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REGULAR ARTICLE

Hydrophilic meso-substituted cyanine dyes in solution and in complexes with serum albumins: spectral properties and molecular docking study

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Abstract. The absorption and fluorescence spectral properties of three *meso*-substituted hydrophilic thiacarbocyanine dyes were studied in solutions and in noncovalent complexes with human and bovine serum albumins (HSA and BSA, respectively). The presence of alkyl substituents at the *meso*-position of the polymethine chain of the dyes determines the occurrence of a *cis–trans* equilibrium. Dyes form aggregates in aqueous media; the effect of electrolyte (NaCl) on aggregation has been studied. The interaction of the dyes with albumins leads to the decomposition of the aggregates and is accompanied by a shift in the isomeric equilibrium. Complexation with HSA leads to accumulation of dye monomers in the *trans*-form. However, in the case of BSA the *cis*-to-*trans* isomeric shift is incomplete. Using the fluorescence data, the effective binding constants of the *trans*-isomers with albumins (K_a) and the detection limits of albumin molecules (LD and LQ) were determined. The data obtained are indicative of high selectivity of some dyes to HSA compared to BSA. The results of molecular docking experiments correspond to the data obtained from the spectra. The influence of the dyes on intrinsic fluorescence of HSA and BSA was also studied, and fluorescence quenching, static in nature, was detected.

Keywords. Polymethine (cyanine) dyes; serum albumins; complexation; *cis–trans* equilibrium; spectral-fluorescent probes; fluorescence growth.

1. Introduction

Polymethine (cyanine) dyes, having a superior range of variation of the absorption, fluorescence, and photochemical properties among known organic dyes, are actively studied and applied in various fields.[1,2] The presence of a flexible polymethine chain in the structure of their molecules causes low quantum yields of cyanine fluorescence in solutions since the energy dissipation of the excited electronic state of cyanine molecules occurs by nonradiative mechanisms (vibrational relaxation, photoisomerization).[3] *Meso*-substituted thiacarbocyanine dyes are characterized by a polarity-dependent equilibrium between the *cis*- and *trans*-isomeric forms.[4,5]

The ability of cyanine dyes to interact noncovalently with biomacromolecules has been actively studied.[6,7] The formation of complexes with proteins and nucleic acids has a noticeable effect on the absorption and fluorescence properties[6–8] and photochemistry of the dyes.[9,10] The significant changes in the photophysical and photochemical properties of cyanine dyes upon binding to biomacromolecules make it possible to use them as spectral-fluorescent probes.[2,6–8] The obvious merits of this analytical method determine the relevance of a search for prospective dye probes. Cyanine dyes have been widely used in biomedical and laboratory research.[6,7]

In this work, the influence of noncovalent interaction with human and bovine serum albumins (HSA and BSA, respectively) on the absorption and fluorescence properties of the *meso*-alkyl-substituted hydrophilic cyanine dyes 3,3′-di-(γ-sulfopropyl)-5,6,5′,6′-di-(methylenedioxy)-9-ethylthiacarbocyanine betaine

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(OXEC), 3,3′-di-(γ-sulfopropyl)-5,5′-dimethoxy-9-ethylthiacarbocyanine betaine (DMEC), and 3,3′-di-(γ-sulfopropyl)-4,5,4′,5′-dibenzo-9-methylthiacarbocyanine betaine (DMC) (Figure 1) was studied in a comparative mode (some data for the interaction of DMC and DMEC with HSA were obtained earlier[11,12]). The influence of the dyes on intrinsic fluorescence of HSA and BSA was also studied. The dye–albumin complexes were modelled *in silico* by molecular docking. Serum albumins were taken as model proteins because they are the main transport proteins for bio-metabolites and can form intermolecular complexes with various compounds,[13] in particular, with cyanine dyes.[7] Some new spectral data were also obtained for the dyes in homogeneous solutions.

We chose these *meso*-substituted polymethine dyes as analogues of the similar *meso*-substituted dye 3,3′-di-(γ-sulfopropyl)-4,5,4′,5′-dibenzo-9-ethylthiacarbocyanine betaine (DEC), which was already used for several years as a sensitive probe for detecting HSA (see, for example[14]).

2. Experimental

2.1 *Materials and methods*

The anionic thiacarbocyanine dyes OXEC, DMEC, and DMC were obtained from research center NIIKHIMPHOTOPROEKT. HSA, BSA, ibuprofen and warfarin were commercially available (Sigma-Aldrich, USA). Ethanol, acetone, acetonitrile, dimethyl sulfoxide (DMSO), 1,4-dioxane (analytical grade), and distilled water were used as solvents. The procedure for preparing dye solutions and the titration is described in[12]. To determine the complexation constants of OXEC and DMEC with albumins, we used lower concentrations of the dyes (see the corresponding section).

The spectra of the dyes were measured on an SF-2000 spectrophotometer (Russia), and a Fluorat-02-Panorama spectrofluorimeter (Russia), using quartz and plastic cells. The fluorescence and fluorescence excitation spectra were corrected for the spectral characteristics of the device (excitation channel) and transmittance of the sample.

The fluorescence quantum yields (Φ_{fl}) of the dyes were measured by comparison with a standard.[15] Cresyl violet perchlorate (Φ_{flst} = 0.54 in ethanol solution) was chosen as the standard.[16]

In fluorescence quenching experiments, the intrinsic fluorescence of albumins (HSA and BSA) was measured in the presence of increasing concentrations of dyes OXEC, DMEC, and DMC both in the normal mode at λ_{ex} = 280 nm and in the synchronous scanning mode. The synchronous fluorescence spectra (SFS) of albumins were measured with a fixed wavelength interval $\Delta\lambda = \lambda_{fl} - \lambda_{ex}$ equal to 15 nm (corresponding to the fluorescence of tyrosine groups) and 60 nm (fluorescence of tryptophan groups),[17,18] at HSA and BSA concentrations of 10–20 μM.

The measurements of dynamic light scattering (DLS) were performed on a Zetasizer Nano-S (Malvern, England) instrument in a standard cell.

