivity of this response toward serum albumins. In addition to the six dioxaborine dyes, two related chromophores — a ketoenol (the hydrolyzed form of a dioxaborine) and a thiobarbituric-based merocyanine — were included for comparison.

Materials and Methods

Dyes **B33** [10], **B36** [10], **M25** [11], **A74** [12], **M22** [12], **M232** [20] and **in4tb** [21] (Fig. 1) were synthesized according to the reported procedures. Dye **H28** was prepared by the hydrolysis of **B33** as follows: a mixture of **B33** (0.50 g, 1.51 mmol) and NaOH (0.36 g, 9.06 mmol) in EtOH/H_2O 5 : 1 mixture (20 mL) was refluxed for 15 min. Then the reaction mixture was cooled down, neutralized with AcOH (0.55 g) and concentrated in vacuo. The residue was partitioned between EtOAc (30 mL) and H_2O (20 mL), the organic phase was separated, dried over Na_2SO_4, and concentrated in vacuo. The residue was triturated with Et_2O (30 mL), the mixture was passed through a small pad of silica and evaporated under reduced pressure. The residue was purified by preparative thin layer chromatography on silica gel (EtOAc/*n*-hexane 1 : 1) to give **H28** (0.18 g, 0.64 mmol 42%) as a red solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 15.99 (s, 1H), 7.93 (dd, *J* = 14.3, 12.8 Hz, 1H), 7.22—7.13 (m, 2H), 6.91 (t, *J* = 7.5 Hz, 1H), 6.69 (d, *J* = 7.5 Hz, 1H), 5.65 (d, *J* = 14.3 Hz, 3H), 5.42 (d, *J* = 12.8 Hz, 6H), 5.40 (s, 1H), 3.17 (s, 3H), 2.06 (s, 2H), 1.60 (s,

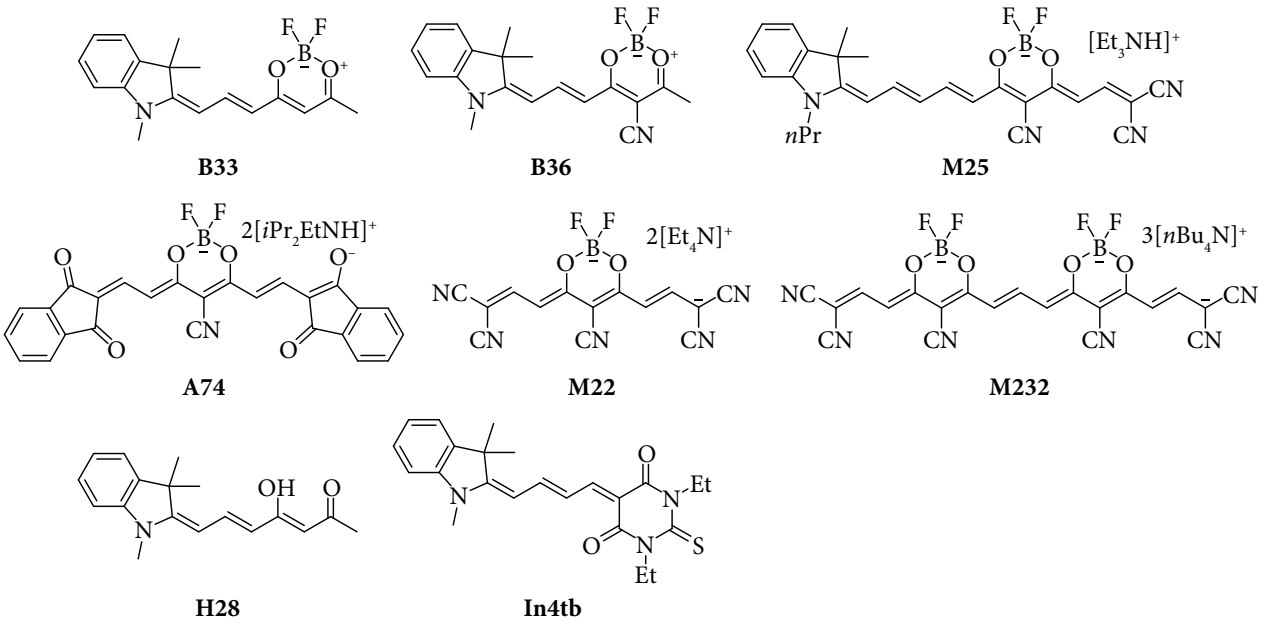

Fig. 1. Structures of the studied dyes

6H) ppm. Elemental analysis: calcd (%) for $C_{18}H_{21}NO_2$: C 76.30, H 7.47, N 4.94; found: C 76.50, H 7.36, N 4.86.

