Molecular Docking simulation and Fluorescence lifetime characteristics of NIR Cyanine Dye Complexes with Albumin

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Abstract The high sensitivity of modern techniques for lifetime detection opens new significant opportunities for investigation of various processes in the biological media. Here, we report on the novel sub-class of cyanine dyes. We examined the effect of electronic coupling on the optical properties of the complex of the dye with the human serum albumin. TCSPC measurements of fluorescence decay provided evidence that the studied dye generates more than two kinds of complexes with albumin. It was established that the effect of “frozen structure” in complex changes the rate of photoisomerization way of the dye’s excited state degradation. Furthermore, by means of molecular docking, the experimentally proposed mechanism of complex formation and the existence of several binding sites were confirmed.

1. Introduction
In recent years the application of cyanine dyes in medicine has tangibly grown due to their good ability to accumulate in tissues and cells, low toxicity and satisfactory fluorescence quantum yields. Nowadays, they are widely used in bioimaging [1], as photosensitizers in photodynamic therapy [2] or as conjugates for selective delivery of drugs in order to improve the bioavailability of the latter [3]. Particularly, utilization of cyanine dyes as fluorescent contrast agents in molecular biology is centered on their ability to accumulate in tissues [4], and now it is a promising technique of bioimaging for visualization of blood vessels and tumors. At present, there are only two cyanine dyes approved by FDA for medical application – fluoresceine and NIR dye – cardio green (ICG). Both of them have a low range of intersystem crossing, which makes them safe, as there is no photosensitizing during irradiation. ICG long polymethine chains contribute to its absorption in NIR, which enables ICG to penetrate the tissues. A crucial role for the biological usage of dyes is devoted to the complex formation with blood proteins, such as albumin and lipoproteins, which ensure the delivery of a dye to the cell target [5]. In [6] it was shown that the interaction of dyes with biomacromolecules leads to an increase of the fluorescence intensity of complex, rigidity and stability of dye structure.

In this paper, we present a new biscardocyanine dye containing two chromophore systems, which further on will be referred to as “BCD”. The molecular docking experiment was also carried out in order to estimate the possible interactions between the dye and the protein and to elicit any special interactions. The obtained data may serve as a basis for further investigation of intercellular accumulation and distribution for fluorescence lifetime imaging and confocal autocorrelation microscopy.

2. Experimental Details
All of the chemicals used in the current study were purchased from commercial vendors and used as received without further purification unless otherwise noted. Human serum albumin (HSA) was used. Experiments in the water phase were carried out in phosphate-buffered saline (PBS) at pH 7.4 at room temperature.

2.1 The binding constant of dye

The binding constant was determined by fluorescence spectroscopy. The interaction of protein and dye corresponds with a bathochromic shift and an increase of fluorescence. The binding constant $K_b$ was calculated from the binding curves according to the equation (1):

$$\theta = \frac{K_b [\text{HSA}]}{1 + K_b [\text{HSA}]}$$

where $\theta = (F - F_0)/(F_w - F_0)$ is the fraction of the dye bound with the protein and $F_0$, $F_w$, and $F$ are the fluorescence intensity at [HSA] = 0 and at the complete and intermediate binding of the dye by protein, respectively.

Steady state spectroscopy

UV-visible absorbance spectra were recorded using the Shimadzu UV-3101PC model in EtOH and PBS buffer solutions with or without human serum albumin HSA. Fluorescence spectra were obtained using the Panorama fluorescence spectrometer.

2.2 TCSPC experiments

Lifetimes of singlet excited state registration were obtained by TCSPC UV-IR spectrofluorometer FluoTime 300 Picoquant. Dye dissolved in EtOH or PBS with albumin and irradiated by 640 nm (LDH-D Picoquant) ps laser 40 MHz rep. rate with vertically polarized beam. Emission detected with a magic angle polarizer to prevent undesired polarization effect. Fluorescence registered in wavelength interval from 750 to 800 nm and obtained data were analyzed using FluoFit software (PicoQuant).