2.2 *Processing and analysis of absorption and fluorescence data*

The contribution of individual components to the absorption spectra of the dyes was estimated using the iterative procedure of deconvolution of the total absorption spectrum into Gaussian components.[19]

The detection and quantitative estimates limits (LD, LQ; μM, μg mL^{-1}) of albumins using the dyes were determined from the dye fluorescence spectra based on the standard deviation (σ, see Equations S2 and S3 in Supplementary Information).[20]

The data on quenching of intrinsic fluorescence of HSA and BSA upon the interaction with the dyes were analyzed using the Stern–Volmer[21] (Equation (1)) and Lehrer–Leavis[22] (Equation (2) dependences:

$$I_0/I = 1 + K_{SV}[Q], \quad (1)$$

$$I_0/(I_0 - I) = 1/\alpha + 1/(\alpha K_{SV}[Q]), \quad (2)$$

where I_0 and I are the fluorescence intensities in the absence and presence of a quencher, respectively; [Q] is the quencher concentration; K_{SV} is the Stern–Volmer quenching constant; and α is the fraction of fluorophores in a

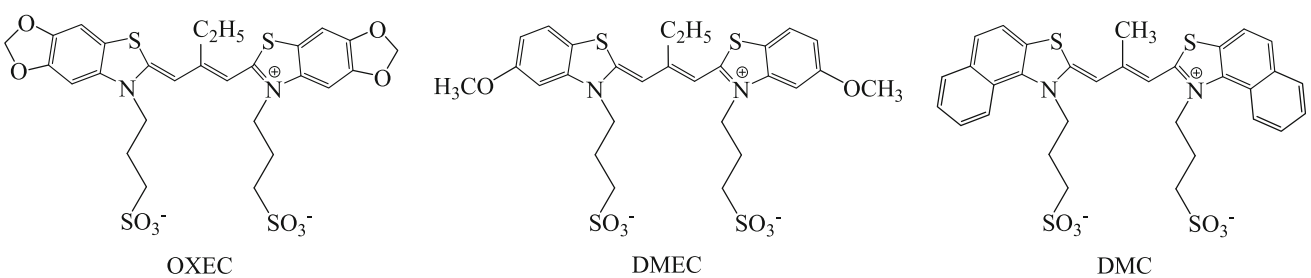

Figure 1. Studied *meso*-substituted cyanine dyes.

biomolecule, which are accessible to a quencher in the Lehrer–Leavis model.[22]

The parameters of Förster resonance energy transfer (FRET) between albumin donor chromophores (tyrosine and tryptophan amino acid residues of HSA and BSA) and acceptor (the dye molecules) were determined from the spectral-fluorescent data using the standard approach (Equations S4, S5).

The estimation of the equilibrium constants of the complexation reaction of dyes OXEC and DMEC with BSA and HSA (K_a, M^{-1}) was carried out from the dye fluorescence spectra of their dilute solutions (see below).[23] The complexation constant of DMC with HSA was taken from the previous work,[12] and that for DMC with BSA was estimated from the dye fluorescence spectra using the approach described therein.

2.3 *Evaluation of hydrophilic/hydrophobic properties of the dyes and molecular docking with albumins*

The octanol-water distribution coefficients (logP) and the polar surface area (TPSA, Å^2) of the dye molecules for evaluation of their hydrophilic/hydrophobic properties were obtained using the www.molinspiration.com website.[24]

Molecular docking of dye-albumin complexes was carried out using DockThor.[25,26] The structures of BSA (4F5S) and HSA (4K2C) were taken from Protein Data Bank.[27,28] Avogadro (http://avogadro.cc/),[29] as well as SwissPdb Viewer (http://www.expasy.org/spdbv/)[30] and UCSF Chimera (http://www.rbvi.ucsf.edu/chimera)[31] were used as 3D visualization and analysis tools.

3. Results and Discussion

3.1 *Absorption and fluorescence properties of the dyes in solutions: isomeric equilibrium and aggregation*

In polar solvents (ethanol, acetonitrile, acetone) dye OXEC, as well as DMEC[32] and DMC,[12] have rather narrow intense absorption bands with peaks in the visible range and a short-wavelength vibronic shoulder (Figure 2a, curve *1*), typical for dye monomers.

The dyes possess a low fluorescence quantum yield in polar media (Table 1). For solutions of *meso*-substituted thiacarbocyanine dyes, which include the dyes under study, a dynamic equilibrium was found between *cis*- and *trans*- isomeric forms, depending on the polarity of the medium. This equilibrium shifts to the *cis*-isomers in polar media and to the *trans*-isomers in low polarity or nonpolar media.[4,5] The *cis*-isomers of thiacarbocyanine dyes are characterized by shorter-wavelength positions of the absorption spectra and the lack of fluorescence in solutions, whereas the fluorescent *trans*-isomers have longer-wavelength spectra. *Meso*-substituted carbocyanines (thia- and oxa-) generally have low Φ_{fl} in polar media, which can be explained by the predominance of non-fluorescent cis-forms of the dyes.[4,5,32]

In ethanol solutions, the maxima of the fluorescence excitation spectra of the dyes are bathochromically shifted relative to the absorption spectra ($\Delta\lambda = \lambda_{ex} - \lambda_{abs}$ = 9–20 nm, see Table 1, Figure 2a, curve *4*).

The difference between the maxima of the fluorescence excitation spectra (which correspond to the *trans*-isomers) and the absorption spectra of the dyes under study (Table 1) is explained by the simultaneous presence in a solution of *cis*- and *trans*-isomers of the dyes[32] with different absorption and fluorescence properties.

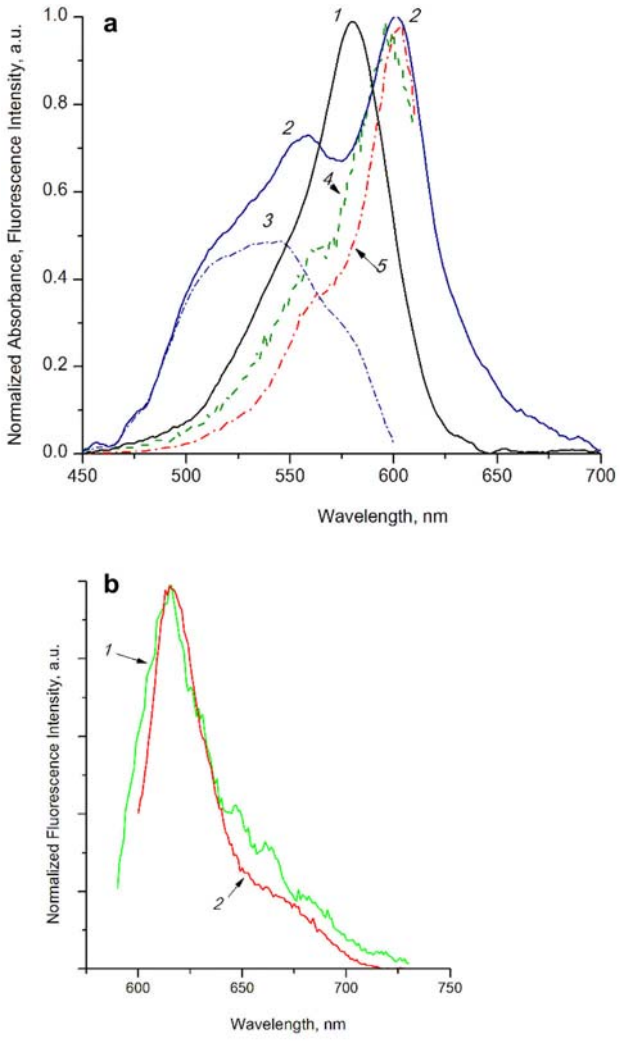

Figure 2. (a) Normalized absorption (*1*, *2*, *3*) and fluorescence excitation spectra (*4*, *5*) of OXEC in polar ethanol (*1*, *4*) and in nonpolar 1,4-dioxane (*2*, *3*, *5*); (b) normalized fluorescence spectra of OXEC in ethanol (*1*) and in nonpolar 1,4-dioxane (*2*).