The structures of the compounds were confirmed by ^{1}H NMR spectroscopy and elemental analysis. Dimethyl sulfoxide (DMSO), methanol, and 0.05 M Tris-HCl buffer (pH 8.0) were used as solvents. Double-stranded DNA (dsDNA) from salmon testes; bovine (BSA), human (HSA), and equine (ESA) serum albumins; as well as ovalbumin (OVA), were purchased from Sigma-Aldrich. Stock solutions of the dyes were prepared in DMSO at a concentration of 2 mM. The stock solution of DNA was prepared in 50 mM Tris-HCl buffer at a concentration of 6 mM base pairs (b.p.). Solutions of HSA, BSA, ESA, and OVA were prepared in 50 mM Tris-HCl buffer at a concentration of 0.2 mg/mL.

Working solutions of free dyes were prepared by diluting the dye stock solution in 50 mM Tris-HCl buffer, methanol, or DMSO. Solutions containing DNA were prepared by adding aliquots of the dye and DNA stock solutions to 50 mM Tris-HCl buffer. Solutions containing HSA, BSA, ESA, or OVA were prepared by adding an aliquot of the dye stock solution to the respective protein solution. Final concentrations in all working solutions were 5 μM for dyes, 60 μM b.p. for DNA, and 0.2 mg/mL for proteins.

Absorption spectra were recorded using a Genesys 20 visible spectrophotometer (Thermo Fisher Scientific, USA). Fluorescence spectra were obtained with a Cary Eclipse fluorescence spectrometer (Varian, Australia). All spectral measurements were performed in 10 × 10 mm quartz cuvettes at room temperature, immediately after preparation of the respective working solutions.

Results and Discussion

Spectral-fluorescent properties of free dyes

The characteristics of the absorption and fluorescence spectra of the studied dyes in organic solvent (DMSO, methanol) and in an aqueous medium (Tris-HCl buffer, pH 8.0) are presented in Table 1.

Due to the substantial structural differences among the dyes, their absorption maxima in

methanol span a wide range, from 456 to 676 nm. At a concentration of 5 μM, their optical densities are relatively high, ranging from 0.34 to 1.75. In DMSO, the overall shape of the absorption bands remains largely unchanged, but the maxima are red-shifted by up to 29 nm. A similar solvatochromic shift of approximately 20 nm for dioxaborine dyes was previously reported in [10], and is typical both for negative solvatochromic chromophores, because of the greater solvation strength of protic methanol toward carbonyl-containing molecules, especially negative-charged ones, and even for less-solvatochromic dyes due to much greater refractive index of DMSO. Notably, some dyes — **B36** in DMSO and **M25** in both solvents — exhibit two distinct absorption maxima.

The fluorescence maxima of the dyes in methanol are observed between 535 and 717 nm, with Stokes shifts ranging from 21 to 79 nm. In DMSO, the emission maxima range from 531 to 734 nm. Fluorescence intensities are particularly high for dioxaborine dyes **M25**, **M22**, **M232**, and merocyanine **In4tb** in both solvents, as well as for dianionic indandione-derived **A74** in DMSO, reaching values between 1727 and 14140 a.u. In contrast, the remaining dyes display moderate fluorescence intensities, ranging from 45 to 535 a.u. The strong fluorescence of dioxaborine dyes has been previously noted in [10, 11, 12].