Characteristic times and the contribution of individual components to the detected fluorescence were calculated using a multi-exponential model with deconvolution based on internal response function (IRF) by the following formula:

$$I(t) = \int_{-\infty}^{t} IRF(t') \sum_{i} A_i \exp\left(-\frac{t - t'}{\tau_i}\right) dt'$$

where $i$ is the number of components, $A$ is the amplitude that determines the contribution of each component, and $\tau$ is the decay time of the components.

The values of $A_i$ and $\tau_i$ can be used to determine the fractional contribution ($Q_i$) of each decay time to the steady-state intensity. This value can be expressed as:

$$Q_i = A_i \tau_i / \sum_j A_j \tau_j$$

where $j$ is the total number of measured components.

The fitting quality of the decays was controlled by the least-squares method ($\chi^2$), weighted residuals, and an autocorrelation function [8]. The residual plot and autocorrelation function are of particular advantage, in that they show where the lack of fitting occurs. The weighted residuals should be below four standard deviations and randomly fluctuate around zero with no obvious trend [8], [9]. The values of the autocorrelation function should be small and randomly distributed around zero for times $>0$.

To resolve the components of a mixture with different lifetimes, the data sets were subjected to global analysis [8–10] with FluoFit software (FluoFit Manual, PicoQuant, 2017). The fluorescence decay kinetic curves were registered sequentially in increments of 10 nm in the selected range of wavelengths using a single fluorescence excitation wavelength. For each data set, three fluorescence spectra were
constructed. Each point in the spectrum corresponded to the integral intensity of one of the three components ($\tau_1$, $\tau_2$ or $\tau_3$) obtained from a single kinetic curve at taken wavelength. The calculation of $\chi^2$ extended over several data sets.

2.3 Molecular Docking

The molecular docking processes of the BCD molecules into the HSA structure were simulated using AutoDock Vina 1.1.2 software [11]. The ligand molecules were analyzed in the ionized form. The atomic charges were calculated using the Gasteiger–Hückel method and geometric parameters were optimized using the Tripos force field in the Sybyl-X 2.1 software [12]. For the analysis and visualization of the results, the UCSF Chimera 1.13.1 [13] and Sybyl-X 2.1 [12] software was used.

3. Results and discussion.

3.1 Optical properties.

Polymethine dyes are a special class of organic compounds: they have the unique property of sensitizing light in visible and NIR range [14]. Polymethine compounds are characterized by relatively narrow intense main absorption bands, indicating a relatively small change in the bond length during excitation [15]. The study of the complexation of cyanine dyes with biomacromolecules is currently in the spotlight, as these dyes are used to detect nucleic acid molecules, and induce sensitized photochemical reactions in chemical and biological systems [16]. A special place among cyanine dyes is occupied by dyes whose molecules contain two conjugated chromophores, which exhibit two effects: dipole-dipole interaction and electron tunneling through the central heterocycle [17]. The result of these effects is the splitting of excited levels, which leads to a strong bathochromic shift in the absorption spectrum and makes these compounds promising photosensitizers [18]. Recently, biscarbocyanine dyes have been investigated as potential photosensitizers for photodynamic therapy, and high photocytotoxicity of these dyes was established on the example of cancer cells (melanoma). This discovery opens up new opportunities for the use of biscarbocyanines, the absorption spectrum of which is strongly shifted to the near-infrared region, as sensitizers in photodynamic therapy in oncology [19]. It was found that the localization of cyanine dyes in cancer cells occurs in the mitochondria, which determines their high photocytotoxicity [20]. An important role in the delivery of photosensitizers to cells is played by the process of complexation of dyes with human serum albumin and further photochemical reactions of excited dyes with proteins belonging to various cell organelles. In this paper, the spectral and kinetic characteristics of excited states of the complexes of biscarbocyanine dye 1,4-dimethyl-2,6-bis-((1E,3E)-3-(1,1,3-trimethyl-1,3-dihydro-2H-benzo[e]indol-2-ylidene)prop-1-ene-1-yl)-pyridin-1-ium iodide (BCD) and albumin were investigated.

The absorption spectrum of BCD in the presence of HSA in PBS is shown in figure 1. According to our studies, the BCD absorption peak is near 660 nm, while a fluorescence peak is near 700 nm. This
indicates that there is only a double chromophore system in this dye, and the effect of Davydov splitting is observed.