Table 1. Absorption and fluorescence properties of dyes OXEC, DMEC, and DMC in solutions: maxima of the absorption (λ_{abs}), fluorescence (λ_{fl}), and fluorescence excitation (λ_{ex}) spectra, as well as fluorescence quantum yields (Φ_{fl}).

	Ethanol				1,4-dioxane				Water						
	λ_{abs}	λ_{fl}	λ_{ex}	Φ_{fl}	λ_{abs}	λ_{fl}	λ_{ex}	Φ_{fl}	λ_{abs} H-	λ_{abs} D-	λ_{abs} M-	λ_{abs} J-	λ_{fl}	λ_{ex}	Φ_{fl}
	nm			%	nm			%	nm						%
OXEC	580	627	597	1.05	602	615	602	9.0	-	535	576	-	609	590	0.5
DMEC	566	598	588	0.3	585	598	586	10	-	524	560	-	583	574	<0.1
DMC	574	606	583	0.2	640, 597*	613	597	2.6	455	534	575-605	656	656	530	-

*Value obtained in a mixture of water: 1,4-dioxane 1: 8.

In nonpolar medium thiacarbocyanine dyes are capable of forming aggregates;[33] the contribution of the absorption of dye aggregates leads to a significant distortion of the shape of the band in the absorption spectrum of the dyes. This is strongly manifested in DMC in 1,4-dioxane;[12] in the case of OXEC and DMEC in 1,4-dioxane, the contribution of aggregates to the absorption spectra is much smaller, and the main contributions are from the absorption bands of monomer molecules (see Figures S2 and S1 in Supplementary Information; λ_{abs}^{max} = 602 and 585 nm for OXEC and DMEC, respectively). To estimate the contribution of aggregates in the absorption spectrum of OXEC in 1,4-dioxane, we subtracted the fluorescence excitation spectrum from the normalized absorption spectrum of the dye, which gave the aggregate absorption spectrum (Figure 2a, spectrum *3*). In a 1,4-dioxane solution, fluorescence increases markedly, in particular, for OXEC, the fluorescence intensity increases by almost 16 times (Table 1). This is due to the contribution of the fluorescent *trans*-isomers of the dyes.

In aqueous solutions, cyanine dyes are also capable of forming molecular associates (aggregates) of various types (in particular, dimers, H- and J-aggregates). Note that both the ability to aggregate and the structural features of carbocyanine aggregates depend, first of all, on the structure of the dye molecules.[34] The formation of dye aggregates in aqueous solutions is controlled by a combination of hydrophobic and polar properties of the molecules and also depends on the concentration of dye molecules in the solution, as well as on the presence of electrolytes and polymer additives.[34]

To evaluate the molecular hydrophobicity of the dyes under study, the octanol-water partition coefficient (logP) was calculated.[24] The values of logP increase in the range OXEC < DMEC < DMC (logP = −2.22, −1.89, and 0.1, respectively), which qualitatively corresponds to a decrease in the polar properties of the molecules estimated on the basis of the polar surface area (TPSA, Å^2) of dye molecules.[24] TPSA for OXEC, DMEC, and DMC were found to be 160, 142, and 123 Å^2, respectively; this range roughly corresponds to a growth in the aggregation ability of the dyes in water.

Indeed, the aggregation properties of DMC are the most pronounced among the dyes studied. In aqueous solutions, the monomeric form (M) of DMC is in equilibrium with the dimeric form (D, λ_{abs}^{max} ∼ 534 nm), and with H- (λ_{abs}^{max} ∼ 455 nm) and J- (λ_{abs}^{max} ∼ 657 nm) aggregates.[12] In the absence of aggregation-promoting additives, the most intense is the D-band (λ_{abs}^{max} ∼ 534 nm), whereas the M-band is weakly expressed as the shoulder with λ_{abs}^{max} ∼ 575–605 nm (see Figure 3, curve 1). The addition of electrolytes to aqueous dye solutions (0.6 M NaCl solution) leads to the formation of the long-wavelength band of J-aggregates of DMC and the rearrangement of its spectra. In particular, at c_{NaCl} = 9.84 mM, the dimer band decreases and the absorption band of J-aggregates (λ_{abs}^{max} ∼ 656 nm) grows (see inset in Figure 3). An increase in the NaCl concentration (to c_{NaCl} = 37.5 mM) leads to further conversion of dye dimers into J-aggregates, which is reflected in further growth in the J-aggregate band (Figure 3, curves *2*–*10*).

For dyes OXEC and DMEC in aqueous solutions, the monomer-dimer equilibrium also occurs (Table 1). The D-bands of OXEC and DMEC is observed even at rather low dye concentrations (c_{DMEC} ∼ c_{OXEC} ∼ 1 μM), but the M-bands are more pronounced. Increasing the electrolyte concentration (up to c_{NaCl} = 70.6 mM) does not lead to an appearance of J-aggregates of dyes DMEC and OXEC. For DMEC, with the addition of NaCl, a certain increase in the D-band (∼ 15%) is observed in the absorption spectra. In the case of OXEC, the addition of NaCl does not affect the

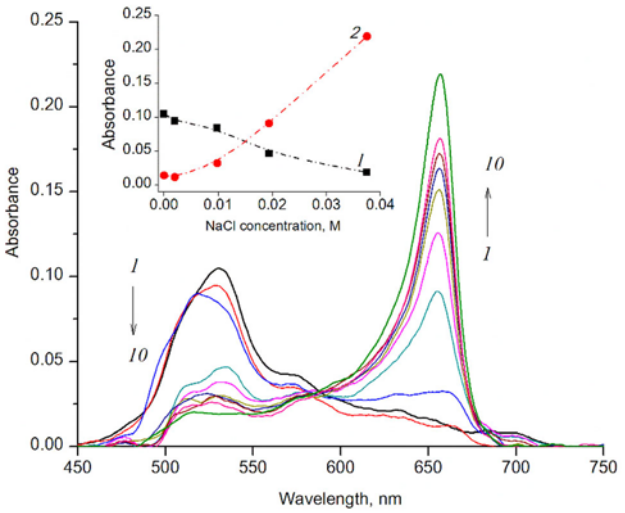

Figure 3. Absorption spectra of DMC in an aqueous solution at various NaCl concentrations (at c_{NaCl} = 0 (*1*); 1.99 (*2*), 9.8 (*3*), 19.4 (*4–9*), and 37.5 (*10*) mM. Spectra 4–9 were recorded in 2, 4, 6, 8, 10, and 14 min, respectively, after adding NaCl to the solution. Inset: dependences of the absorbances at the absorption maxima of dimers (λ_{abs}^{max} = 531 nm) and J-aggregates (λ_{abs}^{max} = 656 nm) of DMC on NaCl concentration (spectra were recorded in 2 min after the addition of NaCl).

monomer-dimer equilibrium in the solution (there is no change in the absorption spectrum).