In 50 mM Tris-HCl buffer, the absorption spectra of dyes **B36**, **A74**, and **In4tb** differ significantly from those in DMSO and methanol. In these cases, short-wavelength maxima (attributable to the formation of molecular aggregates) appear and become dominant. For **M25**, the absorption band in buffer is broader than in DMSO, with an additional short-wavelength shoulder. In contrast, the remaining dyes retain the general shape of their absorption spectra, although the main maxima are shifted by up to 10 nm relative to their positions in DMSO.

Fluorescence maxima for most dyes in buffer are shifted by up to 16 nm compared to DMSO and correspond to the emission of dye monomers. Exceptions include **B36**, whose fluorescence

Table 1. **Spectral characteristics of the dyes (5 μM) in methanol, DMSO and 0.05 M Tris-HCl buffer (pH 8.0)**

Dye	MeOH				DMSO				buffer			
	λ_{abs}	D	λ_{fl}	I_{MeOH}	λ_{abs}	D	λ_{fl}	I_{DMSO}	λ_{abs}	D	λ_{fl}	I_{buff}
B36	530	0.56	552	45	514	0.39	561	56	502	0.32	–	9*
					540	0.44	597	38	530	0.28		
B33	515	0.95	550	209	529	0.76	560	497	530	0.67	557	36
M25	612	0.35	717	1272	631	0.31	734	4090	629	0.21	739	135
	660	0.38			689	0.46			699	0.23		
M22	530	0.97	551	12680	541	1.16	558	14140	536	0.94	561	4818
A74	606	1.66	633	196	627	1.70	648	10090	568	0.90	644	14
									611	0.54		
M232	676	1.75	709	6000	692	1.75	718	9590	694	1.01	728	718
H28	456	0.34	535	535	461	0.33	531	481	452	0.31	504	38
											541	37
In4tb	586	1.08	610	1727	594	1.11	618	4090	541	0.27	603	100
									575	0.26		

λ_{abs}, absorption maximum wavelength (nm); D, optical density at the absorption maximum; λ_{fl}, fluorescence maximum wavelength (nm); I, fluorescence intensity (a.u.); * — since the maximum wavelength could not be recorded, the intensity of the noise is presented.

intensity was too low to determine the maximum, and **H28**, whose spectrum exhibited two maxima, the more intense one being blue-shifted by 27 nm relative to the DMSO spectrum. In general, a strong decrease in fluorescence intensity was observed in buffer for all dyes, with reductions of 2.6- to 17-fold compared to methanol solutions.

Spectral-fluorescent properties of the dyes in the presence of serum albumins

The absorption and fluorescence spectral characteristics of the dyes in the presence of human (HSA), bovine (BSA), and equine (ESA) serum albumins are summarized in Table 2. The presence of any of these serum albumins does not alter the absorption spectral shape of dyes **B33**, **M22**, **M232**, and **H28**, which exist as monomers in the buffer solution, nor that of dye **B36**, where a short-wavelength band associated with molecular aggregates dominates. For these dyes, the absorption maxima shift by no more than 8 nm compared to their spectra in buffer alone.

In contrast, the absorption spectrum of dye **A74** in the presence of serum albumins closely resembles that in DMSO, where the monomer band is dominant. At the same time, the short-wavelength band (attributed to aggregates and dominant in buffer) disappears (Fig. 2), indicating disaggregation upon binding to serum albumins. A similar trend is observed for dye **In4tb**; however, in this case, the aggregate band does not completely

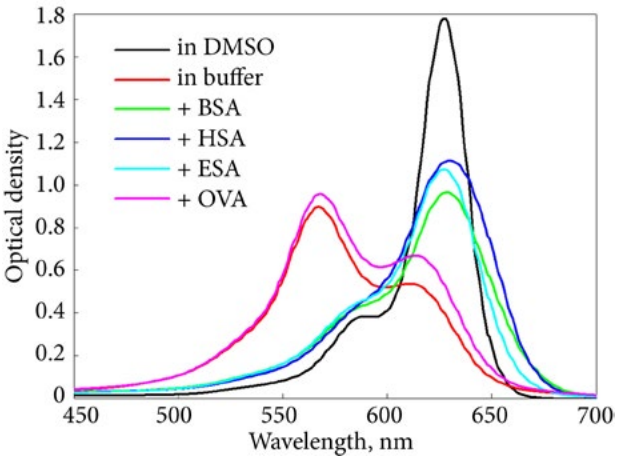

Fig. 2. Absorption spectra of dye **A74** (5 μM) in DMSO, in 50 mM Tris-HCl buffer (pH 8.0) and in the presence of BSA, HSA, ESA and OVA (0.2 mg/mL for all proteins)