Figure 1. Normalized absorbion spectra of BCD in ethanol (\(\lambda_{\text{max}} 668\) nm) (black solid), PBS solution (\(\lambda_{\text{max}} 661\) nm) (red) and in the complex with HSA \(10^{-4}\)M (\(\lambda_{\text{max}} 669\) nm) (blue). monochromophoric analog of BCD – (\(\lambda_{\text{max}} 528\) nm) (dashed line).

The addition of HSA to the dye in PBS solution shifts the absorption and the emission spectra by 10-20 nm to a longer wavelength range. Also, the intensity of fluorescence increased almost ten-fold. The increase of fluorescence while adding HSA indicates that internal conversion proceeds to the ground state from the excited state through the fluorescence pathway more effectively. It is due to the growth of dye’s structure rigidity in complex with protein. The increase of protein concentration results in the reorganization of the complex. The binding constant can be assessed either from the changes in the absorption spectra on adding HSA or from the fluorescence spectra. The determination of a binding constant from the absorption spectra is less accurate mainly because of the aggregation of a dye in PBS. The value of \(K_b\) with HSA estimated \(\sim 10^5 \text{ M}^{-1}\).

3.2 TCSPC analysis of fluorescence kinetics.
Cyanine dyes are generally positively-charged molecules if no special substituents are added. Their structure consists of \(2p_z\) conjugated orbitals with alternated and delocalized electron density. This makes the interaction of cyanine dyes with biomacromolecules quite complicated. [21] The contribution of Coulomb interaction is insignificant, and the contribution of the entropy factor to binding is indeterminate because albumin has different binding sites. The lowest value of fluorescence lifetime characterized by component \(\tau_1\) is constant for all concentrations of protein. It is equal to a characteristic fluorescence lifetime of unbound BCD molecules. In the presence of HSA at high concentrations of protein all BCD molecules are likely to be associated with HSA because of a high value of \(K_d[6]\). Two components of the fluorescence decay of BCD associated with HSA correspond with two types of BCD-HSA complexes[6]. Complex formation characterized by lifetimes of \(\tau_2\) and \(\tau_3\) is associated with different types of complexes. The contribution of these components varies depending on the concentration of HSA. (Table 1).
Table 1 Fluorescence lifetimes $\tau_i$ ns of the mixtures of BCD with different HSA concentrations, the relative amplitudes of the components in % are given in parentheses.

| Concentration | $690$ nm | $10^{-6}$ | 0.20 (96) | 1.26 (4) | – |
|---------------|----------|-----------|-----------|----------|---|
| $10^{-5}$     | 0.19 (40)| 0.92 (22) | 1.68 (38) |
| $10^{-4}$     | 0.22 (13)| 0.96 (36) | 1.65 (51) |

The lifetime value of the long-living component increases sufficiently with the increase of protein concentration. The interchange of amplitudes of components $\tau_2$ and $\tau_3$ is associated with migration of BCD between binding sites and the alteration of complexes’ types (Figure 2-4). We attribute a change of fluorescence wavelength from 660 nm to 690 nm to this process.

Figure 2. Fluorescence decay kinetics of BCD $[1\times10^{-7}$ M] + HSA $[1\times10^{-6}$ M] complex at different wavelengths, excitation 640 nm 70ps pulse.
Figure 3. Fluorescence decay kinetics of BCD $[1 \times 10^{-7} \text{ M}] + \text{HSA} [1 \times 10^{-5} \text{ M}]$ complex at different wavelengths, excitation 640 nm 70ps pulse.

Figure 4. Fluorescence decay kinetics of BCD $[1 \times 10^{-7} \text{ M}] + \text{HSA} [1 \times 10^{-4} \text{ M}]$ complex at different wavelengths, excitation 640 nm 70ps pulse.
The location of the BCD molecule in binding sites is stabilized and refers to the longest fluorescence lifetime. The interaction of BCD and albumin increase the rigidity of dye structure, and the electrostatic π-stacking with aromatic amino acid can change the rate of intersystem crossing to increase the quantum yield of the triplet state. The distance between BCD and tryptophan or tyrosine residues has great value to the possibility of the electron transfer between triplet state of a dye and the aromatic ring of amino acid[22]. Electron phototransfer leads to the formation of a reduced form of BCD, which when interacting with an oxygen molecule forms an intermediate – superoxide anion-radical, which further leads to the formation of highly reactive intermediates that destroy cell structures as a result of necrosis and apoptosis.