In an aqueous solution, OXEC, DMEC, and DMC are characterized by weak fluorescence. DMC exhibits fluorescence of the dimeric form (a weak broad band with $\lambda_{fl}^{max} \sim 660$ nm).[12] The fluorescence excitation spectrum of DMC corresponds to the D-band in the absorption spectrum (see Table 1). The fluorescence spectra of dyes OXEC and DMEC correspond to the emission of their monomeric forms. Note that the dyes under study in aqueous solutions are also characterized by a shift in the *cis–trans* isomeric equilibrium toward the formation of *cis*-forms,[35] and the *trans*-isomers of the dyes are present in a solution in insignificant amounts. The positions of the maxima in the fluorescence excitation spectra of OXEC and DMEC correspond to their *trans*-monomers (see Table 1).

3.2 *Absorption and fluorescence properties of the dyes in complexes with BSA and HSA*

Anionic carbocyanine dyes are known to bind to serum albumins to form complexes.[2,7,11,12,36–38] This results in significant changes in their properties. The absorption and fluorescence spectra of dyes OXEC, DMEC, and DMC were studied at different concentrations of BSA and HSA (c_{BSA} = 0–75.6 μM, c_{HSA} = 0–23.8 μM). The spectra of OXEC–HSA are shown in Figure 4 (the spectra of DMEC and DMC with HSA and BSA are shown in Figures S2–S9 in Supplementary Information).

The complexes of the dyes with serum albumins include dye monomers, which are formed from the decomposition of dye dimers (and other aggregates, if any). In the absorption spectra, it is reflected in a decrease in the intensity of dye D-bands and growth of M-bands upon complexation (Figure 4).

The effects of the interaction of the dyes with albumins are also reflected in changes in their fluorescence properties. The fluorescence and fluorescence excitation spectra of OXEC in the presence of albumins are shown in Figure 5.

In the presence of albumins, the fluorescence spectra of the dyes become narrower and, for OXEC and DMEC, shift bathochromically (for DMC, the fluorescence of dimers is replaced with that of bound monomers[12]). In particular, for OXEC in the presence of HSA, this shift is $\Delta\lambda_{fl}^{max}$ = 19 nm at c_{HSA} = 1.98 μM (see Figure 5). Similar effects occur for DMEC and DMC (fluorescence and fluorescence excitation spectra for DMEC and DMC are given in Figures S4 and S5 of Supplementary Information). DMEC and OXEC are characterized by a long-wavelength shift of the maxima of the fluorescence spectral bands in the presence of BSA (see Table 2 and Figures S6-S9 of Supplementary Information).

In the presence of HSA, the maxima of the fluorescence excitation spectra of the dyes fairly coincide with those of the absorption spectra (Table 2). This confirms the interaction of the dyes with HSA mainly in the form of the fluorescent *trans*-monomers (due to the transition of dye *cis*-isomers to the *trans*-form upon the interaction with albumin). However, for

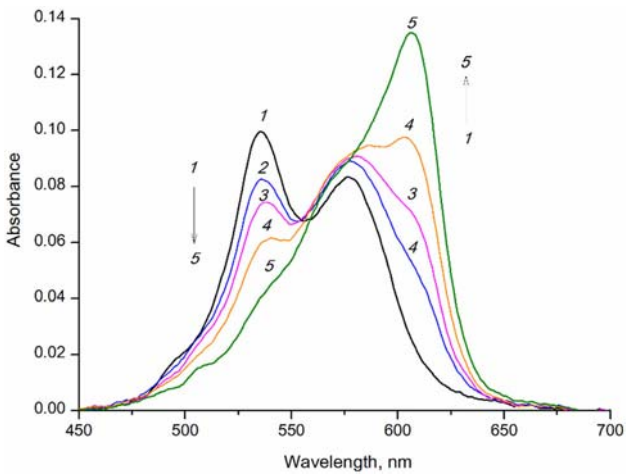

Figure 4. Absorption spectra of OXEC in an aqueous solution at different concentrations of HSA: c_{HSA} = 0 (*1*); 0.99 (*2*), 1.98 (*3*), 5.8 (*4*), 24 (*5*) μM.

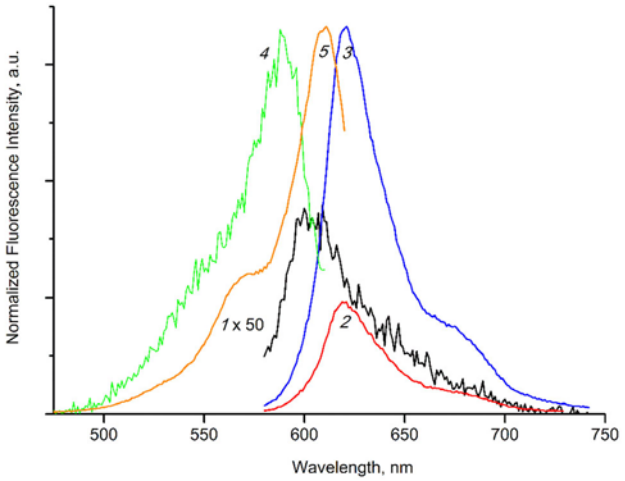

Figure 5. Fluorescence (curves *1–3),* normalized fluorescence excitation spectra (curves *4, 5*) of OXEC (λ_{ex} = 570 nm, λ_{reg} ~ 620, 630 nm) at different concentrations of HSA: c_{HSA} = 0 (*1, 4*), 0.99 (*2*), 24 (*3, 5*) μM.

BSA, this is not always true (see, e.g., Figures S5 Supplementary Information). In particular, for DMC the maxima in the fluorescence excitation spectra satisfactorily correspond to the absorption spectra (λ_{ex} = 586 nm, λ_{abs} = 584 nm at c_{BSA} = 35 μM); however, in the case of DMEC and OXEC, the difference in the positions of the maxima of the absorption and fluorescence excitation spectra is larger (see Table 2, $\Delta\lambda_{max}$ = 17 nm). A comparison of the fluorescence excitation and absorption spectra of the dyes indicates only a partial transition of dyes DMEC and OXEC to the *trans*-isomers bound to BSA. In the presence of serum albumins, apart from changes in the absorption and fluorescence spectra, a significant increase in the dye fluorescence intensity is observed, with a greater increase for HSA than for BSA (Table 2).