Table 2. **Spectral characteristics of the studied dyes in the presence of human (HSA), bovine (BSA), and equine (ESA) serum albumins. Concentrations are 5 μM for dyes and 0.2 mg/mL for proteins**

Dye	HSA					BSA					ESA				
	λ_{abs}	D	λ_{fl}	I_{HSA}	I_{HSA}/I_{buff}	λ_{abs}	D	λ_{fl}	I_{BSA}	I_{BSA}/I_{buff}	λ_{abs}	D	λ_{fl}	I_{ESA}	I_{ESA}/I_{buff}
B36	503 529	0.35 0.31	555	173	19	503 529	0.34 0.30	556	124	14	501 529	0.34 0.29	556	99	11
B33	529	0.57	557	470	13	528	0.45	555	350	9.7	528	0.53	555	397	11
M25	669	0.33	744	910	6.7	630 680	0.31 0.30	740	660	4.9	639 685	0.29 0.28	746	691	5.1
M22	543	0.83	670	11 409	2.4	542	0.78	568	7727	1.6	544	0.82	566	10 318	2.1
A74	630	1.12	663	863	62	628	0.97	653	111	7.9	627	1.08	656	392	28
M232	699	0.98	740	3636	5.1	702	1.12	739	3227	4.5	701	1.10	736	4045	5.6
H28	453	0.36	540	155	4.2	452	0.42	538	148	4	451	0.52	537	188	5.1
In4tb	595	0.64	618	5636	56	590	0.47	613	4682	47	589	0.34	613	2454	25

λ_{abs}, absorption maximum wavelength (nm); D, optical density at the absorption maximum; λ_{fl}, fluorescence maximum wavelength (nm); I, fluorescence intensity (a.u.)

disappear. Notably, the intensity of the **In4tb** monomer band follows the trend HSA > BSA > ESA, indicating differences in binding efficiency or interaction strength among the albumins.

For the mero-anionic dye **M25**, the presence of serum albumins alters the absorption spectral shape. However, only in the case of HSA does a single dominant band (presumably corresponding to the monomeric form) emerge. In contrast, BSA and ESA cause only a partial redistribution of band intensities, suggesting a decrease in the proportion of aggregates, but not complete disaggregation.

Regarding the fluorescence spectra, most dyes exhibit a long-wavelength shift of up to 19 nm in the presence of serum albumins. Exceptions include **B36** (for which the fluorescence maximum could not be detected in buffer), **B33** (shift of up to 2 nm), and **H28**, whose two maxima in buffer collapse into a single peak in albumin-containing solutions, shifted by up to 36 nm relative to the more intense of the two buffer peaks.

In all cases, the presence of HSA, BSA, or ESA results in an increase in fluorescence intensity relative to the corresponding free dye in buffer. For dyes **M25**, **M22**, **M232**, and **H28**, the increase ranges from 1.6- to 6.7-fold, regardless of the albumin type. Dyes **B36** and **B33** show a stronger enhancement (9.7- to 19-fold), while **In4tb** demonstrates a particularly pronounced response, with a 25- to 56-fold increase. Remarkably, **A74** exhibits strong dependence on the type of serum albumin: fluorescence intensities increase by 62-, 28-, and 7.9-fold in the presence of HSA, ESA, and BSA, respectively. This makes **A74** the most selective dye toward a specific albumin (HSA) among those studied.