3.3 Molecular docking

The possible interactions between the BCD molecule and HSA were analyzed using a molecular docking method. All calculations were performed using AutoDock Vina 1.1.2 software [11]. The crystal structure of the human serum albumin was obtained from PDB ID (4L9Q). Among several proposed binding modes two most stable docking models should be noted. In both cases the BCD molecule is located in a wide, moderately polar pocket between the IIA and IIIA albumin subdomains (Figure 5). One substituted indoline fragment is oriented inside deep into the pocket while the other fragment is oriented towards the outer protein surface.

Typically for the albumin ligands, the binding has mostly non-specific nature primarily based on hydrophobic interactions. As far as the second mode is concerned, there is an extra contribution to the binding from weak noncovalent π-π stacking interaction between the Tyr452 residue and the central pyridine ring (fig. 6). In order to estimate the distance between two aromatic rings that interact through the π-π stacking, an approach to calculate the distance between two rings’ centroids (Figure 6) was suggested. The obtained value equals 4.241Å. Though, it is questionable if such π-π stacking distance value is acceptable. However, authors of [23] suggest that the values of 4.1 – 4.4 Å for the distances between two aromatic rings in parallel-displaced π-π stacking interaction counted for centroids are reasonable. Consequently, the obtained value satisfies the range of possible displaced π-π stacking distances as a preliminary estimation. However, it definitely requires further revision as a better approach to calculate this parameter might be devised.
**Figure 6.** Left - π – π stacking interaction between a pyridine ring of the dye and the Tyr452 residue. Right – centroids of Tyr452 benzene ring and the BCD pyridine ring to calculate the distance between the two aromatic rings. The ligand molecule is represented by a grey ball-and-stick model, the Tyr452 residue is represented by a blue ball-and-stick model.

The obtained data from the AutoDock shows that these two modes have slight differences in the binding energy of 0.3 kcal/mol. Consequently, it can be inferred that both binding modes are almost equally possible and, presumably, can interchange. Therefore, the dye molecule may insignificantly alter its conformation within the binding site skipping from one binding mode to another. Moreover, an overlay of two lowest energy conformers in their corresponding conformations in the binding site suggests the need of minor rotation of certain structural moieties for the molecule to transform from one state into another (Figure 7.). The supposition about such energetically low transitions does not conflict with the mechanism concluded from the spectral studies.

**Figure 7.** Overlay of two binding modes’ structures. The conformers’ molecules are represented by a grey and cyan stick models, and nitrogen atoms are colored in dark blue, respectively.

Ultimately, this binding site is the most probable one. However, it should be noted that binding in other albumin sites is also possible. Thus, the analysis of molecular docking results allows proposing a viable mechanism of the complex formation between the BCD molecule and HSA, which correlates well to the mechanism concluded from the spectral and fluorescent experiments.
4. Conclusion

In conclusion, a new bis-carbocyanine dye with an integrated heterocyclic fragment in the polymethine chain was synthesized, the complexation of the dye with HSA was studied using spectrophotometry, spectrofluorimetry, TCPSC, and molecular docking. The complexation of BCD and albumin changes the absorption spectrum and increases the fluorescence intensity. The experimentally determined value of the binding constant (~10^5 M^-1) indicates a high affinity of the dye to albumin. Analysis of fluorescence decays allows us to suggest the existence of at least three types of complexes between BCD and HSA. BCD-albumin complexes form as a result of rearrangements of the initially formed complex by re-distribution between HSA binding sites. A more rigid complex with a longer fluorescence lifetime demonstrates an increase in fluorescence and therefore a decrease in internal conversion by cis-trans-isomerization. The study of the binding site and conclusions about the complex’s nature have been confirmed by molecular docking. The results of this study serve as a basis for further studies of the cellular accumulation and distribution of BCD-HSA using fluorescent imaging and confocal microscopy.

Conflicts of interest

The authors declare no conflict of interest.

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