The fluorescence intensity of the dyes increases in parallel with an increase in the contribution of the long-wavelength M-bands in their absorption spectra. Changes in the fluorescence intensities of dyes OXEC, DMEC, and DMC depending on the concentration of HSA and BSA are shown in Figure 6.

Thus, the interaction of OXEC, DMEC, and DMC with albumins may be expressed by the following general scheme:

$$\mathrm{D} \rightleftarrows 2cis\text{-}\mathrm{Dye_f} \quad (3)$$

$$cis\text{-}\mathrm{Dye_f} + \mathrm{Alb_f} \rightleftarrows [trans\text{-}\mathrm{Dye}...\mathrm{Alb}]_b, \quad (4)$$

$$cis\text{-}\mathrm{Dye_f} + \mathrm{Alb_f} \rightleftarrows [cis\text{-}\mathrm{Dye}...\mathrm{Alb}]_b, \quad (5)$$

where subscript "f" denotes the unbound dye and albumin molecules, and subscript "b" denotes the dye–albumin complex.

The decomposition of dimers (aggregates) under the action of HSA and BSA (Equation (4)) followed by the conversion of the dyes into the *trans*-monomeric forms (Equation (5)) leads to an increase in the fluorescence intensity. To reveal more clearly the changes in the absorption spectra of the dyes under study upon the interaction with human and bovine serum albumins, deconvolution of dye spectra into Gaussian components was carried out,[19] and the contributions of the components corresponding to dimers, *cis*- and *trans*-monomers were obtained (see Figures S6 and S7 of Supplementary Information). The results clearly show the conversion of the *cis*-isomers (the dyes are initially present in this isomeric form in the absence of albumins both as monomers and in dimers) to *trans*-isomers bound into complexes with albumins, see Equations (3) and (4). However, in the case of BSA, a significant contribution of *cis*-isomers to the absorption spectra is noticed even at high BSA concentrations, which indicates complexation of the dyes with BSA also in the *cis*-form. In the case of HSA, this contribution is much smaller.

Changes in the spectral-fluorescent properties of dyes OXEC, DMEC, and DMC observed upon the

Table 2 The absorption, fluorescence, and binding properties of OXEC, DMEC, and DMC in the presence of HSA and BSA: maxima of the absorption (λ_{abs}), fluorescence (λ_{fl}), and fluorescence excitation (λ_{ex}) spectra, as well as relative fluorescence intensities (I_{fl}), detection limits (LD), and quantitative estimates (LQ).

	HSA						BSA					
	λ_{abs}	λ_{fl} nm	λ_{ex}	I_{fl_rel}	LD/LQ μg mL^{-1}	$K_a \times 10^{-6}$ M^{-1}	λ_{abs}	λ_{fl} nm	λ_{ex}	I_{fl_rel}	LD/LQ μg mL^{-1}	$K_a \times 10^{-5}$ M^{-1}
OXEC	606	620	609	119	1.40/4.6	0.43	569	596	586	53	1.73/5.7	4.1
DMEC	592	606	593	183	0.35/1.14	2.1	570	597	587	41	5.8/17.3	0.49
DMC	604*	616*	604*	885*	0.13/0.41	37*	584	608	586	100	5.4/16.6	3.5

*Data were taken from the earlier work[12].

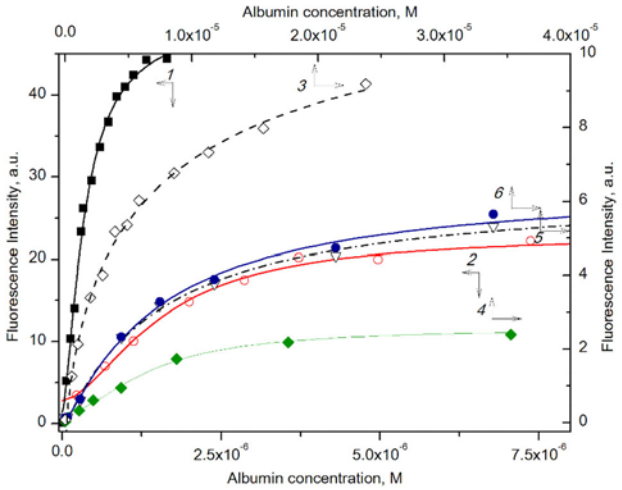

Figure 6. Dependences of changes in the fluorescence intensity of dyes DMC (*1*, *4*), DMEC (*2*, *5*), and OXEC (*3*, *6*) on the concentration of HSA (*1–3*) and BSA (*4–6*).

interaction with serum albumins are explained by shifts in the monomer–dimer and isomeric equilibria. Upon the noncovalent interaction of the dyes with HSA, the dimers decompose and the isomeric equilibrium of the dye monomers shifts toward *trans*-isomers complexed with HSA.

Thus, for HSA processes (3) and (4) are essential, whereas for BSA all processes (3)–(5) play an important role. For DMC with albumins, apart from the decomposition of dimers, decomposition of other aggregates (H- and J-, if any) takes place.

3.3 *Dye-albumin binding constants (K_a) and the analytical limits of albumin detection (LD and LQ)*

Binding of OXEC, DMEC, and DMC with HSA and BSA is a complex process involving both monomeric dye molecules in the forms of *cis*- and *trans*-isomers and dimers (D). In addition, the insufficient completeness of the *cis–trans* conversion of dyes OXEC and DMEC (especially upon complexation with BSA) determines the presence in the system under consideration of a significant content of *cis*-isomers of the dyes (Equations (3)–(5)), which creates a number of additional difficulties for accurate determination of dye–albumin binding constant values. We note that for the use of the dyes as fluorescent probes, it is important to estimate the equilibrium constants for the formation of complexes with albumin of *trans*-isomers of the dyes, which exhibit strong fluorescence (Equation (4)). Note, however, that upon the interaction with BSA, the dyes are complexed also in the nonfluorescent *cis*-form, which permits only rough estimation of the binding constants for BSA from fluorescence data.

It was shown in several works that albumins can form associates at increased concentrations (see, e.g., [39,40]). On checking HSA and BSA solutions by dynamic light scattering, we obtained similar results (data not shown). When we studied dye-albumin complexation by titration of dye solutions with albumins, such albumin associates could be also formed at higher albumin concentrations. In our experiments we obtained the effective values of the binding constants, neglecting the albumin association. This was proven by the fact that for analytical purposes, it is important to obtain the binding constants at low albumin concentrations, at which the albumin association may be neglected.