Spectral-fluorescent properties of the dyes in the presence of DNA and OVA

In the presence of DNA and ovalbumin (OVA), a non-serum albumin protein, most dyes do not

Table 3. **Spectral characteristics of the studied dyes in the presence of DNA and ovalbumin (OVA). Concentrations are 5 μM for dyes, 60 μM b.p. for DNA, and 0.2 mg/mL for OVA**

Dye	DNA					OVA				
	λ_{abs}	D	λ_{flu}	I_{DNA}	I_{DNA}/I_{buff}	λ_{abs}	D	λ_{flu}	I_{OVA}	I_{OVA}/I_{buff}
B36	503	0.31	520	29	3.2	501	0.31	554	27	3
	529	0.28				530	0.27			
B33	528	1.00	559	32	0.89	529	0.52	555	68	1.9
M25	622	0.22	741	179	1.3	623	0.30	719	134	0.99
	696	0.21				690	0.26			
M22	536	0.97	561	4863	1.0	539	0.93	562	5727	1.2
A74	568	0.72	645	17	1.2	568	0.96	646	137	9.8
	613	0.52				614	0.67			
M232	694	1.10	728	676	0.94	696	1.10	727	1636	2.3
H28	452	0.38	505	42	1.1	451	0.39	503	41	1.1
			541	41	1.1			540	42	1.1
In4tb	541	0.25	603	106	1.1	539	0.27	609	191	1.9
	575	0.25								

λ_{abs}, absorption maximum wavelength (nm); D, optical density at the absorption maximum; λ_{fl}, fluorescence maximum wavelength (nm); I, fluorescence intensity (a.u.); * — since the maximum wavelength could not be recorded, the intensity of the noise is presented.

exhibit significant changes in the position and shape of their absorption bands (Table 3). Exceptions include **B36** (with OVA) and **M25** (with both DNA and OVA), where an increase in the short-wavelength band was observed, suggesting enhanced dye aggregation in the presence of these biopolymers.

The fluorescence spectra of most dyes remain largely unchanged upon addition of DNA or OVA, with fluorescence maxima shifting by no more than 2 nm. Notable exceptions are **M25** and **In4tb**, which show red shifts of 20 nm and 6 nm, respectively, in the presence of OVA. For **B36**, the fluorescence maximum was not detected in buffer, but maxima observed in the presence of DNA and OVA differ by 34 nm. Fluorescence intensity increases in the presence of DNA or OVA do not exceed 3.2-fold for most dyes, with the exception of **A74**, which shows a 9.8-fold increase in fluorescence upon addition of OVA. It should be also mentioned that the presence of DNA leads to some decrease in the fluorescence intensity of the dyes **B33** and **M232**; the reason for such behavior is not clear and requires further studies.

Discussion

Based on the obtained spectral-fluorescent characteristics of the studied dyes in the presence of three serum albumins, DNA, and OVA, several conclusions can be drawn. First, all of the dyes exhibit a stronger fluorescence response to serum albumins than to DNA or OVA. This effect cannot be attributed to the differences in electrostatic interactions, as the tested chromophores include both neutral (**B36**, **B33**, **H28**, and **In4tb**) and anionic (**M25**, **M22**, **A74**, and **M232**) species, while all the studied biopolymers carry a negative charge at pH 8.0. Thus, the most plausible explanation is that, indeed, serum albumins are transport proteins whose primary function is to bind small molecules.

Further, a comparison of three structurally similar dyes — **B36**, **B33**, and **H28** (Fig. 1) — reveals additional insights. The data show that: (1) the presence of the difluoroboron bridge in **B36** and **B33** causes a red shift in absorption and fluorescence maxima relative to **H28**; (2) the cyano group in **B36** lowers the fluorescence intensity in both organic solvents and Tris-HCl buffer compared to **B33** and **H28**; and (3) the dioxaborine-containing dyes **B36** and **B33** exhibit a greater fluorescence response to serum albumins than ketoenol-based **H28**. It is also worth noting that **In4tb**, which is thiobarbituric-based, demonstrates much higher fluorescence intensity and a stronger response to serum albumins than the three aforementioned dyes. However, this comparison may not be fully justified, as **In4tb** contains a longer polymethine chain with more methine units.