The complexation constants of the *trans*-isomers of OXEC and DMEC with albumins (K_a, M^{-1}) were obtained from the data on the fluorescence growth in dilute dye solutions upon titration with albumin solutions (see supplementary information). Low dye concentrations (c_{DMEC} = 0.4 μM, c_{OXEC} = 0.6 μM) were chosen to significantly reduce the contribution of dimers. Under these conditions, equilibrium (3) is strongly shifted toward the monomeric forms of the dyes ($[cis\text{-}Dye]_f$). This can be seen in the absorption spectra, in which the D-bands are almost completely absent. If we neglect binding with albumins of the nonfluorescent *cis*-isomer (Equation (5)), then the dye–albumin complexation will be reduced to the equilibrium between the dye monomer molecules (in the *cis*-form) unbound with albumin and the *trans*-isomers of the dyes bound with HSA or BSA in a complex (Equation (4)). The binding constants for OXEC and DMEC were obtained using the analogue of the Scatchard equation described earlier.[12]

Dye DMC is more hydrophobic and more prone to aggregation (dimerization), and a major contribution of the dimer band is observed in the absorption spectra of DMC even at $c_{DMC} \sim 0.3$ μM. Hence, to determine K_a for DMC, Equation (3) should be taken into account (the calculation was described in[12]). The effective complexation constants K_a obtained for the interaction of DMC with BSA is given in Table 2 (the K_a value for the interaction of DMC with HSA was taken from[12]).

From the fluorescence growth of the dyes in the presence of albumins, the analytical limits of albumin detection (LD and LQ values) were also determined (Table 2). It can be seen from the table that high values of K_a correspond to low

values of LD and LQ and vice versa. Dyes DMC and DMEC exhibit low values of LD and LQ (and high values of K_a) for binding to HSA (for DMC $LD_{HSA} = 1.9 \times 10^{-9}$ M[1], for DMEC $LD_{HSA} = 5.2 \times 10^{-9}$ M). Based on this fact, dye DMC was suggested as a sensitive fluorescent probe for detecting HSA[12]. Dye DMEC can be also used as a probe for HSA. In the case of BSA, the LD and LQ values for DMC and DMEC were found to be much higher, which reflects weaker interaction (lower binding constants) of the dyes with BSA. Hence, these dyes possess selectivity in the detection of HSA compared to BSA. For OXEC, the LD values were similar with both albumins (about 2×10^{-8} M, with $K_a \sim 4 \times 10^5$ M^{-1}), and the dye does not show any preference when interacting with HSA or BSA.

Comparing the data obtained in the present work with the literature data available on fluorescent probes for HSA, we can notice higher sensitivity of dyes DMC and DMEC for HSA than the merocyanine dye studied in[41] (LD = 8.6×10^{-9} M) and much higher sensitivity than pentamethine cyanine dyes[42] ($LD < 6 \times 10^{-8}$ M) and novel ratiometric fluorescent probe N-butyl-4-(4-phenyl-benzoyloxy)1,8-naphthalimide (BPBN)[43] (LD = 3.8×10^{-8} M).

We also carried out an experiment on ibuprofen and warfarin displacement of dye molecules bound to albumins and found that both reagents displace to some extent the dyes from the complexes (see Table S1, Supplementary Information). This means that the dyes bind to albumins in both Sudlow's sites II and I, respectively (subdomain IIIa in domain III and subdomain IIa in domain II, respectively).[44,45]

We performed a preliminary study of the effect of a buffer on binding the dyes to albumins. The study carried out for OXEC and HSA in a physiological 0.01 M phosphate buffer-saline solution (pH 7.3–7.5, c_{NaCl} = 0.137 M, c_{KCl} = 0.0027 M) showed a similar effect of dye fluorescence growth in the presence of albumin (see Figures S12 and S13 in Supplementary Information). The value of K_a was found to be $1.3–1.4 \times 10^5$ M^{-1}, that is, about 3 times smaller than that without a buffer (4.3×10^5 M^{-1}, see Table 2). This may be a consequence of diminishing dye–albumin electrostatic interaction with increasing the ionic strength of the solution.

3.4 *Molecular docking of the dyes with HSA and BSA*

The molecular docking experiments of OXEC, DMEC, and DMC in complexes with HSA and BSA were performed on DockThor Server[25,26] (blind docking). Table 3 shows the results of molecular docking: total energy (E_{tot}, kJ/mol), energy of intermolecular interaction (E_{int}, kJ/mol), and its constituent parts—van der Waals energy (E_{vdW}, kJ/mol) and electrostatic interaction (E_{el}, kJ/mol). According to the results obtained, the dyes form complexes on the surface of HSA and BSA molecules, and the Coulomb forces play a significant role in the complex formation.

Since the dyes under study are in the *cis*-form in aqueous solutions, this form of the dyes was chosen as the initial configuration. *In silico* experiments showed that the *trans* conformations are more favorable for binding of dyes DMC and DMEC to the albumins, which confirms *cis*-to-*trans* conversion of the dyes upon complexation with the albumins (see Figures S8 and S9 in Supplementary Information). This configuration corresponds to most of the results of molecular docking of DMC and DMEC with HSA and BSA, and agrees with the results of absorption and fluorescence measurements. In the case of OXEC, it was found that the dye can interact with HSA and BSA in the forms of both *trans*- and *cis*-isomers, while blind docking gives significant contributions of *cis* and twisted (distorted) conformations.

In contrast to the complex DMC–HSA, for which the molecular docking experiments indicated the possibility of binding DMC in domain I of the protein molecule,[12] in the complex of DMC with BSA, the ligand dye is located in domain II, with the distances to the nearest amino acid residues Glu274 and Glu276 of ~6 and ~4.8 Å, respectively (subdomain IIA); the distance to Ser418 is 14.3 Å (Ser418 belongs to subdomain IIB). According to molecular docking, the location of DMEC corresponds to the boundary between domains I and II of HSA/BSA. In particular, in the complex DMEC–HSA, the dye is located near Asp10 and Asp239 residues (the distances from the center of the dye molecule to these residues are 7.5 and 5.3 Å, respectively), which qualitatively corresponds to domains I and II; Tyr242 is located

[1]Earlier[12] we presented a lower value of LD_{HSA} for DMC equal to 7.9×10^{-10} M, which was calculated using the standard relationship **Equation S2**, which is based on the initial linear portion of the plot. However, the very initial portion of the calibration curve for DMC with HSA was found to be not linear (it is s-shaped, see Figure S9); its deviation from linearity is probably due to occurrence of aggregation-deaggregation phenomena[11]. So the actual LD value for DMC is somewhat higher than the value calculated from **Equation S2**.

Table 3. Results of molecular docking of dye–albumin complexes using the DockThor server.