Finally, the dyes **M25**, **M22**, and **M232**, each bearing malononitrile moieties as electron-acceptor groups, show relatively weak fluorescence changes in the presence of serum albumins. In contrast, dye **A74** displays a pronounced response. This observation is consistent with earlier reports showing that the fluorescence of **A74** and other indandione-based polymethines decreases significantly in protic solvents [12]. Therefore, the enhanced fluorescence in the presence of serum albumins likely results from the displacement of water molecules from the dye's solvation shell upon protein binding. An additional notable property of **A74** is its differential sensitivity to various serum albumins: fluorescence intensity increases 62-, 28-, and 7.9-fold in the presence of HSA, ESA, and BSA, respectively. Given that the globular structures of these proteins do not differ substantially, this result suggests that **A74** may be sensitive to subtle conformational variations among serum albumins — a possibility that warrants further investigation. Meanwhile, this dye has a 9.8-fold increase in the presence of OVA. Thus, when studying conformational transitions in certain (e.g. human) serum albumin using **A74** as a fluorescent probe, the absence of other proteins should be provided. On the other hand, the possibility of serum albumin detection with this dye in the presence of other proteins should be attentively studied as well.

Conclusions

The fluorescent response of eight polymethine dyes — including six dioxaborines with varying chromophore charges — was studied in the presence of human, bovine, and equine serum albumins, and compared with their response to DNA and ovalbumin. Dioxaborine merocyanines **B36** and **B33** were found to increase their fluorescence intensity by approximately 10-fold in the presence of serum albumins. Yet, the indanedione-based dianionic dioxaborine **A74** and the thiobarbituric-based merocyanine **In4tb** demonstrated the most promising performance as fluorescent probes for human serum albumin (HSA) detection, with fluorescence intensity increases by 62- and 56-fold, respectively. These dyes warrant further investigation for the potential applications in fluorescent detection and imaging of HSA. Moreover, dioxaborine **A74** exhibited a high degree of selectivity for HSA over other serum albumins, suggesting that it may be sensitive to subtle conformational differences in the globular structure of serum albumin.

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СПЕКТРАЛЬНО-ФЛУОРЕСЦЕНТНИЙ ВІДГУК ДІОКСАБОРИНОВИХ БАРВНИКІВ ЗІ ЗМІННИМ ЗАРЯДОМ ХРОМОФОРА В ПРИСУТНОСТІ СИРОВАТКОВИХ АЛЬБУМІНІВ

Мета. Метою роботи є вивчення спектрально-флуоресцентного відгуку ряду діоксаборинових барвників у присутності сироваткових альбумінів. ***Методи.*** Спектроскопія поглинання в УФ- та видимому діапазонах та флуоресцентна спектроскопія. ***Результати.*** Було вивчено флуоресцентний відгук восьми поліметинових барвників (шість із них на основі діоксаборину) у присутності людського (ЛСА), бичачого (БСА) та кінського (КСА) сироваткових альбумінів та здійснено порівняння з відгуком у присутності ДНК та овальбуміну. Було показано, що діаніонний діоксаборин **A74** і мероціанін на основі тіобарбітурової кислоти **In4tb** демонструють найбільш перспективні властивості як флуоресцентні зонди для виявлення ЛСА, в присутності якого інтенсивність флуоресценції цих сполук зростає у 62 і 56 разів відповідно. Водночас **A74** демонструє найвищу селективність до ЛСА серед інших сироваткових альбумінів**:** у присутності КСА та БСА його інтенсивність зростала лише у 28 разів та 7,9 разів відповідно. Таким чином, можна припустити, що **A74** може бути чутливим до незначних змін у глобулярній конформації сироваткових альбумінів. Крім того, діоксаборинові мероціаніни **B33** і **B36** продемонстрували майже 10-кратне збільшення інтенсивності флуоресценції в присутності сироваткових альбумінів. ***Висновки.*** Слід детальніше вивчати можливість застосування найперспективніших барвників **A74** та **In4tb** в методах, які вимагають флуоресцентного виявлення або візуалізації ЛСА, а барвника **A74** також як зонда, чутливого до незначних змін у глобулярній структурі ЛСА.

***Ключові слова*:** діоксаборини, поліметинові ціанінові барвники, флуоресцентні зонди, сироваткові альбуміни.