		HSA					BSA				
		Run	E_{tot}	E_{int}	E_{vdW}	E_{el}		Run	E_{tot}	E_{int}	E_{vdW}	E_{el}
			kJ/mol						kJ/mol			
DMC	*trans*	9	−3.64	−241	−29.4	−211	*trans*	15	−201	−208	−25.4	−183
DMEC		15	−131.9	−309	68.9	−378		20	−134.9	−309	7.45	−316
OXEC		21	−169.9	−232	−1.439	−231		9	−202	−239	1.531	−241
	cis	17	−164.8	52.4	29.3	−247	*cis*	20	−202	38.6	−2.89	−238

Data for DMC-HSA were taken from the earlier work[12].

somewhat further (at ~13 Å, it belongs to subdomain IIA). For DMEC–BSA, the dye is located near Asp225 (subdomain IB) and Asp244 (subdomain IIA); the distances from DMEC to these amino acids are ~6.9 and 6.2 Å, respectively; Asp13 is ~10.7 Å distant from DMEC. Similar results were obtained for OXEC: when interacting with HSA and BSA, the dye binds to the surface of the protein globule in the region of subdomain IIA and, in part, subdomain IB. In particular, for OXEC–HSA, the distances from the dye *trans*-isomer to Tyr242, Asp238, and His6 are ~10.4, ~6.4, and ~8.1 Å, respectively. The distances to Asp238, Tyr242, and Glu245 of ~7.7, 10.3, and 7.2 Å, respectively, were obtained for the *cis*-isomer of OXEC. In the case of the complex of *trans*-OXEC with BSA, molecular docking showed the distances from the dye to Asp225 and Lys228 of 9.6 and 6.5 Å, respectively; the distance to Leu244 is somewhat longer, ~13.8 Å (subdomain IIA). In the case of the *cis*-isomer of OXEC, the distances to Asp241 and Asp244 are ~6.9 and 7.1 Å, respectively.

3.5 *Synchronous fluorescence spectra of HSA and BSA in the presence of the dyes*

The interaction of the dyes with albumin macromolecules was also studied using SFS spectra. The positions of the maxima of the SFS bands (λ_{fl}^{syn}) demonstrate the sensitivity of the albumin fluorophores to the molecular environment and exhibit shifts when the polarity of the medium changes.[17,18] In the experiments, the SFS spectra of tyrosine and tryptophan amino acid residues of HSA and BSA were measured in the presence of the dyes. The use of such spectra can indicate changes in the polarity of the binding sites of albumin molecules when interacting with the studied dyes.

The SFS experiments did not show appreciable shifts of λ_{fl}^{syn} in the HSA and BSA spectra upon complexation with the dyes, which indicate no evidence of a change in the polarity of the microenvironment of the fluorophore centers of the protein molecules. At the same time, the interaction of the dyes with HSA and BSA led to a decrease in the intensity of the SFS bands of tyrosine and tryptophan, which indicates quenching of fluorescence of the fluorophores in albumin molecules by the dyes. The experimental data on quenching of synchronous fluorescence of HSA and BSA by the dye OXEC are shown in the Supplementary Information (see Figures S10-S13).

The contribution of dynamic quenching to the quenching process was analyzed using the Stern–Volmer and Lehrer–Leavis dependences.[21,22] The experimental dependences in the Stern–Volmer coordinates (Figure 7a; Equation (1)) significantly deviate from linearity, which is especially noticeable on quenching of SFS of BSA at $\Delta\lambda$ = 15 nm (corresponding to the predominant quenching of fluorescence of tyrosine groups). On the other hand, the dependences are well approximated by linear anamorphoses (R^2 ~ 0.95–0.99) in terms of the Lehrer–Leavis model (Figure 7b; Equation (2)), which implies the presence of chromophores inaccessible to a quencher in a protein molecule.[22] To explain this fact, we may assume the presence of a portion (α) of accessible chromophores (mainly a portion of tyrosine residues) in BSA and HSA molecules, whereas the rest of the chromophores (1–α) is inaccessible for quenching. Table 4 contains the obtained parameters (K_{SV}, α) of the Lehrer–Leavis model for quenching of synchronous fluorescence of HSA and BSA by dyes OXEC, DMEC, and DMC.

The values of K_{SV} for different dyes are in the range of $4.60 \times 10^4 - 6.67 \times 10^5$ L mol^{-1}. Since $K_{SV} = k_q\tau_0$, where k_q is the rate constant of the dynamic fluorescence quenching and τ_0 is the fluorescence decay time for HSA and BSA without a quencher ($\tau_0 \sim 10^{-8}$ s)[46], the values of k_q are in the range of $10^{12} - 10^{13}$ L mol^{-1} c^{-1}. However, for the dynamic quenching, the value of k_q should be no more than the diffusion limit (for

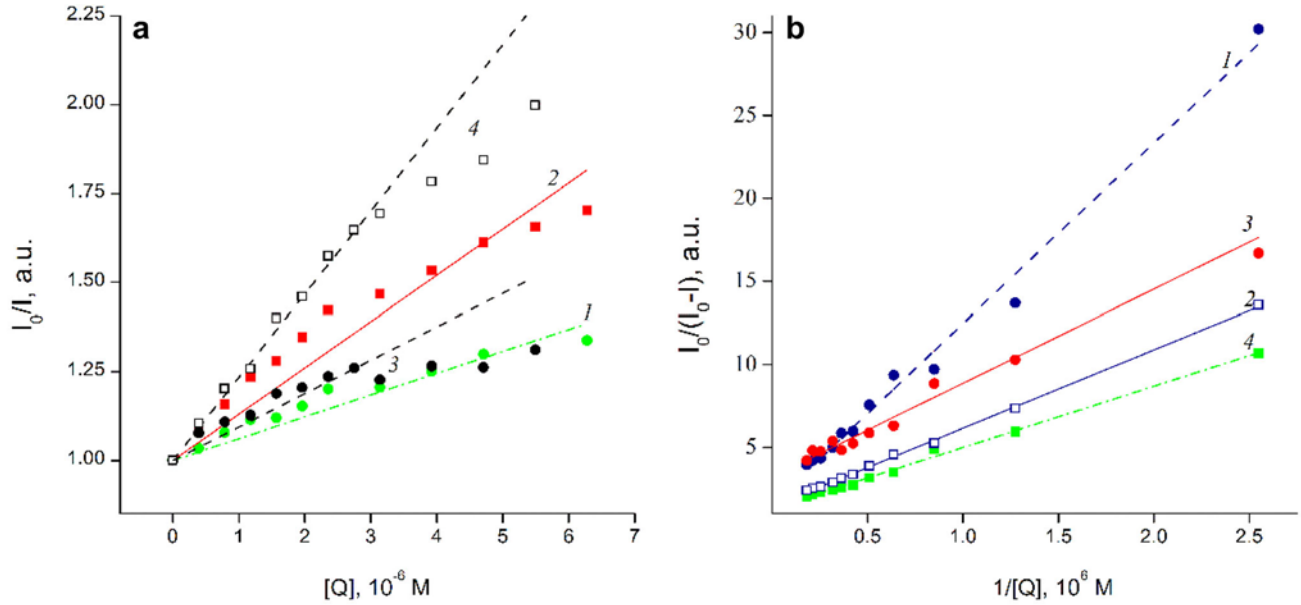

Figure 7. Stern-Volmer (a) and Lehrer-Leavis (b) plots according to the quenching of synchronous fluorescence of HSA (*1, 2*) and BSA (*3, 4*) with OXEC, at Δλ = 15 nm (*1, 3*) and at Δλ = 60 nm (*2, 4*).

Table 4. Parameters of the Lehrer–Leavis model for quenching of synchronous fluorescence of HSA and BSA by dyes OXEC, DMEC, and DMC.

Albumin	SFS Δλ, nm	Fluorophore	OXEC	DMEC	DMC
			K_{SV}, L mol^{-1}; α		
HSA	15	Tyr	1.46×10^5; 0.63	2.03×10^5; 0.57	1.91×10^5; 0.55
	60	Trp	3.04×10^5; 0.7	4.30×10^5; 0.57	1.49×10^5; 0.67
BSA	15	Tyr	5.68×10^5; 0.31	4.60×10^4; 0.34	6.67×10^5; 0.40
	60	Trp	3.56×10^5; 0.76	9.47×10^4; 0.63	5.16×10^5; 0.65

aqueous solutions, $k_{diff} \sim 10^{10}$ L mol^{-1} s^{-1}). This means that the quenching process is not limited by diffusion of a quencher to fluorophore centers;[21,47] that is, the quenching occurs in a complex formed between the fluorophore (albumin) and quencher (dye) molecules, being static in nature.

The quenching of the intrinsic fluorescence of HSA and BSA probably proceeds by FRET from tryptophan and tyrosine donors to dye acceptors. Since the fluorescence spectra of albumins and the absorption spectra of the dyes are overlapped (see Figure S21, Supplementary Information), the FRET process can occur. The formation of dye–albumin complexes bring the acceptor (a dye molecule) closer to the donor moiety (a tryptophan or tyrosine residues), which facilitates FRET. It was possible to estimate the critical distances for quenching of tryptophan and tyrosine fluorophores by the dyes (the Förster critical radius R_0),[21] that is, the distance between the donor fluorophore and the acceptor in the dye-albumin complex at which the probability of quenching is 0.5 (see Equation S5 in Supplementary Information). In particular, for FRET from tryptophan fluorophores to the dye in the systems HSA–OXEC and BSA–OXEC, R_0 = 37 and 36 Å, respectively, with the overlap integrals J (see Equation S4 in Supplementary Information) equal to 1.2×10^{15} and 1.07×10^{15} mol^{-1} cm^{-1} nm^4, respectively.

Note that it was found in the work[48] that albumins can form fluorescent oligomers at higher concentrations. However, we carried out experiments on quenching of albumins fluorescence at rather low their concentrations of about 10^{-5} M, at which the formation of such oligomers could be neglected.

4. Conclusions

The data obtained show both similarities and differences in the behavior of dyes OXEC, DMEC, and DMC in solutions. All these dyes are present in the monomeric form in polar organic solvents, with a large

excess of the *cis*-isomer over the *trans*-isomer, whereas the *trans*-isomer is the main conformation of the dyes in the nonpolar solvent – 1,4-dioxane. Furthermore, in aqueous solutions, the *cis*-monomer is in equilibrium with the dimeric form. The same behavior is characteristic of other *meso*-substituted anionic thiacarbocyanine dyes, in particular, 3,3′-di-(γ-sulfopropyl)-4,5,4′,5′-dibenzo-9-ethylthiacarbocyanine betaine (DEC),[12] which is a close analogue of DMC. However, OXEC, DMEC, and DMC differ in their aggregation ability: in water DMC forms aggregates of various types (dimers, H- and J-aggregates), whereas DMEC and OXEC form only dimers. The aggregation ability in aqueous solutions is in parallel with the hydrophobic properties of the dyes, which are more pronounced for DMC than for DMEC and OXEC (the higher hydrophobicity of DMC is due to the presence of two fused benzene rings in the terminal heterocycles of the DMC molecule, whereas DMEC and OXEC have more hydrophilic side substituents instead). Note that DMC strongly aggregates also in a nonpolar medium of 1,4-dioxane. Noncovalent binding of all these dyes to HSA and BSA is accompanied by the decomposition of dye dimers into monomers and *cis*-to-*trans* isomeric conversion (the same was observed for DEC and other *meso*-substituted cyanines upon complexation with HSA[12]); however, in the case of BSA this conversion is incomplete and the dyes bind to BSA also as *cis*-isomers. This difference could follow from the interplay of energetic and steric factors, which can be revealed by molecular docking modeling. DMC exhibits the highest value of K_a and the lowest limits of HSA detection (LD and LQ), which may be the consequence of higher hydrophobicity of this dye and, hence, stronger interaction with hydrophobic sites of the protein. The high selectivity of DMC and DMEC in the detection of HSA compared to BSA, together with high sensitivity, creates a prerequisite for using these dyes as selective probes for HSA (DMC was recommended as a probe for HSA earlier[12]). In contrast, OXEC, having similar sensitivities both for HSA and BSA, could be suggested as a probe for these albumins, but the lower limits of detection (LD $\sim 2 \times 10^{-8}$ M) restrict its possible application. compared to BSA. The results of molecular docking experiments correspond to the data obtained from the spectra. The influence of the dyes on intrinsic fluorescence of HSA and BSA was also studied, showing no changes in the polarity of the microenvironment of the fluorophore centers; fluorescence quenching, static in nature, was detected. Quenching of albumin fluorescence by the dyes was considered in terms of the FRET model.

Supplementary Information (SI)

Supplementary information includes an additional description of experimental techniques (Equations S1 – S5); absorption and fluorescence spectra of the dyes (Figures S1–S9, S12–S14); deconvolution of the DMEC absorption spectra into Gaussian components (in the presence of HSA and BSA) (Figures S10 and S11); molecular docking results: OXEC – HSA (Figure S15), DMC – BSA (Figure S16); synchronous fluorescence spectra of HSA and BSA in the presence of OXEC (Figures S17 – S20), spectral overlapping for the OXEC- HSA (Figure S21), data on the displacement of dyes by ibuprofen and warfarin from complexes with HSA and BSA (Table S1).
Supplementary information is available at www.ias.ac.in/chemsci.

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